Studies of the Structure and Function of E.coli Aspartate Transcarbamoylase

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Studies of the Structure and Function of \textit{E. coli} Aspartate Transcarbamoylase

Katherine M. Loftus

April 2006
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Abstract

*E. coli* Aspartate transcarbamoylase (ATCase) is the allosteric enzyme that catalyzes the committed step of the *de novo* pyrimidine biosynthesis pathway. ATCase facilitates the reaction between L-aspartate and carbamoyl phosphate to form N-carbamoyl-L-aspartate and inorganic phosphate. The holoenzyme is a dodecamer, consisting of two trimers of catalytic chains, and three dimers of regulatory chains. ATCase is regulated homotropically by its substrates, and heterotropically by the nucleotides ATP, CTP, and UTP. These nucleotides bind to the regulatory chains, and alter the activity of the enzyme at the catalytic site. ATP activates the rate of ATCase’s reaction, while CTP inhibits it. Additionally, UTP and CTP act together to inhibit the enzyme synergistically, each nucleotide enhancing the inhibitory effects of the other. Two classes of CTP binding sites have been observed, one class with a high affinity for CTP, and one with a low affinity. It has been theorized that the asymmetry of the binding sites is intrinsic to each of the three regulatory dimers. It has been hypothesized that the second observed class of CTP binding sites, are actually sites intended for UTP.

To test this hypothesis, and to gain more information about heterotropic regulation of ATCase and signal transmission in allosteric enzymes, the construction of a hybrid regulatory dimer was proposed. In the successfully constructed hybrid, each of the three regulatory dimers in ATCase would contain one regulatory chain with compromised nucleotide binding. This project reports several attempts at constructing the proposed hybrid, but ultimately the hybrid enzyme was not attained.

This project also reports preliminary work on the characterization of the catalytic chain mutant D141A. This residue is conserved in ATCase over a wide array of species, and thus was mutated in order to ascertain its significance.
### Abbreviations Used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>ATCase</td>
<td>Aspartate transcarbamoylase</td>
</tr>
<tr>
<td>T state</td>
<td>Tense state, or the low-affinity, low-activity conformation of ATCase</td>
</tr>
<tr>
<td>R state</td>
<td>Relaxed state, or the high-affinity, high-activity conformation of ATCase</td>
</tr>
<tr>
<td>CA</td>
<td>Carbamoyl aspartate</td>
</tr>
<tr>
<td>PP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>CP</td>
<td>Carbamoyl phosphate</td>
</tr>
<tr>
<td>ASP</td>
<td>L-aspartate</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type aspartate transcarbamoylase</td>
</tr>
<tr>
<td>[S] 0.5</td>
<td>Half Saturation point</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum velocity</td>
</tr>
<tr>
<td>PALA</td>
<td>N-phosphonacetyl-L-aspartate</td>
</tr>
<tr>
<td>K60E/K94E</td>
<td>ATCase regulatory chain with the mutations Lys60→Glu and Lys94→Glu</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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</table>
CHAPTER 1

Introduction
“We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium, a benefit for humanity.”

-Marie Curie

At its most fundamental level, science is a source of truth, and an attempt to expand man’s understanding of the universe. The pursuit of scientific truth extends back into history as far as the philosophers of ancient Greece, and is woven together by society with truths derived from philosophy, religion, and art. The scientist looks at the world as a puzzle, and approaches this puzzle with the belief that a solution exists, and a firm commitment to discovering the solution. Scientists may be motivated by “the excitement of exploring new territory, the hope of finding order, and the drive to test established knowledge.”

Outside of the academic community science is most valued for its practical contributions to society. Science gives rise to technologies and medical advances which extend human life, and make it more comfortable and convenient. The field of biochemistry applies scientific curiosity to life on earth, at its molecular level. Looking at life this closely has affected the accumulation of an expansive volume of information about the way biological organisms function or malfunction. It has also allowed for medical advances which are pertinent not just to a single organ or limb, but to every cell in the body. Rational drug design to combat viruses or cancer, and the synthetic production of proteins which treat various diseases are among the accomplishments of biochemistry.
One of the most important and interesting types of molecules to a biochemist is an enzyme. Enzymes perform the crucial function of speeding up metabolic reactions to rates which can support life. Enzymes interact exclusively with their specified substrates, and in this way the body is able to control which reactions take place. Enzymology becomes more complicated when allosteric enzymes are considered. In addition to control over which reactions take place, organisms need to control when, and how fast these reactions take place. This is achieved through allosteric enzymes, which often have complex structures to carry out their multiple functions. Aspartate transcarbamoylase from \textit{Escherichia coli} is one of the most extensively studied allosteric enzymes, and can in many ways be considered a paradigm for regulatory molecules.

1.1 BASICS OF ASPARTATE TRANSCARBAMOYLASE

Aspartate transcarbamoylase is encoded by the \textit{pyrBI} operon of \textit{Escherichia coli}. This operon is regulated by the pyrimidine nucleotide uridine 5-triphosphate (UTP), in an attenuation control mechanism which allows transcription to proceed when concentrations of UTP are low\textsuperscript{2}. ATCase is a key enzyme in the synthesis of UTP. Therefore, the attenuation mechanism serves to help maintain a relatively stable pool of UTP by allowing ATCase to be transcribed only when additional UTP is needed.

When the twelve polypeptide chains of ATCase are transcribed and translated, they assemble to form a dodecamer. The quaternary structure of ATCase consists of six catalytic and six regulatory polypeptide chains\textsuperscript{3}. Each catalytic chain has a molecular weight of 33,000 Da, and each regulatory chain has a molecular weight of 17,000 Da.
The polypeptide chains are organized into two trimeric catalytic subunits and three dimeric regulatory subunits. The three dimensional arrangement of these subunits is shown below.

