A study of structure and function of two enzymes in pyrimidine biosynthesis

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Boston College

The Graduate School of Arts and Sciences

Department of Chemistry

A STUDY OF STRUCTURE AND FUNCTION OF TWO ENZYMES IN PYRIMIDINE BIOSYNTHESIS

a dissertation

by

WENYUE GUO

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

August 2012
A STUDY OF STRUCTURE AND FUNCTION OF TWO ENZYMES IN
PYRIMIDINE BIOSYNTHESIS

by

Wenyue Guo

Thesis Advisor: Professor Evan R. Kantrowitz

Abstract

Nucleotides, the building blocks for nucleic acids, are essential for cell growth and replication. In *E. coli* the enzyme responsible for the regulation of pyrimidine nucleotide biosynthesis is aspartate transcarbamoylase (ATCase), which catalyzes the committed step in this pathway. ATCase is allosterically inhibited by CTP and UTP in the presence of CTP, the end products of the pyrimidine pathway. ATP, the end product of the purine biosynthetic pathway, acts as an allosteric activator. ATCase undergoes the allosteric transition from the low-activity and low-affinity T state to the high-activity and high-affinity R state upon the binding of the substrates. In this work we were able to trap an intermediate ATCase along the path of the allosteric transition between the T and R states. Both the X-ray crystallography and small-angle X-ray scattering in solution clearly demonstrated that the mutant ATCase (K164E/E239K) exists in an intermediate quaternary structure shifted about one-third toward the canonical R structure from the T structure. The
structure of this intermediate ATCase is helping to understand the mechanism of the allostERIC transition on a molecular basis. In this work we also discovered that a metal ion, such as Mg$^{2+}$, was required for the synergistic inhibition by UTP in the presence of CTP. Therefore, the metal ion also had significant influence on how other nucleotides effect the enzyme. A more physiological relevant model was proposed involving the metal ion. To better understand the allostERIC transition of ATCase, time-resolved small-angle X-ray scattering was utilized to track the conformational changes of the quaternary structure of the enzyme upon reaction with the natural substrates, PALA and nucleotide effectors. The transition rate was increased with an increasing concentration of the natural substrates but became over one order of magnitude slower with addition of PALA. Addition of ATP to the substrates increased the rate of the transition whereas CTP or the combination of CTP and UTP exhibited the opposite effect.

In this work we also studied *E. coli* dihydroorotase (DHOase), which catalyzes the following step of ATCase in the pyrimidine biosynthetic pathway. A virtual high throughput screening system was employed to screen for inhibitors of DHOase, which may become potential anti-proliferation and anti-malarial drug candidates. Upon the discovery of the different conformations of the 100’s loop of DHOase when substrate or product bound at the active site, we’ve genetically incorporated an unnatural fluorescent amino acid to a site on this loop in the hope of obtaining a better understanding of the catalysis that may involve the movement of the 100’s loop.
Acknowledgements

It is impossible for me to have this day without the love, sacrifice, support and prayers of many people God has placed in my life. Even at the end of last year I couldn’t imagine that I would have this opportunity to write the acknowledgements for this doctoral degree. It is truly by the Grace of God that I can be where I am today.

First, my deep gratitude goes to my advisor, Dr. Kantrowitz, known affectionately as Dr. K. I am sincerely grateful to Dr. K for his support to me to finish the doctoral program beyond the normal time range. I’m thankful for his patience, understanding and guidance for the past almost eight years. He never ceases to teach me though I am slow to learn and moreover he is very considerate to me when I went through difficulties along the way. Indeed he is a great advisor I’ve not seen elsewhere.

I would like to thank Dr. Jianmin Gao, Dr. Eranthie Weerapana and Dr. Dunwei Wang for your willingness to be my committee members. Thank you for your time in the midst of your business to read and give comment on this thesis. Thanks for all your advice and suggestions that contribute to complete this work.

It has been a great pleasure and memorable time to work with my current and past labmates and I want to take this chance to thank each one of them. I want to thank Jie Wang, who taught me hand by hand the lab techniques as soon as I joined, and has been a great senior to me since then. Without her help, my transition to the lab would be more difficult. My special thanks go to Sabrina Heng, who was my “neighbor” in the lab for about four years as we sat next to each other. She has become one of my best friends and my dear sister in Christ who shares with me about not only the research progress/ideas
but also our life and faith. She not only gave me a lot of help and insight to my research but also tremendous help to my life when I was in need. I missed her so much after she graduated and moved to Australia. I’m really blessed that I can know her and build up precious moments in life together while at BC. I also want to thank Joby Eldo, Kimberly R. Mendes, James Cardia, Jiarong Xia, Liz O’Day, Jay West, Greg Cockwell, Kate Harris, Yunan Zheng, Yani Zhou and Andrew Dutton whom I have worked with in the lab. Thank you for all your contributions to my work in the lab and also making my time in the lab an enjoyable experience.

I want to thank my parents for their love and sacrifice to raise me up and support my study at a place so far away from them. They always encourage me when I face difficulties in finishing up this degree. They never put any pressure on me but simply support me with all they can and take aside with me. I’m forever grateful to them. I especially thank my mom as it is not easy for her to come to the U.S. but she came, several times, and helped us a lot with taking care of Anna to support my study. I also want to thank my parents-in-law for allowing me to study for a long time and their support and sacrifice and also they came to live us and help us taking care of Anna for more than a year.

I want to thank my husband Dongliang Jiang for his commitment and sacrifice to me. Thank you for your willingness to support me finishing the doctoral program and take the sole responsibility to provide the financial need for our family. Thank you for sharing my burdens and bearing with me in every way as I went through ups and downs through the past years. Especially the past half year despite your heavy load at work, you
still tried to babysit Anna and help more with the housework so that I could have more
time to do the experiments and write the thesis.

I want to specially thank each member of the family of God, ISM (international
student ministry) of Antioch Baptist Church. Their prayers and love carried me thus far.
I’m so blessed to be in this family of God. Especially I’m indebted to Pastor Joseph, Hyejin
SMN, Heonick unni, Jonghoon opa, Irene, Jane and John. Without their prayers, love,
guidance and sacrifice, I would not make this day and be who I am today. They guided me
every step along the way and pouring out their time and resources to love and care for a
undeserving person like me. They strengthened me when I was weak and they carried me
when I cannot walk. Words cannot express my gratitude towards them. I also thank
Pastor Paul and Becky JDSN who founded this church that becomes my “family”. Thanks
for Pastor Paul who always encourages me to not give up and continuously prays for my
study till this day.

Lastly I want to give all the thanks to God, my heavenly Father, who has shown His
amazing grace and His faithfulness to me through this doctoral degree. Though it has not
been easy for me for the past eight years, in the end I am able to say I am blessed and
thankful because He is with me all the way and He has drawn me closer to Him through
the whole process. I give all the honor and glory to God who has led me this far with His
love.
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100's loop          residue 105-115 of DHOase
80's loop            residues 73-88 in the catalytic chain of ATCase
240's loop           residues 230-245 in the catalytic chain of ATCase
5-FOA                 5-fluororotic acid
Abs                   absorbance
ATCase                *E. coli* aspartate transcarbamoylase holoenzyme
ATP                   adenosine-5'-triphosphate
Asp                   L-aspartate
APS                   ammonium persulfate
CA                    N-carbamoyl-L-aspartate
DL-CA                 N-carbamoyl-DL-aspartate
CAPS                  N-cyclohexyl-3-aminopropanesulfonic acid
CP                    carbamoyl phosphate
CTP                   cytidine-5'-triphosphate
DHOase                *E. coli* dihydroorotase
DHO                   L-dihydroorotate
*E. coli*             *Escherichia coli*
EDTA                  Ethylenediaminetetraacetic acid
GA                    genetic algorithm
GTP                   guanosine-5'-triphosphate
HCE-Gly               L-(7-hydroxycoumarin-4-yl)ethylglycine
HCE-Gly114            modified form of DHOase where His114 was site-specifically Altered to HCE-Gly
IC$_{50}$  
  half maximal inhibitory concentration

IPTG  
  isopropyl-$\beta$-D-thiogalactopyranoside

IMAC  
  immobilized metal ion affinity chromatography

KNF  
  Koshland, Nemethy, and Filmer

MWC  
  Monad, Wyman, and Changeux

NTP  
  nucleoside triphosphate

*P. falciparum*  
  *Plasmodium falciparum*

PAGE  
  polyacrylamide gel electrophoresis

PALA  
  $N$-phosphonacetyl-L-aspartate

$P_i$  
  inorganic phosphate

RMSD  
  root mean square deviation

SAXS  
  small angle X-ray scattering

SDS  
  sodium dodecyl sulfate

TR-SAXS  
  time-resolved small angle X-ray scattering

UTP  
  uridine-5'-triphosphate

vHTS  
  virtual high throughput screening
Chapter 1: Introduction
Aspartate Transcarbamoylase

Brief Overview of *E. coli* Aspartate Transcarbamoylase

Many biochemical reactions in a living cell can go both ways, for example, mammalian cells both synthesize and catabolize glucose. The rates at which these reactions proceed must be regulated to maintain homeostasis of cell. The *de novo* biosynthesis of pyrimidine nucleotides in *E. coli* starts with carbamoyl phosphate (CP), which is derived from glutamine, and subsequently proceeds through a series of reactions catalyzed by enzymes to produce the pyrimidine nucleotides, UTP and CTP. The enzyme that exerts control over the rate of this pathway is a classic allosteric enzyme, aspartate transcarbamoylase (EC 2.1.3.2, ATCase), which catalyzes the first step in this pathway: CP reacted with L-aspartate (Asp) to form N-carbamoyl-L-aspartate (CA) and inorganic phosphate (Pi) (see Figure 1.1).\(^1\)\(^-\)\(^3\) ATCase exists as a single monofunctional enzyme in bacteria, such as *E. coli*, whereas in higher organisms ATCase exists as part of a multifunctional enzyme complex. For example, in mammals ATCase contributes as a functional domain in the CAD enzyme complex, which is composed of the first three enzymes, carbamoyl phosphate synthetase, aspartate transcarbamoylase and dihydroorotase, in the pyrimidine biosynthetic pathway.\(^4\)

Allosteric control is a type of enzyme regulation that results from the binding of an effector at a site (allosteric site) other than the active site of the enzyme. An
Figure 1.1. The reaction catalyzed by aspartate transcarbamoylase (ATCase): condensation of carbamoyl phosphate (CP) and L-aspartate (Asp) to form N-carbamoyl-L-aspartate and inorganic phosphate (Pi).
effector that causes an increase in the activity of the enzyme is called an allosteric activator, and an effector that causes a decrease in the activity of the enzyme is an allosteric inhibitor. The influence of an allosteric effector on the activity of the enzyme is called an heterotropic effect. Unlike a heterotropic effect, homotropic effect is caused by the same molecule as the one that will be affected (such as the substrate). Both effects together allow allosteric enzymes to greatly vary the rate of catalysis in response to small changes in substrate or allosteric effector concentration. ATCase, which is regulated both homotropically by its substrate Asp and heterotropically by the pyrimidine and purine nucleotides, becomes an archetypal example of allosteric modulation of control of metabolism.5

ATCase controls the rate of the pyrimidine biosynthesis by altering its rate of catalysis in response to the cellular levels of both pyrimidines and purines.6 When the end-product of the pyrimidine pathway, CTP, is in excess, the rate of catalysis is reduced due to feedback inhibition by allosteric inhibitors CTP and UTP7 in the presence of CTP. When the end-product of the parallel purine pathway, ATP, is in excess, the rate of catalysis is enhanced by the allosteric activator ATP. In addition, the substrate Asp binds cooperatively to the enzyme to increase activity at high concentrations when the end-products accumulate. These regulatory properties of ATCase thus contribute to maintain a balanced pool of pyrimidine and purine nucleotides for the biosynthesis of nucleic acids in the cell.
**Structure of ATCase**

Based on the molecular size, Bethell and Jones\(^8\) first proposed to divide the bacterial ATCases into three classes, namely Class A, Class B and Class C. *E. coli* ATCase is a representative for Class B ATCases, which include the enzymes from the enteric bacteria. Class B ATCases have a molecular weight of approximately 300 kDa, which are composed of the 34 kDa and 17 kDa polypeptide chains. The structural details of this class are described below in detail using *E. coli* ATCase. Class A ATCases are the largest in size with a molecular weight of 480 kDa and mostly found in the pseudomonads. In addition to the 34 kDa polypeptides, larger polypeptides of 45 kDa are found to form the enzymes in this class.\(^9,10\) Class C ATCases only consist of three identical monomers of the 34 kDa polypeptides, giving a total molecular weight of 100 kDa. Unlike ATCases in Class A and B under the regulation of nucleotides, Class C enzymes are not regulated and mostly found from Gram positive bacteria, such as *Bacillus subtilis*, and some from Gram negative bacteria.\(^11\)

The *E. coli* ATCase is an oligomeric protein complex composed of 12 polypeptide chains, which are grouped into two distinct types of subunits, catalytic (c) and regulatory (r). Treatment by heat or reacting with certain mercurial compounds can breakdown the enzyme into these subunits. The catalytic subunit contains the active sites, but is not influenced by CTP or ATP. The regulatory subunit is able to bind the allosteric effectors but has no catalytic activity.\(^12\) The composition of the holoenzyme is c\(_6\)r\(_6\), which was proved from the analysis of the sequence of the
**Figure 1.2.** Schematic representation of the quaternary structure of ATCase viewed along the three-fold axis. The two catalytic trimers (C1-C2-C3 and C4-C5-C6) are shown in blue. The three regulatory dimers (R1-R6, R2-R4 and R3-R5) are shown in yellow. The three red lines indicate the three two-fold axes.\(^\text{15}\)
regulatory chain and also independently from crystallographic symmetry determinations. The ATCase holoenzyme, with a total molecular weight of 310 kDa, is made up of two catalytic trimers (M_r 34,000 Da/chain) that are held together by three regulatory dimers (M_r 17,000 Da/chain). The catalytic trimers and regulatory dimers are related to one another by one three-fold axis and three 2-fold axes as shown in Figure 1.2.

Each catalytic chain is composed of two structural domains, the carboxamidyl phosphate (CP) domain, which contains most of the residues responsible for binding CP (residues 1-135 and 292-310), and the L-aspartate (Asp) domain, which contains most of the residues responsible for binding Asp (residues 136-291). Each regulatory chain is also composed of two structural domains, the allosteric (AL) domain (residues 1-100), which contains the binding site for the allosteric effectors, and the zinc (Zn) domain (residues 101-153), which has the binding site for zinc atom coordinated with four cysteine residues (see Figure 1.3).

Allosteric Transition of ATCase

Most allosteric properties can be explained by the concerted model of Monod, Wyman, Changeux (MWC), or by the sequential model of Koshland, Nemethy, Filmer (KNF). Both models are built on the same basic assumption that enzyme subunits exist in one of two extreme conformational states, the low-activity, low-affinity tense (T) state or the high-activity, high-affinity relaxed (R) state. The two models differ most in their assumption about the process of the T-to-R transition. In the MWC model, the conformational change in one subunit induced
Figure 1.3. Structural domains of catalytic chain and regulatory chain. Each of the six catalytic chains contains two domains: the CP and Asp domains. Each regulatory chain is also composed of two domains: the Al domain and Zn domain. The active site is shown with substrate analogs phosphonoacetamide and malonate bound, and the allosteric site is shown with the nucleotide effector CTP bound. This figure was redrawn with Pymol\textsuperscript{19} using data from PDB ID 8AT1.\textsuperscript{20}
upon ligand binding propagates to all other subunits such that all subunits of the enzyme must exist in the same conformation. Thus, the enzyme is assumed to be either in the T state or the R state, and the transition between the states is concerted. As seen in Figure 1.4 in the absence of ligands, the two states are in equilibrium, which favors the T state. The equilibrium can be shifted to the T or R state upon the binding of one ligand.

Different from the MWC model, the KNF model assumes that the progress from the T to R state is a sequential process. Thus, all enzyme subunits can exist in different conformations and there is no dramatic switch from one state to another. As a result for the KNF model, in the absence of ligands, the enzyme exists only in one state and there is no pre-equilibrium between the two states (see Figure 1.5). The KNF model uses a series of tertiary structural changes during the T-to-R transition, whereas the MWC model uses a quaternary structural change.

ATCase has been shown to undergo an allosteric transition from the T to the R state upon the binding of substrates as well as substrate analogs such as N-phosphonacetyl-L-aspartate (PALA). Studies using a variety of methods have shown that the holoenzyme is more swollen in the R state as compared to the T state. Molecular details of this structural change are revealed from the comparison of the X-ray structures of ATCase in the absence and presence of PALA. The enzyme expands 11 Å along the 3-fold axis during the T-to-R allosteric transition, at the same time the catalytic trimers rotate 12° relative to one another and the regulatory dimers rotate by 15° about the approximate 2-fold axes (see Figure 1.6).
Figure 1.4. The MWC model\textsuperscript{17} for a tetrameric allosteric protein upon binding of ligands. Protein subunits in the T state and the R state are shown as squares and circles, respectively. Unligated protein exists in the equilibrium between the two states. “L” represents ligands.
Figure 1.5. The KNF model\textsuperscript{18} for a tetrameric allostERIC protein upon binding of ligands. Protein subunits in the T state and the R state are shown as squares and circles, respectively. Protein transits from the unligated T state conformation to the ligand-bound R state conformation through a sequential process. “L” represents ligands.
These quaternary changes are accompanied by the tertiary structural alterations in both the catalytic and regulatory chains, the most significant of which involve movement of domains and loops.\textsuperscript{25,28} In particular, the Asp domain and CP domain of the catalytic chain moves toward each other by $\sim 7^\circ$ bringing the substrates in close proximity while the AL domain and Zn domain of the regulatory chain open by $1.7^\circ$.\textsuperscript{29} Besides the motions of domain, there is also a major reorientation of the 240's loop (residue 230 to 245 of the catalytic chains). In the T state, side chains of residues in the 240's loop are involved in the intersubunit interactions which stabilizing the compact form, such as interactions exist at the interfaces c1 (Glu239):c4 (Lys164 and Tyr165), c1 (Asp236):r4 (Lys143), c1 (Ser238):r4 (Asn111) and their symmetry related interfaces. (c1, c4 are catalytic chains and r4 is regulatory chain as shown in Figure 1.2) These interactions are lost after the enzyme expands to the R state, though the salt links of Glu239 to both Lys164 and Tyr165 change from interchain interactions to intrachain interactions. Therefore the allosteric transition involves both the global and local conformational changes, which contribute to breaking the interactions that stabilize the T state and replace with those stabilizing the R state.

Extensive studies on ATCase have proved that the allosteric mechanism of the enzyme can be explained by the MWC model. Recently Fetler \textit{et al.}\textsuperscript{30} were able to detect the pre-existence of the T to R equilibrium using a mutant ATCase. Furthermore, Macol \textit{et al.}\textsuperscript{31} provided direct structural evidence that the binding of
Figure 1.6. Allosteric transition of ATCase from the T state (top) to the R state (bottom). The structure of ATCase in the T state is drawn using data from PDB ID 1ZA1. The structure of ATCase•PALA complex in the R state is drawn using data from PDB ID 1D09.
PALA to just one of the six catalytic sites of ATCase was sufficient to bring the enzyme from the T to the R state, which demonstrated a concerted allosteric transition.

**Catalytic Mechanism and Homotropic Cooperativity for Aspartate**

The active sites are located at the interface between two neighboring catalytic chains in the same trimer. Details of the active site residues involved in substrates binding were revealed from the structural studies of enzyme with the bisubstrate transition state analog PALA bound (see Figure 1.7).33

The reaction catalyzed by ATCase proceeds through an ordered mechanism in which CP binds before Asp and the product CA leaves before Pi.34 X-ray crystallography has been used to obtain snapshots of enzyme at each step in the catalytic cycle to understand not only the catalysis but also the ordered binding and release on a molecular basis.32,35-37 When CP binds, the enzyme remains in the T state but local conformational changes occur which allow the binding of the second substrate Asp.32 The 80's loop from the adjacent catalytic chain moves toward the active site, which positions Ser80 and Lys84 for Asp binding. Along with the motions of 50's loop and 240's loop, these conformational changes create a positively charged binding pocket for Asp, which does not exist in the absence of CP. When Asp binds to the ATCase-CP complex, more conformational changes occur to make the active site more electropositive. The binding of Asp induces a significant reorientation of the 240’s loop which not only assists in the establishment of the high-affinity high-activity R-state active site, but also weakens the T-state stabilizing
**Figure 1.7.** Active site of ATCase with PALA bound. All residues involved in the active site are labeled and those from the adjacent catalytic chain are marked with asterick. This figure was redrawn with Pymol\textsuperscript{19} using data from PDB ID 1D09.\textsuperscript{33}
intersubunit interactions, promoting the quaternary conformational changes required for the enzyme to go to the R state.\textsuperscript{38} Thus the binding of Asp to one active site favors the conversion of the entire enzyme into the R state, resulting in the increased affinity and activity for the other active sites. The cooperativity induced by Asp binding reflects the sigmoidal kinetics of the ATCase reaction as a function of Asp concentration. The sigmoidal Asp saturation curve for ATCase is shown in Figure 1.8.

**Nucleotide Regulation of ATCase**

The nucleotide effectors (such as ATP, CTP and UTP) exhibit heterotropic regulation of ATCase by binding at the allosteric sites. CTP inhibits the enzyme while ATP shows the opposite effect, increasing the catalytic activity. UTP by itself has no effect on the enzyme activity, but in the presence of CTP, UTP causes a further decrease in enzyme activity than CTP alone.\textsuperscript{7} The nucleotide binding sites of ATCase are located in the AL domain of the regulatory chains near the N-terminus and the interface between two regulatory chains in the same dimer. Each active site is at least 60 Å away from where the nucleotides bind (see Figure 1.3). How can the nucleotide effectors influence the catalytic activity at a site 60 Å from the active site?

According to the MWC model, ATCase exists in equilibrium between the low activity T state and the high activity R state. In the absence of any ligand, the T state is favored by a factor of 250 or more.\textsuperscript{39} To explain the heterotropic effect of nucleotides, it has been suggested that the binding of nucleotides shift the equilibrium between the two states, thus changing the net enzyme activity.
Figure 1.8. Asp saturation curves (●) of ATCase in the absence of nucleotides at 25 °C. In the presence of 4 mM ATP (■) the curve shifts to the left as the R state is stabilized by ATP. In the presence of 2 mM CTP (▲) the curve shifts to the right as the T state is stabilized by CTP. All measurements were made in the 50 mM Tris-acetate buffer, pH 8.3. Specific activity is reported in mmoles of CA formed per hour per mg of ATCase.
CTP shifts the equilibrium towards the T state, increasing the [T]/[R] to 1250. The binding of CTP moves the sigmoidal Asp saturation curve to the right, resulting in more substrate needed to reach the same reaction rate in the absence of nucleotides. Conversely, allosteric activator ATP shifts the equilibrium towards the R state, decreasing the [T]/[R] to 70. The Asp saturation curve in the presence of ATP shifts to the left and shows less sigmoidal characteristic as ATP concentration increasing (See Figure 1.8). X-ray crystal structures of ATCase in the T and R states with ATP and CTP bound were solved by Lipscomb and coworkers. The binding of ATP to the T state was found to increase the vertical separation between the catalytic trimers by ~0.5 Å towards the R state, while CTP had the reverse effect of reducing the vertical separation by ~0.5 Å towards the T state.

Though ATP and CTP cause opposite effects, kinetic and binding studies suggest that ATP and CTP bind competitively to the same site. However the six allosteric sites on the enzyme do not all have the same affinity for ATP and CTP. The asymmetry binding of both nucleotides have been interpreted in terms of two distinct classes of binding sites: three high-affinity sites and three low-affinity sites, differing in affinity by about 20-fold. Furthermore, this asymmetry of binding is also observed for the isolated regulatory subunits. Besides CTP, UTP can also synergistically inhibit the enzyme in the presence of CTP, decreasing the catalytic activity lower than that by either nucleotide alone. To understand this synergistic effect, more binding studies have been performed for CTP and UTP. These results indicate that UTP binds to ATCase in the absence of CTP, but not inducing
any inhibition unless CTP is present. CTP enhances the affinity of UTP for the regulatory sites 80-fold, and UTP enhances CTP binding 5-fold. Though CTP binds to all six sites in the absence of UTP, only three high-affinity CTP binding sites are observed when UTP is present. Thus the low-affinity CTP binding sites have been suggested to be the UTP binding sites. This asymmetry binding of nucleotides may be due to the pre-existing structural asymmetry in the allosteric sites on the regulatory dimer which is revealed from the X-ray crystallography studies.

**Research Aspects**

Chapter 2 investigates the allosteric transition by time-resolved small-angle X-ray scattering (TR-SAXS) in the temperature range of 5-25°C. TR-SAXS patterns of ATCase in the presence of substrates (carbamoyl phosphate and L-aspartate) and bi-substrate analogues (N-phosphonacetyl-L-aspartate) are reported. The effects of allosteric effectors, ATP, CTP and UTP on the kinetics of the quaternary structure change of ATCase in the presence of substrates are investigated by TR-SAXS as well.

Chapter 3 uses X-ray crystallography and small-angle X-ray scattering in solution to examine the K164E/E239K ATCase, revealing this mutant exists in an intermediate quaternary structure between the canonical T and R structures. These data indicate that this mutant is not a model for the R state as has been proposed, but rather represents the enzyme trapped along the path of the allosteric transition between the T and R states.

Chapter 4 systematically studies the nucleotide regulation of ATCase in the absence and presence of Mg²⁺ as allosteric effectors, ATP, CTP and GTP, have shown
an altered allosteric effect on the enzyme when combined with Mg$^{2+}$. The effect of
UTP and UTP in the presence of CTP is reexamined with Mg$^{2+}$, since there is no data
regarding the influence of Mg$^{2+}$ either on the independent effect of UTP or its
synergistic inhibitory effect in the presence of CTP. Based on the results a new
model of nucleotide regulation is proposed.
Dihydroorotase

Brief Overview of Dihydroorotase

Dihydroorotase (EC 3.5.2.3, DHOase) is a zinc metalloenzyme that catalyzes the reversible cyclization of N-carbamoyl-L-aspartate (CA) to L-dihydroorotate (DHO) in the third step of de novo pyrimidine biosynthetic pathway (Figure 1.9). At lower pH, the reaction favors the biosynthetic direction to produce DHO. At higher pH, the formation of CA is favored. The equilibrium between CA and DHO reaches unity at pH 6.2.48

In prokaryotes, DHOase is a monofunctional homodimeric enzyme.49 In higher eukaryotes, DHOase is part of a trifunctional enzyme, carbamoyl phosphate synthetase-dihydroorotase-aspartate transcarbamylase (CAD), which catalyzes the first three reactions in the biosynthesis of pyrimidine nucleotides.50 An analysis of the amino acid sequences of DHOase from multiple species reveals two major classes of this enzyme thought to have arisen from ancestral gene duplication.51 Type I DHOases, found in higher organisms, are the most ancient and widely divergent form of the enzyme. There are three subgroups within the Type I DHOases: Type IA is monofunctional and independent (e.g. DHOase from Bacillus caldolyticus);52,53 Type IB only functions when associates non-covalently with one or more other enzymes in the pyrimidine pathway (e.g. DHOase from Aquifex aeolicus);54,55 Type IC is found as part of the multifunctional proteins (e.g. mammalian CAD). Type II DHOases, found in many bacteria and fungi, are a
Figure 1.9. The reversible cyclization of CA to DHO catalyzed by DHOase. The reaction is pH dependent. At lower pH, the forward reaction to form DHO is favored and at higher pH, the reverse reaction to form CA is favored.
more recent evolutionary development from significant changes in sequence. They are all monofunctional proteins, such as DHOase from *E. coli*. Type I DHOases have a larger size (~45 kDa) than the Type II enzymes (~38 kDa). Members of the same type of DHOase have a high-level amino acid sequence identity of more than 40%, while less than 20% sequence identity is observed for members between the two types.

Holm and Sander proposed that DHOase belongs to the amidohydrolase superfamily that comprises a diverse set of hydrolytic enzymes. Members of this superfamily were predicted to have a common structural core consisting of eight alternating β-sheet/α-helices (or TIM-barrel) and a signature pattern of four conserved histidine residues and one aspartate residue. There are two subsets within this superfamily: the first subset consists of enzymes with a binuclear metal center, where the two metal ions are bridged by a solvent-derived hydroxide and a carboxylated lysine (e.g. urease); the second subset consists of enzymes with a single metal ion at the active site in the absence of the carboxylated lysine (e.g. adenosine deaminase). DHOase was first thought to belong to the second subset because only one zinc atom was found at the active site. However the first structure of a DHOase, that from *E. coli*, solved to a resolution of 1.7 Å by Thoden *et al.*, (PDB ID 1J79) showed a binuclear zinc center at the active site, placing the *E. coli* enzyme in the first subset.
Structure of *E. coli* DHOase

*E. coli* DHOase was first purified by Washabaugh *et al.* The native molecular weight was determined to be 80,900 Da by equilibrium sedimentation while molecular weight of subunit was determined to be 38,400 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was thus a dimer. More than 15 years later the first structure of *E. coli* DHOase was solved and reported by Thoden *et al.*, which was also the first structure of any DHOase (PDB ID 1J79). As proposed by Holm and Sander, the architecture of the *E. coli* DHOase resembles the structure core of urease. As shown in Figure 1.10 each subunit of the protein folds into a TIM-barrel motif, with eight strands of parallel β-sheets flanked on the outer surface by α-helices.

The asymmetric unit contained a single homodimer. Though the enzyme was crystallized in the presence of racemic substrate D,L-CA, the structure showed the substrate CA bound at the active site of one subunit (subunit B) and the product DHO bound at the active site of the other (subunit A). No explanation for the unusual structure was given and the residues B109-B112 were omitted. Later, Lee *et al.* were able to resolve the conformations of residues 109-112 for both subunits by solving the structure of *E. coli* DHOase crystallized in the presence of DHO (PDB ID 1XGE). They found that the residues B109-B112 missing in the first structure comprised part of the 100’s loop (residues 105-115), which adopted different conformations for each subunit of the dimer. For the CA-bound subunit, this loop reached toward the active site and made direct contact with the substrate. For the
Figure 1.10. Structure of *E. coli* DHase. Each monomer of the dimer folds into the TIM barrel. The substrate CA and product DHO are shown in the active sites with two zinc atoms shown as magenta spheres. This figure was drawn with Pymol\textsuperscript{19} using data from PDB ID 1J79.\textsuperscript{58}
Figure 1.11. Superposition of the 100’s loop (residues 105-115) from subunit A (green) and subunit B (blue) of DHOase. In DHO-bound subunit A, the loop has the “out” conformation. In CA-bound subunit B, the loop has the “in” conformation. CA is shown with its carbon atoms in yellow. When loop reaches in toward the active site, Thr109 and Thr110 shown in thick lines have hydrogen-bonding interactions with CA. The zinc atoms in the active site are shown as magenta spheres. This figure was redrawn with Pymol\textsuperscript{19} using data from PDB ID 1XGE.\textsuperscript{59}
DHO-bound subunit, this loop moved away from the active site forming part of the protein surface (see Figure 1.11).

**Active Site of *E. coli* DHOase**

As a member of the amidohydrolase superfamily, the signature pattern of four histidine and one aspartate residues was observed in the active site. Though DHOase was originally proposed to belong to the second subset of this superfamily, in the structure of *E. coli* DHOase it clearly showed that the active sites of both subunits contained a binuclear zinc center, which suggested this enzyme belong to the first subset. This was also consistent with the observation that the carboxylated Lys102 served as a bridging ligand between the two metals. In the active site with DHO bound a water or hydroxide ion also served to bridge the two zinc ions. One of the zinc ions was tetrahedrally coordinated with His139, His177, Lys102, and the bridging hydroxide, while the other zinc ion was trigonal bipyramidal coordinated with His16, His18, Asp250, Lys102 and the bridging hydroxide. The binuclear zinc center was arranged in the similar fashion in the active site with CA bound except the replacement of the bridging hydroxide with the carboxylate oxygen of the substrate CA.