**Figure 1.** View of Aspartate transcarbamoylase along the threefold axis.\(^4\)

The enzyme’s active sites are located at the junctions between the catalytic chains. This amounts to three active sites per catalytic trimer, and six per molecule. Each active site contains a domain for each of the enzyme’s two substrates, L-aspartate (Asp), and carbamoyl phosphate (CP). The catalysis of the reaction between these two molecules to form N-carbamoyl-L-aspartate and inorganic phosphate is the basic function of ATCase. The carbamoyl group of carbamoyl phosphate is transferred to the \(\alpha\)-amino group of L-Asp. This reaction is the first step unique to the pyrimidine biosynthesis pathway. With the help of several other enzymes, carbamoyl aspartate, ATCase’s product, is eventually converted to UTP and to cytidine 5’-triphosphate (CTP).
Figure 2. Mechanism of the reaction between carbamoyl phosphate and aspartate to form carbamoyl aspartate and inorganic phosphate

The binding of ATCase’s substrates is ordered. Carbamoyl phosphate binds first to its site on the catalytic chain, and the binding of L-aspartate follows. The proposed mechanism for the reaction is that the leaving phosphate group of carbamoyl phosphate helps remove a proton from the NH$_2$ group of aspartate. This nitrogen can then readily attack the carbonyl carbon of CP. The reaction then proceeds through a tetrahedral intermediate before the inorganic phosphate ion leaves. When the reaction is complete, the enzyme first releases carbamoyl-aspartate and second inorganic phosphate.

1.1 HOMOTROPIC REGULATION

In 1965, Monod, Wyman, and Changeux proposed a model stating that allosteric enzymes exist in two conformational states that are in equilibrium with each other. These states are known as the tense (T) state, and the relaxed (R) state. The T state is characterized by a structural constraint which impedes ligand binding. The R state represents a release from this structural constraint, allowing the ligand to bind to the molecule more easily. When no ligand is bound to the enzyme, the equilibrium is
shifted towards the T state. However, ligand binding at even one subunit of a multi-subunit enzyme causes a concerted transition from the T to the R state in all subunits. ATCase adheres to this model of global conformational change upon ligand binding. For ATCase, the T to R transition involves an 11 Å expansion and rotation of the catalytic subunits about the enzyme’s 3-fold axis (Figure 3). The aspartate and carbamoyl phosphate domains undergo domain closure, while the two domains of the regulatory chain undergo domain opening. The global change in quaternary structure is also accompanied by changes in the enzyme’s tertiary structure, which help to form the active site. The transition to the R state is essential to the formation of the fully catalytically effective active site.

Figure 3. T (left) and R (right) structural states of ATCase. Catalytic chains are shown in red and regulatory chains are shown in grey.

The two state model accounts for the homotropic cooperativity and the sigmoidal kinetics of aspartate binding to ATCase. When assays of the kinetic properties of the enzyme are performed, as the concentration of aspartate is steadily increased the
enzyme’s velocity increases in a manner that results in an “S”-shaped, or sigmoidal curve\textsuperscript{12}.

![Aspartate Saturation Curve](image)

**Figure 4.** L-aspartate saturation curve resulting from a colorimetric assay performed in 0.05M Tris-acetate buffer at saturating concentrations of CP. Specific activity in mmoles N-carbamoyl-L-aspartate formed per hour per mg of ATCase\textsuperscript{21}

The sigmoidal shape can be explained by the fact that aspartate binds preferentially to the R state of the enzyme which causes a shift of the equilibrium towards the R conformation. When a small amount of the substrate is present, it promotes the T to R transition. The enzyme is then in its more active conformation, and so a more rapid increase in velocity is observed\textsuperscript{13}.

A similar effect to positive cooperativity of substrate binding is observed when the bi-substrate analogue N-(phosphonacetyl)-L-asparate (PALA) binds to ATCase. PALA combines the structural features of both of the two natural substrates of the enzyme into one molecule. Figure five illustrates the structural similarities.
Figure 5. Structures of N-(Phosphonacetyl)-L-Aspartate and the substrates of the reaction catalyzed by aspartate transcarbamoylase.\textsuperscript{15}

PALA binds simultaneously to the CP and Asp site, and it binds about one thousand times more tightly than does carbamoyl phosphate. This makes PALA a highly potent competitive inhibitor.

PALA mimics the substrates of ATCase not only by binding at the same site, but also by causing the enzyme to undergo the same global conformational change from the T to the R state. The binding of one PALA molecule to ATCase transforms all active sites to the more catalytically proficient R-state conformation. Therefore, at low concentrations, PALA serves to activate the enzyme, increasing its maximal velocity of catalysis, rather than inhibiting it. As concentrations of PALA increase, it out-competes CP at all of the active sites, and effectively prevents catalysis\textsuperscript{14}. 
1.3 HETEROTROPIC REGULATION: THE REGULATORY CHAINS

Aspartate transcarbamoylase’s catalytic subunits are of great importance because they carry out the catalysis of carbamoyl aspartate formation, and respond with great sensitivity to the presence of substrate. However, its regulatory chains make ATCase more biochemically and metabolically interesting. The regulatory chains determine the pattern of the heterotropic regulation of the molecule. In a study done by Wild et. al. in 1989, hybrid ATCase molecules were constructed that had catalytic chains from *Escherichia coli* and regulatory chains from *Serratia marcescens*. The regulatory phenotype of this hybrid molecule was that of the *S. marcescens*, proving that it was in fact the regulatory dimers, and not the catalytic trimers which allow ATCase to be controlled by its heterotropic effectors, adenosine 5’- triphosphate (ATP) and cytosine 5’-triphosphate (CTP)\(^1\). Even without nucleotides bound, the regulatory chains place a certain amount of constraint on the catalytic chains. Evidence for this is found in the fact that the maximum activity of the holoenzyme reaches only two thirds of the activity of the isolated catalytic trimer\(^3\).

ATCase’s nucleotide effectors bind to the molecule some 60 Å away from ATCase’s catalytic site, and yet they are able to cause changes which alter the way that substrates bind at the catalytic site\(^1\). This characteristic raises questions about signal transmission in the enzyme. The mechanism by which the nucleotides effect changes at the active site, and thereby modulate catalytic activity, is not well understood. The study of ATCase’s regulatory chain can be seen as an opportunity to learn more about the ways in which information is passed from subunit to subunit within one molecule. Additionally, the regulatory chains make possible control of the enzyme by feedback inhibition.
Like the catalytic chains, the regulatory chains have two functional domains. The first is the allosteric domain where nucleotide effectors bind (Figure 6a). The allosteric domain is formed by a ten-strand anti-parallel β-sheet, flanked on one side by a row of α-helices (Figure 6b)\(^ {17}\). The second domain is the zinc domain and is important for the affinity of the two regulatory chains in each regulatory subunit for each other, and for the complexing of the regulatory chains with the catalytic chains\(^ {18}\). There are six Zn\(^ {2+}\) ions present per molecule of ATCase, and each zinc is coordinated to the molecule by the sulfhydryl groups of four cysteine residues. The presence of Zn\(^ {2+}\) is important to the stability of the molecule\(^ {17}\).

The N-terminus of the regulatory chain also has several significant features. It is important for preventing aggregation of regulatory chains, and evidence exists that it is involved in the formation of the nucleotide binding sites\(^ {18}\).

**Figure 6.** a. View of the quaternary structure of ATCase with domains of the catalytic and regulatory chains labeled as followed al, allosteric domain; Zn, zinc domain; cp, carbamoyl phosphate binding domain; asp, aspartate binding domain. b. View of the allosteric domains of a regulatory dimer (R1-R6). Shown with CTP bound.\(^ {18}\)
ATCase’s regulatory chains interact with the nucleotides CTP, UTP, and ATP, to affect a regulation of the molecule’s activity. Understanding the binding characteristics of these ligands is important to the understanding of the enzyme’s mechanism.

**Figure 7.** Molecular Structure of ATCase’s three nucleotide effectors

Early studies of the binding of CTP to ATCase revealed the presence of six total binding sites for CTP, which could be divided into two discrete classes. One set of binding sites had a much greater affinity for CTP than the second site. This was revealed by equilibrium dialysis studies, and the resulting Scatchard Plots which deviated from a straight line. These plots were indicative of two classes of binding sites, one with high affinity and one with low affinity for CTP (Figure 8).
Figure 8. CTP binding isotherms plotted as \( r/(\text{CTP}) \) vs. \( r \), where \( r \) is the number of moles of ligand bound per mole of enzyme and \( (\text{CTP}) \) is the concentration of free ligand\(^{20}\).

Winlund and Chamberlin found that not only was CTP binding heterogeneous to the ATCase holoenzyme, but it was heterogeneous to each regulatory dimer. This meant that to each regulatory subunit, CTP could bind once with high affinity and once with low affinity. However, whether or not the observed nonequivalence of the site was pre-existing and due to some asymmetry in the polypeptide, or was induced upon binding of the first CTP molecule was unknown\(^{20}\).

The phenomenon in which it becomes harder for a ligand to bind with each subsequent ligand that is bound, is known as negative cooperativity. CTP binding to ATCase is an archetypal example of this characteristic. A proposed evolutionary reason for such a development is that it widens the range of ligand concentration which can be effective for regulation of the molecule. A protein is most sensitive to effector concentrations which approximate the dissociation constant, \( K_d \), of the protein-effector complex. For this reason, the presence of two discrete classes of binding sites, with \( K_d \)'s
that differ by at least an order of magnitude, greatly enhances the range of concentrations for which ATCase can be sensitive to the presence of CTP\textsuperscript{20}.

Studies with UTP as a heterotropic effector revealed that UTP and CTP work together synergistically to inhibit the enzyme. UTP on its own is not an effective inhibitor of ATCase. However, when both pyrimidine nucleotides are present, they work together to inhibit activity to a greater extent than either of the two can independently.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure9}
\caption{Influence of nucleotide triphosphates on the activity of ATCase. Colorimetric assays were performed in 0.1 M imidazole-acetate buffer in the presence of increasing concentration of a) CTP, and b) CTP (open circles) and CTP and UTP (closed circles).\textsuperscript{21}}
\end{figure}
Based on this information, a theory was developed that explained the behavior of CTP and UTP in regards to ATCase. The three so-called low affinity CTP sites are actually sites designated by ATCase for UTP. By this measure, a full inhibition of the enzyme is only possible when both nucleotides are present to fill their sites.

ATP also has a regulatory effect on ATCase. Rather than inhibiting the activity of the enzyme, ATP acts as an activator, increasing the enzyme’s activity\textsuperscript{12} (Figure 10). ATP and CTP show competitive behavior in binding to the regulatory chain, indicating that these two nucleotides bind at the same site. ATP however, binds to the site much less strongly than CTP.

\textbf{Figure 10.} Graph of kinetic data displaying the effect of ATP and CTP on reaction velocity of ATCase. Specific activity in units of mmols N-carbamoyl-L-aspartate produced per hour per mg of protein. Activating effect of ATP (red), Aspartate saturation curve in the absence of any nucleotides (blue), Inhibiting effect of CTP (green).

While it is clear that ATP, CTP, and UTP bind to and regulate aspartate transcarbamoylase, the mechanism by which they do so is ambiguous. Homotropic regulation in ATCase adheres well to the two-state model proposed by Monod, Wyman,
and Changeux. Heterotropic regulation however, can not be described by this model as easily. It remains unclear whether binding of nucleotide effectors brings about regulation by way of a global conformation change, and a shift in the T to R equilibrium caused by stabilization of one of the two forms of the enzyme, or by way of more localized structural changes that propagate the regulatory signal to the active site\textsuperscript{10}.

1.4 METABOLIC CONTROL

The volume of information about aspartate transcarbamoylase which has been accumulated is immense. What makes this molecule such a focal point of scientists’ attention? One of the undercurrents of the study of ATCase is the theme of control. ATCase is controlled in three distinct and important ways. The first of which is the attenuation control mechanism which allows ATCase to be transcribed from its gene only when UTP, the end-product of the pathway, is needed. This conserves resources, ensuring that the cell does not waste the energy required for the transcription and translation of a large macromolecule if it will not be immediately necessary.