As can be seen in Figure 1.12 A, DHO is in close proximity to the binuclear metal center. Backbone atoms of Leu222 and Ala266 formed hydrogen bonds with DHO. The side chains of Arg20, Asn44 and His254 help to anchor the carboxylate group of DHO. CA also has interactions with these residues as shown in Figure 1.12
Figure 1.12. Active sites of *E. coli* DHOase. (A) The active site of subunit A. DHO is shown with its carbon atoms in yellow. The oxygen atom of a water molecule bridging the two zinc atoms is shown as red sphere. (B) The active site of subunit B. CA is shown with its carbon atoms in yellow. Only the side chains of Thr109 and Thr110 are shown as thin lines. The zinc atoms in both active sites are shown as magenta spheres. This figure was drawn with Pymol\textsuperscript{19} using data from PDB ID 1XGE.\textsuperscript{59}
B. In addition, Thr109 and Thr110, the tip of the 100’s loop hydrogen bond to both carboxylate groups of CA.59

Catalytic Mechanism

The detailed reaction mechanism of E. coli DHOase was first proposed based on the three-dimensional crystal structure of the enzyme in the presence of an equilibrium mixture of the reaction products.58 The proposed mechanism was improved later on the basis of an analysis of the effects of pH, metal substitution, solvent isotope effects, mutant proteins, and alternative substrates.60

Presented in Figure 1.13 is the mechanism of the reversible reaction catalyzed by DHOase involving the binuclear metal center and the proton transfer facilitator Asp250 in the active site.60 For the biosynthesis of DHO which is favored at lower pH, the substrate CA binds to the protonated form of the enzyme with the release of a bound water molecule, which results from the protonation of the bridging hydroxide associated with the binuclear metal center. The reaction is initiated by the abstraction of a proton from the amide nitrogen of CA by the carboxylate of Asp250 residue. Meanwhile the ring closure occurs with the nucleophilic attack on the carbon center of the carboxylate of CA by the same amide nitrogen to form the product DHO. The binuclear metal center is recharged with the bridging solvent hydroxide. In the reverse direction to form CA, the hydroxide bridging the two divalent cations is required for the catalysis. The polarization of the carbonyl oxygen of DHO through the interaction with the β-metal promotes the nucleophilic attack of the bridging hydroxide on the carbon center. Asp250 transfers
Figure 1.13. Proposed mechanism of the catalysis by *E. coli* DHOase. This figure was redrawn with modification of the scheme presented by Porter *et al.*[60]
the proton from the bridging hydroxide to the amide nitrogen via the formation of the tetrahedral intermediate. Formation of CA takes place when the tetrahedral intermediate collapsed with the cleavage of the carbon-nitrogen bond.

Research Aspects

Chapter 5 uses a virtual high-throughput screening (vHTS) system developed in our lab to identify inhibitors of DHOase, which may become lead compounds as potential drug candidates. In particular, we first screened about 3,000,000 compounds from the ZINC database using the docking program Surflex through this system. The top five hundred compounds were then docked and scored using 3 other docking tools: AutoDock3.0, DOCK5.0, and GOLD. An independent scoring program XScore was used to rank the docking poses from all 4 tools. Among the top fifty poses, five candidates were purchased and their inhibitory effects against *E. coli* DHOase were tested.

Chapter 6 uses unnatural amino acid mutagenesis to genetically incorporate a fluorescent amino acid into the 100’s loop (residue 105-115) of DHOase to investigate the role of loop motion for catalysis and cooperativity. 5-fluoroorotate, a known inhibitor of DHOase, was used to probe the fluorescence change upon loop movement. The inhibition curve of FOA against fluorescent DHOase was determined by colorimetric assay. The fluorescence response was measured upon the binding of FOA and examined with comparison to the inhibition curve.
References


Chapter 2: Time Evolution of the Quaternary Structure of
\textit{Escherichia coli} Aspartate Transcarbamoylase upon
Reaction with the Natural Substrates and a Slow Tight
Binding Inhibitor
Time Evolution of the Quaternary Structure of *Escherichia coli*

Aspartate Transcarbamoylase upon Reaction with the Natural Substrates and a Slow Tight Binding Inhibitor

An article published in Journal of Molecular Biology

by

Jay M. West, Jiarong Xia, Hiro Tsuruta, Wenyue Guo, Elizabeth M. O’Day and Evan R. Kantrowitz

*Contribution to work:* Purified wild-type ATCase holoenzyme and carried out the kinetic study of ATCase in the absence and presence of ethylene glycol, and at both 25° C and 5° C.
Introduction

Aspartate transcarbamoylase (ATCase) catalyzes one of the first steps in pyrimidine nucleotide biosynthesis, the reaction of carbamoyl phosphate (CP), with L-aspartate (Asp) to form N-carbamoyl-L-aspartate and inorganic phosphate (P\textsubscript{i}).\textsuperscript{1} In many prokaryotes such as Escherichia coli this reaction is the committed step in pyrimidine nucleotide biosynthesis. E. coli ATCase is composed of two types of subunits. The two larger or catalytic subunits are each composed of three identical polypeptide chains (M\textsubscript{r} 34,000), while the three smaller or regulatory subunits are each composed of two identical polypeptide chains (M\textsubscript{r} 17,000). Each of the six active sites is located at the interface between two adjacent catalytic chains, and side chains required for catalysis are recruited to the active site from both chains.\textsuperscript{2} The enzyme demonstrates homotropic cooperativity for the substrate Asp and is heterotropically regulated by the effectors ATP, CTP,\textsuperscript{3} and UTP in the presence of CTP.\textsuperscript{4}

The structures of the low-activity T state (in the absence of substrates)\textsuperscript{5,6} and high-activity R state (in the presence of substrates or substrate analogues such as N-phosphonacetyl-L-aspartate, PALA)\textsuperscript{7-9} have been determined by X-ray crystallography. A comparison of the T and R structures reveals that during the T → R transition, the two catalytic trimers increase their separation along the 3-fold axis by about 11 Å and rotate about 5° around the same axis, while the regulatory dimers rotate about 15° around their respective 2-fold axes.\textsuperscript{10} The 11 Å expansion of the
enzyme observed during the T → R transition is easily monitored by small-angle X-ray scattering (SAXS).\textsuperscript{11} Thus, the SAXS pattern is a sensitive and specific probe to study the quaternary conformational changes of the enzyme.

By using SAXS as a structural probe in stopped-flow experiments, the time-evolution of the quaternary conformational changes of ATCase have been monitored.\textsuperscript{12,13} These studies showed that the enzyme when mixed with substrates is very quickly converted from the T to the R state, the enzyme remains in the R state until substrates are exhausted, and then the enzyme reverts back to the T state. These early studies required integration of the signal over time intervals of 100 - 200 ms and averaging over many runs to improve the signal to noise ratio.\textsuperscript{12,13} Because of the relatively long time window for each point it was necessary to slow the reaction rate, which was done by performing the reaction at −5° C in a buffer containing 20% ethylene glycol.

Dreyfus \textit{et al.}\textsuperscript{14} showed that a variety of alcohols such as methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and 2-methyl-2-propanol have a significant influence on the activity and homotropic cooperativity of ATCase. For example, 20% methanol or ethanol reduced the activity of ATCase by approximately 90%. Additional studies with 15% ethanol showed a shift in the pH optimum of the reaction and an alteration in the Hill coefficient. Their interpretation of these solvent effects was that the cosolvent preferentially stabilized the T or R state of the enzyme depending upon the relative concentration and polarity of the cosolvent. For the simple alcohols, the primary effect was stabilization of the T state. Although
ethylene glycol was not investigated by Dreyfus et al., one would predict that ethylene glycol with its two hydroxyl groups would behave similarly to methanol and ethanol.

Here we reinvestigate the time-evolution of the quaternary conformational changes of ATCase in the absence of ethylene glycol. This was made possible by significant instrumental developments including a fast CCD X-ray detector and a high-flux X-ray beam via a multilayer monochromator, with an increase in beam brightness due to the update of the synchrotron storage ring at Stanford Synchrotron Radiation Laboratory (SSRL) to SPEAR3, a third generation source. These improvements allowed the collection of time-resolved SAXS data at a time resolution as short as 5 ms. Using this system we were able to study the quaternary conformational changes of ATCase in the temperature range of 5-22°C in the absence of ethylene glycol.
**Results**

**Kinetics of the ATCase Reaction In the Presence of Ethylene Glycol**

Since previous TR-SAXS experiments were performed in the presence of 20% ethylene glycol,\textsuperscript{12,13} and Dreyfus \textit{et al.}\textsuperscript{14} showed that a variety of alcohols at concentrations of 20% or less can dramatically alter the catalytic turnover rate and cooperativity of ATCase, kinetic assays were performed in the presence of ethylene glycol to determine if it had any influence on the ATCase reaction. As shown in Figure 2.1 (a), ethylene glycol dramatically reduced the activity of ATCase at 5° C. In the presence of 20% ethylene glycol the activity of the enzyme was reduced by 75%. In addition to reducing the activity of the enzyme, ethylene glycol had a small influence on homotropic cooperativity (data not shown). Ethylene glycol also has a significant influence on the ability of the heterotropic effectors to modulate enzyme activity. As shown in Figure 2.1 (b) at 5° C the presence of 20% ethylene glycol increased the activation of the enzyme by ATP, while reducing the inhibition by CTP. The maximal activation by ATP increased from 220% to 274% in the presence of 20% ethylene glycol. The residual activity at a saturating concentration of CTP was 29% as compared to 48% in the presence of ethylene glycol. The value of $K_{\text{ATP}}$ (K is the nucleotide concentration required at 50% maximal activation or inhibition of the enzyme) increased from 0.47 mM to 0.72 mM in the presence of 20% ethylene glycol. The value of $K_{\text{CTP}}$ increased from 7.8 µM to 14.5 µM in the presence of 20% ethylene glycol.
Figure 2.1. (a) The dependence of activity of ATCase on the concentration of ethylene glycol. All measurements were made at 5° C in the presence of 50 mM Tris, 2 mM DTT, pH 8.3. (b) Influence of the nucleotide effectors ATP and CTP on the activity of ATCase in the presence and absence of 20% ethylene glycol. Colorimetric assays were performed at 5° C in 50 mM Tris acetate buffer (pH 8.3) at a subsaturating concentration of L-Asp (2.4 mM) and saturating CP concentration (4.8 mM), in the presence of ATP and 20% ethylene glycol (v/v) (○), in the presence of ATP and in the absence of ethylene glycol (●), in the presence of CTP and 20% ethylene glycol (v/v) (□), in the presence of CTP and in the absence of ethylene glycol (v/v) (■).
The ability of UTP to act as a synergistic inhibitor of ATCase in the presence of CTP\textsuperscript{4} was also tested at 5° C in the absence and presence of 20% ethylene glycol. In the absence of ethylene glycol the combination of 4 mM CTP and 4 mM UTP (CTP/UTP) yielded a residual activity of the enzyme of 22%, whereas in the presence of ethylene glycol the combination of CTP/UTP yielded a residual activity of the enzyme of 28%.

**Kinetics of the ATCase Reaction at 5° C**

Because the TR-SAXS experiments reported here were performed at temperatures as low as 5° C, the kinetic properties of ATCase were fully characterized at 5° C in the same buffers used for the TR-SAXS experiments (Figure 2.2). As shown in Table 2.1, many aspects of catalysis at 25° C are not only quantitatively different at 5° C, but in some cases qualitatively different. As would be expected, the maximal observed velocity was reduced by nearly 5-fold. This reduction in velocity was accompanied by an almost 3-fold decrease in the concentration of Asp required for half-maximal activity ([Asp]\textsubscript{0.5}), and a decrease in the Hill coefficient (n\textsubscript{H}) from 2.6 to 2.0. The observed changes in [Asp]\textsubscript{0.5} and n\textsubscript{H} are similar to the changes observed when Asp saturating curves are determined in the presence of ATP, suggesting that, like ATP, lower temperatures shift the equilibrium towards the R state. This phenomenon was demonstrated in a previous SAXS study by the shift in the structural equilibrium of the mutant D236A enzyme in the direction of the R state with decreasing temperature. At 5° C the [Asp]\textsubscript{0.5} decreased nearly 2-fold in the presence of ATP; increased 20% in the presence of UTP;
Table 2.1. Kinetic parameters of ATCase with nucleotide effectors at 25 and 5 °C

<table>
<thead>
<tr>
<th>Conditions</th>
<th>(V_{\text{max}}) (^b)</th>
<th>(\text{[Asp]}_{0.5}) (^c)</th>
<th>(n_{H}) (^d)</th>
<th>(V_{\text{max}}) (^b)</th>
<th>(\text{[Asp]}_{0.5}) (^c)</th>
<th>(n_{H}) (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>5 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No NTPs</td>
<td>19.1 ± 0.3</td>
<td>12.7</td>
<td>2.6 ± 0.2</td>
<td>4.2 ± 0.5</td>
<td>5.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>5mM ATP</td>
<td>19.8 ± 0.7</td>
<td>6.1</td>
<td>1.4 ± 0.1</td>
<td>5.4 ± 0.2</td>
<td>2.9</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>4mM CTP</td>
<td>17.9 ± 0.9</td>
<td>21.2</td>
<td>2.4 ± 0.1</td>
<td>3.6 ± 0.2</td>
<td>11.3</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>4mM UTP</td>
<td>18.4 ± 0.2</td>
<td>13.5</td>
<td>2.3 ± 0.1</td>
<td>4.1 ± 0.6</td>
<td>5.9</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>4mM</td>
<td>17.3 ± 0.4</td>
<td>28.6</td>
<td>2.9 ± 0.1</td>
<td>3.0 ± 0.4</td>
<td>12.5</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>CTP/UTP</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\(^a\) All experiments were performed in 50 mM Tris-acetate buffer (pH 8.3) at a saturating CP concentration (4.8 mM) 

\(^b\) The maximal observed velocity (mmol•h⁻¹•mg⁻¹) 

\(^c\) The observed concentration of L-Asp (mM) which produces one-half the observed maximal velocity 

\(^d\) Hill coefficient
Figure 2.2. L-Asp saturation kinetics in the absence and presence of the nucleotide effectors at (a) 5° C and (b) 25° C. Reactions were carried out in 50 mM Tris buffer, pH 8.3. The kinetic curves were determined in the presence of 5 mM ATP (Δ), 4 mM CTP (□), 4 mM UTP (■), 4 mM CTP and 4 mM UTP (▲) as compared to the saturation kinetics in the absence of nucleotide (○). Specific activity is reported in units of mmoles of carbamoyl aspartate formed per mg per hr.
increased over 2-fold in the presence of CTP; and increased by a factor of 2.5 in the presence of CTP/UTP. A similar trend was also observed at 25° C. The $[\text{Asp}]_{0.5}$ decreased over 2-fold in the presence of ATP; increased 10% in the presence of UTP; increased nearly 2-fold in the presence of CTP; and increased by 2.3-fold in the presence of CTP/UTP.

The kinetic data at 5° C was plotted as $v/V_{\text{max}}$ vs. $[\text{Asp}]$ as shown in Figure 2.3 and fitted by the theoretical curve of $\bar{Y}_{\text{Asp}}$ (fraction of active sites occupied by Asp) vs. $\alpha K_R(\text{Asp})$, which is derived from the equations of the theoretical two-state allosteric transition model proposed by Monod et al.$^{17,18}$

$$\bar{Y}_{\text{Asp}} = \frac{\alpha (1 + \alpha)^{n-1} + L' \alpha c_{\text{Asp}} (1 + \alpha c_{\text{Asp}})^n}{(1 + \alpha)^n + L' (1 + \alpha c_{\text{Asp}})^n}$$

where $\alpha = [\text{Asp}] / K_R(\text{Asp})$, $L'$ is the allosteric equilibrium constant in the presence of saturating CP, and $c_{\text{Asp}} = K_R(\text{Asp}) / K_T(\text{Asp})$. $K_T(\text{Asp})$ and $K_R(\text{Asp})$ are the dissociation constants of Asp for the T and R allosteric states, respectively. A value of $L' = 10$ was obtained from the static SAXS data shown in Figure 2.4, according to the methods of Tsuruta et al.$^{13}$ The starting point used for $K_R(\text{Asp})$ was the $[\text{Asp}]_{0.5} = 5.1$ mM value obtained from the kinetic data. After obtaining a suitable fit for the data the values of $K_R(\text{Asp}) = 3.5 \pm 0.5$ mM and $K_T(\text{Asp}) = 40 \pm 10$ mM were derived.

**Time-Resolved X-ray Scattering: Effect of Substrates and Substrate Analogues**

TR-SAXS was used to monitor the quaternary conformational changes that ATCase undergoes when the enzyme is mixed with substrates. Using the high beam flux obtained by the multilayer monochromator and fast CCD detector at SSRL,
Figure 2.3. L-Asp saturation kinetics at 5° C fitted by a theoretical curve calculated from a modified Monod et al. equation and using parameters listed in the text. Reactions were carried out in 50 mM Tris buffer, pH 8.3.
Figure 2.4. Steady-state SAXS patterns of ATCase without ligands (○), with 50mM CP (●), and with 5mM PALA (□). The allosteric equilibrium constant was calculated from these curves as $L' = 10$. 
practical time resolution of the TR-SAXS data collection was improved from 100 - 200 ms\textsuperscript{19} to as low as 5 ms in a single mixing event. However, the rate constants for the T → R transition presented here were obtained from 19 ms collection rate data, because at this rate the signal to noise ratio was significantly better and fast enough to record much of the transition. This improvement in detection allowed reactions to be monitored in the temperature range between 5° C and 22° C, rather than at −5 °C as previously reported.\textsuperscript{19}

Shown in Figure 2.5 (a) are a series of SAXS patterns recorded upon mixing 1.5 mM ATCase (in active sites) plus 50 mM CP in one syringe with 100 mM Asp plus 50 mM CP in the second syringe at 5° C. Immediately after mixing the enzyme concentration was 0.75 mM in active sites (37.5 mg/ml), while the CP and Asp concentrations were each 50 mM. The SAXS patterns shown in Figure 2.5 (a) are at 38 ms, 380 ms, and 3800 ms. The scattering pattern at 38 ms does not correspond to either the T or R states as the enzyme population is in the process of undergoing the quaternary conformational change. The pattern at 380 ms is essentially identical to the curve of the enzyme in the presence of PALA (R state), while the curve at 3800 ms is essentially the same as that observed in the presence of D-Asp and CP (T state). To determine if the curve at 38 ms corresponded to the formation of a transient intermediate on the pathway between the T and R states, a curve was generated from a sum of fractions of the T (33%) and R (67%) state curves corresponding to a value of $L = 0.5$.\textsuperscript{13} This generated curve is a near match to the X-ray scattering curve recorded at $t = 38$ ms after mixing the enzyme with substrates.
Figure 2.5. (a) A time series of SAXS patterns of 0.75 mM ATCase (in active sites) mixed with 50 mM substrates (CP and L-Asp), and 50 mM CP plus 50 mM D-Asp as a T state control, at 5° C. The SAXS patterns of the enzyme with the substrates are shown for 38 ms (O), 380 ms (□), and 3800 ms (●) after mixing. The SAXS pattern of the enzyme with CP and D-asp is shown for 3800 ms (Δ, short dashed curve) after mixing. The long dashed curve (■) is calculated for an L = 0.5, from the sum of 0.33 x (T state curve) and 0.67 x (R state curve). (b) Time-courses of the quaternary structure change after mixing with substrates (solid line) and 50 mM CP plus 50 mM D-Asp (short dashed line), as monitored by the scattering intensity integrated over the s-range 0.085-0.152 Å⁻¹. Inset: First 300 ms of the structural change after mixing with substrates shown along with the curve fit (two exponential) to the data.
In order to help visualize the time course of structural change, the area under the curves between \( s = 0.085 \ \text{Å}^{-1} \) and \( s = 0.152 \ \text{Å}^{-1} \) was integrated and plotted as a function of time. This integration converts observed scattering intensity to relative concentration of the enzyme species on the basis that solution scattering intensity reflects relative concentration of each species linearly, in the absence of oligomeric state changes. Figure 2.5 (b) shows the time-dependent change in the integrated intensity of the SAXS pattern for this experiment. At \( t \approx 5 - 10 \text{ ms} \), \( t = 0 \) as shown on the plot. The dead time of the stopped-flow mixer is approximately 5 - 10 ms) the enzyme population is nearly a equal mixture of T and R state molecules. Between \( t = 100 \text{ ms} \) and \( t = 1500 \text{ ms} \) 95% of the enzyme population is in the R-state as the enzyme catalyzes the reaction converting Asp and CP into carbamoyl aspartate and \( P_i \). After \( t = 1500 \text{ ms} \) the enzyme population is returning to the T state as the substrates are depleted, and after 3000 ms virtually the entire enzyme population is back in the T state. At \( 5^\circ C \) the turnover rate at maximal velocity of the holoenzyme is \( 350 \pm 40 \text{ s}^{-1} \). At a substrate concentration of 50 mM and an active site concentration of 0.75 mM the substrate:holoenzyme ratio is 400, so it should take 1 - 1.3 seconds to consume the substrate. The R-state plateau, defined here as the region of >95% of the peak amplitude of the integrated scattering curve, has a total duration of 1.4 seconds, which demonstrates that enzyme quickly reverts to the T state after depleting the substrates.

When ATCase is mixed with D-Asp and CP, (final concentrations 0.75 mM active sites, \( [\text{D-Asp}] = [\text{CP}] = 50 \text{ mM} \)) the TR-SAXS curve showed virtually no change
**Figure 2.6.** Time-course of the quaternary structure change of 0.75 mM ATCase (in active sites) as monitored by the scattering intensity integrated over the $s$-range 0.085-0.152 Å$^{-1}$. The final substrate or substrate analog concentrations after mixing were $[CP] = [L$-Asp] = 50 mM (O), $[CP] = 50$ mM and $[PALA] = 5$ mM (●), and $[CP] = [D$-Asp] = 50 mM (□). Data displayed are from a 36 ms collection rate. Inset: First 1000 ms of the structural change after mixing with substrates or substrate analogs. Data displayed are from a 19 ms collection rate.
(see Figures 2.5 and 2.6). The use of D-Asp thus provides a control for the L-Asp experiments with a compound with equal scattering potential or electron density, and as a control for the T-state scattering curve as previously described. The integrated intensity at the end of experiment when L-Asp and CP are mixed was practically identical to that observed when D-Asp and CP are mixed with enzyme. Because the TR-SAXS curve returns to the level observed in the presence of D-Asp and CP, it is clear that virtually the entire enzyme population has reverted back to the T state after the substrates have been depleted.

The initial time-course of the structural change after combining the enzyme with substrates or substrate analogs appeared to fit to either a single or double exponential depending upon the experimental conditions. Therefore each set of data was fit to both exponential fits and the number of rate constants (represented as $k_{T \rightarrow R(1)}$ for the first or fast phase and $k_{T \rightarrow R(2)}$ for the second or slow phase, where applicable) reported reflects which fit was superior. As demonstrated by the rate constant data for the quaternary structural change in Table 2.2, increasing the substrate concentration from 25 mM to 50 mM and doubling the enzyme concentration increased the rate of the fast phase of the transition from 18.3 s$^{-1}$ to 51 s$^{-1}$. In this experiment it was necessary to use a lower enzyme concentration at the lower substrate concentration in order to observe the full T to R conversion of the enzyme population before significant depletion of the substrates. Lowering the enzyme concentration by one half, to 18 mg/ml, and half again to, 9 mg/ml, while keeping the substrate concentration at 50 mM did not change the rate of the fast or
Table 2.2. Kinetic parameters for the T to R allosteric transition of ATCase at 5 °C

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$k_{T\rightarrow R1}$ (s$^{-1}$)</th>
<th>$k_{T\rightarrow R2}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM substrates$^d$</td>
<td>18.3 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>50 mM substrates</td>
<td>51 ± 4</td>
<td>7.6 ± 1</td>
</tr>
<tr>
<td>100 mM substrates</td>
<td>51 ± 5</td>
<td>6.3 ± 0.9</td>
</tr>
<tr>
<td>50 mM substrates + 5 mM ATP</td>
<td>89 ± 20</td>
<td>7.9 ± 1</td>
</tr>
<tr>
<td>50 mM substrates + 4 mM UTP</td>
<td>52 ± 8</td>
<td>14.8 ± 1.8</td>
</tr>
<tr>
<td>50 mM substrates + 4 mM CTP</td>
<td>12 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>50 mM substrates + 4 mM CTP / 4 mM UTP</td>
<td>10.3 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>50 mM succinate + 50 mM CP</td>
<td>38 ± 1</td>
<td></td>
</tr>
<tr>
<td>5 mM PALA + 50 mM CP</td>
<td>1.53 ± 0.19</td>
<td>0.31 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$ These experiments were performed in 50 mM Tris acetate buffer (pH 8.3), final enzyme concentration of 37.5 mg/ml after mixing, equal concentrations of substrates ([L-Asp] = [CP]) unless otherwise stated, and data points recorded at 19ms intervals

$^b$ Rate constant of fast phase or first phase of two-exponential fit

$^c$ Rate constant of slow phase or second phase of two-exponential fit, where applicable

$^d$ Final enzyme concentration used was 18.75 mg/ml because at this low substrate concentration (and a relatively high enzyme concentration) the substrates were exhausted before the enzyme fully attained the R state
slow phase, within error, of the T → R transition (data not shown). Therefore an 
enzyme concentration of 37.5 mg/ml was used for all experiments, with the 
previous exception, for a superior signal to noise ratio for the scattering data. 
Increasing the substrate concentration further to 100 mM while maintaining the 
same enzyme concentration (37.5 mg/ml) did not change the rate of transition for 
the fast or slow phases, as might be expected since this concentration was much 
higher than the [Asp]₀.₅ value of 5.1 mM.

In order to establish the value for the integrated intensity and scattering 
pattern of the R-state structure of ATCase in a TR-SAXS experiment, PALA was 
mixed with enzyme and CP (final concentrations 0.75 mM active sites, 5 mM PALA, 
50 mM CP). As seen in Figure 2.6, the integrated intensity observed immediately 
after mixing was shifted towards the R-state value. The rate constants for the fast 
and slow phases after mixing with PALA were both more than an order of 
magnitude slower than the corresponding T → R transition rate constants observed 
in the presence of a saturating concentration of the natural substrates, L-Asp and CP. 
The single fast phase rate constant for the structural change with 50 mM succinate 
and 50 mM CP was 38 s⁻¹, similar to the rate of the fast phase with the natural 
substrates at a saturating concentration.

**Effect of Nucleotides**

The time-course of the quaternary structural change in the presence of ATP 
is shown in Figure 2.7 and is similar to that observed in the absence of nucleotides. 
However, in the presence of ATP, the duration that the enzyme remains in the R-
Figure 2.7. Time evolution of the quaternary structure change of 0.75 mM ATCase (in active sites) as monitored by the scattering intensity integrated over the s-range 0.085-0.152 Å⁻¹ after mixing with 50 mM substrates (Asp and CP) and without nucleotides (○) and with [ATP] = 5 mM (●). Inset: First 300 ms of the structural change after mixing with substrates shown along with the curve fits (two exponential) to the data.
state plateau is shorter than in the absence of nucleotides and the $k_{T \rightarrow R(1)}$ increased from 51 $s^{-1}$ to 89 $s^{-1}$. The time-course of the structural change for ATCase in the presence of the nucleotide inhibitors are shown in Figure 2.8. The rate of $T \rightarrow R$ transition in the presence CTP and CTP/UTP decreased significantly, to 12 $s^{-1}$ and 10.3 $s^{-1}$ respectively. The integrated scattering intensity of the R-state plateau in the presence of CTP and CTP/UTP is lower than that observed in the absence of nucleotides, and the duration of the plateau in the presence of CTP and CTP/UTP is much shorter than in the absence of nucleotides.

The results with UTP alone were unexpected. The rate of the fast phase of the $T \rightarrow R$ transition in the presence of UTP was identical to that in the absence of nucleotides. The integrated scattering intensity of the R-state plateau in the presence of UTP is also the same as in the absence of nucleotides. However, the duration of the R-state plateau phase in the presence of UTP is only half of that observed in the absence of nucleotides, suggesting a slight destabilization of the R state by UTP.

**Determination of the Activation Energy of ATCase**

The kinetics of the quaternary structural change were observed at a series of temperatures between 5° C and 22° C in order to calculate the activation energy of the quaternary conformational changes of ATCase both from the T to the R state and from the R to the T state. Unfortunately, above 10° C the $T \rightarrow R$ transition rate is so fast that our instrumentation was unable to follow it. However, the rate constants for the $R_0 \rightarrow T_0$ (unliganded states) transition after the substrates were exhausted
**Figure 2.8.** Time evolution of the quaternary structure change of 0.75 mM ATCase (in active sites) as monitored by the scattering intensity integrated over the s-range 0.085-0.152 Å⁻¹ after mixing with 50 mM substrates (Asp and CP) and without nucleotides (○), with [CTP] = 4 mM (◊), with [UTP] = 4 mM (□, dashed curve), and with [CTP] = [UTP] = 4 mM (■, dashed curve). Inset: First 500 ms of the structural change shown after mixing with substrates along with the curve fits to the data for without nucleotides (○, two exponential) and with CTP and UTP (■, one exponential).
Figure 2.9. Arrhenius plot of the temperature dependence of the R $\rightarrow$ T transition.

The rate constants for the R to T transition ($k_{R\rightarrow T}$) at 5, 10, 16, and 22 °C were 2.08 ± 0.03, 3.7 ± 0.1, 5.1 ± 0.1, and 8.3 ± 0.1 s$^{-1}$, respectively.
were obtained by fitting the lower half of the return phase to a single exponential fit. The rate constants for the $R_0 \rightarrow T_0$ transition ($k_{R \rightarrow T}$) at 5, 10, 16, and 22° C were 2.08 ± 0.03, 3.7 ± 0.1, 5.1 ± 0.1, and 8.3 ± 0.1 s$^{-1}$ respectively. An Arhennius plot of these data is shown in Figure 2.9, which yielded an activation energy for the $R_0 \rightarrow T_0$ transition of 13.0 ± 0.4 kcal/mol.
Discussion

TR-SAXS experiments investigating the time-evolution of the quaternary structural change of ATCase induced by the binding of the natural substrates CP and L-Asp were performed here in the absence of ethylene glycol. In previous studies\(^{19}\) 20% ethylene glycol was added to all solutions to allow the experiment to be performed at \(-5^\circ\) C. Here we demonstrate that ethylene glycol dramatically alters the homotropic and heterotropic kinetics of the enzyme, as is the case with many other alcohols.\(^{14}\) Therefore these new studies were important not only in monitoring the quaternary structural change at temperatures closer to physiological, but also because ethylene glycol was eliminated from the reaction.

In order to better correlate the time-resolved structural results reported here at \(5^\circ\) C to the functional characteristics of ATCase at this temperature, a complete kinetic characterization of the enzyme was performed at \(5^\circ\) C. As would be expected, the maximal velocity of the enzyme at \(5^\circ\) C in the absence and presence of the nucleotide effectors was reduced nearly five-fold as compared to the maximal velocity at \(25^\circ\) C. The \([\text{Asp}]_{0.5}\) at \(5^\circ\) C in the absence of nucleotides was also reduced nearly 3-fold, with a concomitant reduction in the cooperativity for Asp (see Table 2.1). Similarly in the presence of nucleotides at \(5^\circ\) C, the \([\text{Asp}]_{0.5}\) was reduced but with no change in cooperativity, except when CTP was present, where the cooperativity increased. The results of these kinetic experiments suggest that the structural results obtained from the TR-SAXS experiments performed at \(5^\circ\) C should
strongly correlate with the structure and function of the enzyme at higher temperatures.

In agreement with previous results,\textsuperscript{19} when ATCase is mixed with its natural substrates, CP and L-Asp, there is a rapid structural transition of the enzyme from the T to the R state. The preponderance of the enzyme population remains in the R state as the enzyme reacts with the substrates, and then reverts to the T state when the substrates are exhausted (see Figure 2.5 (b)). This clearly demonstrates that the allosteric transition is not the rate-limiting step in catalysis, as has been previously suggested.\textsuperscript{20} Therefore, under conditions of saturating substrates the enzyme remains in the R-quaternary structure until the substrates are essentially exhausted and then reverts to the T-quaternary structure.

As shown in the inset to Figure 2.5 (b), the T → R structural transition upon addition of the natural substrates to the enzyme appears to be a biphasic exponential process, with a fast and slow phase. In this particular case the fast phase accounts for approximately 75\% of the total amplitude of the curve, representing the change in integrated intensity between the mixture of T and R states at the first recorded time point and R state at the curve plateau. Considering the evidence against the formation of a structural intermediate as demonstrated in Figure 2.5 (a), along with the structural transition being a single-phase exponential process in the presence of allosteric inhibitors, as shown in Figure 2.8, we determined that the curve monitoring the structural change may represent a composite of T-state species with different ligation states, each with its own
particular rate of transition to the R state, as has been previously suggested in studies of the rate of the structural change in aspartate transcarbamoylase.\textsuperscript{13,21} In the case of the allosteric protein hemoglobin, it is well documented that not only different ligation states but also configurational isomers, or asymmetric ligation states, exhibit a wide range of structural transition rates that originate from different activation energies for the structural change.\textsuperscript{22,23} When all the CP binding sites of T-state ATCase are saturated, as they are under our experimental conditions, there are thirteen possible species or ligation states with aspartate bound in the six binding sites when taking into account the T-state interactions between the C1 and C4 chains on opposing catalytic trimers. Therefore the two rates we observe are the composites of up to thirteen or more individual rates for the T → R transition when accounting for these configurational isomers. The observed rate of the T → R transition increases about 3-fold when doubling the aspartate concentration from 25 mM, a concentration well in excess of the $K_{R(Asp)}$ value of 3.5 ± 0.5 mM, to 50 mM, while also doubling the enzyme concentration. Therefore, we are confident that the structural transition is triggered by aspartate binding to the T state, and is not a simple shift in the preexisting equilibrium between the T and R states towards the R state caused by aspartate binding only to that state and “locking” it into that state. If the structural transition were simply a population shift to the R state, then the rate would not change or change very little when increasing the aspartate concentration from 25 mM to 50 mM as both concentrations are well in excess of $K_{R(Asp)}$. This can be accounted for using a kinetic version of the two-state model, which would
suggest 14 individual rates for the allostERIC transition, defining the T to R
equilibrium constants at the 7 ligation states.\textsuperscript{24} In order to approximate the average
number of aspartate molecules bound to the T state during the structural transition,
we used the equation derived for the two-state allostERIC model of Monod \textit{et al.},
simplified by assuming a saturating concentration of CP in the manner previously
described to fit our aspartate saturation data at 5° C.\textsuperscript{17,18} After obtaining a
reasonable fit of the data and extracting the appropriate parameters, we obtained a
\( K_{T(Asp)} \) value of 40 ± 10 mM. With an active site concentration of 0.75 mM and an
aspartate concentration of 25 mM an average of 33 - 46% of the active sites are
ligated with aspartate (2 - 2.5 per holoenzyme). Similarly, at an aspartate
concentration of 50 mM an average of 50 - 67% of the active sites are ligated with
aspartate (3 - 4 per holoenzyme) and at an aspartate concentration of 100 mM an
average of 67 - 77% of the active sites are ligated with aspartate (4 - 4.6 per
holoenzyme). These observations compare favorably with the previous SAXS
studies of Fetler \textit{et al.}\textsuperscript{25} that two PALA molecules per ATCase holoenzyme molecule
are necessary to shift the T to R equilibrium in favor of the R state, and four PALA
molecules are necessary to shift the entire enzyme population to the R state. Macol
\textit{et al.}\textsuperscript{26} demonstrated that the binding of one PALA molecule could shift the entire
holoenzyme population to the R state, however, the holoenzyme was comprised of
five chains with the R105A mutation. In either case the enzyme clearly does not
need to be saturated with substrate analogs or presumably substrates, in order to
shift the equilibrium towards the R state. Our findings suggest that the faster
observed rate is the composite of rates of the structural transition for the highly liganded species, with three or more active sites filled. Likewise, the slower observed rate may be the composite of structural transition rates of the least liganded species, with two or fewer active sites filled. At an aspartate concentration of 25 mM we observe only one exponential fit to the data, which according to our model would be the composites of rates of the least liganded species along with the rates of the highly liganded species containing three or more aspartate present as a small fraction of the mixed population. Increasing the aspartate concentration from 50 mM to 100 mM yielded identical rates for both observed phases, suggesting that once three or more aspartate molecules are bound to the holoenzyme the rate for the structural transition is near a maximum, or that highly liganded T-state molecules bound with four or more aspartate are present as only a small fraction of the population even at high aspartate concentrations. Further evidence for this model is provided by comparison of the ratio of the fast phase amplitude to the slow phase amplitude at 50 mM aspartate and 100 mM aspartate concentrations; at the higher aspartate concentration the ratio is higher, suggesting a shift in population towards the highly liganded molecules.

There are some substantial qualitative differences between these TR-SAXS results and the data obtained previously in the presence of ethylene glycol, such as the time-course of the TR-SAXS pattern of the enzyme in the presence of ATP, shown in Figure 2.7. We observed that the R-state plateau region in the presence of ATP, during which most of the substrates are being converted to products, is shorter than
in the absence of ATP. This was not unexpected since ATP, an activator of the enzyme, at 5° C increased the \( V_{\text{max}} \) value from 4.2 mmol\( \cdot \)h\(^{-1}\)\cdot mg\(^{-1}\) to 5.4 mmol\( \cdot \)h\(^{-1}\)\cdot mg\(^{-1}\) (Table 2.1) and also the apparent binding affinity of L-Asp, as the \([\text{Asp}]_{0.5}\) value decreased from 5.1 mM to 2.9 mM. However, in the presence of 20% ethylene glycol, a longer R-state plateau was observed in the presence of ATP than in its absence.\(^\text{19}\) To explain this it was proposed that ATP, in addition to being an activator may possibly become an inhibitor by “increasing the chance of making L-Asp bind to the active site before \( P_i \) leaves and locking the active site into an unproductive cycle with no alteration of the quaternary structure”.\(^\text{19}\) In that experiment the activity of enzyme was dramatically reduced not only by the presence of 20% ethylene glycol, which as demonstrated in Figure 2.1(a) reduces the activity by 75%, but also by performing the TR-SAXS at -5° C. The similar phenomenon of “substrate inhibition” has been observed for the isolated catalytic subunit and for the incomplete complexes \( C_6R_4 \) and \( C_3R_6 \), as well as for the holoenzyme \( C_6R_6 \),\(^\text{27-29}\) where a high aspartate concentration ostensibly acts as an inhibitor by binding to the CP binding site.

As detailed in Table 2.2, ATP increased the rate of the \( T \rightarrow R \) transition, specifically the rate of the fast phase, by approximately 75%. It should be noted that in the presence of ATP the allosteric transition is so rapid that with the current instrumentation the experimental error is somewhat large. As discussed previously, saturating the enzyme with aspartate beyond a concentration of 50 mM did not increase the rate of the structural transition. Therefore one possible interpretation
is that ATP increases the on rate of aspartate to the T state, suggesting that aspartate binding is a rate-limiting step in the allosteric transition. However, a more elegant explanation, which is in accord with the data from the inhibitors CTP and CTP/UTP and numerous studies on the effects of the heterotropic nucleotides, can be derived using some aspects of the two-state model of Monod et al. At pH 7.0 Howlett et al. observed that ATP increases the stability of the R state relative to the T state by 0.8 kcal and reduces the T to R equilibrium constant from 250 to 7 in the absence of substrates. The R to T transition rate with ATP was reduced by only approximately 15% (Figure 2.7), suggesting a slight stabilization of the R state, so therefore the large increase in the T → R rate would suggest a significant T state destabilization by ATP. Here the two-state model suggests that ATP binds more tightly to the R state and therefore shifts the structural equilibrium in favor of the R state. However, our observation of a possibly different mechanism for ATP was suggested by the crystal structures of the T and R states of the enzyme in the presence of ATP; the T state structure showed a slight shift towards the R state with ATP present, whereas globally the R state structure was unchanged in the presence of ATP. It should be noted that under these conditions with ATP the V_max did increase even though the structural state at saturating substrate levels appears to be the same R state as with no ATP present, with the caveat that the V_max value is difficult to obtain precisely from steady-state kinetics curves which exhibit substrate inhibition. This suggests that the previously observed increase in the Asp to CA exchange rate with ATP may influence the catalytic mechanism to a modest
degree. However our time-resolved structural study indicates that perturbation of T to R equilibrium specifically via T-state destabilization may be the most significant mechanism of ATP activation of ATCase.

The time-courses of the quaternary structural change in the presence of CTP or CTP/UTP are similar in the presence and absence of ethylene glycol (see Figure 2.8). For both cases, in the presence of CTP or CTP/UTP the duration of the R-state shifted plateau is shorter and the integrated intensity of the plateau is lower than in the absence of nucleotides, and the observed return to the T-state quaternary structure is a slow exponential decline as the remaining substrates are consumed as the enzyme population shifts towards the T state. The integrated intensity of the peak with CTP present and with CTP/UTP present was approximately 80% and 75% respectively of that when no nucleotides were present, indicative that the percent of high-activity R-state molecules at a saturating substrate concentration was reduced to a likewise value. This is in excellent agreement with the enzymatic kinetic data, in which the $V_{\text{max}}$ was, within error, reduced by the same amount as the integrated scattering peak in the presence of CTP and CTP/UTP as compared to when no nucleotides were present. These allosteric inhibitors also reduced the rate of the structural transition by over 4-fold. Similar to the condition when no nucleotides were present and the substrate concentration was 25 mM, the initial time-course of the structural change fits to a single exponential. This suggests that the least liganded species have a rate that is so reduced that we do not observe them before the shortened plateau starts its reversion towards the T state, or that the $R \rightarrow T$ rate
for these species is nearly equal to the T → R transition rate. As with the case of ATP, our experimental observations are not in complete agreement with some of the tenets of the two-state model; the much reduced T → R transition rate suggests the nucleotide inhibitors stabilize the T state, and the incomplete conversion of the enzyme population to the R state at a saturating substrate concentration and shift in equilibrium towards the T state long before the substrates are exhausted caused by the collective R → T rates becoming greater than the T → R rates suggest that they destabilize the R state. Using the two-state model as a theoretical framework, Howlett et al.\textsuperscript{17,31} observed that CTP increases the stability of the T state relative to the R state by 0.9 kcal and increases the T to R equilibrium constant from 250 to 1250 in the absence of substrates. In addition, the two-state model suggests that CTP (and UTP) exert their influence by binding more tightly to the T state and shifting the structural equilibrium in favor of the T state. However, again as with ATP our observations are somewhat at variance with this model, but are supported by the crystal structures of the T and R states of the enzyme in the presence of CTP; the T state structure globally was unchanged in the presence of CTP,\textsuperscript{5} and R state structure was shifted slightly in the direction of the T state with CTP present.\textsuperscript{33}

In the case of UTP, by itself it had no influence on the kinetics of the enzyme with the exception of raising the [Asp]\textsubscript{0.5} slightly. It also had no influence on the kinetics of the T → R transition, except for increasing the rate of the slow phase for reasons that are unclear. However, UTP did have a noticeable effect on the length of time the enzyme spent in the R-state plateau region, causing the enzyme population
to begin reverting to the T state before the substrates were exhausted. This indicates that UTP may slightly destabilize the R state, but otherwise does not have an appreciable effect on the allosteric behavior of ATCase under these conditions.

The activation energy of the R → T transition was determined by fitting the bottom half of the return curve, after the enzyme had exhausted the substrates, to a single exponential rate, as opposed to the upper half of the curve where presumably not all enzyme molecules have completely exhausted the substrate bound to the active sites. By fitting the bottom half of the return curve mainly the rate of $R_0 \rightarrow T_0$ should be observed, which is supported by the very low error in the $k_{R \rightarrow T}$ values obtained. As shown in Figure 2.9, the $E_{aR \rightarrow T}$ was calculated to be $13.0 \pm 1.4$ kcal/mol from the slope of the Arrhenius plot. At temperatures above 10 °C the T → R rate was so rapid that the data could not give rates with a reasonable error. Moreover these rates are composites of the multiple species with different ligation states, making interpretation of a single activation energy value problematic. However by making some assumptions, an approximate value for the T → R activation energy was derived for the unliganded T and R states. Assuming that the respective transition states in both the T → R transition and R → T transition are the same, and the free energy difference between the T and R states is $3.3$ kcal/mol,$^{17,31}$ then the activation energy for the T → R transition should be in the range of 15–18 kcal/mol. The charged-charged hydrogen bonds between Glu239 and Lys164 and non-charged hydrogen bonds between Glu239 and Tyr165 are critical for the
stabilization of both the T and R state conformations of the enzyme, which are interchain in the T state and intrachain in the R state. Sakash et al. showed that three of the six stabilizing interactions between catalytic chains on opposing subunits involving Glu239 are sufficient to stabilize the enzyme in the T state conformation. Thus at least three of the hydrogen bonds involving Glu239 must be broken during the allosteric transition. Considering a typical charged-charged hydrogen bond energy is approximately 4 kcal/mol and a non-charged hydrogen bond energy is approximately 0.5 - 1.5 kcal/mol, the minimal energy required for the allosteric transition of the enzyme is 13.5 - 16.5 kcal/mol. The results reported here are consistent with these calculations.

The rate of the allosteric transition was also measured when ATCase is mixed with two substrate analogs; succinate, an aspartate analog that promotes the T → R state transition when combined with CP, and PALA, a bisubstrate analog that binds at nanomolar affinity and also causes the T → R state transition. The structural transition rate with succinate and CP at a concentration of 50 mM each was slightly lower than the rate with the natural substrates at equivalent concentration and was observed to be a single exponential process. This may be because succinate binds approximately one order of magnitude more tightly than aspartate and therefore the bulk of the T state molecules should be highly liganded during the transition to the R state. However the structural transition rate with PALA was over one order of magnitude slower than with the natural substrates. The kinetics of the interaction of PALA with the isolated catalytic subunit at pH 7.0 and 25° C has been

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studied using stopped flow kinetics and $^{31}$P saturation transfer NMR by Cohen and Schachman.\textsuperscript{39} They observed a rapid binding of PALA followed by a much slower isomerization of the complex with a forward rate constant of 0.18 s$^{-1}$, similar to the rate constant of the slow phase we observed by TR-SAXS of 0.31 s$^{-1}$. However, the catalytic subunit by itself does not undergo a T $\rightarrow$ R transition so it is unclear whether the similarity of these values implies a similar mechanism between the change in the tertiary structure following PALA binding to the catalytic subunit and change in quaternary structure following PALA binding to the holoenzyme. The rate constant data and integrated scattering intensity presented here were obtained after mixing PALA with enzyme premixed with CP, to be consistent with the other experimental conditions. In addition experiments were performed where PALA was mixed with only the enzyme and the rate constants for the T $\rightarrow$ R transition were essentially identical (data not shown), which suggests the slow allosteric transition after PALA binding is not caused by having to displace CP from the active site. Because PALA combines elements of both substrates into one covalently linked bisubstrate analog, when the CP moiety of PALA initially binds the aspartate moiety may not be able to bind well to the aspartate binding site with the domains open, as they are in the T state. Subsequently the enzyme may undergo a slow conformational change in order for the aspartate moiety to bind tightly and then complete the T $\rightarrow$ R structural conversion. In this regard PALA appears to fit in the category of a slow tight-binding inhibitor.\textsuperscript{40} However the phenomenon of PALA being a very tight binding bisubstrate analog that causes an initial conformational
change followed by a much slower conformational change needs further investigation to be better understood.

In summary, our data showed ethylene glycol had a profound influence on the kinetics and behavior of ATCase, so therefore the time-evolution of the allostERIC transition of ATCase was reinvestigated in the absence of ethylene glycol by SAXS revealing several important new insights. Experimentally, these studies demonstrate that SAXS is now capable of monitoring relatively rapid structural changes at temperatures approaching physiological. The allostERIC transition is not the rate-limiting step in ATCase catalysis, and the rate of allostERIC transition is increased with increasing substrate concentration up to 50 mM. ATP appears to destabilize the T state and have little effect on the R state. CTP and the combination of CTP/UTP appear to destabilize the R state and stabilize the T state. PALA causes a very slow conformational change as compared to the natural substrates. In the future, novel TR-SAXS experiments could be performed with hybrid ATCase molecules that bind one, two, three, four, or five aspartate molecules to determine the individual rate constants and activation energies for each one of these species. Such studies will be facilitated by further instrumental upgrades that are being made to improve time-resolution. We believe we have begun to arrive at a clear understanding of the allostERIC mechanism of ATCase, and further studies into its dynamic behavior utilizing such time-resolved techniques as employed here will be invaluable in this.
References


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Chapter 3: Trapping and Structure Determination of an Intermediate in the Allosteric Transition of Aspartate Transcarbamoylase
Trapping and Structure Determination of an Intermediate in the
Allosteric Transition of Aspartate Transcarbamoylase

An article published in Proceedings of the National Academy of Sciences

by

Wenyue Guo, Jay M. West, Andrew Dutton, Hiro Tsuruta and Evan R. Kantrowitz

*Contribution to work: Conducted all experiments except the small angle X-ray scattering experiments
**Introduction**

Although many aspects of allosteric regulation and cooperativity have been established for *Escherichia coli* aspartate transcarbamoylase (ATCase), the enzyme that catalyzes the committed step in pyrimidine nucleotide biosynthesis, many of the details of how the active sites change from low-activity, low-affinity to high-activity, high-affinity during the T to R transition have not been delineated. The major limitation has been that stabilization of the enzyme in the R state requires the presence of active site ligands, thus making interpretation of the structural rearrangements upon the T to R transition, in the absence of ligands, difficult. Newell and Schachman have concluded, from sedimentation velocity experiments, that the quaternary structures of ATCase with the catalytic chain mutation K164E and the catalytic chain double-mutation K164E and E239K exist in the R-state, irrespective of the presence of active site ligands. The enzymatic properties of the double mutant (K164E/E239K ATCase) include a lack of homotropic cooperativity and an inability to be activated by ATP or inhibited by CTP: properties coincident of an enzyme that cannot transition between the two allosteric states. More recently, Velyvis et al. demonstrated that the partially labeled K164E/E239K ATCase exhibited certain chemical shifts in solution NMR studies that they conclude are characteristic of the R state of the enzyme. These same chemical shifts were also observed when the wild-type enzyme was bound with the bisubstrate analog N-phosphonacetyl-L-aspartate (PALA), which is known to stabilize fully the R-state of the enzyme. Thus, a crystal structure of K164E/E239K ATCase should provide us a
means to obtain the structural details of an R-state active site in the absence of ligands. However, here we report the X-ray crystal structure and the solution SAXS data for the K164E/E239K ATCase, which clearly demonstrates that this double mutant enzyme is not in the R-quaternary structure. Instead this mutant enzyme is in a unique intermediate state on the path between the T and R structural states.
Results and Discussion

Crystal Structure of K164E/E239K ATCase is Different from the Wild-type R State Structure

The K164E/E239K ATCase was purified and subsequently crystallized in the absence of ligands as described in Chapter 7. The detailed statistics of data collection and refinement are given in Table 3.1. The crystals were determined to be in the P2_12_12_1 space group with unit cell dimensions of a = 124.1, b = 144.8, c = 203.4 with α = β = γ = 90°. A previously determined structure of R-state ATCase in the presence of the PALA has also been solved in the P2_12_12_1 space group.\(^5\) However, the unit cell dimensions (a = 125.5, b = 153.5, c = 185.7 with α = β = γ = 90°) vary significantly from those determined for the K164E/E239K ATCase. In the crystal of the PALA-ATCase complex the molecular three-fold axis is parallel to the crystallographic b-axis.\(^5\) The almost 9 Å shorter b-axis of the K164E/E239K ATCase crystals was the first indication that the structure of this double mutant enzyme may not be an R-state structure.

In order to quantitatively compare the quaternary conformation of the K164E/E239K structure to the canonical ATCase T and R structures, the vertical separation\(^6\) between the upper and lower catalytic subunits, planar angle between allosteric domains of the regulatory dimer, and the rotation about the 3-fold axis (Figure 3.1) of the K164E/E239K structure were determined and compared to the corresponding values for the published T and R structures (see Figure 3.2). The vertical separation for the K164E/E239K structure was 50.5 Å as compared to 47.3
**Table 3.1.** Data collection and refinement statistics for the K164E/E239K ATCase structure

**Data collection**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>Cell Dimensions</td>
<td></td>
</tr>
<tr>
<td>$a, b, c$ (Å)</td>
<td>124.1, 144.8, 203.4</td>
</tr>
<tr>
<td>$\alpha, \beta, \gamma$ (°)</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>50.00–2.80 (2.90–2.80)</td>
</tr>
<tr>
<td>$R_{sym}$ (%)</td>
<td>0.079 (0.473)</td>
</tr>
<tr>
<td>Average $(I/\sigma)$</td>
<td>8.9 (2.2)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>92.4 (92.4)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.33 (3.35)</td>
</tr>
</tbody>
</table>

**Refinement**

<table>
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<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
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<tr>
<td>Reflections</td>
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</tr>
<tr>
<td>$R_{work}/R_{free}$</td>
<td>0.226 / 0.276</td>
</tr>
<tr>
<td>Number of atoms</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>21258</td>
</tr>
<tr>
<td>Waters</td>
<td>506</td>
</tr>
<tr>
<td>r.m.s. deviations</td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.014</td>
</tr>
<tr>
<td>Angles (°)</td>
<td>1.51</td>
</tr>
<tr>
<td>Mean B value (Å²)</td>
<td>81.5</td>
</tr>
</tbody>
</table>

Values in parentheses are for the highest resolution shell.
Figure 3.1. The ATCase holoenzyme showing the three-fold and two-fold axes. The asymmetric unit (c1-r1-r6-c6), corresponding to one-third of the molecule, is represented as a ribbon diagram. The other two-thirds of the molecule is shown without secondary structure. Planar angles between the CP and ASP domains of the catalytic chain (PA1) and the allosteric domains of the r1-r6 regulatory chains (PA2) are also shown. Insets, the c1 and r1-r6 chains with the corresponding PA1 and PA2 are shown in an orientation to better visualize the planar angle between the domains as well as the centers of gravity of these domains. Also indicated is the vertical separation between the centers of gravity of an upper and lower catalytic chain. This figure was produced using MOLSCRIPT7 and RASTER3D8.
Figure 3.2. Comparison of the structures of (A) wild-type ATCase in the T state (PDB ID code 1ZA1), (B) wild-type ATCase in the R state (PDB ID code 1D09), and (C) the K164E/E239K mutant ATCase (PDB ID code 4E2F). The vertical separation between the center of masses of the upper and lower catalytic trimers are indicated. The catalytic chains are dark and the regulatory chains are light in color. This figure was drawn with PyMol.\textsuperscript{9}
Å and 57.9 Å for the T-state (PDB ID code 1ZA1 used throughout) and R-state (PDB ID code 1D09 used throughout), respectively. The average planar angle between allostERIC domains of the regulatory dimer in the K164E/E239K structure was 153°, which compares to 152.0° and 155.8° for the T and R structures, respectively, of the wild-type enzyme. For the wild-type enzyme the relative rotation around the molecular three-fold axis between the T and R structures is 12°, whereas the rotation from the wild-type T structure to the K164E/E239K structure is only 4.8°. The vertical separation, the angle between the allostERIC domains of the regulatory dimer, and the rotation of the catalytic subunits about the 3-fold axis all indicate that the K164E/E239K structure is not globally in either the wild-type T or R structure.

The analysis of the global conformation of the K164E/E239K structure by means of X-ray crystallography does not agree with the previously reported data as determined by sedimentation velocity.\textsuperscript{2} Therefore, we used the program HydroPRO\textsuperscript{10} to calculate the hydrodynamic properties based upon the wild-type and K164E/E239K structures. In particular, the radius of gyration (R\textsubscript{g}) was compared to the corresponding values for wild-type ATCase in the T and R states. The R\textsubscript{g} values of the wild-type T and R-states were calculated to be 46.4 and 49.1 Å, respectively, which compares well with the reported values of 46.6 ± 0.3 and 49.3 ± 0.2 Å, respectively.\textsuperscript{11} The calculated R\textsubscript{g} for the K164E/E239K structure was 47.2 Å, a value closer to the T than the R state of the wild-type enzyme.
K164E/E239K ATCase Exists in a Different Structural State from the Wild-type R-state in Solution

For the wild-type enzyme, the percent change in the sedimentation coefficient between the T- and R-state structures has been reported to be -3.8%. The corresponding percent difference calculated between the K164E/E239K and wild-type R-state structures was -0.4%. These data suggest that the structure of K164E/E239K enzyme may be different in solution than it is in the crystal. Therefore, we used small-angle X-ray scattering (SAXS) to obtain additional structural data on the K164E/E239K ATCase in solution.12,13

SAXS data were recorded using Beamline 4-2 at the Stanford Synchrotron Research Laboratory at pH values 8.3, 7.5 and 7.0. In addition to recording SAXS data for the K164E/E239K enzyme, data were also recorded for the wild-type ATCase in the absence of ligands (T-state control) and in the presence of PALA (R-state control). The SAXS patterns (Figure 3.3), plotted as normalized Kratky Plots,14 for the T and R-states of the wild-type enzyme are dramatically different. The SAXS pattern for the K164E/E239K enzyme at pH 8.3 (Figure 3.3 A) is intermediate between the T and R-states observed for the wild-type enzyme. The intermediate curve observed for the K164E/E239K enzyme can be explained in at least two ways. First, the K164E/E239K enzyme exists in a new structural state that is different from the wild-type T or R-state; or second, in solution the K164E/E239K enzyme is a mixture of two or more structures. The second explanation is consistent with the
Figure 3.3. Small-angle X-ray scattering of the wild-type and the K164E/E239K enzymes at pH 8.3 (A), pH 7.5 (B) and pH 7.0 (C). At each pH the wild-type enzyme in the absence of ligands is shown in black and in the presence of PALA in blue. The K164E/E239K enzyme in the absence of ligands is shown in red and in the presence of PALA in orange. The data are plotted as normalized Kratky Plots. The green dash line indicates the best fit of a combination of the wild-type T pattern and the K164E/E239K PALA R pattern to the K164E/E239K pattern in the absence of ligands. Each scattering curve corresponds to 100 data points connected by linear line segments.
K164E/E239K enzyme having a T to R equilibrium shifted from the T-state dominated equilibrium observed for the wild-type enzyme.

If the SAXS pattern of the K164E/E239K enzyme is a mixture of wild-type T and R structures, it should be possible to generate the SAXS pattern of the double mutant enzyme by a linear combination of the wild-type T and R-state patterns. The dotted line in Figure 3.3 A, B and C corresponds to the best fit of the SAXS pattern of the K164E/E239K enzyme using the wild-type T and R curves. At each pH, the best-fit curve does not match that observed for the K164E/E239K enzyme. In particular, the position and amplitude of the first subsidiary minimum is not predicted correctly. The inability to deconvolute the curve into T and R components suggests that in solution the K164E/E239K enzyme is neither the T or R-quaternary structure of the wild-type enzyme or a mixture thereof.

As opposed to the wild-type enzyme in the absence or presence of PALA, the SAXS pattern for the K164E/E239K enzyme is pH dependent (compare Figure 3.3 A, B, C). Thus, by altering the pH either the quaternary structure of the K164E/E239K enzyme is shifting or the population of molecules in the R-state is increasing relative to the T-state. According to these SAXS data, at no pH between 7.0 and 8.3 does the unliganded K164E/E239K enzyme exist in a structure that would be considered the same as the R structure of the wild-type enzyme. As the pH is varied from 8.3 to 7.0 the SAXS pattern of the unliganded K164E/E239K enzyme shifts towards the wild-type R structure. This shift in structure could be modeled based on the SAXS pattern of the K164E/E239K enzyme at pH 8.3 in the presence and absence of PALA,
suggesting that this shift in the SAXS pattern was due to a change in the relative population of the two structures in solution. The SAXS data for the wild-type and the K164E/E239K enzymes are also plotted as scattering intensity and the log of the scattering intensity versus the momentum transfer (q) (Figure 3.4 and Figure 3.5).

These results suggest that the previously reported findings that the K164E/E239K ATCase is in the R-state may need to be re-evaluated.\textsuperscript{2,3} The evidence presented here clearly demonstrates the quaternary structure of the K164E/E239K ATCase is not identical to the wild-type ATCase•PALA complex, the typical structure used to define the R-state. The sedimentation velocity experiments on the K164E/E239K ATCase, which indicated R-state like sedimentation values were performed at 20° C and pH 7.0,\textsuperscript{2} a temperature and pH that may perturb the enzyme toward a more R-like structure. The SAXS data in Figure 3.3 show that lowering the pH from 8.3 to 7.0 causes a shift in the structure toward the R-state. The solution NMR report by Velyvis \textit{et al.} \textsuperscript{3} also asserts the K164E/E239K ATCase is in the R-state and concludes the Monod, Wyman, and Changeux (MWC) model\textsuperscript{15} can fully account for the allosteric properties of ATCase. The experiments performed by Velyvis \textit{et al.}\textsuperscript{3} were carried out at 37° C and pH 7.5. At this pH value and 25°C, the K164E/E239K ATCase is not in the canonical R-state structure (Figure 3.3 B). Although the higher temperature of the NMR experiments may cause a shift in the structure towards the R-state, the temperature dependence of SAXS data has been determined for the wild-type and the D236A enzyme. The wild-type enzyme showed no change in structure by SAXS through the temperature range of 4° C to 55° C, while the D236A
Figure 3.4. Small-angle X-ray scattering of the wild-type and the K164E/E239K enzymes at pH 8.3 (A), pH 7.5 (B) and pH 7.0 (C). The data are plotted as scattering intensity versus momentum transfer (Q). Scattering intensity was normalized to take into account the small differences in enzyme concentration between samples. At each pH the wild-type enzyme in the absence of ligands is shown in black, and in the presence of PALA in blue. The K164E/E239K enzyme in the absence of ligands is shown in red and in the presence of PALA in orange. The green dash line indicates the best fit of a combination of the wild-type T pattern and the K164E/E239K PALA R pattern to the K164E/E239K pattern in the absence of ligands. Each scattering curve corresponds to 100 data points connected by linear line segments.
Figure 3.5. Small-angle X-ray scattering of the wild-type and the K164E/E239K enzymes at pH 8.3 (A), pH 7.5 (B) and pH 7.0 (C). The data are plotted as scattering intensity versus momentum transfer (Q). Scattering intensity was normalized to take into account the small differences in enzyme concentration between samples. At each pH the wild-type enzyme in the absence of ligands is shown in black, and in the presence of PALA in blue. The K164E/E239K enzyme in the absence of ligands is shown in red and in the presence of PALA in orange. The green dash line indicates the best fit of a combination of the wild-type T pattern and the K164E/E239K.
ATCase exhibited a large shift towards the T-state between 4° C and 30° C with a minor shift back towards the R-state between 30° C and 45° C. These data suggest that under the NMR conditions (37° C), the K164E/E239K ATCase, which has similar properties to the D236A ATCase, would not be shifted toward the R-state. This study underscores the need for extreme caution when making inferences about the allosteric mechanism of a wild-type protein when working with a model system, which in this case is the double-mutant enzyme.