The second mechanism of control is the homotropic regulation by the substrate aspartate. Aspartate binds to ATCase with positive cooperativity, meaning binding is enhanced with each subsequent ligand bound. ATCase is regulated by being shifted into a more active conformation, and increasing its maximal velocity when substrate is present. Positive cooperativity gives a molecule the metabolic advantage of signal amplification. ATCase is more sensitive, and can respond more efficiently to the presence of aspartate because of this property of homotropic regulation.
The third way in which ATCase is controlled is by a mechanism known as feedback inhibition. In feedback inhibition, an end product of a pathway imposes control by stopping the production of more of itself. The production of CTP and UTP by ATCase is controlled by feedback inhibition (Figure 12)\textsuperscript{12}.

\textbf{Figure 12.} Schematic diagram of the feedback inhibition of pyrimidine biosynthesis in E.coli. Heavy broken arrow indicates feedback inhibition\textsuperscript{12}.

Feedback inhibition dictates that additional end product is not made unless existing end product is being consumed. This is beneficial to an organism because it conserves resources and prevents the excessive buildup of any given end product.

Analyzing the synergistic inhibition of ATCase by CTP and UTP within the context of feedback inhibition reveals a new layer of logic in the way that ATCase functions. Elevated levels of CTP, in addition to the partial inhibition of ATCase, cause a full inhibition of the enzyme CTP synthetase, effectively shutting down the production of any more CTP. However, ATCase is left partially active, and thus the production of UTP is still possible. For the biosynthesis of UTP to be prevented, UTP levels must also be
elevated, to complete the inhibition of ATCase\textsuperscript{22}. That ATP would activate ATCase is also logical. Cellular levels of purine and pyrimidine nucleotides must be kept relatively even for organisms to be able to effectively produce nucleic acids. Therefore, an excess of ATP, a purine nucleotide, indicates to the cell that more pyrimidines should be produced. The fact that ATP competes for the same site as CTP, but binds much less strongly, ensures that regulation by ATP and CTP do not cancel each other out. Cellular levels of ATP must be significantly higher than cellular levels of CTP in order to effectively inhibit ATCase.

The importance of metabolic control is most noticed in its absence. When cells lose their ability to control their rate of growth and division, the result is cancerous growth. Due to the fact that ATCase plays a crucial role in the production of nucleotides, creating a drug which could stop the catalytic activity of ATCase is an attractive possibility as a way to impose control upon cancerous cells. Denying a cell the building blocks for DNA synthesis will stop its progress through the mitotic cycle. As mentioned earlier, PALA is a highly potent inhibitor of ATCase. For this reason it was given serious consideration as an antiproliferative drug, to be used in conjunction with an inhibitor of the pyrimidine nucleotide salvage pathway. Studies were able to demonstrate that PALA could effectively inhibit \textit{de novo} pyrimidine nucleotide synthesis in mammalian cells in culture\textsuperscript{23}. PALA’s toxicity prove to be too high for medicinal use, but it has sparked researchers to work towards the creation of a similarly effective, but less toxic inhibitor.
1.5 OBJECTIVES

In hopes of gaining more answers about the mechanism of signal transmission in the regulatory chain, the creation of a hybrid regulatory subunit has been proposed. The hybrid would be composed of one wild-type regulatory polypeptide chain, and one which had been mutated so that it was incapable of binding any of the nucleotide effectors. This hybrid subunit would then be combined with wild-type catalytic trimers to reconstitute the holoenzyme. An ATCase molecule in which half of the regulatory chains are non-functional would afford us with the opportunity to observe the relationship between the two polypeptide chains which comprise the regulatory dimer. Kinetic studies done on the hybrid would potentially be able to answer several questions about nucleotide binding and effects, and signal transmission in allosteric enzymes. Such studies would allow for the examination of whether or not both CTP and UTP are able to bind and act as inhibitors and whether or not the enzyme is able to respond differently to these two nucleotides, whether or not ATP can still bind and activate the enzyme, and finally, whether or not proper functioning of both subunits of the regulatory dimer is essential to signal transmission through the molecule.

Figure 13. Schematic representation of regulatory chain hybrids. Holoenzyme consists of two catalytic chain trimers, and three regulatory dimers, (two are shown) composed of one wild type (WT) and one mutant (M) regulatory chain.
Previous work in the Kantrowitz lab has made many attempts at the creation of regulatory chain hybrids. In this project, several new strategies were conceived in hopes of successfully attaining the hybrid. The first of these strategies was the creation of a plasmid which contained a histidine tail attached to the N-terminus of a wild type regulatory chain gene, followed by a double mutant regulatory chain gene. The theory was that because the two genes would be translated by the same ribosome, one immediately following the other, at their formation the two protein monomers would be in close proximity to each other. They therefore would have a high likelihood of dimerizing as a hybrid. The histidine tag would serve as a chromatographic handle aiding in the isolation of the hybrid molecule, which uniquely would have only one histidine tag (as opposed to two or zero in the wild type or mutant dimers, respectively). (Figure 14).

**Figure 14.** Cartoon Representing the three types of regulatory chain dimers which should form from the proposed plasmid. *Left,* Mutant dimer, no histidine tags. *Middle,* Hybrid dimer, one histidine tag. *Right,* Wild type dimer, two histidine tags.

In a second approach, a signal sequence from the enzyme alkaline phosphotase (AP) was attached to the N-terminus of the ATCase regulatory chain. In AP this signal sequence targets the enzyme to the periplasmic space of the bacteria. To get to the periplasm the enzyme must be pulled through the cell membrane, one polypeptide chain at a time. By co-transforming an *E.coli* cell with a plasmid containing a wild-type regulatory chain with a signal sequence, and a plasmid containing a mutant regulatory
chain with a signal sequence, it seemed possible that wild-type and mutant regulatory chains could be pulled through the cell membrane as monomers, and would then dimerize to form a hybrid when they meet in the periplasm.

A third strategy for creating a hybrid involved the synthesis of a small polypeptide designed to bind to the dimerization domain of the regulatory chain, and therefore block the formation of a regulatory chain dimer. If dimerization of wild-type enzyme could successfully be blocked, it might then be possible to use mutant regulatory chain in a high molar excess to out-compete the peptide for the binding site, and form a hybrid dimer.