**K164E/E239K ATCase is in an Intermediate State during the Allosteric Transition from the T to the R-state**

Both the X-ray crystallographic and SAXS data indicate that the structure of the K164E/E239K enzyme in the absence of ligands is not in the R structure, but rather it is an intermediate structure between the canonical T and R structures. Instead of providing a model for the R-state, the K164E/E239K enzyme gives us structural information on the pathway of the T to the R-state transition of ATCase. When wild-type ATCase undergoes the allosteric transition to the R-state, the enzyme expands 10.6 Å and the upper catalytic trimer rotates 12° relative to the lower trimer along the 3-fold axis. Compared to the wild-type T-state structure the K164E/E239K enzyme only expands 2.8 Å and the upper catalytic trimer rotates only 4.4° relative to the lower trimer (Figure 3.6 A). Previous studies revealed that the planar angle between the allosteric domains of the regulatory dimer changes almost linearly with changes in the size of the molecule, which increases from 152.0° to 155.8° as the enzyme transitions from the T to the R state. The fact that in
Figure 3.6. (A) The percent change in quaternary structural properties of the K164E/E239K ATCase. As reference, the wild-type enzyme characteristics go from 0% (T state) to 100% (R state). (B) The T structure of one catalytic chain of wild-type ATCase (PDB ID code 1ZA1). (C) The R structure of one catalytic chain of wild-type ATCase (PDB ID code 1D09). In B and C the diameter of the tube corresponds to the (α-carbon) RMSD between the wild-type and the K164E/E239K structures. The portions of the tube highlighted in black correspond to those with RSMD greater than 1.5 Å.
the K164E/E239K structure this planar angle is only 153° suggests that the size of the double mutant is between the size of the wild-type T and R-state enzymes. As seen in Figure 3.6 A, the percent change in the vertical separation, the angle between the allosteric domains of the regulatory dimer, the rotation about the 3-fold axis and the radius of gyration observed in the K164E/E239K structure are all about one-third of the difference in the parameters observed between the T and R states of the wild-type enzyme. Therefore the K164E/E239K exists in an intermediate structure shifted about one-third towards the wild-type R structure from the T structure.

Not only is the quaternary conformation of the K164E/E239K structure intermediate between the canonical ATCase T and R structures but also the tertiary structure of the individual chains is intermediate as well. As seen in Figure 3.6 B and 3.6 C, the root mean square deviation (RMSD) of the Cα in the catalytic chain between the wild-type and the K164E/E239K structures is reflected in the diameter of the tube. The catalytic chain of the K164E/E239K structure has less RMSD to that of the T-state structure than the R-state structure. Thus, the tertiary structure of the double mutant represents an intermediate state closer to the T-state.

Another prominent feature of the T to R transition is the closure of the two domains of each catalytic chain to rearrange the active site residues so as to push the substrates toward one another in the bimolecular reaction. However, the active site of the K164E/E239K ATCase more closely resembles the T-state than the R-state active site (Figure 3.7).
Figure 3.7. Stereoview of the K164E/E239K ATCase 2 $F_o - F_c$ electron density map of the active site region contoured at $1.3 \sigma$. Overlaid onto the electron density are the side chains known to be involved in substrate binding and catalysis. Side chains are shown for the K164E/E239K (cyan carbons), wild-type T-state (magenta carbons) and wild-type T-state (orange) ATCases. The three structures were overlaid based upon the $\alpha$-carbon positions of the active site residues.
A comparison of the active site region of the K164E/E239K ATCase with the T and R-states of the wild-type enzyme is shown with electron density in Figure 3.7. Key differences include significant alterations in the positions of Arg54 and Arg167 that are two key residues involved in catalysis. Both Arg54 and Arg167 swing out of the active site in the K164E/E239K ATCase structure. These alterations in side chain position may be responsible for the reduced catalytic activity and increased [Asp]0.5 of the K164E/E239K compared to the wild-type enzyme.2

In addition to the K164E/E239K enzyme reported here, SAXS experiments have shown that the E239Q and D236A enzymes also exhibit patterns intermediate between the T and R patterns of the wild-type enzyme.11,16 These mutants break the T-state interchain c1-c4 interactions between Glu239 and both Lys164 and Tyr165 or the c1-r4 interaction between Asp236c and Lys143r. In order for ATCase to transition from the T- to the R-quaternary structure, these interchain interactions must break (see Figure 3.8 A, C), although for the wild-type enzyme in the R-state, c1 intrachain interactions are formed between Glu239 to both Lys164 and Tyr165 (see Figure 3.8 C). In the case of the K164E/E239K enzyme, the possible salt link between Glu164 and Lys239 does not form (average distance between Glu164 Oε and Lys239 Nζ is 4.78 ± 0.7 Å). Furthermore, the salt-link between Asp236 (c1/c4) and Lys143 (r4/r1) observed in the T-state structure (Figure 3.8 A) is broken in the K164E/E239K enzyme with an average distance between the Asp236 Oδ and the Lys143 Nζ of 8.17 ± 3.5 Å (Figure 3.8 B).
Figure 3.8. The c1-c4, c1-r4 and c4-r1 interfaces. (A) The T-state residue positions and interactions of the wild-type enzyme (PDB code 1ZA1), (B) the same residue positions in the K164E/E239K ATCase reported here and (C) the R-state residue positions and interactions of the wild-type enzyme (PDB code 1D09). Shown are the interactions involving Glu239 (wild-type T state (A) and R state (C) or Lys239 in the K164E/E239K structure (B)). Also shown are the interactions between Asp236 of the catalytic chains and Lys143 of the regulatory chains. For clarity Tyr165 is not labeled.
Most interestingly, the scattering curve of the K164E/E239K ATCase at pH 8.3 is comparable to the intermediate curve of the structure at the end of the early fast-rising phase of the time-resolved stop-flow X-ray scattering of ATCase upon mixing with substrates carbamoyl phosphate and L-aspartate. The elongation of the enzyme is a necessary part of the allosteric transition, which requires the breaking of the T-state intersubunit interactions involving both Glu239 and Asp236; thus the K164E/E239K structure, lacking these interactions, is a unique structure along the path of the quaternary structural transition from the T to the R-state. As such, these findings suggest that there are transiently stable intermediate structures, such as the K164E/E239K, that the protein passes through and which lower the overall activation energy of the allosteric transition.
References


Chapter 4: A Model for Nucleotide Regulation of ATCase involving Metal Ions
Introduction

*Escherichia coli* aspartate transcarbamoylase (ATCase, EC 2.1.3.2), which has been established as a classic model for allosteric regulation,\(^1\) catalyzes the first committed step in the *de novo* pyrimidine biosynthetic pathway, the carboxamoylation of the amino group of L-aspartate (Asp) by carbamoyl phosphate (CP) to form N-carbamoyl-L-aspartate (CA). This enzyme is highly regulated by the nucleotide effectors, which bind at the allosteric sites about 60 Å away from the catalytic sites.\(^2\) The end-product of the pyrimidine pathway, CTP, feedback inhibits the enzyme, whereas ATP, the end-product of the parallel purine pathway, exerts the opposite effect, activating the enzyme. However, both nucleotides have been shown to competitively bind at the same allosteric sites.\(^3\) Other purine nucleotides, such as GTP and ITP, inhibit the enzyme (25-30%) but less than that by CTP (50-70%).\(^4\) UTP, another end-product of the pyrimidine pathway, is a synergistic inhibitor of ATCase in the presence of CTP, however UTP has no independent effect at pH 7.0.\(^5\) The inhibition (90-95%) by CTP and UTP is much more than that by either nucleotide alone. However, this synergistic inhibitory effect is not as pronounced at pH 8.3 when UTP alone slightly inhibits the enzyme.\(^5\)

Extensive work, including but not limited to enzyme kinetics\(^4,6-8\) and binding studies\(^9-13\) of both the wild-type and mutant ATCases, have been performed to understand the allosteric regulation of the enzyme by nucleotide effectors. Most of these studies simply used the sodium salts of the nucleotides for their experiments, though it was reported as early as 1966 by Kleppe and Spaeren\(^14\) that the Mg\(^{2+}\)•ATP
and Mg$^{2+}$•CTP complexes had modified regulatory effects on the enzyme relative to the sodium nucleotides. More than 10 years later, both the work of Christopherson et al.$^{15}$ and Honzatko et al.$^{16}$ indicated an enhanced enzymatic activity by the complexes of nucleotides (ATP, CTP and GTP) and several metals (Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, Al$^{3+}$ and Gd$^{3+}$) relative to that produced by the nucleotides free of these metals. Later Fetler et al.$^{17}$ investigated solution X-ray small-angle scattering curves of E. coli ATCase in the absence and presence of Mg$^{2+}$•ATP, which appeared to further expand the R solution structure of the enzyme along the 3-fold axis. However, the details of the role of metal ions in combination with the nucleotides on the allosteric effect of ATCase remain to be elucidated.

Almost all intracellular ATP is present as Mg$^{2+}$•ATP complexes.$^{18}$ The other nucleotides most likely exist in complex with Mg$^{2+}$ under physiological conditions, as the stability constants of their complexes with Mg$^{2+}$ have been found close to that for Mg$^{2+}$•ATP.$^{19}$ In the present study we focus on examining the influence of Mg$^{2+}$ on the allosteric regulation of E. coli ATCase by nucleotide effectors, especially UTP, the information of which is lacked from previous studies of the metal-nucleotide complexes.
Results

Comparison of Effect of Nucleotides on the Activity of ATCase in the Absence and Presence of Mg\(^{2+}\)

Colorimetric assays were performed with various purine and pyrimidine nucleotide triphosphates independently and in combination in the absence and presence of Mg\(^{2+}\). The contamination of metal ions in some commercial nucleotides was reported to reverse the effect of the nucleotides reported in previous studies.\(^4\)

In our studies all the commercial nucleotides were pretreated with Chelex 100 resin (Bio-Rad) to remove metals prior to assays. In order to compare our results with those reported by Wild et al.,\(^5\) ATCase activity was measured under the same conditions at saturating carbamoyl phosphate (2 mM) and 5 mM aspartate, the concentration required for half-maximal activity \([S]_{0.5}\) at pH 7.0, 28° C.

Interestingly, as shown in Table 4.1 the relative activities reported by Wild et al.\(^5\) were in agreement with our results for the nucleotides and their combinations, except for CTP, ATP/CTP and CTP/GTP, in the presence of Mg\(^{2+}\) rather than those in the absence of Mg\(^{2+}\). The effect of CTP to reverse the activation by ATP, as previously reported,\(^5,20\) is possible only in the absence of Mg\(^{2+}\). In the presence of Mg\(^{2+}\), CTP/ATP increases enzyme activity 125%, which is lower than the activation by ATP alone (129%). GTP alone inhibits the enzyme about 5% and UTP alone has no effect on the enzyme. However, in the absence of Mg\(^{2+}\) GTP/UTP together inhibit the enzyme about 30, much more than either alone. Addition of Mg\(^{2+}\) slightly reduces this inhibition to 25%. Either GTP or UTP when paired with ATP enhances the
Table 4.1. Relative activities\(^a\) of ATCase for nucleotide effectors in the absence and presence of Mg\(^{2+}\) at pH 7.0

<table>
<thead>
<tr>
<th>NTP(^b)</th>
<th>Reported Value(^c)</th>
<th>Mg(^{2+}) (mM)(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>1.35</td>
<td>1.29 ± 0.071</td>
</tr>
<tr>
<td>CTP</td>
<td>0.43</td>
<td>0.49 ± 0.011</td>
</tr>
<tr>
<td>UTP</td>
<td>0.95</td>
<td>0.99 ± 0.0068</td>
</tr>
<tr>
<td>GTP</td>
<td>0.71</td>
<td>0.95 ± 0.051</td>
</tr>
<tr>
<td>ATP/CTP</td>
<td>0.85</td>
<td>0.87 ± 0.023</td>
</tr>
<tr>
<td>ATP/UTP</td>
<td>1.52</td>
<td>1.40 ± 0.055</td>
</tr>
<tr>
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<td>1.58</td>
<td>1.38 ± 0.041</td>
</tr>
<tr>
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</tr>
<tr>
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<td>0.61 ± 0.080</td>
</tr>
<tr>
<td>UTP/GTP</td>
<td>0.84</td>
<td>0.68 ± 0.011</td>
</tr>
</tbody>
</table>

\(^a\) Relative activities are determined by dividing the values of specific activities of ATCase in the presence of nucleotide(s) by that in the absence of nucleotide(s).

\(^b\) Concentration is 2 mM for each nucleotide.

\(^c\) The relative activities reported by Wild et al.\(^5\)

\(^d\) All experiments were performed at 28 °C in 20 mM bis-Tris, 20 mM Tris, and 20 mM Caps buffer, pH 7.0. Saturating carbamoyl phosphate (2 mM) and 5 mM aspartate were used throughout the assays. The values reported are the average of four determinations.
activation by ATP alone, which is even more enhanced with Mg$^{2+}$. Among all the
effect of nucleotides with and without Mg$^{2+}$, the maximal increase in activity (157%) is from the combination of ATP, GTP and Mg$^{2+}$, despite the fact that 2 mM GTP inhibits the enzyme about 40% with 1 mM Mg$^{2+}$ and 15% with 2 mM Mg$^{2+}$. In the absence of Mg$^{2+}$, GTP reduces the CTP inhibition from 50% to 40%. 2 mM Mg$^{2+}$ further reduces the inhibition by CTP/GTP to only 20%. Though UTP alone has no effect on the enzyme, 2 mM UTP•Mg$^{2+}$ (ratio 1:1) inhibits the enzyme about 10%.

Mg$^{2+}$ reduces the CTP inhibition. At 2 mM CTP•Mg$^{2+}$ (ratio 1:1) there is only 3% inhibition, however the addition of Mg$^{2+}$ to CTP/UTP increases the CTP inhibition from 50% to 88%, which is very similar to the 94% synergistic inhibition of the enzyme by CTP and UTP reported first by Wild et al.$^5$ On the contrary, the combination of CTP and UTP in the absence of Mg$^{2+}$ only inhibits the enzyme 53%, which is closer to the inhibition by CTP alone (50%) rather than the previous reported synergistic inhibition by CTP and UTP.

**Mg$^{2+}$ is Required for the Synergistic Inhibition by CTP and UTP**

To understand the role of Mg$^{2+}$ on the synergistic inhibition of ATCase, saturation studies with CTP, UTP and their Mg$^{2+}$-complexes were performed. As shown in Figure 4.1 A, de-metaled CTP is able to reduce the activity of ATCase about 50% but de-metaled UTP is not able to enhance this inhibition. However, as shown in Figure 4.1 B, UTP•Mg$^{2+}$ (1:1 ratio) has the ability to synergistically inhibit ATCase in the presence of CTP. There is no inhibitory effect on ATCase observed from de-metaled UTP alone whereas UTP•Mg$^{2+}$ exhibits slight inhibition (see Figure 4.2). In
Figure 4.1. Influence of CTP and UTP in the presence of CTP on the activity of ATCase (A) in the absence of Mg\textsuperscript{2+} and (B) in the presence of Mg\textsuperscript{2+}. Each data point is the average of three determinations with a deviation of ±8.1%. Measurements were made at pH 7.0 in the same conditions as described in Table 4.1. (A) The independent effect of CTP (●) was determined up to 4 mM. The effect of UTP plus CTP (○) was determined by adding increasing concentrations of UTP (0-2 mM) to the enzyme while the CTP concentration was held constant at 2 mM. (B) The independent effect of CTP (●) was determined up to 4 mM. The effect of UTP•Mg\textsuperscript{2+} plus CTP (○) was determined by adding increasing concentrations of UTP•Mg\textsuperscript{2+} (0-2 mM) to the enzyme while the CTP concentration was held constant at 2 mM.
Figure 4.2. Influence of UTP and CTP in the presence of UTP on the activity of ATCase (A) in the absence of Mg²⁺ and (B) in the presence of Mg²⁺. Each data point is the average of three determinations with a deviation of ±6.5%. Measurements were made at pH 7.0 under the same conditions as described in Table 4.1. (A) The independent effect of UTP (●) was determined up to 4 mM. The effect of CTP plus UTP (○) was determined by adding increasing concentrations of CTP (0-2 mM) to the enzyme while UTP concentration was held constant at 2 mM. (B) The independent effect of UTP•Mg²⁺ (●) was determined up to 4 mM. The effect of CTP plus UTP•Mg²⁺ (○) was determined by adding increasing concentrations of CTP (0-2 mM) to the enzyme while the UTP•Mg²⁺ concentration was held constant at 2 mM.
the presence of 2 mM de-metaled UTP, the inhibition curve of CTP (Figure 4.2 A) is similar to that of CTP alone (Figure 4.1 A). The maximal inhibition percentages of both curves fall in the range of 50-60%. The CTP concentrations required to reach half of the maximal inhibition were 0.136 ± 0.042 mM in the absence of UTP, which agrees with the reported value (0.14 mM) by Wild et al., and 0.099 ± 0.034 mM in the presence of UTP. The inhibition curve of CTP changes drastically in the presence of 2 mM UTP•Mg²⁺ (see Figure 4.2 B). The maximal inhibition of CTP in this curve increases to 90%, which reaches the same maximum level of inhibition by UTP•Mg²⁺ in the presence of 2 mM CTP. Moreover the CTP concentration required for half maximal inhibition is reduced to 0.029 ± 0.0041 mM in the presence of UTP•Mg²⁺.

Compared to that in the presence of de-metaled UTP, the CTP concentration required for half maximal inhibition in the presence of UTP•Mg²⁺ is closer to that reported by Wild et al. for the synergistic inhibition by CTP and UTP.

The synergistic inhibition is not observed when de-metaled CTP and UTP are used. But in the presence of Mg²⁺, CTP and UTP exhibited the synergistic effect on ATCase activity, as previously reported. It is possible that in previous studies there was sufficient metal ion contamination in the nucleotide solutions to allow this effect. These results suggest that a metal ion such as Mg²⁺ is indispensable for the synergism inhibition by CTP and UTP. Furthermore, it appears that Mg²⁺ not only serves to improve the inhibitory effects by CTP/UTP, but also promotes the binding of CTP as the affinity of the enzyme for CTP in the presence of UTP•Mg²⁺ is about 5-fold higher to that for CTP alone.
At pH 8.3, UTP and CTP Can Synergistically Inhibit ATCase in the Presence of Mg$^{2+}$

Wild et al. stated that the synergistic effect observed at pH 7.0 was unnoticeable at pH 8.3. However, UTP alone can inhibit the enzyme by 15-20% at pH 8.3.\textsuperscript{5} We found that at pH 8.3 UTP and CTP can also synergistically inhibit the enzyme when Mg$^{2+}$ was present. Figure 4.3 shows that UTP•Mg$^{2+}$ can further reduce the activity of the enzyme saturated with CTP to only 10% residual activity, which is significantly lower than that by UTP alone. It is worthwhile to note the differences between the synergistic effect at pH 8.3 and pH 7.0. A 2-fold higher concentration of UTP•Mg$^{2+}$ was needed at pH 8.3 than at pH 7 to reach the maximal synergistic inhibition in combination with 2 mM CTP. UTP•Mg$^{2+}$ inhibits the enzyme in the presence of CTP significantly more at pH 7 than at pH 8.3 when the concentration of UTP•Mg$^{2+}$ is less than 0.5 mM. At 0.5 mM UTP•Mg$^{2+}$, the enzyme activity at 2 mM CTP is further decreased by about 60% at pH 7 compared to only about 12% at pH 8.3. This difference of the influence of Mg$^{2+}$ at different pH values is most likely due to the differences in the strength of the interactions between Mg$^{2+}$ and nucleotides, as the ionization of the nucleotides vary according to pH.
Figure 4.3. Influence of CTP and UTP in the presence of CTP on the activity of ATCase (A) in the absence of Mg\(^{2+}\) and (B) in the presence of Mg\(^{2+}\) at pH 8.3. Each data point is the average of three determinations with a deviation of ±4.4%. Measurements were made in the same condition as described in Table 4.1 except pH. (A) Independent effect of CTP (●) was determined up to 4 mM. The effect of UTP plus CTP (○) was determined by adding increasing concentrations of UTP (0-2 mM) to the enzyme while CTP concentration was held constant at 2 mM. (B) Independent effect of CTP (●) was determined up to 6 mM. The effect of UTP•Mg\(^{2+}\) plus CTP (○) was determined by adding increasing concentrations of UTP•Mg\(^{2+}\) (0-4 mM) to the enzyme while CTP concentration was held constant at 2 mM.
Discussion

The influence of metal ions on the activity of *E. coli* ATCase was first examined by Kleppe and Spaeren. They reported that the divalent metal ions inhibited the holoenzyme while the alkali metals had no effect. They also reported that the activation of ATCase by ATP and inhibition by CTP were greatly dependent on the concentration of Mg$^{2+}$. Additional kinetic studies were carried out to understand the regulatory properties of ATCase with metal-NTP complexes. The results of these studies showed an enhanced enzyme activity by ATP, CTP and GTP in the presence of Mg$^{2+}$ relative to that produced by the nucleotides without addition of Mg$^{2+}$. It was noted that the contamination of heavy metal ions in the commercial supply of nucleotides may alter the nucleotide effects opposite to what was previously reported. However, the above studies of the influence of metal ions on the nucleotide regulation of ATCase overlooked the metal contamination of the nucleotides. Thus the reported enzyme activity measured with sodium-salt nucleotide solutions, which were used without the removal of trace metals, may include the effect of their complexes with non-sodium metal ions, such as Mg$^{2+}$. Our results should provide a more accurate comparison between the effect of nucleotides on the enzyme in the absence and presence of Mg$^{2+}$ since all the nucleotide solutions used in the present experiments have been de-metaled before use. Since its discovery by Wild *et al.* in 1989, there has been no data published regarding the influence of Mg$^{2+}$ on the synergistic inhibitory effect of UTP in the presence of CTP. Thus we have made here a systematic study of the influence of
Mg$^{2+}$ with ATP, CTP, GTP and UTP to fill this gap, providing a more complete picture of regulation of ATCase by the Mg$^{2+}$•nucleotide complexes.

**A Model for Nucleotide Regulation of ATCase Including Mg$^{2+}$**

An early model of nucleotide regulation of ATCase without considering the influence of metal ions was proposed by London and Schmidt based on the assumption that each regulatory chain contained one nucleotide binding site, which was equivalent to the other sites and existed at least in two different conformations. Different nucleotides have different affinities for the different conformations of the binding site, which is related to the quaternary conformation of the enzyme. When the quaternary conformation is more open and the enzyme is more active, the binding site favors a less contracted conformation, and vice versa. According to this model ATP activation is the consequence of ATP only binding to the expanded conformation of the binding site, which leads to a more open conformation of the enzyme having more activity. Recently Peterson et al. discovered a novel allosteric site for UTP binding on each regulatory chain by determining the X-ray structure of the ATCase•UTP complex. The UTP was bound to a site (referred to as the B site) adjacent to the known allosteric site (referred to as the A site) where ATP and CTP bind. Thus each regulatory chain contains two distinct nucleotide-binding sites, which naturally requires the need to modify the previous model in which only one nucleotide-binding site per regulatory chain is considered.

Though the synergistic inhibition of CTP and UTP is suggested by Peterson et al. as a result of the concurrent binding of CTP at the A site and UTP at the B site,
the kinetic studies reported here clearly demonstrate that this synergistic inhibition can be observed only upon addition of Mg$_{2+}$ to the combination of CTP and UTP. This indicates that Mg$_{2+}$ is essential for the synergistic inhibition to occur. Mg$_{2+}$$\cdot$ATP is observed in many crystal structures that have been deposited in the Protein Data Bank.$^{23-25}$ Mg$_{2+}$ is found mostly forming bidentate complexes with Mg$_{2+}$ interacting with the $\beta$ and $\gamma$ phosphate groups.$^{17}$ In the presence of CTP, replacing UTP with UDP results in the loss of synergistic inhibition.$^{26}$ These observations may indicate that Mg$_{2+}$ coordinates with the oxygen atoms of the $\beta$ and $\gamma$ phosphate groups of CTP and UTP, aligning both nucleotides in the proper orientation for binding to the enzyme at the combined AB site. Without Mg$_{2+}$, CTP and UTP are probably not favored to bind simultaneously to the AB site due to the repulsion of their negative charge phosphates. Therefore, the combined synergistic inhibitory effect is only possible when CTP binds to the A site and UTP binds to the B site through the connection of Mg$_{2+}$. Since UTP by itself binds exclusively to the B site$^{21}$ without having any effect on the enzyme while the A site is left empty, these results suggest that for the synergistic inhibition UTP in the B site only acts to magnify the inhibition by CTP bound in the A site when CTP$\cdot$Mg$_{2+}$$\cdot$UTP is bound together in the combined AB site.

From this understanding of the CTP/UTP synergism, we propose a revised model for nucleotide regulation of ATCase that can provide a probable explanation for the observed nucleotide effects in the absence and presence of Mg$_{2+}$ reported in this study. In this model each of the six regulatory chains contains two neighboring
nucleotide binding sites, the A site which has been identified as the only nucleotide binding site in the past, and the B site where UTP was discovered to bind (see Figure 4.5).\textsuperscript{21} The nucleotides have different favoritism of binding to the A site or the B site as the residues involved in binding interactions are different between the two sites.\textsuperscript{21} Not only that, binding of the same nucleotide acts differently in allosteric control of the enzyme at the A site from that at the B site: the nucleotide bound at the A site directly affects enzyme activity, either activating (ATP) or inhibiting (CTP), whereas binding of the nucleotide in the B site only serves to magnify or modify the effect of the nucleotide at the A site, but has no direct effect on the enzyme. In the crystal structure of the ATCase•CTP•UTP•Mg\textsuperscript{2+} complex,\textsuperscript{27} CTP binds to the A site, UTP binds in the B site and Mg\textsuperscript{2+} acts as a bridge between the β and γ phosphate groups of the two nucleotides. In the absence of metal ions, the repulsion of the negatively charged phosphate oxygens would prevent the binding of any two nucleotide molecules to the A and B sites at the same time. Therefore, the apparent effect of nucleotide(s) is determined by the total effect of nucleotide(s) binding at the A site, the B site or the AB site, which depends on the presence of metal ions and the relative affinities of the nucleotide(s) for the A and B sites.
**Figure 4.4.** The A and B nucleotide-binding sites of one regulatory chain. (A) The A site was shown with CTP bound (PDB ID 8AT1) and (B) the B site was shown with UTP bound (PDB ID 4F04) from the same viewpoint.
Reevaluation of Nucleotide Effects on ATCase Based on the Proposed Model

Unfortunately many of the previous studies providing the binding and kinetic data for the nucleotide effectors on ATCase are questionable since the extent of metal ion contamination in these experiments is unknown. Here we reevaluate the functional effects of the nucleotides and their combinations on ATCase based on the proposed model using our kinetic results in the absence and presence of Mg$^{2+}$.

**CTP and UTP**

UTP alone greatly favors binding to the B site over the A site, which is due to a more favorable electrostatic interaction between the 4-keto group of uracil ring and Lys60 revealed in the structure of ATCase•UTP complex.\(^{21}\) According to our model, a nucleotide bound at the B site has little or no effect on the enzyme activity. As a result, UTP in the absence of metals has no effect on enzyme activity, which agrees with our kinetic data for UTP without Mg$^{2+}$. However in the presence of Mg$^{2+}$, up to 10% inhibition is observed for 2 mM UTP. Similar to the synergistic inhibition induced by the combined binding of CTP and UTP with Mg$^{2+}$ in the AB allosteric site, this altered effect could be explained by the simultaneous binding of two UTP molecules linked by a Mg$^{2+}$ ion, one at the A site and the other at the B site. The UTP bound to the A site would inhibit the enzyme and this inhibition would be amplified by the UTP bound in the B site. This also explains the increasing trend of the inhibition when more Mg$^{2+}$ is added to the same amount of UTP. In the mixture of UTP and Mg$^{2+}$ solution, more UTP would complex with Mg$^{2+}$ with less free UTP left
as the amount of Mg$^{2+}$ increases, resulting in a larger apparent inhibitory effect observed for 2 mM UTP with 2 mM Mg$^{2+}$ than with 1 mM Mg$^{2+}$.

Previous binding studies suggested six binding sites on the enzyme for CTP, three high-affinity and three low-affinity sites. With the discovery of the B site on each regulatory chain, there are a total of twelve possible binding sites for nucleotides. These two results are not comparable as all of the previous binding experiments performed with CTP did not control for the metal contamination. New binding experiments with consideration of the influence of metal ions are required to better understand the binding of CTP. Despite this difference in the number of binding sites for CTP, it is well known from kinetic and structural studies that CTP binds to the A site and inhibits the enzyme about 50%.$^{3,22,29,30}$ This reported inhibition is about the same as our data for CTP inhibition in the absence of Mg$^{2+}$. As seen in Table 4.1, the addition of Mg$^{2+}$ diminishes instead of increases the inhibition by 2 mM CTP, which is almost completely relieved when Mg$^{2+}$ concentration reaches to the same level as CTP. Additionally, the electrostatic attraction observed between UTP and Lys60 at the B site would become a positive charge-charge repulsion when the 4-keto group of UTP is replaced by the 4-amino group of CTP, which may prevent CTP from binding to the B site.$^{21}$ Since even as little as 0.1 mM (5% of 2 mM) free CTP can inhibit the enzyme about 15% as seen in Figure 4.1 B, the less than 10% inhibition by 2 mM CTP in the presence of 2 mM Mg$^{2+}$ may be explained by the exclusion of CTP•Mg$^{2+}$•CTP binding in the AB site due to the inability of CTP to bind to the B site and the preference of free CTP binding to the Mg$^{2+}$ than to the enzyme.
Not surprisingly the synergistic inhibition by CTP and UTP is achieved when both nucleotides are allowed to bind to the enzyme with their favorable interactions, CTP to the A site and UTP to the B site, through the coordination of Mg$^{2+}$ as seen in the structure of the ATCase•CTP•UTP•Mg$^{2+}$ complex.$^{27}$ The combined binding of CTP•Mg$^{2+}$•UTP to the AB allosteric site could stabilize the T state even more than CTP alone, shifting the T to R equilibrium further towards the T state.

*ATP and its combination with other nucleotides*

In the same fashion, ATP-induced activation could be enhanced by Mg$^{2+}$ through the combined binding of ATP•Mg$^{2+}$•ATP in the allosteric site. The response of the ATP in the A site would be amplified by the binding of ATP in the B site. Though ATP was only observed in the A site from previous X-ray crystallography,$^{22,30}$ the possibility of ATP binding to the B site can not be ruled out since Mg$^{2+}$ was not present in these studies. Binding of UTP or GTP to the B site can also amplify the activating effect of ATP in the A site despite the presence of Mg$^{2+}$, though to a larger extend in the presence of Mg$^{2+}$. This supports the role of Mg$^{2+}$ in assisting or stabilizing the simultaneous binding of two nucleotides in the AB site by eliminating the repulsion between the phosphate oxygens of the two nucleotides. When ATP in the A site binds to the enzyme with another nucleotide (ATP, GTP or UTP) binding to the B site in the presence of Mg$^{2+}$, the R state could be stabilized more than that by ATP alone and thereby the T to R equilibrium could be further shifted to the R state than in the presence of ATP alone. Among all the nucleotide combinations, ATP/GTP with Mg$^{2+}$ induces the largest allosteric activation. The
combined binding of ATP\(\text{•}Mg^{2+}\text{•}GTP\) appears to act in a similar way as CTP\(\text{•}Mg^{2+}\text{•}UTP\) but has the opposite effect on the enzyme. Different from GTP and UTP, CTP can reverse or reduce the activation by ATP in the absence and presence of Mg\(^{2+}\), respectively. CTP is known to compete with ATP for binding to the A site with a higher affinity, which explains the inhibitory effect on the enzyme by the metal-free combination of ATP/CTP. As discussed above, addition of Mg\(^{2+}\) can allow the combined binding of ATP\(\text{•}Mg^{2+}\text{•}ATP\) in the allosteric site, promoting the stimulation of the enzyme, whereas the ability of Mg\(^{2+}\) to complex with CTP could prevent CTP from binding to the A site, relieving the inhibition of the enzyme. Therefore the total effect of the ATP/CTP with Mg\(^{2+}\) retains the activating effect of ATP with a slight decrease, which is most likely due to a small portion of the CTP in the nucleotide mixture bound to the enzyme not to the Mg\(^{2+}\).

**GTP and its combinations with other nucleotides**

Though GTP interacts with ATP and Mg\(^{2+}\) to activate the enzyme, GTP on its own is a weak inhibitor. Similar to UTP, Mg\(^{2+}\) enhances the inhibition by GTP but in a different fashion. When more Mg\(^{2+}\) is added to a fixed concentration of UTP, an increase in inhibition is observed which can only be explained by more UTP\(\text{•}Mg^{2+}\text{•}UTP\) binding to the AB site according to our model, since UTP alone only binds to the B site and has no inhibitory effect. This may lead us to one possible explanation for the different trend in the GTP inhibition when Mg\(^{2+}\) is added: Unlike UTP, GTP alone can bind to both the A site and the B site. The slight inhibition by GTP results from the ability of GTP to bind to the A site. This is also supported by the
fact that GTP can enhance ATP activation and diminish CTP inhibition in the absence of Mg\textsuperscript{2+}. These results indicate that GTP can bind to the B site in the presence of ATP and compete with CTP for the A site. Due to the difference in the relative binding affinities of Mg\textsuperscript{2+} and ATCase for GTP in the A or B site, 2 mM GTP could produce a larger inhibitory effect with 1 mM Mg\textsuperscript{2+} than with 2 mM Mg\textsuperscript{2+}. The mechanism of the de-metaled GTP/UTP inhibiting the enzyme better than either nucleotide alone is not clearly explained by our current model. Binding and structural studies for GTP with ATCase are needed in the absence and presence of the metal ions to better understand how GTP functions to regulate ATCase activity.

**The Role of Mg\textsuperscript{2+} in the Allosteric Site**

From our model, UTP alone in the B site has no effect on enzyme activity, but when Mg\textsuperscript{2+} is present UTP in the B site can magnify the inhibition by the CTP in the A site. The kinetics of CTP saturation curves (see Figure 4.1 and 4.2) shows that the binding affinity of the enzyme for CTP in the presence of UTP•Mg\textsuperscript{2+} is about 5-fold and 4-fold higher than that for CTP alone and for CTP in the presence of UTP, respectively. These results suggest that this reinforcement by the B site nucleotide on the effect of the nucleotide in the A site is accomplished by enhancing the binding of the nucleotide in the A site, which depends on the presence of the metal ion. In addition to relieving the repulsion of the phosphate oxygens of the two nucleotides which would prevent both binding simultaneously at the AB site, Mg\textsuperscript{2+} may help to create more interactions between the nucleotide in the A site and the enzyme. Asp19 of the regulatory chain, which interacts with CTP in the A site\textsuperscript{22} but not with
UTP in the B site,\textsuperscript{21} may be involved in the binding of the metal ions in the allosteric site. The kinetic and binding experiments of the D19A mutant were performed in parallel with the wild-type enzyme.\textsuperscript{13} In these studies the wild-type enzyme exhibited synergistic inhibition by UTP in the presence of CTP and UTP enhanced the binding of CTP for the wild-type enzyme. These findings agree with our results in the presence of Mg\textsuperscript{2+}, indicating that the UTP used in these studies must contain contamination of the metal ions. Not only the synergistic inhibition but also the enhanced binding of CTP by UTP was not observed for the D19A mutant. This was explained by the binding of UTP to the mutant enzyme not being detected in the presence of CTP. However UTP alone was still able to bind to the enzyme. If the same UTP was used for the wild-type enzyme as was used for the D19A mutant, one explanation to account for all these data based on our model is that Asp19 is critical for the binding of the metal ion. Without the metal ion, the binding of UTP to the B site is not favored in the presence of CTP thus the synergistic effect is lost. Therefore the metal ion, Mg\textsuperscript{2+} in our study, is not only required for the enhanced nucleotide effect but also the nucleotide binding at the AB allosteric site.

In conclusion metal ions, such as Mg\textsuperscript{2+} studied here, have a significant influence on the allosteric regulation of ATCase, which was not previously realized. In our experiments all nucleotide solutions were de-metaled before use to ensure an accurate comparison between the nucleotide effects in the absence and presence of Mg\textsuperscript{2+}. Interestingly, most of the previous reported nucleotide effects agree with the
nucleotide effects determined with Mg\textsuperscript{2+} in our study rather than those without Mg\textsuperscript{2+}.\textsuperscript{5} Along with the recent discovery of a novel UTP binding site on each regulatory chain,\textsuperscript{21} we propose a modified model for nucleotide regulation involving metal ions in which there are two adjacent but not overlapping nucleotide binding sites on each regulatory chain. In this model, the nucleotides either inhibit or activate the enzyme when binding to the A site but have no direct effect when binding to the B site. In the presence of the metal ions, two nucleotides can bind in the combined AB site, the nucleotide in the B site amplifying the effect of the nucleotide in the A site. Since nucleotides are most likely present as nucleotide-metal complexes in cells, our model would provide a more relevant picture of the allosteric control of ATCase under physiological conditions. Based on this model, CTP alone binds to the A site and inhibits the enzyme but with Mg\textsuperscript{2+} this inhibition is greatly weakened as the binding of CTP to the B site is not favored. UTP alone binds to the B site and thus has no effect on the enzyme. Mg\textsuperscript{2+} enhances the binding of UTP to the A site and allows another UTP to bind in the B site at the same time, producing a slight inhibitory effect on ATCase. In the presence of Mg\textsuperscript{2+}, CTP binds to the A site and UTP synergistically inhibits the enzyme by binding to the B site which enhances the CTP inhibition. The inhibition by CTP•UTP•Mg\textsuperscript{2+} may be of biologically importance as it compensates for the weak inhibition by CTP•Mg\textsuperscript{2+}, which would be the dominant form of CTP under physiological conditions. Therefore, the synergism of CTP and UTP is necessary to maintain the feedback inhibition of ATCase. ATP alone favors binding to the A site and activates the enzyme. This activation is
enhanced when ATP, UTP or GTP binds to the B site with addition of Mg$^{2+}$. The combined binding of ATP•Mg$^{2+}$•GTP activates the enzyme the most. Therefore, the combination of ATP and GTP, end products of the purine biosynthetic pathway, provides a synergistic control that activates ATCase to produce CTP and UTP, the end products of the parallel pathway for pyrimidine biosynthesis, when the level of the purine nucleotides is elevated in *E. coli*. In this way, the pool of purine and pyrimidine nucleotides can be balanced for the biosynthesis of nucleic acids in *E. coli*. To better understand the mechanism of allosteric control of enzyme by nucleotide effectors, new binding and structural studies of ATCase with nucleotides in the absence and presence of metal ions would be extremely helpful.
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Chapter 5: Drug Discovery of Dihydroorotase using a Virtual High Throughput Screening System
Drug Discovery of Dihydroorotase using a Virtual

High Throughput Screening System

A poster presented at the 236th ACS national meeting

by

Wenyue Guo and Evan R. Kantrowitz

*Contribution to work: Conducted all experiments including the virtual screening of the compounds, analysis of the docking results and biochemical validation of the candidates
Introduction

Pyrimidine nucleotide metabolism is critical for the survival of the cell as it provides the pyrimidine nucleotides which are not only used for the biosynthesis of the nucleic acids, but are also involved in assembly of cell membranes, protein glycosylation and glycogen synthesis\(^1,2\) in which sugar nucleotides act as glycosyl donors. There are two ways that cells obtain pyrimidines and pyrimidine nucleotides, one is from the *de novo* biosynthesis, and the other is from the catabolism of foodstuffs via a salvage pathway. Since the pyrimidine nucleotides are necessary for nucleic acid synthesis, the enzymes of pyrimidine production, both in the *de novo* and salvage pathways, have become targets for anti-proliferation drugs. The inhibitors used clinically as salvage pathway inhibitors are methotrexate, 5-fluorouracil, and 6-azauridine.\(^3\) However, all these compounds have clinical side effects. Therefore, there is a need to develop a new class of lead compounds for blocking the *de novo* pathway. Dihydroorotase (DHOase) becomes an attractive target for drug design as it catalyzes the reversible cyclization of N-carbamoyl-L-aspartate (CA) to L-dihydroorotate (DHO) in the pyrimidine biosynthesis (see Figure 1.9).\(^4\) The inhibitors of DHOase may also become potential anti-malarial drugs since the malarial parasite *Plasmodium falciparum* does not have a salvage pathway for pyrimidines.

There are two general classes of DHOase: the class I enzymes are found in higher organisms as multifunctional proteins such as mammalian CAD or monofunctional proteins in gram positive bacteria; the class II enzymes are all
monofunctional proteins from gram negative bacteria and yeast. There are no high-resolution structures available for the DHOase component of CAD and *P. falciparum* DHOase. However, sequence alignments of *E. coli* DHOases with these two DHOases show highly conserved regions corresponding to the residues involved in catalysis and metal binding. Therefore, the 1.9 Å X-ray crystal structure of *E. coli* DHOase can serve as the target structure for drug discovery for both the human and *P. falciparum* enzymes.

Conventional drug discovery often involves experimental high-throughput screening of millions of compounds. However, as this screening is both cost-inefficient and time-consuming, virtual high-throughput screening (vHTS), which is a computational screening method applied to evaluate the ability of a large number of compounds to form complexes with the target receptor *in silico*, has emerged as an alternative and complementary method over the past decade. With the rapid increase in the number of available macromolecular three-dimensional structures, vHTS has been successfully used to identify inhibitors against targets such as HIV-1 protease, protein kinase, and aldose reductase.

Though it is relatively straightforward to perform a small virtual screening (<100 compounds) using a single docking program, it remains a challenge to perform vHTS (>1,000,000 compounds) with different docking programs. Dr. Kantrowitz has developed an easy to use vHTS system, which is able to screen large virtual libraries of compounds. Based on the crystal structure of *E. coli* DHOase, I
utilized this vHTS system to identify new lead inhibitors for further drug development as anti-proliferation and anti-malarial drugs.
Results

Identification of DHOase Inhibitors through the vHTS System

During this project, I used the vHTS system (set up of the system is described in details in Chapter 7) to find inhibitors against the DHO-bound subunit of DHOase by screening about 1,500,000 compounds obtained from the ZINC database (http://blaster.docking.org/zinc/). Before screening these ligands I first validated four docking programs (AutoDock 3.0\textsuperscript{11}, DOCK 5.0\textsuperscript{12}, Surflex\textsuperscript{13} and GOLD\textsuperscript{14}) to determine the parameters used to predict the docking pose of DHO closest to that from the available X-ray structure. The quality of the results from virtual screening was directly related to the ability of the docking program to position the ligands into the receptor target with accuracy. For each docking program, the X-ray structure of subunit with DHO bound\textsuperscript{6} (PDB ID 1XGE) was used as receptor target. DHO in the active site was first removed, and its coordinates were altered so that DHO was in a different spatial position, unrelated to actual binding site. Then the docking of product DHO was performed with the four programs. The four docking programs gave acceptable DHO conformations compared to that determined by the X-ray crystallography (See Table 5.1). In order to screen a large scale of compounds against DHO efficiently within a reasonable time, we initially used Surflex\textsuperscript{13} because it utilized the shortest time, compared to the other three docking programs, about 5 seconds to dock and evaluate one ligand. After completion of docking 1,500,000 compounds, the top 500 hits that were generated from the energy scoring function of Surflex were docked using three other docking programs that employed different
Table 5.1. RMSD (non hydrogen atoms, in Å) between docked conformations of substrate CA and product DHO (top solution of each docking program) of *E.coli* DHOase and their conformations in the X-ray crystal structure (PDB ID 1XGE)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>AutoDock3.0</th>
<th>DOCK5.0</th>
<th>GOLD</th>
<th>Surflex</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHO</td>
<td>0.03</td>
<td>0.67</td>
<td>0.38</td>
<td>1.57</td>
</tr>
<tr>
<td>CA</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.54</td>
</tr>
</tbody>
</table>
scoring functions. Lastly the Xscore\textsuperscript{15} program was used to reevaluate the docking results of the top 500 compounds with the four docking programs by calculating the binding score for the protein-ligand complex structures. The top 50 poses ranked by Xscore\textsuperscript{15} were selected and analyzed. Among the top 50 poses, certain compounds were selected more than once by different programs as the Xscore\textsuperscript{15} evaluated the docking pose independently. For example, the docking pose of zinc902674 from Surflex was ranked first by Xscore and the docking pose of the same compound from GOLD was ranked second by Xscore. Among the top 10 compounds ranked by Xscore from the results using different docking programs, three compounds were available to be purchased immediately at a reasonable cost: zinc902674, zinc903866 and zinc391516. These compounds were obtained for experimental validation. Shown in Figure 5.1 A are the structures of zinc902674, zinc903866 and zinc391516.

The active site of CA-bound subunit was much less open compared to that of the DHO-bound subunit due to the closure of the 100s loop.\textsuperscript{6} Therefore, I performed a separate test screening to find inhibitors against the CA-bound subunit. Validation of Surflex\textsuperscript{13} program was performed for CA in the similar manner as that for DHO. The RMS deviations between the coordinates from docking and crystal structure were less than 2 Å (see Table 5.1). For inhibitors targeting the CA binding site, we finished screening about 750,000 compounds using Surflex. Surflex\textsuperscript{13} ranked the docking results by pK\textsubscript{i} score. The higher the pK\textsubscript{i} value was, the higher the binding affinity of the docked ligand was to the protein target. The pK\textsubscript{i} score of the top five
Figure 5.1. The chemical structure of the hits identified through the vHTS system. (A) Three hits targeting DHO binding site of DHOase; (B) Two hits targeting CA binding site of DHOase.
docking poses varied from 10.37 to 11.31, which were higher than the pKᵢ of docked CA (pKᵢ = 10.10). Two out of the five compounds were available to be purchased immediately at a reasonable cost: zinc394318 and zinc402968. These compounds were obtained for experimental validation. Shown in Figure 5.1 B are the structures of zinc394318 and zinc402968.

**Biochemical Validation of DHOase Inhibitors Identified through the vHTS System**

The three hits (zinc902674, zinc903866 and zinc391516) identified through the vHTS system targeting the DHO-bound active site were further characterized for their ability to inhibit DHOase. For simplicity the enzymatic activity in the reverse direction was measured using the colorimetric assay described in Chapter 7. Zinc902674 and zinc903866 showed no significant inhibition while zinc391516 decreased the enzyme activity by 80% at 2 mM (See Figure 5.2). The fact that the activity of DHOase was not brought down to zero at the saturating concentration of zinc391516 indicated that zinc391516 was not a competitive inhibitor. In order to understand the inhibition of zinc391516 against DHOase, DHO saturation curves were performed at 0, 200, 600 and 1400 uM of zinc391516. Both the overlay of DHO saturation curves and the corresponding double reciprocal curves showed that the Kᵣ remained constant but the Vₑ max decreased as the concentration of zinc391516 increased (see Figure 5.3). Therefore, zinc391516 is a noncompetitive inhibitor of DHOase with a Kᵢ of 1.41 mM.
Figure 5.2. Inhibition effect of (A) zinc902674, (B) zinc903866 and (C) zinc391516 on the activity of DHOase. Each data point was determined in duplicate, and the data points shown are the average. All measurements were made at 25°C in the presence of 50 mM Tris-phosphate, pH 8.0 with 0.08 mM DHO and 0.25 μg E. coli DHOase.
Figure 5.3. Specific activity of DHOase in the presence of 0.2 mM (●), 0.6 mM (■), 1.4 mM (▲) and in the absence (○) of inhibitor zinc391516 (A) plotted versus concentration of DHO and (B) its corresponding Lineweaver-Burk plot. All measurements were made at 25° C in the presence of 50 mM Tris-phosphate, pH 8.0 with 0.08 mM DHO and 0.25 μg E. coli DHOase. Specific activity is reported in mmoles of carbamoyl aspartate formed per hour per mg of DHOase. The data points shown are the average of four determinations with a deviation of ± 6.5%.
The two hits (zinc394318 and zinc402968) identified through the vHTS system targeting the CA-bound active site were further characterized for their inhibition of DHOase in the biosynthetic direction using the direct assay described in Chapter 7. Zinc394318 fully inhibited the enzyme at 2 mM while zinc402968 inhibited about 50% at 2 mM (see Figure 5.4). Zinc394318 is an inhibitor versus the enzyme substrate CA with an IC\textsubscript{50} of 16.2 μM. Though zinc402968 doesn’t fully inhibit the enzyme, the compound has a lower IC\textsubscript{50} of 14.1 μM.
**Figure 5.4.** Inhibition effect of (A) zinc394318 and (B) zinc402968 on the activity of DHOase. Each data point was determined in duplicate, and the data points shown are the average. All measurements were made at 37° C in the presence of 75 mM Na₂HPO₄ buffer, pH 5.5 with 1 mM DL-CA and 50 μg *E. coli* DHOase.


**Discussion and Future Prospects**

We have applied the vHTS system developed in our lab to screen more than a million compounds but unfortunately no compounds tested can be considered as an acceptable lead for drugs as the IC$_{50}$ of all candidates are larger than 10 μM.\(^{16}\) Several explanations could account for this result as I examine our docking experiments as a comparison to those giving successful results.\(^{8-10}\) Firstly, the number of compounds tested is too small. Vangrevelinghe *et al* tested 12 candidates out of 400,000 compounds screened, four of which turned out to inhibit protein kinase CK2.\(^{9}\) In another docking study to find inhibitors of aldose reductase, 36 candidates were tested out of 120,000 compounds screened. 10 out of 36 compounds showed more than 40% inhibition.\(^{10}\) For inhibitions against DHO, we only tested three candidates out of 1,500,000 compounds screened and only one inhibited in the millimolar range. Better results may appear if we increase the number of compounds tested. Secondly, the virtual screening procedure we used may not be a powerful method to identify potential candidate inhibitors. Docking methods and the scoring function have shown dependence on the target selected, for example GOLD and Surflex ranks better with more reliable scores for highly polar active sites, whereas the scoring function of DOCK was found to perform better for hydrophobic active sites.\(^{17}\) Since DHO binding site is highly polar, the scoring function of GOLD and Surflex may rank candidates more accurate than DOCK. Moreover no clear relationships could be found between rank of compounds by docking and scoring.\(^{17}\) For example, one compound docks better with a smaller
RMS deviation than another compound by GOLD may have a worse score. To have a more efficient procedure for virtual screening of DHOase inhibitors, a test screening of a smaller set of compounds containing a few known ligands can first be used to select the optimal docking and scoring combinations from the available programs. Then the scheme giving the highest hit rate can be applied to screen the entire set of compounds. Lastly, DHOase may not be an ideal target for docking in the discovery of inhibitors. Compared to the enzymes used as targets in the successful docking examples,8-10 DHOase has much less binding interactions with the substrate DHO. This may restrict the possible orientations of ligand at the binding site.

Though we were only able to identify one moderate noncompetitive inhibitor, zinc391516, against the DHO-bound subunit, the chemical structure and binding mode of zinc391516 to the active site of DHO revealed to us a novel class of inhibitors against DHOase. First, the chemical structure of zinc391516 is distinctive from the existing inhibitors targeting DHO binding site of E. coli DHOase (see Figure 5.5).18 The size of the molecule is much larger and it is not a substrate/product analog or a transition-state analog, which are the two categories for the existing inhibitors. The best pose of zinc391516 shown in Figure 5.6 shows that the molecule actually binds at the area above the pocket where DHO binds. The binding mode of zinc391516 agrees with the kinetic results that this compound is a noncompetitive inhibitor, binding at a different location from where DHO binds. Through this we discovered that the active site for DHO is unusual, as it is actually a pocket within a pocket. The product DHO binds at the bottom of the big pocket with
Figure 5.5. Structure comparison of inhibitor zinc391516 to the existing inhibitors designed to target the DHO bound active site of *E. coli* DHOase.¹⁴
Figure 5.6. The best docked pose of zinc391516 (thick lines) in the active site where DHO bound with protein surface shown in yellow and DHO (thin lines). Both zinc391516 and DHO are colored according to atom types (carbon atoms in white, nitrogen atoms in blue and oxygen atoms in red)
the upper pocket empty, which makes a way for the release of the product after the reaction. The upper pocket provides a novel binding site for the potential inhibitors to DHOase. A new class of inhibitor can be either a molecule filling up the space of the upper pocket or a fragment binding at the upper pocket while connecting to a DHO like portion. On the other hand, the active site for CA is rather small and closed. There is no open area above the site where CA binds. As seen in Figure 5.2 B the two inhibitors found through our vHTS system resembles similar structure to the substrate CA.

For future work there are two ways to improve the inhibition of zinc391516: (i) zinc391516 doesn’t fill up the entire upper pocket above DHO and there is still empty space left. The inhibition may be improved by extending the molecule to cover the whole pocket. An approached for this is to perform the docking to the binding pocket with DHO bound. It also might be possible to divide the upper pocket to sub-binding sites and use these binding sites separately as the target to search for the best fragment that can bind at a specific part of the large pocket. After all the fragments are found, they can be combined together and tested as one inhibitor. (ii) The part of zinc391516 trying to reach into the bottom of the pocket is a benzene ring at one end of the molecule. However, the DHO ring is a non-planar six-member ring, which has a different conformation from the benzene ring. The inhibition might be improved by modifying the benzene ring to a ring similar to DHO. When we modified the ring in silico and docked the modified zinc391516 (see Figure 5.7)
Figure 5.7. Modification of the benzene ring at one end of zinc391516 to a DHO-like ring improves the binding energy to DHOase calculated by AutoDock3.0.
using AUTODOCK3.0, the binding energy was improved from -14.57 kcal/mol to -15.41 kcal/mol.
References


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Chapter 6: Approach to Investigate the Loop Movement in

*Escherichia coli* Dihydroorotase Using a Genetically

Encoded Fluorescent Amino Acid
**Introduction**

Dihydroorotase (DHOase), which catalyzes the reversible cyclization of N-carbamyl-L-aspartate (CA) to L-dihydroorotate (DHO), belongs to the amidohydrolase superfamily, which comprises of a variety of hydrolytic enzymes of the (β/α)_{8}-barrel (or TIM-barrel) fold. The first structure of DHOase from *E. coli* was reported to a resolution of 1.7 Å by Thoden *et al.* (PDB ID 1J79) Although the *E. coli* DHOase has been reported to contain one zinc at the active site, this high resolution structure clearly showed a binuclear zinc center with a carboxylated lysine residue bridging the two metals. The crystals were grown in the presence of the racemic substrate, DL-CA, however surprisingly, the structure, which had a dimer in the asymmetric unit, revealed that one subunit (subunit B) had the substrate CA bound in its active site, while the other subunit (subunit A) had the product DHO bound in its active site. No explanation was given for this unusual structure. Subsequently, Lee *et al.* reported the structure of *E. coli* DHOase crystallized in the presence of the optically pure product, L-DHO, refined at 1.9 Å resolution (PDB ID 1XGE). Though the enzyme was crystallized in the presence of product DHO instead of the substrate CA, the structure confirmed the previous results. Importantly, this structure provided an explanation for the observation of CA bound to the active site of subunit B and DHO bound to the active site of subunit A. Comparison between the two subunits revealed that except for one loop, the 100’s loop comprised of residues 105-115, the rest of the two subunits were nearly
identical, with an RMS deviation of \( \alpha \)-carbon atoms of only 0.26 Å. The 100’s loop had significantly different conformation in each subunit. In subunit B, this loop reached in toward the active site (Figure 6.1), with two residues Thr109 and Thr110 directly making hydrogen-bonding contact with the bound substrate, CA ("loop-in" conformation). In subunit A, the 100’s loop flipped out of the active site (Figure 6.1), not interacting with the bound product DHO, and instead forming part of the protein surface ("loop-out" conformation).

Gerstein et al.\(^6\) proposed a mechanism of the movement of the loops during enzymatic reaction. They stated that the states of enzyme in the loop-in or loop-out conformations were only slightly different in energy and were in dynamic equilibrium at room temperature. The loops that constitute active site lids usually had different conformations depending on the catalytic state of the enzyme and the relative stabilities of the closed and open states of the loops depended upon the substrate bound in the active site. When substrate bound, the loop formed contacts with the substrate or the enzyme that stabilized the closed state of the loop. When product was formed, these interactions may be lessened thus making the open state of the loop more stable, which caused the release of the product favorable. Such loop movements involved in the reaction of enzyme were critical to the mechanism of catalysis.

The crystallography method has the limitation to probe the dynamics of the loop motion as the crystal structure can only capture a static state of the enzyme. In the crystal structure of subunit A with loop-out (PDB ID 1XGE), residues of the loop
Figure 6.1. The 100's loop conformations. The “loop-in” (shown in cyan) conformation in subunit B is observed when CA is bound at the active site and faces away from the active site as the “loop-out” (shown in green) conformation in subunit A when DHO is bound at the active site (not shown here). Thr109 and Thr110 form direct contact with CA when the 100’s loop reaches into the active site. Subunit A and B are compared after superposition.
were found to make hydrogen-bonding interactions with symmetry related molecules in the crystal. These interactions, involving only this subunit, would be sufficient to align the dimer in the crystal and made the two subunits distinguishable. Therefore there is a possibility that the observed “loop-in” and “loop-out” conformations were due to crystal packing forces. In order to determine if the loop positions observed in the structure occur in solution and the role of 100’s loop motion for catalysis, we employed the system developed by Schultz and co-workers, specifically the methodology of Wang et al. to genetically incorporate the fluorescent amino acid L-(7-hydroxycoumarin-4-yl)ethylglycine (HCE-Gly), which is sensitive to solvent polarity and pH, site-specifically on the 100’s loop of DHOase. These experiments validate the usefulness of the methods developed by Schultz and co-workers to incorporate unnatural amino acids into the structure of proteins as a useful probe for many biological studies.
Results and Discussion

Selection of HCE-Gly Incorporation Site

One of the challenges is the selection of the appropriate location to place HCE-Gly, where it does not influence the function of the enzyme but the movement of the loop should perturb the local environment of the HCE-Gly in such a fashion that its fluorescence would be altered. After visual examination of the DHOase structure with in silico mutation of the protein residue to HCE-Gly in Pymol and sequence alignment of the amino acid sequence of DHOases, four sites were selected for incorporation of HCE-Gly: Asn107, Ser113, His114 and Ala46. The first three positions are within the 100's loop. Alignment of ~500 DHOase sequences was carried out using ClustalW. Shown in Figure 6.2 is a representation of the conserved residues in the 100's loop region of DHOase from the alignment result. Asn107, Ser113 and His114 were least conserved and would be the first candidates for a site to introduce the fluorescent probe. Ala46, a residue that was not conserved located on the edge of the active site pocket and so predicted to be sensitive to the movement of the loop, was another possible location for the fluorescent probe.

Expression and Purification of HCE-Gly DHOase

Our initial attempt to express the HCE-Gly DHOase using plasmid, pSup-CouRS-D8, which had been constructed by the Schultz Lab for a more efficient incorporation of the unnatural amino acid, was not successful. Instead two plasmids were utilized to successfully incorporate HCE-Gly into DHOase. The first plasmid
Figure 6.2. Alignment of the 100's loop (residues 105-115) region from about 500 DHOase sequences. The larger the letter the more conserved the residue is. This figure was created with WebLogo.10
pBK-CouRS-D8\textsuperscript{7} contained the gene for a special \textit{Methanococcus jannaschii} tyrosyl-tRNA synthetase, which was paired with a mutant \textit{M. jannaschii} tyrosyl amber suppressor tRNA\textsuperscript{7} to uniquely insert HCE-Gly in response to a TAG amber stop codon. The tRNA was supplied by the second plasmid, pEK701, a derivative of pBAD/JYAMB-4TAG-Myo,\textsuperscript{7} in which the myoglobin gene was replaced by the \textit{E. coli} \textit{pyrC} gene, encoding DHOase. The codons at position 107 or 114 of DHOase were altered to the amber codon TAG by site-directed mutagenesis but attempts to alter the other two selected sites 46 and 113 were not successful. The expression was performed by growth of Top10 cells (Invitrogen) containing both of the above plasmids in glycerol minimal media supplemented by 1.0 mM HCE-Gly, which was synthesized as described previously.\textsuperscript{7} Protein was purified by the Ni\textsuperscript{2+} affinity chromatography as a His\textsubscript{6} tag was introduced at the C-terminal of the DHOase sequence. The success of incorporation was confirmed directly by gel electrophoresis, since the modified protein is fluorescent (see Figure 6.3). The level of suppression of the TAG codon was significantly better at position 114 than 107. We were unable to obtain sufficient HCE-Gly107 DHOase for further investigations.

\textbf{Kinetic and Fluorescence Analysis of HCE-Gly114 DHOase}

From the results of the colorimetric assay of DHOase carried in the reverse reaction from DHO to CA, the HCE-Gly114 DHOase exhibited about 10-fold less activity than the His-tag DHOase into which the HCE-Gly was incorporated (See Table 6.1). From the sequence alignment of the 100’s loop from \~500 DHOases (See Figure 6.2) 8 out of 11 residues comprising the 100’s loop were highly conserved,
**Figure 6.3.** HCE-Gly114 and His-tag DHOases are visualized on the SDS-PAGE gel stained by Coomassie blue (right). The fluorescence of HCE-Gly114 DHOase on the same SDS-PAGE gel can be visualized under UV light (left).
### Table 6.1. Kinetic parameters of the His-tag and HCE-Gly114 DHOases

<table>
<thead>
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<th>$V_{\text{max}}$ $^a$</th>
<th>$[S]_{0.5}$$^b$</th>
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<tbody>
<tr>
<td>His-tag DHOase</td>
<td>7.79 ± 1.11</td>
<td>0.27 ± 0.075</td>
</tr>
<tr>
<td>HCE-Gly114 DHOase</td>
<td>0.73 ± 0.022</td>
<td>0.15 ± 0.019</td>
</tr>
</tbody>
</table>

All experiments were performed at 25 °C in 50 mM Tris-phosphate buffer at pH 8.0. The values reported are the average deviation of three determinations.

$^a$The maximal observed velocity of CA formation (mmol hr$^{-1}$ mg$^{-1}$)

$^b$Substrate concentration [DHO] at half of the maximal observed velocity (mM)
which indicate the importance of the 100’s loop for the function of DHOase. Though His114 was not conserved and didn’t form direct contacts with either Zn atoms or substrate CA at the active site, the hydrogen-bonding interaction between His114 and Tyr79 was critical for anchoring the 100’s loop to the remainder of the chain. Replacement of His114 with HCE-Gly might eliminate this interaction destabilizing the 100’s loop, which could possibly cause the Thr109 and Thr110 not positioned correctly to CA and influence the catalysis. Interestingly, DHO appeared to have a higher binding affinity to HCE-Gly114 DHOase than His-tag DHOase based on kinetic data. As seen in Table 6.1, the half-saturating concentration of DHO for the fluorescent DHOase was about half of that for the His-tag DHOase. Despite the fact that the incorporation of the HCE-Gly at position 114 was not as expected to produce a functional DHOase with kinetic properties very similar to the His-tag DHOase, the fluorescence measurements of HCE-Gly114 DHOase were performed since HCE-Gly114 DHOase was the only successful incorporation at the desired location in DHOase among the four selected sites.