Finally, experiments were conducted using only EDTA to promote monomerization. Wild-type and mutant monomers were then combined, in an environment that would facilitate re-dimerization, in hopes that this procedure would yield some hybrid dimers.

Due to the fact that the hybrid project was unsuccessful, a second project was begun. This second, less extensive project, is the kinetic characterization of a catalytic chain mutant of ATCase. Evolutionarily conserved residues in ATCase were searched for and located by the Kantrowitz group through extensive comparison of the ATCase amino acid sequences from many different species. Residues which remain unchanged across a spectrum of organisms ranging from bacteria to humans are assumed to have a functional significance. The goal of this project is to mutate these residues and then examine the changes in the enzyme’s behavior. Colorimetric assays of the mutant enzyme’s kinetic behavior should help reveal whether or not the conserved amino acid in question is critical to any of the functions of aspartate transcarbamoylase.
CHAPTER 2

Materials and Methods
Materials—Adenosine-5’-triphosphate, agar, agarose, ammonium persulfate, ampicillin, bromophenol blue, cytidine-5’-triphosphate, L-aspartic acid, nickel sulfate, sodium chloride, sodium dodecyl sulfate, sodium EDTA, sucrose, and 2-mercaptoethanol were purchased from Sigma Chemical Co. Tris was purchased from MP Biomedicals. 2,3-butanedionemonoxime was purchased from Acros Organics. Dialysis tubing, glacial acetic acid, hydrochloric acid, sulfuric acid, tryptone, and yeast extract were purchased from Fisher Scientific. Q-Sepharose Fast Flow (QFF) resin was purchased from Pharmacia. Bis, coomassie blue, TEMED, and UnoSphere Q Ion Exchange resin was purchased from BioRad. Acrylamide and enzyme grade ammonium sulfate were purchased from ICN Biomedicals. Kanamycin sulfate was purchased from USB Corporation. Chelating Sepharose Fast Flow was purchased from Amersham Biosciences. Oligonucleotide primers and reagents for DNA purification and gel extraction were purchased from Qiagen and were used according to supplier’s recommendations. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs.

Strains — E. coli strain EK1611 [Δ(mcrA)183, Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lacF’ proAB, lacZΔM15, Tn10(Tet^r)], based on XL1-Blue MRF’ was obtained from Stratagene (La Jolla, CA). The strain BLR(DE3) [F’ dcm ompT hsdS(rB^-mB^-) gal λ(DE3)] was also obtained from Stratagene. The strain EK1594 [F’ ara, thi, Δpro-lac, ΔpyrB, pyrF^t, rpsL λ(DE3)] was previously constructed in this laboratory.
**Methods**—Construction of a plasmid containing a histidine tag on the N-terminus of the wild-type pyrI gene, followed by the mutant pyrI gene, K60E/K94E.

The *E. coli* ATCase plasmid pEK67 carries the wild-type *pyrI* gene, preceded by a sequence coding for a histidine tag. It is controlled by a T7 promoter allowing for protein over-expression in the presence of IPTG, and contains a gene for kanamycin resistance. Plasmid pEK599 carries the *E. coli* regulatory chain double mutant K60E/K94E, known to exhibit compromised nucleotide binding, and a -4 charge difference from the wild-type enzyme when reconstituted with the catalytic subunits.

**Figure 15.** Cartoon of plasmid containing a wild-type *pyrI* gene with a histidine tag at the N-terminus, followed by a double mutant *pyrI* gene

In order to construct a plasmid with a histidine tag followed by wild-type *pyrI* and double mutant *pyrI*, the two plasmids were digested with restriction enzymes NdeI and XhoI and electrophoresed on a 1% agarose and ethidium bromide gel. The larger fragment from pEK647 and the smaller fragment from pEK599 were cut out of the gel.
and ligated using T7 DNA ligase. EK1611 competent cells, designed for plasmid over-expression were then transformed with the ligated DNA and grown overnight on a YT-kanamycin plate. A colony was then selected from this plate and a 5 mL culture of YT media with 0.05 mg/mL kanamycin was grown overnight. The desired plasmid was purified from this culture using a Qiagen plasmid prep kit. Competent BLR cells were then transformed with the new plasmid. The strain BLR contains a deletion in the recA gene, which serves to prevent homologous recombination and provides high-level protein expression. Transformed BLR cells were plated on YT-kanamycin plates. Single colonies were then used to make 5 mL overnight cultures. The overnight cultures were used to inoculate 2L of YT-media with kanamycin. Cells were grown at 37° to approximately A₅₆₆=0.6, and then over-expression of the protein was induced by addition of 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). The culture then continued to grow overnight to an optical density of about 1.2, allowing for the cells to reach stationary phase. The culture was harvested by centrifugation (5000 rpm for 20 minutes). The pellets were re-suspended in 40 mL of ice-cold lysis buffer (0.1 M Tris-Cl, 0.1 mM zinc acetate, pH 8.3). The cells were then disrupted by sonication for 10 cycles of 30-second intervals at 50% duty. This process breaks the cell wall and releases intercellular proteins. The lysate was then centrifuged at 15000 rpm for 20 minutes. The pellet, which contained the cellular membranes, membrane proteins, DNA, and RNA was discarded. The supernatant contained cellular proteins.
Purification of regulatory chain hybrids using immobilized metal affinity chromatography (IMAC).

A nickel affinity column was prepared by washing the chelating sepharose fast flow column, first with distilled water, then with a 0.3 M nickel sulfate solution in order to apply the nickel to the column. Excess nickel was rinsed off with water. Several column volumes of binding buffer (0.05M Tris-HCl, 0.5M NaCl, pH 7.0) were then applied to the column. The mixture of cellular proteins which was the supernatant after the sonication step was then carefully applied to the prepared IMAC column. The immobilized nickel chelates the histidine tag on the proteins of interest. The column was once again rinsed with binding buffer to remove any non-bound protein. Protein of interest was eluted using an imidizole containing elution buffer. First a 0.05 M imidizole buffer was used for more weakly bound proteins, (0.05 M Tris-HCl, 0.05 M imidizole, 0.5 M NaCl, pH 7.5). and second a 0.4 M Imidizole Buffer was applied in order to elute any proteins that may have been bound more strongly. The material being eluted from the column was collected in fractions. The fractions were then analyzed by 15% native polyacrylamide gel electrophoresis (PAGE).