The structure of E. coli DHOase crystallized in the presence of DHO (PDB ID 1XGE) revealed that the 100’s loop adopted the loop-out conformation when DHO bound at the active site and the loop-in conformation when CA bound at the active site. However, for the DHOase dimers in solution there can be many combinations of loop conformations that were possible since the reaction of CA to DHO was reversible. Therefore it would be hard to depict the loop motion if we measure the fluorescence changes of HCE-Gly114 DHOase in the presence of either DHO or CA.
The structure of *E. coli* DHOase complexed with 5-fluororate (FOA), a product DHO-like inhibitor of DHOase, showed that in subunit A the loop was out when the active site was fully occupied by FOA while in subunit B parts of the 100’s loop (residues 108-114) were disordered when the active site was partially occupied by FOA. When FOA bound to the enzyme the loop-in conformation was not feasible because FOA cannot make hydrogen bonding to Thr109 and Thr110 of the 100’s loop, which was required for the loop-in conformation. Therefore fluorescence changes caused by the movement of the 100’s loop with addition of FOA were monitored. (Methods described in Chapter 7) As the concentration of FOA increased up to 30 mM, the fluorescence intensity at 455 nm decreased significantly with almost no shift in the emission maximum (see Figure 6.4 A) The relative fluorescence was plotted as a function of FOA concentration, and the concentration of FOA required for half-maximal reduction in the relative fluorescence is 8.84 ± 1.63 mM (see Figure 6.4 B). As a comparison, the inhibition of FOA was measured for the HCE-Gly114 DHOase at the same pH and buffer (see Figure 6.5). The concentration of FOA required for half-maximal inhibition (IC\textsubscript{50}) is 4.04 ± 0.27 mM. If the alteration of fluorescence intensity reflects the binding of FOA to the enzyme, the concentration of FOA required for half-maximal reduction in relative fluorescence should agree with the IC\textsubscript{50} value from the kinetic data. However from our experiments, the concentration of FOA when the relative fluorescence reaches half-maximum is about two-fold greater than the IC\textsubscript{50} obtained from the protein assay of HCE-Gly114 DHOase in the presence of FOA. The fluorescence response to
Figure 6.4. Monitoring inhibitor FOA binding to HCE-Gly114 DHOase by fluorescence. (A) Fluorescence spectrum of HCE-Gly114 DHOase as a function of the FOA concentrations. HCE-Gly114 DHOase (32 µg/ml) in 50 mM Tris-phosphate, pH 8.0 at 25°C was excited at 355 nm, and the emission spectrum was measured from 400 – 550 nm. As the FOA concentration was increased from 0 mM (open circle) to 30 mM (solid circle), the emission intensity at 455 nm decreased significantly. (B) Corresponding plot of the fluorescence intensity at 455 nm normalized to the highest value (0 mM FOA) versus the FOA concentration. The data points shown are the average of five determinations.
Figure 6.5. Inhibition of FOA to HCE-Gly114 DHOase measured by colorimetric assay. All measurements were made at 30° C in 50 mM Tris-phosphate buffer, pH 8.0 with 0.06 mM DHO and 4 μg HCE-Gly114 DHOase. Each data point shown is the average of three determinations.
the binding of FOA reveals a weaker apparent FOA affinity to the enzyme than that from the kinetic study. The fact that the replacement of HCE-Gly at position 114 greatly affects the function of the enzyme might be responsible for the disagreement between fluorescence and kinetic data. Therefore position 114 is not an ideal location for fluorescent probe HCE-Gly to monitor the binding of ligand at the active site. For future studies of DHOase with HCE-Gly the position of incorporation should not be on the 100's loop but another site outside the loop that would still be sensitive to the loop motion.
References


Chapter 7: Materials and Methods
**Materials and General Methods**

Ampicillin, kanamycin, tetracycline, magnesium chloride hexahydrate, zinc chloride, ATP, UTP, glycine, glucose, PEG4000, EDTA, agar, L-aspartate, N-carbamoyl-L-aspartate, 2-mercaptoethanol, uracil, ethidium bromide, arabinose and bis-Tris were obtained from Sigma-Aldrich. Compounds tested for virtual screening (zinc391516, zinc902674, zinc903866, zinc402968, zinc394318) were also purchased from Sigma-Aldrich. CTP was from Santa Cruz Biotechnology Inc. Tris, imidazole, CAPS, electrophoresis-grade acrylamide and enzyme-grade ammonium sulfate and GTP were from MP Biomedicals. Antipyrine, diacetylmonoxime, sodium molybdate dihydrate, tryptone and yeast extract were obtained from Fisher. Casamino acid was from Becton, Dickinson and Company. Agarose, ammonium persulfate (APS), sodium dodecyl sulfate, coomassie brilliant blue, Chelex 100 resin, the protein assay dye and the Bio-Prep SE-1000/17 column for size exclusion chromatography were purchased from Bio-Rad. L-dihydroorotic acid was obtained from Chem-Impex International. 5-fluoroorotic acid (5-FOA) was from Research Products International Corp. The oligonucleotides required for site-specific mutagenesis and sequencing were obtained from Operon Technologies. Crystallization VDX™ plates, siliconized glass circle cover slides and cryo-mounting loops were purchased from Hampton Research. Carbamoyl phosphate dilithium salt obtained from Sigma was purified before use by precipitation from 50% (v/v) ethanol and was stored desiccated at -20°C.¹
Protein Expression and Purification

Wild-type ATCase

Wild-type ATCase holoenzyme was overexpressed utilizing *E. coli* strain EK1104 [F *ara, thi, Δ(pro-lac), ΔpyrB, pyrF*, *rpsL]*\(^2\) containing plasmid pEK152.\(^3\) The enzyme was isolated based on the procedure described previously\(^2\) and purified by isoelectric precipitation at pH 5.8, ion-exchange and hydrophobic interaction chromatography following ammonium sulfate precipitation. For the ion-exchange purification, a Q-Sepharose Fast Flow (GE Healthcare) column (11 cm x 2.5 cm) was employed. The column was first equilibrated with 0.05 M Tris-acetate, 2 mM 2-mercaptoethanol, pH 8.3 (Low Q buffer) at a flow rate of 1.0 mL/min. Protein sample was loaded onto the column after being dialyzed against two changes of 4 liters of Low Q buffer for 12 hrs per change. The column was washed with 60 mL Low Q buffer, and the protein was eluted using a linear gradient developed from 100-mL volume of Low Q buffer and Low Q buffer plus 0.5 M sodium chloride for every 1 liter of culture harvested. 5.0 mL fractions were collected at a flow rate of 1.0 mL/min. For the hydrophobic interaction chromatography, a Phenyl Sepharose High Performance (GE Healthcare) column (8.5 cm x 2 cm) was employed. After the ion-exchange chromatography, ATCase containing fractions were dialyzed into Low Q and brought to 20% saturation with ammonium sulfate and loaded onto the Phenyl Sepharose column pre-equilibrated with Low Q buffer plus 20% saturating ammonium sulfate. The column was washed with 60 mL of Low Q buffer plus 20% saturating ammonium sulfate at a flow rate of 1.0 mL/min. After that the protein
was eluted using a linear gradient from Low Q buffer plus 20% saturating ammonium sulfate to Low Q buffer with a total volume of 300 mL at the same flow. The purity of the enzyme was checked by SDS-PAGE and non-denaturing PAGE. The concentration of the purified wild-type ATCase was determined by absorption at 280 nm with an extinction coefficient of 0.59 cm²mg⁻¹.

**K164E/E239K ATCase**

The mutations K164E/E239K were introduced to the catalytic chains of ATCase on plasmid pEK152 by site-specific mutagenesis employing the Quick-Change Site-Directed Mutagenesis Kit (Stratagene) to produce plasmid pEK695. The double mutant was isolated based on the procedure described previously, from over-producing *E. coli* strain EK1104, containing the plasmid pEK695. The enzyme was purified by isoelectric precipitation, ion-exchange and hydrophobic interaction chromatography as indicated above for the wild-type enzyme. The homogeneity of the protein was checked by SDS-PAGE. The concentration of purified double-mutant enzyme was determined by the Bio-Rad version of Bradford's dye-binding assay using wild-type ATCase as the standard.

**Wild-type DHOase**

Wild-type DHOase was overexpressed from utilizing *E. coli* strain EK2110 (also named as X7014a which lacks a functional gene for DHOase) containing plasmid pEK662 which is a gift from Prof. Frank M. Raushel of Texas A&M University. Cells in YT media containing 150µg/mL ampicillin were incubated at 37°C until they reached mid-log phase, at which point IPTG was added to a
concentration of 0.4 mM. After overnight incubation, the cells were harvested by centrifugation, sonication, and purified as described previously\(^9\) with the modification of employing the Q-Sepharose Fast Flow (GE Healthcare) column (11 cm x 2.5 cm) followed by Phenyl Sepharose High Performance (GE Healthcare) column (8.5 cm x 2 cm) after the ammonium sulfate precipitation step. The procedure of chromatography is similar to that for ATCase purification described above. For the ion-exchange purification, the column was first equilibrated with 50 mM Tris-phosphate, 100 μM ZnCl\(_2\), pH 7.0, (Low D buffer) at a flow rate of 1.0 mL/min. After the protein was loaded the column was washed with 60 mL Low D buffer. The protein was eluted from the column at the same flow rate using a linear gradient from Low D buffer to Low D buffer plus 1.0 M sodium chloride in a total volume of 100 mL for every 1 liter of culture harvested. After the ion-exchange chromatography, DHOase containing fractions were dialyzed into Low D and brought to 30% saturation with ammonium sulfate and loaded onto the Phenyl Sepharose column pre-equilibrated with Low D buffer plus 30% saturating ammonium sulfate. The protein was eluted using a linear gradient from Low D buffer plus 30% saturating ammonium sulfate to Low D buffer in a total volume of 300 mL at flow rate of 1.0 mL/min. The purity of the enzyme was checked by SDS-PAGE\(^4\) and non-denaturing PAGE\(^5,6\). The concentration of the purified wild-type DHOase was determined by absorption at 278 nm with an extinction coefficient of 0.638 cm\(^2\)mg\(^{-1}\).\(^{10}\)
HCE-Gly114 DHOase

The DHOase gene of the plasmid pEK755, which was used to express the fluorescent DHOase, was originated from that of plasmid pEK668. This plasmid contains the *E. coli pyrC* gene with a C-terminal His_{6} tag, which was inserted by site-specific mutagenesis (Quick-Change Site-Directed Mutagenesis Kit from Stratagene) using pEK662\(^9\) as template. A Kpn I site, which was downstream to the 6 His-tail, and a Nco I site overlapping the ATG initiation codon of the *pyrC* gene were introduced on plasmid pEK668 by site-specific mutagenesis employing the same method as described above to produce plasmid pEK700. Then both pEK700 and pBAD/JYAMB-4TAG-Myo\(^{11}\) were digested with Kpn I and Nco I for 2.5 hrs at 37\(^\circ\) C. The fragment from pEK700 carrying the *pyrC* gene and the backbone of pBAD/JYAMB-4TAG-Myo\(^{11}\) were extracted and purified after DNA agarose gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen). The fragment and backbone were then mixed and treated with T4 DNA Ligase in 1 x T4 DNA ligase reaction buffer (New England Biolabs) at 16\(^\circ\) C for 16 hrs. Competent Top10 cells (Invitrogen) were transformed with the ligation reaction. Cells containing the ligation products were selected on YT plates containing tetracycline. Individual colonies were used to inoculate 5 mL YT cultures, which were grown at 37\(^\circ\) C overnight. The plasmids were isolated from the overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen). After digested with Kpn I and Nco I, the plasmids showing both bands for the *pyrC* gene and the backbone of pBAD/JYAMB-4TAG-Myo on the DNA agarose gel were sent for sequencing. The successful ligation
products were verified by the sequencing results and stored as pEK701. Finally, site-directed mutagenesis was performed on plasmid pEK701 to substitute His114 in DHOase for an amber stop codon TAG to create pEK755. This expression plasmid pEK755 contains the pyrC gene under the control of the arabinose promoter, the gene for the Methanococcus janaschii tyrosyl amber suppressor tRNA that specifically inserts unnatural amino acid HCE-Gly in response to the TAG codon and the tetracycline-resistant gene.

Plasmid pEK755 was cotransformed with pBK-CouRS-D8 into competent Top10 cells (Invitrogen) and plated on 2 LB plates with 12.5 µg/ml tetracycline and 50 µg/ml kanamycin. Cells were amplified in 2 LB media (5 ml) with the same antibiotics. Starter culture (1 ml) was used to inoculate 500 ml of liquid M9 glycerol minimal media supplemented with 0.5% casamino acids (Bacto), 12.5 µg/ml tetracycline, 50 µg/ml kanamycin, and 1.0 mM HCE-Gly. Cells were then grown at 37°C to an OD₆₀₀ of 0.5 and protein expression was induced by the addition of 0.2% (g/ml) arabinose. After further incubation at 37°C for 12 hrs, cells were harvested by centrifugation and lysed by sonication. The HCE-Gly114 DHOase was then purified by immobilized metal ion affinity chromatography (IMAC) employing Chelating Sepharose™ Fast Flow (GE Healthcare) column (2 cm x 1.5 cm) charged with Ni²⁺. The column was pre-equilibrated with 50 mM Tris-phosphate, 200 mM NaCl, pH 7.0 at 1.5 mL/min. After protein sample was loaded, the column was sequentially washed with 40 mM (6 mL), 100 mM (6 mL), 200 mM (12 mL) and 400 mM (8 mL) imidazole in 50 mM Tris-phosphate, 200 mM NaCl, pH 7.0 at 1.0
mL/min. The pure HCE-Gly114 DHOase enzyme was found to elute with 200 mM imidazole. Purity was verified by SDS PAGE.\textsuperscript{4}

**Aspartate Transcarbamoylase Assay**

**Kinetic characterization at 25° C and 5° C**

The activity of ATCase was measured by the colorimetric method\textsuperscript{12} in the 50 mM Tris acetate buffer, pH 8.3, and in the presence of 4.8 mM CP. L-Asp saturation curves in the absence and presence of nucleotide effectors (ATP, CTP and UTP) were performed at both 25° C and 5° C. Influence of ATP and CTP on the activity of ATCase in the absence and presence of 20% ethylene glycol were observed at 5° C. All data were collected in duplicate and averaged. The data analysis was carried out as described previously.\textsuperscript{13}

**Nucleotide Saturation Curves**

Saturation curves of nucleotide effectors ATP, CTP, GTP and UTP were performed in the absence and presence of Mg\textsuperscript{2+} at 28° C. Pretreatment of nucleotides with Chelex 100 resin (Bio-Rad) were carried out to remove any metal contaminations in the commercial supply. 0.05 g of Chelex 100 resin was added to every 1.0 mL nucleotide solution. The mixture was stirred gently on ice for 1 hr. Resin was removed by centrifugation and the nucleotide solution with metal removed was obtained as supernatant. Spectrophotometry was used to determine the concentrations of NTP (ATP, CTP, GTP and UTP) solutions: at pH 7.0, $\varepsilon_{\text{ATP}} = 15400$ at 259 nm, $\varepsilon_{\text{CTP}} = 9000$ at 271 nm, $\varepsilon_{\text{GTP}} = 13700$ at 253 nm, and $\varepsilon_{\text{UTP}} = 10000$ at 262 nm.\textsuperscript{14} Mg-NTP solutions were made with addition of MgCl\textsubscript{2}•6H\textsubscript{2}O. Due to the
critical dependence of enzyme activity on pH\textsuperscript{15} all NTP and Mg-NTP solutions were carefully adjusted to pH 7.0 ± 0.05 before use. Colorimetric assays\textsuperscript{12} were performed in a tri-part buffer at either pH 7.0 or pH 8.3, containing 20 mM Bis-Tris, 20 mM Tris, and 20 mM CAPS, in the presence of a saturating concentration of carbamoyl phosphate (2.0 mM). In all assays the aspartate concentration was 5.0 mM (the value of $[S]_{0.5}$ at pH 7.0)$^{16}$ and the concentration of wild-type ATCase varied from 6 x 10$^{-5}$ to 30 x 10$^{-5}$ mg/ml. Data analysis of the steady-state kinetics was carried out as described previously.$^{13}$

**Dihydroorotase Assay**

**Kinetic Characterization**

The degradative activity of DHOase and inhibition of FOA against DHOase were measured by the rate of CA formation using modified colorimetric assay procedures,$^{12}$ which had been used many years for the kinetic studies of ATCase. Each reaction was initiated with addition of buffer containing DHOase instead of CP in the assay for ATCase. All colorimetric assays were performed at 25° C in 50 mM Tris-phosphate buffer at pH 8.0.

The direct assay used to measure the biosynthetic conversion of CA to DHO was performed as described by Sander \textit{et al.}$^{17}$ The reaction mixture contained 75 mM Na$_2$HPO$_4$ buffer (pH 5.5) and 1 mM DL-CA in a total volume of 1.0 ml. The reaction was initiated by the addition of enzyme at 37° C. The linear increase in absorbance at 230 nm due the formation of DHO ($\varepsilon_{230\text{nm}} = 1.17$ mM$^{-1}$ cm$^{-1}$) was followed. The concentration of inhibitors ranged from 0 to 2 mM in the assays.
Fluorescence Measurements

For the HCE-Gly114 DHOase (Chapter 6), all steady-state measurements were performed using a JASCO spectrofluorometer FP-6300 in a quartz SUPRASIL fluorescence cell (Hellma) with a slit width of 5 nm at 25°C in 50 mM Tris-phosphate buffer at pH 8.0 and 32 μg/ml HCE-Gly114 DHOase. An excitation spectrum from 250-420 nm was obtained to determine the maximum excitation wavelength. Using an excitation wavelength of 355 nm at pH 8.0, a fluorescence spectrum from 400-600 nm was obtained in the absence of the inhibitor FOA with maximum emission at 455 nm. Microliter amounts of the inhibitor FOA was added to the fluorescence cell up to a concentration of 30 mM. At each concentration of FOA, a fluorescence spectrum was recorded and the maximum fluorescence intensity was determined. Data was adjusted by dilution factor as more inhibitor FOA was added to the fluorescence cell.

Crystallography

Crystallization and Data Collection

A sample of 10 mg/ml purified K164E/E239K ATCase was sent to the Hauptman-Woodward Institute for high-throughput screening of crystallization conditions. The final crystallization condition was developed based upon one of the screening results. The mutant enzyme was crystallized by the hanging-drop vapor diffusion method. 2 μl of enzyme (10 mg/ml) in 50 mM Tris-acetate, pH 8.3 was mixed with 2 μl of crystallization buffer (16% (w/v) PEG 4000, 0.04 M Na₂MoO₄•2H₂O, 0.04 M CAPS and 0.03 M Tris-acetate at pH 8.75) and equilibrated
over a reservoir of 1.0 ml of crystallization buffer at 20° C. Bar-shaped crystals grew to average dimensions of 0.3 x 0.3 x 0.4 mm in about two weeks.

Crystals were transferred into a freezing solution containing 20% (v/v) ethylene glycol in crystallization buffer for 1 min prior to freezing in liquid N₂. Data were collected in the Boston College Crystallography Facility. X-rays were generated using a Rigaku MicroMax-007HF rotating-anode generator operating at 40 kV and 30 mA. The diffraction data were integrated, scaled and averaged using d*TREK.¹⁹

**Structure Determination**

Attempts to solve the structure of the K164E/E239K ATCase by molecular replacement were successful using the T-state ATCase structure (PDB ID 1ZA1) rather than the R-state PALA-ATCase structure in the same space group (PDB ID 1Q95). First, one catalytic trimer (c₃) of the double mutant was found by using the c₃ of the T-state structure (PDB ID 1ZA1) in MOLREP²⁰ as implemented in CCP4.²¹ MOLREP was also able to locate the second c₃. MOLREP was then used to locate the three regulatory dimers (r₂) by fixing the coordinates of the two c₃ subunits. Combining the two c₃ and three r₂ subunits positioned by MOLREP the initial model of the holoenzyme (c₆r₆) in the asymmetric unit was established. Refinement of the structure was performed using PHENIX.²² The 80’s loops (residues 77 to 84) and 240’s loops (residues 231 to 245) of all the catalytic chains were rebuilt into the refined structure using the Autobuild protocol in PHENIX.²² The loop positions were further checked using composite omit maps, calculated using RESOLVE²³ within PHENIX, and displayed in COOT²⁴,²⁵ averaging over the 6-fold non-crystallography
symmetry. The 80’s and 240’s loops of the catalytic chains had elevated B-factors, which were higher than in the T- or R-state structure of the wild-type enzyme. The higher B-factors are most likely due to loss of a number of loop-stabilizing interactions. Even though the density of these loops was weak and the B-factors high, the atomic coordinates have been included for completeness. Water molecules were added to the structure using PHENIX on the basis of the \( F_o-F_c \) map at or above the 3.0 \( \sigma \) level. After completion of the refinement, the final \( R_{\text{work}} \) and \( R_{\text{free}} \) were 0.214 and 0.274, respectively. The model was checked for errors using PROCHECK. Residues 1-9 in the regulatory chains were omitted from the deposited structure due to weak electron density.

**Virtual High Throughput Screening**

**Setup of the Virtual High Throughput Screening System (vHTS)**

In order to perform the virtual screening with a large scale of compounds, MySQL software (http://www.mysql.com) was selected both as the database for the storage of the information of compounds for screening, as well as the database for the storage of docking results. As seen in Figure 7.1, three databases had been set up on the MySQL server. (1) The **Compounds** database was the storage location for the information on each of the compounds in their appropriate formats (mol2 or pdbq format) required for the screening programs. All the data for the compounds were obtained from the non-commercial public ZINC database.
Figure 7.1. Organization of the MySQL databases and tables for the vHTS system. Databases are indicated in yellow and the tables in green.
(http://blaster.docking.org/zinc/), which contained a large number of small molecule compounds that were purchasable. About 3,000,000 compounds were downloaded and rearranged to four tables named all_buy1 to all_buy4, each of which contained about 750,000 compounds. (2) The Setup database was the storage location for the files required for docking, which were program dependent. There was also a table containing pointers to program executable (exe) tar files, which included the specific information the vHTS system needed to execute a particular docking program. (3) The Results database was the storage location for the output of the docking runs. The tables within Results database were set up according to the particular docking program used and the target for docking.

The entire virtual high throughput screening (vHTS) system was controlled by a shell script written by Dr. Kantrowitz. Shown in Figure 7.2 is a schematic of how the vHTS system works. Execution of the vHTS was performed on the Boston College Linux Cluster. The shell script would first determine the type of computer and operating system. Based upon this information, the User Input file would then direct the script to download the files from the Setup database required for the docking to the particular receptor with the program selected by the user. The User Input file would also provide the parameters to select the particular set of compounds from the Compounds database. After the docking was performed, the results was extracted from the log files and then input to the designated table in the Results database specified in the User Input file.
Figure 7.2. Schematic of the vHTS system showing the program flow of the shell script based upon the **User Input** file. The MySQL databases are shown in yellow connected with the tables. In this example, the docking of compounds in the **all_buy1** table is performed on the active site of DHO-bound subunit of DHOase using the Surflex program.
**Preparation of Protein Coordinates**

Target protein coordinates used for docking were taken from the 1.9 Å X-ray structure of *E. coli* DHOase (PDB ID 1XGE) in complex with substrate CA in one subunit and product DHO in the other subunit. Since the surface of the active site was different between the two subunits due to the position of the 100s loop (residue 105-115), the CA-bound subunit was target for the screening of inhibitors against CA while the DHO-bound subunit was target for the screening of inhibitors against DHO. Prior to the virtual screening the bound substrate CA and product DHO were removed from the active site.

**Docking and Scoring of Ligands**

Molecular docking has been widely used to computationally predict the binding mode of a small molecule (ligand) to a receptor (often protein). During the docking process a number of binding modes for each ligand are generated and for each binding mode a score is given based on the evaluation of the intermolecular interactions between the ligand and the protein receptor. The aim of the docking is to find the optimized docking conformation for each ligand to the receptor with the minimum free energy of binding. The differences among docking programs thus mainly reside in these two parts: the methods employed to search for the optimized binding mode and the scoring functions. Here in our study the four docking programs used for the virtual screening were AutoDock3.0, DOCK5.0, Surflex and GOLD. Xscore was used as a score function for the docking results from the
four docking programs with default parameters. The details about docking are described below.

*AutoDock3.0*

AutoDock3.0 uses a new hybrid genetic algorithm search method, referred as the Lamarckian genetic algorithm, in combination with an empirical binding free energy function to predict the binding mode of flexible ligands to non-flexible macromolecular receptors. Unlike most genetic algorithms that mimic the Darwinian evolution, the hybrid genetic algorithm employs a traditional genetic algorithm as global optimization with local search technique, and resembles Jean-Baptiste Lamarck’s idea of evolution: phenotypic characteristics of an individual acquired during its lifetime that can be inherited by its offspring. The traditional genetic algorithm will be described in more detail in the section for GOLD. Here in AutoDock, the ligand’s state, which is used to describe the translation, orientation and conformation of the ligand to the protein, corresponds to the genotype. The ligand’s atomic coordinates and energy calculated from that represent the phenotype. In Lamarckian genetic algorithm, new genotype can be obtained from a phenotype found by local search whereas in the traditional genetic algorithm new genotype can only be obtained from another genotype. The scoring function used to evaluate the docking conformations in AutoDock3.0 is improved from previous versions. Besides the typical molecular mechanics terms for Van der Waals potential, hydrogen bonding, and electrostatics, two new terms have been included: desolvation free energy of the ligand and restriction of conformational degrees of
freedom of the ligand upon binding. The coefficients of all the terms in the free energy function are empirically derived using linear regression analysis from a set of 30 protein-ligand complexes with known structures and binding constants. To calculate the free energy of binding, AutoDock requires potential grid maps pre-calculated by AutoGrid, for each atom type of the ligands. A grid map is a cubic lattice composed of grid points with designated spacing. For each atom type the potential energy at each grid point is determined by the set of parameters used for that particular atom type.

In our docking for DHOase, The grid box was set at 40 Å³ centered at the middle of the active site with a grid spacing of 0.275 Å between grid points. For each ligand, 25 Lamarckian genetic algorithm runs were carried out with a maximum of 500,000 energy evaluations performed on a single population of 50 individuals. For the zinc atoms located in the active site of DHOase, atomic affinity grid maps were generated with equilibrium internuclear separation $R_{ij}$ and energy well depth $\varepsilon_{ij}$ at $R_{ij}$ set as 1.95 Å and 0.25 kcal/mol, respectively. Other parameters used for docking and scoring were standard.

**DOCK5.0**

In DOCK5.0 the ligand was docked to the binding site of the protein via a geometric matching method. In this method both ligand and protein are treated to be rigid. The solvent accessible protein surface area of binding site is calculated and spheres are created to fill the empty space complementary to this calculated binding surface. Prior to docking the energy grid (a cubic lattice covering the binding site) is
calculated. Every point on the lattice is assigned a score on the basis of its contacts with protein atoms. The ligand atoms are then matched to sphere centers and scored against the energy grid by mapping onto the nearest points on the lattice.

In our docking for DHOase, a maximum of 1000 orientations were cycled through the matching to the receptor spheres for each ligand. Once the largest rigid substructure of the ligand was fixed by matching to the receptor spheres, 10 conformations of the remaining portion of the ligand with altered torsion angles were tested and scored. The best-scored conformation was carried to the next cycle. The search cycled until the top three solutions were within 1.5 Å rmsd of each other. Other parameters used for docking and scoring were standard.

**Surflex**

Surflex operates by a surface shape-based method, aligning each test ligand to the idealized binding site ligand (called a protomol) generated from the protein structure. Each ligand being docked is first divided into 1 to 10 molecular fragments. Alignment of each conformation of each fragment is performed to identify the poses that have the most molecular similarity to the protomol. The aligned fragments are scored and the whole molecule approach is used to construct full molecules from the aligned fragments. The poses with best score are then output along with their scores. The function used to score the docking poses is a continuous differentiable function which contains a hydrophobic term, polar terms (hydrogen bonding and electrostatics), entropic terms and solvation terms.
The coefficients of a linear combination of these terms are empirically determined by a set of 34 protein-ligand complexes with known binding affinities.\textsuperscript{33}

In our docking for DHOase, because the active site volume of DHO-bound subunit is larger than that of the CA-bound subunit, “protomol” was generated using two different methods. The “protomol” for the screening of inhibitors against DHO was generated using the residue-based method. The residues Tyr104, Pro105, Glu141, His139 and Leu222 in the active site of the DHO-bound subunit were selected. The “protomol” for the screening of inhibitors against CA was generated using the ligand-based method, where the crystal structure of CA bound in the active site was served as ligand. Standard parameters were used for docking and scoring.

\textit{GOLD}

GOLD, standing for “Genetic Optimisation for Ligand Docking”, uses a genetic algorithm method to search for the optimized binding mode of ligand and protein with the allowance of full range flexibility of ligand and limited protein flexibility in the binding site.\textsuperscript{31} The search process employed genetic algorithm imitates natural evolution. This method starts with a randomly created population of solutions represented by chromosomes. Each chromosome codes for the values of a possible docking pose of ligand within the binding site of protein, such as the translation, orientation and conformation information of the ligand. A scoring function, which measures the energy of hydrogen bonding and the hydrophobic interactions involved in binding and the internal energy of the ligand, is used to evaluate the fitness of each docking solution. A new population is then created following these
steps: parent chromosomes are selected based on the linear-normalized fitness scores; genetic operators are applied to form child chromosomes, fitness scores of which are then calculated. If the solution is new to the population, then it replaces the least fit solution in the population. The search cycles until a designated number of operations are applied. The most important factor in generating the new solution is the genetic operator. In GOLD three genetic operators, crossover (or recombination), mutation and migration, are used. Crossover selects genes from parent chromosomes and recombines to form child chromosomes. Mutation changes any site on the chromosomes. Migration copies and moves individual chromosomes among the subpopulations.

In our docking for DHOase, there were 10 independent genetic algorithm (GA) searches for each ligand. Algorithm performed on a single population of 100 solutions and terminated after the application of 10,000 genetic operations, outputting the docking poses with best fitness scores. Operator weights, based on which the operators were chosen, were set to 100, 100 and 0 respectively for crossover, mutation and migration. The common metal ion coordination parameters have already been incorporated into the GOLD docking program. The coordination geometries of two zinc atoms in the active site of DHOase were set to 4 (tetrahedral) and 5 (trigonal bipyramidal) respectively according to their interactions found in the crystal structure of DHOase. Other parameters used for docking and scoring were standard.
References


Appendix: Published Papers
Here, we present a study of the conformational changes of the quaternary structure of *Escherichia coli* aspartate transcarbamoylase, as monitored by time-resolved small-angle X-ray scattering, upon combining with substrates, substrate analogs, and nucleotide effectors at temperatures between 5 and 22 °C, obviating the need for ethylene glycol. Time-resolved small-angle X-ray scattering time courses tracking the $T \rightarrow R$ structural change after mixing with substrates or substrate analogs appeared to be a single phase under some conditions and biphasic under other conditions, which we ascribe to multiple ligation states producing a time course composed of multiple rates. Increasing the concentration of substrates up to a certain point increased the $T \rightarrow R$ transition rate, with no further increase in rate beyond that point. Most strikingly, after addition of $N$-phosphonacetyl-L-aspartate to the enzyme, the transition rate was more than 1 order of magnitude slower than with the natural substrates. These results on the homotropic mechanism are consistent with a concerted transition between structural and functional states of either low affinity, low activity or high affinity, high activity for aspartate. Addition of ATP along with the substrates increased the rate of the transition from the $T$ to the $R$ state and also decreased the duration of the $R$-state steady-state phase. Addition of CTP or the combination of CTP/UTP to the substrates significantly decreased the rate of the $T \rightarrow R$ transition and caused a shift in the enzyme population towards the $T$ state even at saturating substrate concentrations. These results on the heterotropic mechanism suggest a destabilization of the $T$ state by ATP and a destabilization of the $R$ state by CTP and CTP/UTP, consistent with the $T$ and $R$ state crystallographic structures of aspartate transcarbamoylase in the presence of the heterotropic effectors.

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**Keywords:** cooperativity; quaternary structure; allosteric transition; small-angle X-ray scattering

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**Introduction**

Aspartate transcarbamoylase (E.C.2.1.3.2, ATCase) catalyzes one of the first steps in pyrimidine nucleotide biosynthesis, the reaction of carbamoyl phosphate (CP), with l-aspartate (Asp) to form $N$-carbamoyl-l-aspartate and inorganic phosphate ($P_i$). In many prokaryotes such as *Escherichia coli*, this reaction is the committed step in pyrimidine nucleotide biosynthesis. *E. coli* ATCase is composed of two types of subunits. The two larger or catalytic subunits are each composed of three identical poly-

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Abbreviations used: ATCase, aspartate transcarbamoylase (EC 2.1.3.2, aspartate carbamoyltransferase); CA, carbamoyl aspartate; CP, carbamoyl phosphate; NIH, National Institutes of Health; PALA, $N$-phosphonacetyl-l-aspartate; $P_i$, inorganic phosphate; SSRL, Stanford Synchrotron Radiation Laboratory; TR-SAXS, time-resolved small-angle X-ray scattering.
peptide chains ($M_r = 34,000$), while the three smaller or regulatory subunits are each composed of two identical polypeptide chains ($M_r = 17,000$). Each of the six active sites is located at the interface between two adjacent catalytic chains, and side chains required for catalysis are recruited to the active site from both chains. The enzyme demonstrates homotropic cooperativity for the substrate Asp and is heterotropically regulated by the effectors ATP, CTP, and UTP in the presence of CTP. A comparison of the T and R structures reveals that during the T→R transition, the two catalytic trimers increase their separation along the 3-fold axis by about 11 Å and rotate about 5° around the same axis, while the regulatory dimers rotate about 15° around their respective 2-fold axes. The 11-Å expansion of the enzyme observed during the T→R transition is easily monitored by small-angle X-ray scattering (SAXS). Thus, the SAXS pattern is a sensitive and specific probe to study the quaternary conformational changes of the enzyme.

By using SAXS as a structural probe in stopped-flow experiments, the time evolution of the quaternary conformational changes of ATCase has been monitored. These studies showed that the enzyme when mixed with substrates is very quickly converted from the T to the R state, the enzyme remains in the R state until substrates are exhausted, and then the enzyme reverts to the T state. These early studies required integration of the signal over time intervals of 100–200 ms and averaging over many runs to improve the signal-to-noise ratio. Because of the relatively long time window for each point, it was necessary to slow the reaction rate, which was done by performing the reaction at −5 °C in a buffer containing 20% ethylene glycol.

Dreyfus et al. showed that a variety of alcohols such as methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and 2-methyl-2-propanol have a significant influence on the activity and homotropic cooperativity of ATCase. For example, 20% methanol or ethanol reduced the activity of ATCase by approximately 90%. Additional studies with 15% ethanol showed a shift in the pH optimum of the reaction and an alteration in the Hill coefficient. Their interpretation of these solvent effects was that the cosolvent preferentially stabilized the T or R state of the enzyme depending upon the relative concentration and polarity of the cosolvent. For the simple alcohols, the primary effect was stabilization of the T state. Although ethylene glycol was not investigated by Dreyfus et al., one would predict that ethylene glycol with its two hydroxyl groups would behave similarly to methanol and ethanol.

Here, we reinvestigate the time evolution of the quaternary conformational changes of ATCase in the absence of ethylene glycol. This was made possible by significant instrumental developments including a fast CCD X-ray detector and a high-flux X-ray beam via a monochromator, with an increase in beam brightness due to the update of the synchrotron storage ring at Stanford Synchrotron Radiation Laboratory (SSRL) to SPEAR3, a third-generation source. These improvements allowed the collection of time-resolved SAXS (TR-SAXS) data at a time resolution as short as 5 ms. Using this system, we were able to study the quaternary conformational changes of ATCase in the temperature range of 5–22 °C in the absence of ethylene glycol.

### Results

#### Kinetics of the ATCase reaction in the presence of ethylene glycol

Since previous TR-SAXS experiments were performed in the presence of 20% ethylene glycol, and Dreyfus et al. showed that a variety of alcohols at concentrations of 20% or less can dramatically alter the catalytic turnover rate and cooperativity of

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**Fig. 1.** (a) The dependence of activity of ATCase on the concentration of ethylene glycol. All measurements were made at 5 °C in the presence of 50 mM Tris and 2 mM DTT, pH 8.3. (b) Influence of the nucleotide effectors ATP and CTP on the activity of ATCase in the presence and absence of 20% ethylene glycol. Colorimetric assays were performed at 5 °C in 50 mM Tris acetate buffer (pH 8.3) at a saturating concentration of L-Asp (2.4 mM) and saturating CP concentration (4.8 mM), in the presence of ATP and 20% ethylene glycol (○), in the presence of ATP and in the presence of ethylene glycol (●), in the presence of CTP and 20% ethylene glycol (△), and in the presence of CTP and in the absence of ethylene glycol (▽).
ATCase, kinetic assays were performed in the presence of ethylene glycol to determine if it had any influence on the ATCase reaction. As shown in Fig. 1a, ethylene glycol dramatically reduced the activity of ATCase at 5 °C. In the presence of 20% ethylene glycol, the activity of the enzyme was reduced by 75%. In addition to reducing the activity of the enzyme, ethylene glycol had a small influence on homotropic cooperativity (data not shown).

Ethylene glycol also has a significant influence on the ability of the heterotropic effectors to modulate enzyme activity. As shown in Fig. 1b at 5 °C, the presence of 20% ethylene glycol increased the activation of the enzyme by ATP while reducing the inhibition by CTP. The maximal activation by ATP increased from 220% to 274% in the presence of 20% ethylene glycol. The residual activity at a saturating concentration of CTP was 29% as compared to 48% in the presence of ethylene glycol. The value of $K_{ATP}$ (K is the nucleotide concentration required at 50% maximal activation or inhibition of the enzyme) increased from 0.47 to 0.72 mM in the presence of 20% ethylene glycol. The value of $K_{CTP}$ increased from 7.8 to 14.5 μM in the presence of 20% ethylene glycol.

The ability of UTP to act as a synergistic inhibitor of ATCase in the presence of CTP was also tested at 5 °C in the absence and presence of 20% ethylene glycol. In the absence of ethylene glycol, the combination of 4 mM CTP and 4 mM UTP (CTP/UTP) yielded a residual activity of the enzyme of 22%, whereas in the presence of ethylene glycol, the combination of CTP/UTP yielded a residual activity of the enzyme of 28%.

**Kinetics of the ATCase reaction at 5 °C**

Because the TR-SAXS experiments reported here were performed at temperatures as low as 5 °C, the kinetic properties of ATCase were fully characterized at 5 °C in the same buffers used for the TR-SAXS experiments (Fig. 2). As shown in Table 1, many aspects of catalysis at 25 °C are not only quantitatively different at 5 °C but also, in some cases, qualitatively different. As would be expected, the maximal observed velocity was reduced by nearly 5-fold. This reduction in velocity was accompanied by an almost 3-fold decrease in the concentration of Asp required for half-maximal activation ([Asp]0.5) and a decrease in the Hill coefficient ($n_H$) from 2.6 to 2.0. The observed changes in [Asp]0.5 and $n_H$ are similar to the changes observed when Asp saturating curves are determined in the presence of ATP, suggesting that, similar to ATP, lower temperatures shift the equilibrium towards the R state. This phenomenon was demonstrated in a previous SAXS study by the shift in the structural equilibrium of the mutant D236A enzyme in the direction of the R state with decreasing temperature. At 5 °C, the [Asp]0.5 decreased nearly 2-fold in the presence of ATP, increased 20% in the presence of UTP, increased more than 2-fold in the presence of CTP, and increased by a factor of 2.5 in the presence of CTP/UTP. A similar trend was also observed at 25 °C. The [Asp]0.5 decreased more than 2-fold in the presence of ATP, increased 10% in the presence of UTP, increased nearly 2-fold in the presence of CTP, and increased by a factor of 2.5 in the presence of CTP/UTP.

**Table 1. Kinetic parameters of ATCase with nucleotide effectors at 25 and 5 °C**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$V_{max}$ b</th>
<th>[Asp]0.5c, 25 °C</th>
<th>$n_H$ d</th>
<th>$V_{max}$ b</th>
<th>[Asp]0.5c, 5° C</th>
<th>$n_H$ d</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NTPs</td>
<td>19.1±0.3</td>
<td>12.7</td>
<td>2.6±0.2</td>
<td>4.2±0.5</td>
<td>5.1</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>5 mM ATP</td>
<td>19.8±0.7</td>
<td>6.1</td>
<td>1.4±0.1</td>
<td>5.4±0.2</td>
<td>2.9</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>4 mM CTP</td>
<td>17.9±0.9</td>
<td>21.2</td>
<td>2.4±0.1</td>
<td>3.6±0.2</td>
<td>11.3</td>
<td>3.0±0.1</td>
</tr>
<tr>
<td>4 mM UTP</td>
<td>18.4±0.2</td>
<td>13.5</td>
<td>2.3±0.1</td>
<td>4.1±0.6</td>
<td>5.9</td>
<td>2.2±0.1</td>
</tr>
<tr>
<td>4 mM CTP/UTP</td>
<td>17.3±0.4</td>
<td>28.6</td>
<td>2.9±0.1</td>
<td>3.0±0.4</td>
<td>12.5</td>
<td>3.0±0.1</td>
</tr>
</tbody>
</table>

* All experiments were performed in 50 mM Tris-acetate buffer (pH8.3) at a saturating CP concentration (4.8 mM).
* b The maximal observed velocity (in millimoles per hour per milligram).
* c The observed concentration of l-Asp (in millimolar) that produces one-half the observed maximal velocity.
* d Hill coefficient.
CTP, and increased by 2.3-fold in the presence of CTP/UTP.

The kinetic data at 5 °C were plotted as \( v / V_{\text{max}} \) versus [Asp] as shown in Fig. 3 and fitted by the theoretical curve calculated from a modified Monod et al. equation and using parameters listed in the text. Reactions were carried out in 50 mM Tris buffer, pH 8.3.

\[
Y_{\text{Asp}} = \frac{\alpha(1 + \alpha)^{n-1} + L'\alpha c_{\text{Asp}} (1 + \alpha c_{\text{Asp}})^{n-1}}{(1 + \alpha)^n + L' (1 + \alpha c_{\text{Asp}})^n}
\]

where \( \alpha = [\text{Asp}] / K_{R(\text{Asp})} \), \( L' \) is the allosteric equilibrium constant in the presence of saturating CP, and \( c_{\text{Asp}} = K_{R(\text{Asp})}/K_{T(\text{Asp})} \). \( K_{T(\text{Asp})} \) and \( K_{R(\text{Asp})} \) are the dissociation constants of Asp for the T and R allosteric states, respectively. A value of \( L' = 10 \) was observed from the static SAXS data shown in Fig. 4, according to the methods of Tsuruta et al. The starting point used for \( K_{R(\text{Asp})} \) was the \([\text{Asp}]_{0.5} = 5.1 \) mM value obtained from the kinetic data. After obtaining a suitable fit for the data, the values of \( K_{R(\text{Asp})} = 3.5 \pm 0.5 \) mM and \( K_{T(\text{Asp})} = 40 \pm 10 \) mM were derived.

**Time-resolved X-ray scattering: Effect of substrates and substrate analogs**

TR-SAXS was used to monitor the quaternary conformational changes that ATCase undergoes when the enzyme is mixed with substrates. Using the high beam flux obtained by the multilayer monochromator and fast CCD detector at SSRL, practical time resolution of the TR-SAXS data collection was improved from 100–200 ms to as low as 5 ms in a single mixing event. However, the rate constants for the T→R transition presented here were obtained from the 19-ms collection rate data, because at this rate, the signal-to-noise ratio was significantly better and fast enough to record much of the transition. This improvement in detection allowed reactions to be monitored in the temperature range between 5 and 22 °C, rather than at −5 °C as previously reported.

Shown in Fig. 5a are a series of SAXS patterns recorded upon mixing 1.5 mM ATCase (in active sites) plus 50 mM CP in one syringe with 100 mM CP, and 50 mM CP plus 50 mM D-Asp as a T-state control, at 5 °C. The SAXS patterns of the enzyme with the substrates are shown for 38 ms (○), 380 ms (□), and 3800 ms (●) after mixing. The SAXS pattern of the enzyme with CP and D-Asp is shown for 3800 ms (△, short dashed curve), and the long dashed curve (▪) is calculated for an \( L = 0.5 \), from the sum of 0.33×(T-state curve) and 0.67×(R-state curve). (b) Time courses of the quaternary structure change after mixing with substrates (continuous line) and 50 mM CP plus 50 mM D-Asp (broken line), as monitored by the scattering intensity integrated over the \( s \)-range 0.01–0.02 Å\(^{-1}\). Inset: First 300 ms of the structural change after mixing with substrates shown along with the curve fit (two-exponential) to the data.
Asp plus 50 mM CP in the second syringe at 5 °C. Immediately after mixing, the enzyme concentration was 0.75 mM in active sites (37.5 mg/ml), while the CP and Asp concentrations were each 50 mM. The SAXS patterns shown in Fig. 5a are at 38, 380, and 3800 ms. The scattering pattern at 38 ms does not correspond to either the T or R states as the enzyme population is in the process of undergoing the quaternary conformational change. The pattern at 380 ms is essentially identical with the curve of the enzyme in the presence of PALA (R state), while the curve at 3800 ms is essentially the same as that observed in the presence of D-Asp and CP (T state).

To determine if the curve at 38 ms corresponded to a transient intermediate on the pathway between the T and R states, we generated a curve from a sum of fractions of the T-state (33%) and R-state (67%) curves corresponding to a value of L = 0.5.15 This generated curve is a near match to the X-ray scattering curve recorded at t = 38 ms after mixing the enzyme with substrates.

The area under the curves between s = 0.01 Å⁻¹ and s = 0.02 Å⁻¹ was integrated and plotted as a function of time in order to help visualize the time course of structural change. This integration converts observed scattering intensity to relative concentration of the enzyme species on the basis that solution scattering intensity reflects relative concentration of each species linearly, in the absence of oligomeric state changes. Figure 5b shows the time-dependent change in the integrated intensity of the SAXS pattern for this experiment. At t = 5–10 ms (t = 0 as shown on the plot; the dead time of the stopped-flow mixer is approximately 5–10 ms), the enzyme population is nearly an equal mixture of T and R state molecules. Between t = 100 ms and t = 1500 ms, 95% of the enzyme population has reverted to the T state after depleting the substrates.

The initial time course of the structural change after combining the enzyme with substrates or substrate analogs appeared to fit to either a single or double exponential depending upon the experimental conditions. Therefore, each set of data was fit to both exponential fits and the number of rate constants [represented as k_{T→R(1)} for the first or fast reaction converting Asp and CP into carbamoyl aspartate (CA) and P_i. After t = 1500 ms, the enzyme population returns to the T state as the substrates are depleted, and after 3000 ms, virtually the entire enzyme population is back in the T state. At 5 °C, the turnover rate at maximal velocity of the holoenzyme is 350±40 s⁻¹. At a substrate concentration of 50 mM and an active-site concentration of 0.75 mM, the substrate:holoenzyme ratio is 400; hence, it should take 1–1.3 s to consume the substrate. The R-state plateau, defined here as the region of >95% of the peak amplitude of the integrated scattering curve, has a total duration of 1.4 s, which demonstrates that enzyme quickly reverts to the T state after depleting the substrates.

When ATCase is mixed with D-Asp and CP (final concentrations, 0.75 mM active sites; [D-Asp] = [CP] = 50 mM), the TR-SAXS curve showed virtually no change (see Figs. 5 and 6). The use of D-Asp thus provides a control for the L-Asp experiments with a compound with equal scattering potential or electron density and as a control for the T-state scattering curve as previously described.19 The integrated intensity at the end of the experiment when L-Asp and CP are mixed was practically identical with that observed when D-Asp and CP are mixed with enzyme. Because the TR-SAXS curve returns to the level observed in the presence of D-Asp and CP, it is clear that virtually the entire enzyme population has reverted to the T state after the substrates have been depleted.

### Table 2. Kinetic parameters for the T-to-R allosteric transition of ATCase at 5 °C

<table>
<thead>
<tr>
<th>Conditions</th>
<th>k_{T→R(b)} (s⁻¹)</th>
<th>k_{T→R(c)} (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM substrates</td>
<td>18.3±0.6</td>
<td>7.6±1</td>
</tr>
<tr>
<td>50 mM substrates</td>
<td>51±4</td>
<td>6.3±0.9</td>
</tr>
<tr>
<td>100 mM substrates</td>
<td>51±5</td>
<td>7.9±1</td>
</tr>
<tr>
<td>50 mM substrates + 5 mM ATP</td>
<td>89±20</td>
<td>14.8±1.8</td>
</tr>
<tr>
<td>50 mM substrates + 4 mM UTP</td>
<td>52±8</td>
<td>12.0±3</td>
</tr>
<tr>
<td>50 mM substrates + 4 mM CTP</td>
<td>10.3±0.3</td>
<td>10.3±0.3</td>
</tr>
<tr>
<td>50 mM substrates + 4 mM UTP</td>
<td>38±1</td>
<td>38±1</td>
</tr>
<tr>
<td>50 mM succinate + 50 mM CP</td>
<td>5 mM PALA + 50 mM CP</td>
<td>1.53±0.19</td>
</tr>
</tbody>
</table>

* These experiments were performed in 50 mM Tris-acetate buffer (pH 8.3), with a final enzyme concentration of 37.5 mg/ml after mixing. Equal concentrations of substrates ([L-Asp]= [CP]) were used, unless otherwise stated, and data points recorded at 19-ms intervals.

a Rate constant of fast phase or first phase of two-exponential fit.
b Rate constant of slow phase or second phase of two-exponential fit, where applicable.

c Equal concentration of substrates, [L-Asp]= [CP].

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**Fig. 6.** Time course of the quaternary structure change of 0.75 mM ATCase (in active sites) as monitored by the scattering intensity integrated over the s-range 0.01–0.02 Å⁻¹. The final substrate or substrate analog concentrations after mixing were [CP]=[L-Asp]=50 mM (○), [CP]=50 mM and [PALA]=5 mM (●), and [CP]=[D-Asp]=50 mM (□). Data displayed are from a 36-ms collection rate. Inset: First 1000 ms of the structural change after mixing with substrates or substrate analogs. Data displayed are from a 19-ms collection rate.
phase and $k_{T\rightarrow R(2)}$ for the second or slow phase, where applicable] reported reflects which fit was superior. As demonstrated by the rate constant data for the quaternary structural change in Table 2, increasing the substrate concentration from 25 to 50 mM and doubling the enzyme concentration increased the rate of the fast phase of the transition from 18.3 to 51 s$^{-1}$. In this experiment, it was necessary to use a lower enzyme concentration at the lower substrate concentration in order to observe the full T-to-R conversion of the enzyme population before significant depletion of the substrates. Lowering the enzyme concentration by one-half, to 18 mg/ml, and half again, to 9 mg/ml, while keeping the substrate concentration at 50 mM, did not change the rate of the fast or slow phase, within error, of the T→R transition (data not shown). Therefore, an enzyme concentration of 37.5 mg/ml was used for all experiments, with the previous exception, for a superior signal-to-noise ratio for the scattering data. Increasing the substrate concentration further to 100 mM while maintaining the same enzyme concentration (37.5 mg/ml) did not change the rate of transition for the fast or slow phases, as might be expected since this concentration was much higher than the [Asp]$^{0.5}$ value of 5.1 mM.

In order to establish the value for the integrated intensity and scattering pattern of the R-state structure of ATCase in a TR-SAXS experiment, we mixed PALA with enzyme and CP (final concentrations, 0.75 mM active sites, 5 mM PALA, 50 mM CP). As seen in Fig. 6, the integrated intensity observed immediately after mixing was shifted towards the R-state value. The rate constants for the fast and slow phases after mixing with PALA were both more than 1 order of magnitude slower than the corresponding T→R transition rate constants observed in the presence of a saturating concentration of the natural substrates, i.e. Asp and CP. The single fast phase rate constant for the structural change with 50 mM succinate and 50 mM CP was 38 s$^{-1}$, similar to the rate of the fast phase with the natural substrates at a saturating concentration.

**Effect of nucleotides**

The time course of the quaternary structural change in the presence of ATP is shown in Fig. 7 and is similar to that observed in the absence of nucleotides. However, in the presence of ATP, the duration that the enzyme remains in the R-state plateau is shorter than that in the absence of nucleotides and the $k_{T\rightarrow R(1)}$ increased from 51 to 89 s$^{-1}$. The time course of the structural change for ATCase in the presence of the nucleotide inhibitors is shown in Fig. 8. The rate of T→R transition in the presence of CTP and CTP/UTP decreased significantly to 12 and 10.3 s$^{-1}$, respectively. The integrated scattering intensity of the R-state plateau in the presence of CTP and CTP/UTP is lower than that observed in the absence of nucleotides, and the duration of the plateau in the presence of CTP and CTP/UTP is much shorter than that in the absence of nucleotides. The results with UTP alone were unexpected. The rate of the fast phase of the T→R transition in the presence of UTP was identical with that in the absence of nucleotides. The integrated scattering intensity of the R-state plateau in the presence of UTP is also the same as that in the absence of nucleotides. However, the duration of the R-state plateau phase in the presence of UTP is only half of that observed in the absence of nucleotides, suggesting a slight destabilization of the R state by UTP.

**Determination of the activation energy of ATCase**

The kinetics of the quaternary structural change was observed at a series of temperatures between 5 and 22 °C in order to calculate the activation energy.
of the quaternary conformational changes of ATCase both from the T to the R state and from the R to the T state. Unfortunately, above 10 °C, the T→R transition rate is so fast that our instrumentation was unable to follow it. However, the rate constants for the R-to-T transition (k_R→T) at 5, 10, 16, and 22 °C were 2.08±0.03, 3.7±0.1, 5.1±0.1, and 8.3±0.1 s⁻¹, respectively. An Arrhenius plot of these data is shown in Fig. 9, which yielded an activation energy for the R0→T0 transition of 13.0±1.4 kcal/mol.

Discussion

TR-SAXS experiments investigating the time evolution of the quaternary structural change of ATCase induced by the binding of the natural substrates CP and L-Asp were performed here in the absence of ethylene glycol. In previous studies, 20% ethylene glycol was added to all solutions to allow the experiment to be performed at −5 °C. Here, we demonstrate that ethylene glycol dramatically alters the homotropic and heterotropic kinetics of the enzyme, as is the case with many other alcohols. Therefore, these new studies were important not only in monitoring the quaternary structural change at temperatures closer to physiological but also because ethylene glycol was eliminated from the reaction.

In order to better correlate the time-resolved structural results reported here at 5 °C to the functional characteristics of ATCase at this temperature, we performed a complete kinetic characterization of the enzyme at 5 °C. As would be expected, the maximal velocity of the enzyme at 5 °C in the absence of nucleotides was also reduced nearly threefold, with a concomitant reduction in the cooperativity for Asp (see Table 1). Similarly, in the presence of nucleotides at 5 °C, the [Asp]₀.₅ was reduced but with no change in cooperativity, except when CTP was present, where the cooperativity increased. The results of these kinetic experiments suggest that the structural results obtained from the TR-SAXS experiments performed at 5 °C should strongly correlate with the structure and function of the enzyme at higher temperatures.

In agreement with previous results, when ATCase is mixed with its natural substrates, CP and L-Asp, there is a rapid structural transition of the enzyme from the T to the R state. The preponderance of the enzyme population remains in the R state as the enzyme reacts with the substrates and then reverts to the T state when the substrates are exhausted (see Fig. 5b). This clearly demonstrates that the allosteric transition is not the rate-limiting step in catalysis, as has been previously suggested. Therefore, under conditions of saturating substrates, the enzyme remains in the R-quaternary structure until the substrates are essentially exhausted and then reverts to the T-quaternary structure.

As shown in the inset to Fig. 5b, the T→R structural transition upon addition of the natural substrates to the enzyme appears to be a biphasic exponential process, with a fast and slow phase. In this particular case, the fast phase accounts for approximately 75% of the total amplitude of the curve, representing the change in integrated intensity between the mixture of T and R states at the first recorded time point and R state at the curve plateau. Considering the evidence against the formation of a structural intermediate as demonstrated in Fig. 5a, along with the structural transition being a single-phase exponential process in the presence of allosteric inhibitors, as shown in Fig. 8, we determined that the curve monitoring the structural change may represent a composite of T-state species with different ligation states, each with its own particular rate of transition to the R state, as has been previously suggested in studies of the rate of the structural change in ATCase. In the case of the allosteric protein hemoglobin, it is well documented that not only different ligation states but also configurational isomers, or asymmetric ligation states, exhibit a wide range of structural transition rates that originate from different activation energies for the structural change. When all the CP binding sites of T-state ATCase are saturated, as they are under our experimental conditions, there are 13 possible species or ligation states with aspartate bound in the six binding sites when taking into account the T-state interactions between the C1 and C4 chains on opposing catalytic trimers. Therefore, the two rates we observe are the composites of up to 13 or more individual rates for the T→R transition when accounting for these configurational isomers. The observed rate of the T→R transition increases about threefold when doubling the aspartate concentration from 25 mM, a concentration well in ex-
cess of the $K_{R(Asp)}$ value of $3.5 \pm 0.5 \text{mM}$, to $50 \text{mM}$, while also doubling the enzyme concentration. Therefore, we are confident that the structural transition is triggered by aspartate binding to the T state and is not a simple shift in the preexisting equilibrium between the T and R states towards the R state caused by aspartate binding only to that state and “locking” it into that state. If the structural transition were simply a population shift to the R state, then the rate would not change or change very little when the aspartate concentration is increased from 25 to $50 \text{mM}$ as both concentrations are well in excess of $K_{R(Asp)}$. This can be accounted for using a kinetic version of the two-state model, which would suggest 14 individual rates for the allosteric transition, defining the T-to-R equilibrium constants at the 7 ligation states. In order to approximate the average number of aspartate molecules bound to the T state during the structural transition, we used the equation derived for the two-state allosteric model of Monod et al., simplified by assuming a saturating concentration of CP in the manner previously described to fit our aspartate saturation data at $5 \text{°C}$. After obtaining a reasonable fit of the data and extracting the appropriate parameters, we obtained a $K_{T(Asp)}$ value of $40 \pm 10 \text{mM}$. With an active-site concentration of $0.75 \text{mM}$ and an aspartate concentration of $25 \text{mM}$, an average of $33-46\%$ of the active sites are ligated with aspartate (2–2.5 per holoenzyme). Similarly, at an aspartate concentration of $50 \text{mM}$, an average of $50-67\%$ of the active sites are ligated with aspartate (3–4 per holoenzyme), and at an aspartate concentration of $100 \text{mM}$, an average of $67-77\%$ of the active sites are ligated with aspartate (4–4.6 per holoenzyme). These observations compare favorably with the previous SAXS studies of Fetler et al. that show that two PALA molecules per ATCase holoenzyme molecule are necessary to shift the T-to-R equilibrium in favor of the R state and that four PALA molecules are necessary to shift the entire enzyme population to the R state. Macol et al. demonstrated that the binding of one PALA molecule could shift the entire holoenzyme population to the R state; however, the holoenzyme was composed of five chains with the R105A mutation. In either case, the enzyme clearly does not need to be saturated with substrate analogs or presumably substrates, in order to shift the equilibrium towards the R state. Our findings suggest that the faster observed rate is the composite of rates of the structural transition for the highly liganded species, with three or more active sites filled. Likewise, the slower observed rate may be the composite of structural transition rates of the least liganded species, with two or fewer active sites filled. At an aspartate concentration of $25 \text{mM}$, we observe only one exponential fit to the data, which, according to our model, would be the composites of rates of the least liganded species along with the rates of the highly liganded species containing three or more aspartate present as a small fraction of the mixed population. Increasing the aspartate concentration from 50 to $100 \text{mM}$ yielded identical rates for both observed phases, suggesting that once three or more aspartate molecules are bound to the holoenzyme, the rate for the structural transition is near a maximum, or that highly liganded T-state molecules bound with four or more aspartate are present as only a small fraction of the population even at high aspartate concentrations. Further evidence for this model is provided by comparison of the ratio of the fast-phase amplitude to the slow-phase amplitude at $50 \text{mM}$ aspartate and $100 \text{mM}$ aspartate concentrations; at the higher aspartate concentration, the ratio is higher, suggesting a shift in population towards the highly liganded molecules.

There are some substantial qualitative differences between these TR-SAXS results and the data obtained previously in the presence of ethylene glycol, such as the time course of the TR-SAXS pattern of the enzyme in the presence of ATP, shown in Fig. 7. We observed that the R-state plateau region in the presence of ATP, during which most of the substrates are being converted to products, is shorter than that in the absence of ATP. This was not unexpected since ATP, an activator of the enzyme, at $5 \text{°C}$ increased the $V_{\text{max}}$ value from 4.2 to $5.4 \text{mmol h}^{-1} \text{mg}^{-1}$ (Table 1) as well as the apparent binding affinity of L-Asp, as the $[\text{Asp}]_{0.5}$ value decreased from 5.1 to 2.9 mM. However, in the presence of 20% ethylene glycol, a longer R-state plateau was observed in the presence of ATP than in its absence. To explain this, it was proposed that ATP, in addition to being an activator, may possibly become an inhibitor by “increasing the chance of making L-Asp bind to the active site before $P_i$ leaves and locking the active site into an unproductive cycle with no alteration of the quaternary structure”. In that experiment, the activity of the enzyme was dramatically reduced not only by the presence of 20% ethylene glycol, which, as demonstrated in Fig. 1a, reduces the activity by 75%, but also by performing the TR-SAXS at $-5 \text{°C}$. The similar phenomenon of “substrate inhibition” has been observed for the isolated catalytic subunit and for the incomplete complexes C$_i$R$_6$ and C$_6$R$_i$, as well as for the holoenzyme C$_6$R$_6$, where a high aspartate concentration ostensibly acts as an inhibitor by binding to the CP binding site. As detailed in Table 2, ATP increased the rate of the T→R transition, specifically the rate of the fast phase, by approximately 75%. It should be noted that in the presence of ATP, the allosteric transition is so rapid that with the current instrumentation, the experimental error is somewhat large. As discussed previously, saturating the enzyme with aspartate beyond a concentration of $50 \text{mM}$ did not increase the rate of the structural transition. Therefore, one possible interpretation is that ATP increases the on rate of aspartate to the T state, suggesting that aspartate binding is a rate-limiting step in the allosteric transition. However, a more elegant explanation, which is in accord with the data from the inhibitors CTP and CTP/UTP and numerous studies on the effects of the heterotropic nucleotides, can be derived using some aspects of the two-state
model of Monod et al. At pH 7.0, Howlett et al. observed that ATP increases the stability of the R state relative to the T state by 0.8 kcal and reduces the T-to-R equilibrium constant from 250 to 7 in the absence of substrates. The R-to-T transition rate with ATP was reduced by only approximately 15% (Fig. 7), suggesting a slight stabilization of the R state; therefore, the large increase in the T → R rate would suggest a significant T state destabilization by ATP. Here, the two-state model suggests that ATP binds more tightly to the R state and therefore shifts the structural equilibrium in favor of the R state. However, our observation of a possibly different mechanism for ATP was suggested by the crystal structures of the T and R states of the enzyme in the presence of ATP; the T-state structure showed a slight shift towards the R state with ATP present. Whereas, globally, the R-state structure was unchanged in the presence of ATP; the T-state structure showed a similar shift in the presence and absence of ethylene glycol (see Fig. 6). For both cases, in the presence of CTP or CTP/UTP, the duration of the R-state-shifted plateau is shorter and the integrated intensity of the plateau is lower than that in the absence of nucleotides, and the observed return to the T-state quaternary structure is slow for reasons that are unclear. However, UTP did have a noticeable effect on the kinetics of the T → R transition, except for increasing the rate of the slow phase for reasons that are unclear. However, UTP did have a noticeable effect on the length of time the enzyme spent in the R-state plateau region, causing the enzyme population to begin reverting to the T state before the substrates were exhausted. This indicates that UTP may slightly destabilize the R state but otherwise does not have an appreciable effect on the allosteric behavior of ATCase under these conditions.

The activation energy of the R → T transition was determined by fitting the bottom half of the return curve, after the enzyme had exhausted the substrates, to a single exponential rate, as opposed to the upper half of the curve where presumably not all enzyme molecules have completely exhausted the substrate bound to the active sites. By fitting the bottom half of the return curve, mainly the rate of $R_0 \rightarrow T_0$ should be observed, which is supported by the very low error in the $k_{R \rightarrow T}$ values obtained. As shown in Fig. 9, the $E_{\text{ATCase}}$ was calculated to be $13.0 \pm 1.4 \text{kcal/mol}$ from the slope of the Arrhenius plot. At temperatures above 10 °C, the T → R rate was so rapid that the data could not give rates with a reasonable error. Moreover, these rates are compos-
ganded T and R states. Assuming that the respective transition states in both the T→R transition and R→T transition are the same, and the free energy difference between the T and R states is 3.3 kcal/mol, \(^{-17,31}\) then the activation energy for the T→R transition should be in the range of 15–18 kcal/mol. The charged–charged hydrogen bonds between Glu239 and Lys164 and noncharged hydrogen bonds between Glu239 and Tyr165 are critical for the stabilization of both the T and R state conformations of the enzyme, which are interchain in the T state and intrachain in the R state.\(^ {7}\) Sakash et al. showed that three of the six stabilizing interactions between catalytic chains on opposing subunits involving Glu239 are sufficient to stabilize the enzyme in the T-state conformation.\(^ {14}\) Thus, at least three of the hydrogen bonds involving Glu239 must be broken during the allosteric transition. Considering a typical charged–charged hydrogen bond energy is approximately 4 kcal/mol and a noncharged hydrogen bond energy is approximately 0.5–1.5 kcal/mol,\(^ {35,36}\) the minimal energy required for the allosteric transition of the enzyme is 13.5–16.5 kcal/mol. The results reported here are consistent with these calculations.