Construction of a plasmid containing an alkaline phosphotase signal sequence attached to the pyrI gene.

The E. coli ATCase plasmid pEK592 contains the wild-type pyrI gene. The alkaline phosphotase (AP) plasmid pEK196 contains the wild-type gene for AP, which is preceded by a signal sequence that targets AP for the periplasm. In order to transfer this signal sequence to the ATCase regulatory chain, the wild-type pyrI gene was cut from pEK592, and used to replace the wild type AP gene in pEK 196 (Figure 16). The two
plasmids were digested with restriction enzymes XmaI, and XhoI, and electrophoresed on a 1% agarose gel stained with ethidium bromide. The larger fragment from pEK196 and the smaller fragment from pEK592 were cut out of the gel, excess agarose was dissolved according to the protocol of the Qiagen Qiaquick gel extraction kit, and the DNA fragments were ligated using T7 DNA polymerase.

EK1611 competent cells were transformed with the ligated DNA, and successfully transformed cells were selected for on a YT-ampicillin plate. 5 mL overnight cultures were prepared from single colonies, and the desired plasmid was isolated from these cultures according to the protocol of the Quiagen Mini-Prep kit. EK1594 competent cells, a cell line which has the gene for ATCase deleted on its chromosome, were then transformed with the newly prepared plasmid, pEK649. Successfully transformed cells were selected for on a YT-ampicillin plate.

**Figure 16.** Cartoon depiction of plasmids and restriction enzymes required to create a plasmid with the AP signal sequence attached to a wild-type pyrI gene.
5 mL overnight cultures were prepared from single colonies. The overnight cultures were used to inoculate 50 mL trial cultures of YT-media with ampicillin. At an A\textsubscript{566} of 0.6, trial cultures were induced with 0.4 mM IPTG and allowed to grow either for 4 hours at 37° or overnight at 25°. After the growth period, cells were harvested by centrifugation at 5000 rpm for 20 minutes. The pellet was then resuspended in buffer containing 20% sucrose, 0.03 M Tris, 0.005 M EDTA, pH 7.4. The cells were then vortexed for five minutes and centrifuged for five minutes. The supernatent was removed, and the pellet was resuspended in cold water, vortexed, and centrifuged. This procedure is known as osmotic shock, it allows for the isolation of all proteins in the periplasmic space of a gram negative bacterium, while leaving the intracellular membrane in tact with all cytoplasmic proteins sequestered inside. At the end of the procedure the contents of the periplasm is in the supernatent, while the rest of the cell remains in the pellet. The cells in the pellet were then resuspended in Low Q buffer (50 mM Tris-acetate, 2 mM 2-mercaptoethanol, 0.1 mM zinc acetate pH 8.3), and subjected to sonication for 9 cycles of 30 second intervals at 50% duty. The lysate was then centrifuged at 15000 rpm for 20 minutes, and the supernatent containing the cytoplasmic proteins was saved. An identical osmotic shock followed by isolation of periplasmic and cytoplasmic proteins procedure was performed on cells that overexpressed the \textit{pyrI} gene without a signal sequence attached. The protein isolated from these cells served as a control. The four protein samples, periplasmic, cytoplasmic, control periplasmic, and control cytoplasmic, were analyzed by 15% Sodium dodecyl sulfate (SDS)-PAGE. SDS is an anionic detergent that coats proteins with a negative charge proportional to its
molecular weight. Each protein’s electrophoretic mobility is thus dependent only on its molecular weight, simplifying analysis.

Blocking dimer formation with a synthetic polypeptide

A member of the Kantrowitz Laboratory synthesized the pentapeptide Isoleucine-Threonine-Isoleucine-Glycine-Leucine. The polypeptide was designed to mimic the interface of regulatory chain dimerization. Examination of the sequence of the pentapeptide and of the regulatory chain showed that the pentapeptide would form six hydrogen bonds with the regulatory chain at the interface. Wild-type regulatory chain enzyme was mixed with varied ratios of polypeptide in molar excess and with 10 mM EDTA. The mixture was allowed to equilibrate overnight at 4°C. The mixture was then electrophoresed on a 15% native gel to visualize any band shifts as a result of addition of the polypeptide or of EDTA.

Sucrose Density Gradient Experiments

In order to determine more conclusively if the polypeptide caused the protein to exist as a monomer, sucrose gradients were created ranging from 6 to 20 % sucrose. 20 % sucrose was first added to a centrifuge tube, and then an equal volume of the 6 % sucrose was carefully added on top of the 20 % sucrose. A small volume of regulatory chain enzyme or enzyme mixed with the polypeptide, or with the polypeptide and EDTA, was added carefully to the top of the sucrose gradient. Separate tubes with the enzymes carbonic anhydrase (29kDa) and myoglobin (17kDa) were also prepared to use as molecular weight standards. All tubes were then centrifuged at 47,000 rpm for 20 hours. At the end of the 20 hour period, a hole was poked in the bottom of the tube, and a
solution of crystal violet blue dye was used to push the contents out of each tube and transfer it sequentially into 200 µL wells. The absorbance at 595 nm of each well was then read, providing data about where in the tube protein was located. Additional trials of this experiment were performed with varied concentrations of EDTA mixed with the regulatory chain enzyme.

**EDTA dialysis experiments**

Wild-type and K60E/K94E double mutant regulatory chain protein were combined in 60 µL dialysis buttons and allowed to dialyze overnight at 4°C against 1 L of a buffer containing 10 mM EDTA, 50 mM Tris-acetate, and 2 mM 2-mercaptoethanol, pH 8.3. Following dialysis in buffer with EDTA, the dialysis buttons were transferred to 1 L of a buffer containing 0.1mM zinc-acetate, 50 mM Tris-acetate, and 2 mM 2-mercaptoethanol, pH 8.3. Samples were dialyzed in this second buffer overnight at 4°C. Following dialysis in each buffer, protein samples were electrophoresed on 12% native gels for visualization of band shifting.