The rate of the allosteric transition was also measured when ATCase is mixed with two substrate analogs: succinate, an aspartate analog that promotes the T→R state transition when combined with CP, and PALA, a bisubstrate analog that binds at nanomolar affinity and also causes the T→R state transition. The structural transition rate with succinate and CP at a concentration of 50 mM each was slightly lower than the rate with the natural substrates at equivalent concentration and was observed to be a single exponential process. This may be because succinate binds approximately 1 order of magnitude more tightly than aspartate,\(^ {17,37,38}\) and therefore, the bulk of the T-state molecules should be highly liganded during the transition to the R state. However, the structural transition rate with PALA was more than 1 order of magnitude slower than that with the natural substrates. The kinetics of the interaction of PALA with the isolated catalytic subunit at pH7.0 and 25 °C has been studied using stopped-flow kinetics and \(^ {31}\)P saturation transfer NMR by Cohen and Schachman.\(^ {39}\) They observed a rapid binding of PALA followed by a much slower isomerization of the complex with a forward rate constant of 0.18 s\(^ {-1}\), similar to the rate constant of the slow phase we observed by TR-SAXS of 0.31 s\(^ {-1}\). However, the catalytic subunit by itself does not undergo a T→R transition; hence, it is unclear whether the similarity of these values implies a similar mechanism between the change in the tertiary structure following PALA binding to the catalytic subunit and the change in the quaternary structure following PALA binding to the holoenzyme. The rate constant data and integrated scattering intensity presented here were obtained after mixing PALA with enzyme premixed with CP, to be consistent with the other experimental conditions. In addition, experiments were performed where PALA was mixed with only the enzyme and the rate constants for the T→R transition were essentially identical (data not shown), which suggests that the slow allosteric transition after PALA binding is not caused by having to displace CP from the active site. Because PALA combines elements of both substrates into one covalently linked bisubstrate analog, when the CP moiety of PALA initially binds, the aspartate moiety may not be able to bind well to the aspartate binding site with the domains open, as they are in the T state. Subsequently, the enzyme may undergo a slow conformational change in order for the aspartate moiety to bind tightly and then complete the T→R structural conversion. In this regard, PALA appears to fit in the category of a slow, tight-binding inhibitor.\(^ {40}\) However, the phenomenon of PALA being a very tight-binding bisubstrate analog that causes an initial conformational change followed by a much slower conformational change needs further investigation to be better understood.

In summary, our data showed that ethylene glycol had a profound influence on the kinetics and behavior of ATCase; therefore, the time evolution of the allosteric transition of ATCase was reinvestigated in the absence of ethylene glycol by SAXS, revealing several important new insights. Experimentally, these studies demonstrate that SAXS is now capable of monitoring relatively rapid structural changes at temperatures approaching physiological. The allosteric transition is not the rate-limiting step in ATCase catalysis, and the rate of allosteric transition is increased with increasing substrate concentration up to 50 mM. ATP appears to destabilize the T state and to have little effect on the R state. CTP and the combination of CTP/UTP appear to destabilize the R state and stabilize the T state. PALA causes a very slow conformational change as compared to the natural substrates. In the future, novel TR-SAXS experiments could be performed with hybrid ATCase molecules that bind one, two, three, four, or five aspartate molecules to determine the individual rate constants and activation energies for each one of these species. Such studies will be facilitated by further instrumental upgrades that are being made to improve time resolution. We believe that we have begun to arrive at a clear understanding of the allosteric mechanism of ATCase, and further studies into its dynamic behavior utilizing such time-resolved techniques as employed here will be invaluable in this.

### Experimental Procedures

**Materials**

ATP, CTP, UTP, CP, L-aspartate, D-aspartate, N-carbamoyl-L-aspartate, potassium dihydrogen phosphate, sodium azide, succinate, and uracil were obtained from Sigma Chemical Co. (St. Louis, MO). Ammonium sulfate, electrophoresis-grade acrylamide, and trishydroxymethylaminomethane (Tris) were purchased from ICN (Costa Mesa, CA).
PALA was obtained from the National Cancer Institute, National Institutes of Health (NIH). All commercially available starting materials and solvents were reagent grade or better and used without further purification. CP dilithium salt was purified before use by precipitation from 50% (v/v) ethanol and was stored desiccated at -20 °C.

Enzyme preparation

The E. coli ATCase was overexpressed utilizing E. coli strain EK1104 containing plasmid pEKL32.\(^{42}\) The isolation and purification were as described previously.\(^{43}\) The purity of the enzyme was checked by SDS-PAGE\(^{44,45}\) and nondenaturing PAGE.\(^{44,45}\) The concentration of wild-type enzyme was determined by absorbance measurements at 280 nm with an extinction coefficient of 0.59 cm\(^{-1}\) mg\(^{-1}\).\(^{46}\) The purified enzyme was dialyzed into a buffer solution containing 50 mM Tris and 2 mM DTT, pH 8.3, and concentrated to approximately 75 mg/ml. The pH of the buffer solution was readjusted to be approximately 8.3 at 5 °C.

Enzyme kinetics

The activity of the enzyme was measured by the colorimetric method at either 5 or 25 °C. Colorimetric assays were performed at a pH of 8.3, in 50 mM Tris acetate buffer. All of the saturation kinetics was performed in duplicate, and the data points shown are the average values. Data analysis for the steady-state kinetics was carried out as previously described.\(^{47}\) The experimental data were fit to theoretical equations using nonlinear regression. When significant substrate inhibition was present, the data were analyzed using an extension of the Hill equation that includes a term for substrate inhibition.\(^{28}\) The nucleotide saturation curves were fit to a hyperbolic binding isotherm by nonlinear regression.

Small-angle X-ray scattering

Time-resolved X-ray scattering experiments were performed on beamline 4-2 at SSRL, Menlo Park, CA. Synchrotron radiation from a 20-pole 2-T wiggler was focused by a bent crystal mirror and monochromatized (X-ray wavelength, 1.38 Å) by a pair of synthetic W/B\(_{4}\)C multi-layer diffraction elements.\(^{15}\) A stopped-flow mixer injected 0.1 ml of enzyme solution and an equal volume of another solution, typically containing substrates or substrate analogs, into an observation cell via a mixing chamber. The observation cell was kept at a constant temperature within an error of ±0.5 °C. The dead time of our stopped-flow apparatus for X-ray scattering is approximately 5–10 ms, which is the earliest time point that one can obtain after initiation of the enzyme reaction. All stopped-flow experiments were performed at a pH of 8.3, in 50 mM Tris acetate buffer containing 2 mM DTT. The time-resolved measurements were done by mixing solutions from two syringes: one syringe containing the substrate CP, nucleotides where applicable, and enzyme and the other containing the substrates CP and L-Asp (or substrate analogs where applicable) and nucleotides where applicable. L-Asp was substituted for L-Asp for recording the T-state scattering curves without enzyme catalysis as well as for the background correction with identical electron density contrast.

A series of successive measurements of 2D scattering data and corresponding beam intensities were synchronized with the completion of sample mixing. The data sampling rate for all experiments was 19 ms, with the exception of the curves displayed in Fig. 5, in which the rate was 36 ms. The scattering data were recorded by an image-intensified interline CCD X-ray detector system (Hamamatsu Photonics C4880-80-14A & V5445P),\(^{49}\) located at ~85 cm from the observation cell. The beam intensities incident on the sample were integrated during a series of CCD exposures by the European Molecular Biology Laboratory data collection system.\(^{49}\) The detector channel numbers were converted to S = 2sinθ/λ, where θ is the scattering angle and λ is the X-ray wavelength (1.38 Å), by recording the position of the (100) and related reflections of a cholesterol myristate powder sample placed at the sample position. Image distortion correction of 2D data was performed using the program Fit2D.\(^{50}\) Radial integration, intensity scaling, background subtraction, and correction for nonuniformity of detector response were done by MarParse, developed at SSRL.\(^{51}\)

Each reported set of T → R rate constants was obtained from averaging three individual runs under the same conditions, with each run inspected for consistency and any experimental anomalies. The time courses of the initial quaternary structural change were subject to both one-exponential curve fits and two-exponential curve fits with terms for separate amplitudes and time constants. The number of reported rate constants reflects which least-squares fit yielded the highest R value (correlation coefficient) or goodness of fit under each experimental condition. The time evolution of the structural change displayed in Figs. 4–7 is from individual single-mixing runs, and the reported R → T rate constants were obtained from the same individual runs.

Acknowledgements

This work was supported in part by Grant GM26237 from the NIH. The SSRL is operated by the Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Biology Resource is supported by the NIH, National Center for Research Resources (P41RR01209), and by the Department of Energy, Office of Biological and Environmental Research. The content is solely the responsibility of the authors and does not necessarily reflect the official views of the National Center for Research Resources or NIH.

References


Trapping and structure determination of an intermediate in the allosteric transition of aspartate transcarbamoylase

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X-ray crystallography and small-angle X-ray scattering (SAXS) in solution have been used to show that a mutant aspartate transcarbamoylase (ATCase), the enzyme that catalyzes the same chemical shifts were also observed when the wild-type ATCase was crystallized in the absence of active site ligands, thus making interpretation of the structural details of an R-state active site in the absence of ligands possible. The mutant enzyme is in a unique intermediate state on the path of the allosteric transition between the T and R states.

Although many aspects of allosteric regulation and cooperativity have been established for Escherichia coli aspartate transcarbamoylase (ATCase), the enzyme that catalyzes the committed step in pyrimidine nucleotide biosynthesis, many of the details of how the active sites change from low-activity, low-affinity to high-activity, high-affinity during the T to R transition have not been delineated (1). The major limitation has been that stabilization of the enzyme in the R state requires the presence of active site ligands. Thus, making interpretation of the structural rearrangements upon the T to R transition, in the absence of ligands, difficult. Newell and Schachman (2) have concluded, from sedimentation velocity experiments, that the quaternary structures of ATCase with the catalytic chain mutation K164E and the catalytic chain double-mutation K164E and E239K exist in the R state, irrespective of the presence of active site ligands. The enzymatic properties of the double mutant (K164E/E239K ATCase) include a lack of homotropic cooperativity and an inability to be activated by ATP or inhibited by CTP: properties coincident of an enzyme that cannot transition between the two allosteric states (2). More recently, Velyvis et al. (3) demonstrated that the partially labeled K164E/E239K ATCase exhibited certain chemical shifts in solution NMR studies that they conclude are characteristic of the R state of the enzyme. These same chemical shifts were also observed when the wild-type enzyme was bound with the bisubstrate analog N-phosphonacetyl-L-aspartate (PALA) (3), which is known to stabilize fully the R state of the enzyme (4). Thus, a crystal structure of K164E/E239K ATCase should provide us a means to obtain the structural details of an R-state active site in the absence of ligands. However, we report the X-ray crystal structure and the solution small-angle X-ray scattering (SAXS) data for the K164E/E239K ATCase, which clearly demonstrate that this double-mutant enzyme is not in the R-quaternary structure. Instead this mutant enzyme is in a unique intermediate state on the path between the T and R structural states.

Results and Discussion

Crystal Structure of K164E/E239K ATCase Is Different from the Wild-Type R State Structure. The K164E/E239K ATCase was purified and subsequently crystallized in the absence of ligands as described in Methods. The crystals were determined to be in the P2₁₂₁₂ space group with unit cell dimensions of a = 124.1 Å, b = 144.8 Å, and c = 203.4 Å with α = β = γ = 90°. A previously determined structure of R-state ATCase in the presence of PALA has also been solved in the P2₁₂₁₂ space group (5). However, the unit cell dimensions (a = 125.5 Å, b = 153.5 Å, c = 185.7 Å with α = β = γ = 90°) vary significantly from those determined for the K164E/E239K ATCase. In the crystal of the PALA–ATCase complex the molecular threefold axis is parallel to the crystallographic b-axis (5). The almost 9 Å shorter b-axis of the K164E/E239K ATCase crystals was the first indication that the structure of this double-mutant enzyme may not be an R-state structure.

To quantitatively compare the quaternary conformation of the K164E/E239K structure with the canonical ATCase T and R structures, the vertical separation (6) between the upper and lower catalytic subunits, planar angle between allosteric domains of the regulatory dimer, and the rotation about the threefold axis (Fig. 1) of the K164E/E239K structure were determined and compared with the corresponding values for the published T and R structures (Fig. 2). The vertical separation for the K164E/E239K structure was 50.5 Å, compared with 47.3 Å and 57.9 Å for the T state [Protein Data Bank (PDB) ID 1ZA1 used throughout] and R state [PDB ID 1D09 used throughout], respectively. The average planar angle between allosteric domains of the regulatory dimer in the K164E/E239K structure was 153°, which compares to 152.0° and 155.8° for the T and R structures, respectively, of the wild-type enzyme. For the wild-type enzyme the relative rotation around the molecular threefold axis between the T and R structures is 12°, whereas the rotation from the wild-type T structure to the K164E/E239K structure is only 4.8°. The vertical separation, the angle between the allosteric domains of the regulatory dimer, and the rotation of the catalytic subunits about the threefold axis all indicate that the K164E/E239K structure is not globally in either the wild-type T or R structure.

The analysis of the global conformation of the K164E/E239K structure by means of X-ray crystallography does not agree with the previously reported data as determined by sedimentation velocity (2). Therefore, we used the program HydroPRO (7) to calculate the radius of gyration (Rg) on the basis of the wild-type and K164E/E239K structures. These values have some uncen-
tainty because the highly mobile portions of the structures are not reported in the structural data. The $R_g$ values of the wild-type T and R states were calculated to be 46.4 and 49.1 Å, respectively, which compares well with the reported values of 46.6 ± 0.3 Å and 49.3 ± 0.2 Å, respectively (8). The calculated $R_g$ for the K164E/E239K structure was 47.2 Å, a value closer to the T than the R state of the wild-type enzyme.

K164E/E239K ATCase Exists in a Structural State Different from the Wild-Type R State in Solution. For the wild-type enzyme, the percent change in the sedimentation coefficient between the T- and R-state structures has been reported to be −3.8% (2). The corresponding percent difference calculated between the K164E/E239K and wild-type R-state structures was −0.4% (2). These data suggest that the structure of K164E/E239K enzyme may be different in solution than it is in the crystal. Therefore, we used SAXS to obtain additional structural data on the K164E/E239K ATCase in solution (9, 10).

SAXS data were recorded using Beamline 4-2 at the Stanford Synchrotron Research Laboratory at pH values 8.3, 7.5, and 7.0. In addition to recording SAXS data for the K164E/E239K enzyme, data were also recorded for the wild-type ATCase in the absence of ligands (T-state control) and in the presence of PALA (R-state control). The SAXS patterns (Fig. 3), plotted as normalized Kratky Plots (11), for the T and R states of the wild-type enzyme are dramatically different. The SAXS pattern for the K164E/E239K enzyme at pH 8.3 (Fig. 3A) is intermediate between the T and R states observed for the wild-type enzyme. This clear difference in the scattering patterns between the T and R states and the K164E/E239K enzyme can also be visualized when the data are plotted as $I(q)$ vs. $I$ (Fig. S1A) and as log $I(q)$ vs. $I$ (Fig. S2A). The intermediate curve observed for the K164E/E239K enzyme can be explained in at least two ways. First, the K164E/E239K enzyme exists in a new structural state that is different from the wild-type T or R states; or second, in solution the K164E/E239K enzyme is a mixture of two or more structures. The second explanation is consistent with the K164E/E239K enzyme having a T to R equilibrium shifted from the T state-dominated equilibrium observed for the wild-type enzyme.

If the SAXS pattern of the K164E/E239K enzyme is a mixture of wild-type T and R structures, it should be possible to generate the SAXS pattern of the double-mutant enzyme by a linear combination of the wild-type T- and R-state patterns. The dotted line in Fig. 3A–C corresponds to the best fit of the SAXS pattern of the K164E/E239K enzyme using the wild-type T and R curves. At each pH, the best-fit curve does not match that observed for the K164E/E239K enzyme. In particular, the position and amplitude of the first subsidiary minimum is not predicted correctly. The inability to deconvolute the curve into T and R components suggests that in solution the K164E/E239K enzyme is neither the
The Rg values can be determined directly from the SAXS data for the wild-type or mutant enzymes. At the two other pHs tested this trend was the same: the Rg was greater ($0.5 \pm 0.1$ Å) for the unliganded mutant before the addition of PALA and conversion to the R state. This discrepancy between the Rg as determined in solution by SAXS and the crystal structure suggest that the ground state structure of the mutant enzyme is much more flexible than that of the wild-type; however, whatever the explanation may be the crystal structure and SAXS data both support the conclusion that the mutant without ligands is not in an R-state structure.

These results suggest that the previously reported findings that the K164E/E239K ATCase is in the R state may need to be reevaluated (2, 3). The evidence presented here clearly demonstrates that the quaternary structure of the K164E/E239K ATCase is not identical to the wild-type ATCase–PALA complex, the typical structure used to define the R state. The sedimentation velocity experiments on the K164E/E239K ATCase, which indicated R state-like sedimentation values, were performed at 20 °C and pH 7.0 (2), a temperature and pH that may perturb the enzyme toward a more R-like structure. The SAXS data in Fig. 3 show that lowering the pH from 8.3 to 7.0 causes a shift in the structure toward the R state. The solution NMR report by Velyvis et al. (3) also asserts the K164E/E239K ATCase is in the R structure and concludes that the Monod, Wyman, and Changeux model (13) can fully account for the allosteric properties of ATCase. The experiments performed by Velyvis et al. (3) were carried out at 37 °C and pH 7.5. At this pH value and 25 °C, the K164E/E239K ATCase is not in the canonical R-state structure (Fig. 3B). Although the higher temperature of the NMR experiments may cause a shift in the structure toward the R state, the temperature dependence of SAXS data has been determined for the wild-type and the D236A enzyme. The wild-type enzyme showed no change in structure by SAXS through the temperature range of 4° to 55 °C, whereas the D236A ATCase exhibited a large shift toward the T state between 4 and 30 °C, with a minor shift back toward the R state between 30 °C and 45 °C (14). These data suggest that under the NMR conditions (37 °C) the enzyme, 48.1 ± 0.1 Å and 51.9 ± 0.1 Å for the T and R states, respectively, are similar to the previously determined Rg values in solution of 47.3 ± 0.2 Å and 49.9 ± 0.2 Å for the T and R states (12).

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K164E/E239K ATCase, which has properties similar to those of D236A ATCase, would not be shifted toward the R state. This study underscores the need for extreme caution when making inferences about the allosteric mechanism of a wild-type protein when working with a model system, which in this case is the double-mutant enzyme.

K164E/E239K ATCase Is in an Intermediate State During the Allosteric Transition from the T to the R State. Both the X-ray crystallographic and SAXS data indicate that the structure of the K164E/E239K enzyme in the absence of ligands is not in the R structure, but rather it is an intermediate structure between the canonical T and R structures. Instead of providing a model for the R state, the K164E/E239K enzyme gives us structural information on the pathway of the T- to R-state transition of ATCase. When wild-type ATCase undergoes the allosteric transition to the R state, the enzyme expands 10.6 Å and the upper catalytic trimer rotates 12° relative to the lower trimer along the threefold axis. Compared with the wild-type T-state structure the K164E/E239K enzyme only expands 2.8 Å and the upper catalytic trimer rotates only 4.4° relative to the lower trimer (Fig. 4A). Previous studies revealed that the planar angle between the allosteric domains of the regulatory dimer changes almost linearly with changes in the size of the molecule (6), which increases from 152.0° to 155.8° as the enzyme transitions from the T to the R state. The fact that in the K164E/E239K structure this planar angle is only 153° suggests that the size of the double mutant is between the size of the wild-type T- and R-state enzymes. As seen in Fig. 4A, the percent change in the vertical separation, the angle between the allosteric domains of the regulatory dimer, the rotation about the threefold axis, and the radius of gyration observed in the K164E/E239K structure are all approximately one-third of the difference in the parameters observed between the T and R states of the wild-type enzyme. These data suggest that the K164E/E239K enzyme exists in an intermediate structure shifted approximately one-third toward the wild-type R structure from the T structure.

Not only is the quaternary conformation of the K164E/E239K structure intermediate between the canonical ATCase T and R
E239K enzyme, the possible salt link between Glu164 and reduced catalytic activity and increased [Asp]0.5 of the K164E/E239K ATCase structure is shown. The catalytic chain of the K164E/E239K structure has less rmsd than the R-state active site (Fig. 5).

A comparison of the active site region of the K164E/E239K ATCase with the T and R states of the wild-type enzyme is shown with electron density in Fig. 5. Key differences include significant alterations in the positions of Arg54 and Arg167, which are two key residues involved in catalysis. Both Arg54 and Arg167 swing out of the active site in the K164E/E239K ATCase structure. These alterations in side chain position may be responsible for the tertiary structure of the double mutant represents an intermediate state closer to the T state.

Another prominent feature of the T to R transition is the closure of the two domains of each catalytic chain to rearrange the active site residues so as to push the substrates toward another in the bimolecular reaction. However, the active site of the K164E/E239K ATCase more closely resembles the T-state structure (PDB ID 1ZA1) rather than the R-state PALA-ATCase structure in the catalytic chain between the wild-type and the K164E/E239K ATCase structures is reflected in the diameter of the tube. The catalytic chain of the K164E/E239K structure has less rmsd to that of the T-state structure than the R-state structure. Thus, the tertiary structure of the double mutant represents an intermediate state closer to the T state.

In addition to the K164E/E239K enzyme reported here, SAXS data collected on the K164E/E239K ATCase at pH 8.3 is comparable to the intermediate curve of the structure at the end of the early fast-rising phase of the time-resolved stop-flow X-ray scattering of ATCase upon mixing with substrates carbamoyl phosphate and 1-aspartate (15). The elongation of the enzyme is a necessary part of the allosteric transition, which requires the breaking of the T-state inter-subunit interactions involving both Glu239 and Asp236; thus the K164E/E239K structure, lacking these interactions, is a unique structure along the path of the quaternary structural transition from the T to the R state. As such, these findings suggest that there may be transiently stable intermediate structure(s) that the protein passes through during the allosteric transition.

**Table 1. Data collection and refinement statistics for the K164E/E239K ATCase structure**

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Space group</th>
<th>Cell dimensions</th>
<th>a, b, c (Å)</th>
<th>α, β, γ (°)</th>
<th>Resolution (Å)</th>
<th>R_{free} (%)</th>
<th>Completeness (%)</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>P2_1,2_1</td>
<td></td>
<td>124.1, 144.8, 203.4</td>
<td></td>
<td>90, 90, 90</td>
<td>29.89–2.80</td>
<td>92.4 (92.4)</td>
<td>3.33 (3.35)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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Values in parentheses are for the highest resolution shell.

Most interestingly, the scattering curve of the K164E/E239K ATCase at pH 8.3 is comparable to the intermediate curve of the structure at the end of the early fast-rising phase of the time-resolved stop-flow X-ray scattering of ATCase upon mixing with substrates carbamoyl phosphate and 1-aspartate (15).

**Methods**

**Enzyme Preparation.** The mutations K164E/E239K were introduced to the catalytic chains of ATCase on plasmid pEK152 (16) by site-specific mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) to produce plasmid pEK695. The double mutant was isolated according to the procedure described by Nowlan and Kantrowitz (17), from overproducing E. coli strain EK1104 (A ara, thi, l-pro-lac, ΔpyrB, pyrF+, rpsL), containing the plasmid pEK695. The enzyme was purified by isoelectric precipitation (17), ion exchange, and hydrophobic interaction chromatography. For the ion-exchange purification, a Q-Sepharose Fast Flow (GE Healthcare) column (11 cm × 2.5 cm) was used. The protein was eluted using a linear gradient from 0.05 M Tris-acetate, 2 mM 2-mercaptoethanol, pH 8.3 (Low Q buffer) to Low Q buffer plus 0.3 M sodium chloride. For hydrophobic interaction chromatography, a Phenyl Sepharose High Performance (GE Healthcare) column (8.5 cm × 2 cm) was used. After the ion-exchange chromatography, ATCase-containing fractions were dialyzed into Low Q and brought to 20% saturation with ammonium sulfate and loaded onto the Phenyl Sepharose column. The protein was eluted using a linear gradient from Low Q buffer plus 20% saturating ammonium sulfate to Low Q buffer. The homogeneity of the catalytic chain was checked by SDS-PAGE (18), and the concentration of purified double-mutant enzyme was determined by the Bio-Rad version of Bradford's dye-binding assay using wild-type ATCase as the standard (21).

**Crystallization and Data Collection.** A sample of 10 mg/mL purified K164E/E239K ATCase was sent to the Hauptman-Woodward Institute for high-throughput screening of crystallization conditions (22). The final crystallization condition was developed on the basis of one of the screening results. The mutant enzyme was crystallized by the hanging-drop vapor diffusion method. Two microliters of enzyme (10 mg/mL) in 50 mM Tris-acetate (pH 8.3) was mixed with 2 μL of crystallization buffer [0.2 M sodium phosphate buffer, 8.3% PEG 4000, 0.04 M Na2MoO4, 0.04 M N-cyclonexyl-3-aminopropanesulfonic acid, 2 mM 2-mercaptoethanol, pH 8.3] (Low Q buffer) in a hanging drop over a reservoir of 1.0 mL of crystallization buffer at 20 °C. Bar-shaped crystals grew to average dimensions of 0.3 × 0.3 × 0.4 mm in approximately 2 wk.

Crystals were transferred into a freezing solution containing 20% (vol/vol) ethylene glycol in crystallization buffer for 1 min before freezing in liquid N2. Data were collected in the Boston College Crystallography Facility. X-rays were generated using a Rigaku MicroMax-007HF rotating-anode generator operating at 40 kV and 30 mA, and data collected using a Rigaku K-axis IV++ detector. The diffraction data were integrated, scaled, and averaged using d*TREK (23).

**Structure Determination.** Attempts to solve the structure of the K164E/E239K ATCase by molecular replacement succeeded using the T-state ATCase structure (PDB ID 1ZA1) rather than the R-state PALA-ATCase structure in the same space group (PDB ID 1Q95). First, one catalytic trimmer (c3) of the double mutant was found by using the c3 of the T-state structure (PDB ID 1ZA1) in the MOLREP (24) as implemented in CCP4 (25). The double mutant was isolated according to the procedure described by Nowlan and Kantrowitz (17), from overproducing E. coli strain EK1104 (A ara, thi, l-pro-lac, ΔpyrB, pyrF+, rpsL), containing the plasmid pEK695. The enzyme was purified by isoelectric precipitation (17), ion exchange, and hydrophobic interaction chromatography. For the ion-exchange purification, a Q-Sepharose Fast Flow (GE Healthcare) column (11 cm × 2.5 cm) was used. The protein was eluted using a linear gradient from 0.05 M Tris-acetate, 2 mM 2-mercaptoethanol, pH 8.3 (Low Q buffer) to Low Q buffer plus 0.3 M sodium chloride. For hydrophobic interaction chromatography, a Phenyl Sepharose High Performance (GE Healthcare) column (8.5 cm × 2 cm) was used. After the ion-exchange chromatography, ATCase-containing fractions were dialyzed into Low Q and brought to 20% saturation with ammonium sulfate and loaded onto the Phenyl Sepharose column. The protein was eluted using a linear gradient from Low Q buffer plus 20% saturating ammonium sulfate to Low Q buffer. The homogeneity of the catalytic chain was checked by SDS-PAGE (18), and the concentration of purified double-mutant enzyme was determined by the Bio-Rad version of Bradford's dye-binding assay using wild-type ATCase as the standard (21).

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Values in parentheses are for the highest resolution shell.
noncrystallography symmetry. The 80’s and 240’s loops of the catalytic chains had elevated b-factors, which were higher than in the T- or R-state structures of the wild-type enzyme. The higher b-factors are most likely due to loss of the density of these loops was weak and the b-factors high, the atomic coordinates have been included for completeness. Water molecules were added to the structure using PHENIX (26) on the basis of the F-c, F-map at or above the 3.0 λ level. After completion of the refinement, the final R_total and R_free were 0.214 and 0.274, respectively. The model was checked for errors using PROCHECK (30). The detailed statistics of data collection and refinement are given in Table 1. Residues 1–9 in the regulatory chains were omitted from the deposited structure owing to extremely weak electron density. Coordinates for the K164E/E239K ATCase structure have been deposited with the PDB (PDB ID 4E2F).

Small-Angle X-Ray Scattering. The SAXS experiments were performed at Beamline 4-2 at the Stanford Synchrotron Radiation Laboratory (3.0 GeV, 50–100 mA). The experimental setup and procedures were performed as described by Sakash et al. (31). For experiments at pH 8.3 the buffer used was 0.05 M Tris-acetate and 10 mM 2-mercaptoethanol, whereas for pH 7.0 and pH 7.5 the buffer used was 0.05 M Hepes buffer, 50 mM KCl, and 20 mM 2-mercaptoethanol.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health (NIH) Grant GM026237 (to ERK) and Grant CHE-0923264 from the National Science Foundation. The Stanford Synchrotron Radiation Laboratory (SSRL) is operated by the Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Biology Resource is supported by NIH, National Center for Research Resources (P41 RR01208) and by the Department of Energy, Office of Biological and Environmental Research.
Supporting Information

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Fig. S1. Small-angle X-ray scattering of the wild-type and the K164E/E239K enzymes at (A) pH 8.3, (B) pH 7.5, and (C) pH 7.0. The data are plotted as scattering intensity (I) vs. momentum transfer (q). Scattering intensity was normalized to take into account the small differences in enzyme concentration between samples. At each pH the wild-type enzyme in the absence of ligands is shown in black and in the presence of PALA in blue. The K164E/E239K enzyme in the absence of ligands is shown in red and in the presence of PALA in orange. The green dashed line indicates the best fit of a combination of the wild-type T pattern and the K164E/E239K PALA R pattern to the K164E/E239K pattern in the absence of ligands. Each scattering curve corresponds to 100 data points connected by linear line segments.

$q = 4 \pi \sin \theta / \lambda (\text{Å}^{-1})$
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Table S1. Radius of gyration (R_g) as determined by Guinier analysis of SAXS data at pH 8.3

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<th>Enzyme</th>
<th>Ligand</th>
<th>R_g (Å)</th>
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<tr>
<td>Wild-type</td>
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Fig. S3. Size-exclusion chromatography of the (A) wild-type and the (B) K164E/E239K ATCases on a Bio-Prep SE1000/17 column (BioRad Laboratories). The column was equilibrated with 0.05 M Tris acetate buffer (pH 8.3) at a flow rate of 0.25 mL/min. At zero, the sample in a total volume of 300 μL was injected, and the sample was eluted with the same buffer and at the same flow rate.
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Fig. S2. Small-angle X-ray scattering of the wild-type and the K164E/E239K enzymes at (A) pH 8.3, (B) pH 7.5, and (C) pH 7.0. The data are plotted as scattering intensity (I) vs. momentum transfer (q). Scattering intensity was normalized to take into account the small differences in enzyme concentration between samples. At each pH the wild-type enzyme in the absence of ligands is shown in black and in the presence of PALA in blue. The K164E/E239K enzyme in the absence of ligands is shown in red and in the presence of PALA in orange. The green dashed line indicates the best fit of a combination of the wild-type T pattern and the K164E/E239K PALA R pattern to the K164E/E239K pattern in the absence of ligands. Each scattering curve corresponds to 100 data points connected by linear line segments.
Table S1. Radius of gyration ($R_g$) as determined by Guinier analysis of SAXS data at pH 8.3

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