**Protein purification**

All ATCase regulatory chain enzyme for the polyptide, sucrose gradient, and EDTA dialysis experiments was purified according to the following method: EK594 competent cells are transformed with pEK592 or pEK599, and are plated on YT-ampicillin plates. A 5 mL overnight culture in YT media with 0.15 µg/mL ampicillin. 2 L of YT-media with 0.15 µg/mL ampicillin is inoculated with 1 mL of the overnight culture. The culture is grown at 37° to A566 of 0.6 and then over-expression of the regulatory chain protein is induced by addition of 4mM IPTG. Growth is allowed to
continue over night until cells reach $A_{566}$ of approximately 1.2. The culture is then harvested by centrifugation (5000 rpm for 20 minutes). The pellets are re-suspended in 40 mL of ice-cold lysis buffer (0.1 M Tris-Cl, 0.1 mM zinc acetate, pH 8.3). The cells are then disrupted by sonication on ice for 10 cycles of 30-second intervals. The lysate is then centrifuged at 15000 rpm for 20 minutes. Ammonium sulfate is slowly added to the supernatent to 65% saturation (0.431 g/mL) at 4ºC while stirring. *E. coli* ATCase precipitates at this concentration of salt, facilitating separation from other proteins which precipitate at higher saturation. Centrifugation concentrates the precipitated protein into the pellet. The pellet is then dissolved in 50 mM Tris-acetate, 2 mM 2-mercaptoethanol, 0.1 mM zinc acetate (pH 8.3). The re-dissolved pellet is dialyzed for 24 hours with two buffer changes, which served to remove the ammonium sulfate from the protein. Following dialysis, anion-exchange chromatography with a QFF column was performed and the pooled fractions were analyzed by SDS-PAGE. $A_{280}$ was recorded for each fraction and the location of the desired protein was identified. The corresponding fractions were collected, and protein concentration could then be determined via UV spectroscopy at 280 nm. Protein was stored at 4ºC.

*Colorimetric Assays*

All assays of the enzyme’s kinetics are performed according to the following procedure: All reagents except for carbamoyl phosphate are added to disposable glass culture tubes. Tubes are placed in a 25°C water bath. Carbamoyl phosphate is added to the tubes sequentially, and the tubes are vortexed. Tubes are incubated for 10 minutes, and then acid quenched with 1 mL of ice cold color mix (50 % sulfuric acid, 5 % acetic acid, 300 mg 2,3-butanedionemonoxime). Tubes are then immediately stoppered and
placed in a box protected from light. A set of standards is prepared which can be quenched with no time limit. The box is placed in a dark drawer overnight at room temperature. After the assay is allowed to develop overnight, the tubes are one by one unstoppered and placed into a 45° water bath with a fluorescent light positioned directly above them. The tubes incubate in the water bath for 24 minutes. At the end of this period, the tubes are re-stoppered and placed into an ice filled box that is protected from light. $A_{466}$ of the tubes is then determined. The spectrophotometer is blanked with water.

The assay measures the amount of carbamoyl aspartate that the enzyme is able to produce in 10 minutes. Carbamoyl aspartate reacts with the color mix to produce color with absorbance at 466 nm in proportion to the amount of carbamoyl aspartate present. In the standards, a set amount of carbamoyl aspartate is added to each tube.

*Aspartate saturation curve*

Added to each reaction tube was L-aspartate, ranging in concentration from 0 to 60 mM, 0.86 µg D141A mutant ATCase in 500 µL of 0.1M Tris-acetate buffer pH 8.3, 100 µL of 48mM carbamoyl phosphate, water to raise volume to 1 mL.

*CTP saturation curve*

Added to each reaction tube was CTP, ranging in concentration from 0 to 2.0 mM, 3.08 µg D141A mutant ATCase in 500 µl of 0.1M Tris-acetate buffer pH 8.3, 100 µl of 48 mM carbamoyl phosphate, 100 µL of 40 mM aspartate, water to raise volume to 1mL.
**ATP saturation curve**

Added to each reaction tube was ATP, ranging in concentration from 0 to 4.0 mM, 1.54 µg D141 A mutant ATCase in 500 µL of 0.1 M Tris-acetate buffer pH 8.3, 100 µL of 48 mM carbamoyl phosphate, 100 µL of 40 mM aspartate, water to raise volume to 1 mL.

**Standard Curves**

Added to each reaction tube was carbamoyl aspartate ranging in concentration from 0 to 0.12 mM, 500 µL of 0.1 M Tris-acetate buffer pH 8.3, and water to raise volume to 1 mL.
CHAPTER 3

*ATCase Regulatory Chain Hybrid Project: Results & Discussion*
This project reports that the proposed aspartate transcarbamoylase regulatory chain hybrid was not successfully created. In the various methods employed in the pursuit of the hybrid, several loose ends can be found. The possibilities for creation of a regulatory chain hybrid were not exhausted by this project. However, after extensive and prolonged efforts by the Kantrowitz lab, a decision was made that continued labor on the problematic hybrid construction would not be the most efficient use of the lab’s temporal or financial resources. For this reason, work on the multiple approaches to the hybrid was cut short.

*Use of a histidine tag as a chromatographic handle*

In order to create the plasmid containing a histidine tag on the N-terminus of the wild-type *pyrI* gene, followed by the mutant *pyrI* gene K60E/K94E a restriction digest was performed on the plasmids pEK647 and pEK599. After digestion with NdeI and XhoI the vectors were electrophoresed on a 1% agarose/EtBr gel. Figure 17 depicts the resulting fragments.

![Figure 17. 1% Agarose/EtBr gel electrophoresis of the restriction digest products of vector pEK647 with NdeI and XhoI (lanes 1 and 2), pEK647 undigested (lane 3), the digest products of pEK599 with NdeI and XhoI (lanes 4 and 5), and pEK599 undigested (lane 6).](image-url)
The lesser mobility of the uncut fragments indicates that the digest was successful. The two larger (top) pieces of DNA from the pEK647 digest (lanes one and two), along with the smaller (bottom) piece of DNA from the pEK599 digest (lane five) were excised from the gel purified, and ligated. The digest product in lane four was not used because the bands were not distinct. The success of the ligation was confirmed by a second restriction digest in which the newly created plasmid was cut either zero, one, or two times and these products were run on an agarose/EtBr gel for comparison. As Figure 18 illustrates, the uncut plasmid runs the shortest distance; the single cut plasmid travels slightly farther due to the greater ease of mobility of a linear DNA fragment as compared to a circular fragment. The double cut plasmid travels the furthest through the gel because a small piece of the vector, (the double mutant pyrI gene) has been cut away from the rest of the DNA. The lowered molecular weight of this fragment allows it to travel the furthest.

![Lane 1 2 3](image)

Figure 18. 1% Agarose/EtBr gel electrophoresis of the NdeI and XhoI digest of plasmid pEK648 (lane 1), the NdeI digest of plasmid pEK648 (lane 2), and plasmid pEK648 on its own.

After BLR competent cells were transformed with the successfully prepared plasmid, this cell line was used to over-express the wild-type and mutant ATCase regulatory chain protein encoded by the plasmid. In order to purify the ATCase regulatory chain protein, a nickel affinity column was used. Protein containing fractions
collected from the column were electrophoresed on a native polyacrylamide gel which is depicted below.

**Figure 19.** 15% Native PAGE of the crude protein extract which was loaded onto the nickel affinity column (lane 1), protein which did not stick to the column (lane 2), protein eluted after the addition of 0.05 M imidazole (lanes 3, 4, and 5), protein eluted after the addition of 0.4 M imidazole (lanes 6, 7, and 8), and a wild-type regulatory chain standard (lane 9).

This gel revealed the absence of a band which would indicate the successful over-expression of the mutant regulatory chain protein. The K60E/K94E mutant is known to run lower on a native polyacrylamide gel than does the wild-type protein. A bulk of protein from the crude sample lines up with the wild-type standard, however there is no large band which runs lower than the wild-type protein. If the experiment had gone ideally, we would have looked to see large bands from wild-type and mutant protein, and a smaller band from the hybrid dimer. The hybrid dimer, with one histidine tag would be eluted with a lower concentration of imidizole than was necessary to elute the wild-type dimers, which each possess two histidine tags. Without over-production of the mutant enzyme however, the possibility of hybrid formation is categorically excluded. This result was confirmed by a second attempt to grow and purify the protein encoded by the newly created plasmid (Figure 20).
Figure 20. 15% Native PAGE of the crude protein sample loaded onto the nickel affinity column (lane 1), protein which did not stick to the column (lane 2), protein eluted by 0.05M imidizole (lanes 3 and 4), and wild-type ATCase regulatory chain standard (lane 5).

This gel again illustrates that the crude protein sample contains a large amount of wild-type regulatory chain protein, as the large bands in lanes one and two line up with the standard in lane five, however there is no significant presence of any other protein.

The approach towards creating a regulatory chain hybrid by way of a plasmid containing a histidine tag attached to a wild-type pyrI gene, followed by the K60E/K94E mutant pyrI gene was unsuccessful. The results showed that while the histidine tag was able to isolate the wild-type ATCase regulatory chain protein from the bulk of the protein collected from the E.coli cells, the K60E/K94E mutant protein was not successfully over-expressed. A potential reason for this failure is an unforeseen problem with the plasmid sequence that interferes with the mechanism of transcription of the second pyrI gene.

Alkaline phosphotase signal sequence as a means of exporting regulatory chain monomers to the periplasm

In order to construct a plasmid coding for a wild-type ATCase regulatory chain protein with an alkaline phosphotase signal sequence attached, a double restriction digest of the plasmids pEK196 and pEK592 was performed. The products of this digestion were electrophoresed on a 1% agarose/EtBr gel. The resulting band pattern is displayed.
in Figure 21. From lane one, the top band — the larger vector fragment containing all of pEK196 except for the excised AP gene — was cut from the gel. From lane three, the bottom band—the smaller vector fragment containing only the wild-type pyrI gene — was cut from the gel. These excised DNA fragments were purified and ligated to reform a circular plasmid.

![Figure 21](image)

**Figure 21.** 1% Agarose/EtBr gel electrophoresis of the XmaI and XhoI restriction digest of pEK196 (lane 1), pEK196 undigested (lane 2), XmaI and XhoI digest of pEK592 (lane 3), and pEK592 undigested (lane 4).

The success of the ligation was confirmed by the performance of a second restriction digest in which the newly created plasmid was digested zero, one, or two times.

![Figure 22](image)

**Figure 22.** 1% agarose/EtBr gel electrophoresis of the restriction digest products of the signal sequence/pyrI plasmid cut with XmaI and XhoI (lane 1), plasmid cut only with XmaI (lane 2), plasmid uncut (lane 3).
Figure 22 displays that the double-cut plasmid travels farthest through the gel due to its lowered molecular weight, the vector cut only one time travels further than the circular vector due to the increased ease of mobility of a linear fragment.

After purification and over-expression of the signal sequence/pyrI plasmid, followed by transformation of EK1594 competent cells, the osmotic shock procedure was performed. The procedure was performed on cells transformed with the signal sequence containing plasmid, as well as on cells transformed with wild-type pyrI plasmid (pEK592). The contents of the periplasm and of the cytoplasm were isolated from each of these cell lines. The isolated protein was electrophoresed on a 15% native polyacrylamide gel to facilitate visualization of the contents of the sample. The gel revealed that ATCase regulatory chain protein was present in the cytoplasmic samples, but was not present to any significant extent in the periplasmic samples. From the cells transformed with pEK592, the wild-type regulatory chain protein was seen, as expected, in the cytoplasmic samples and not in the periplasmic samples (Figure 23).

![Figure 23. 15% Native-PAGE of the protein isolated from the periplasm of EK1594 cells transformed with the signal sequence / pyrI plasmid (lanes 1 and 2), protein isolated from the cytoplasm of these cells (lanes 3 and 4), protein isolated from the periplasm of cells transformed with pEK592 (lane 5), and protein isolated from the cytoplasm of these cells (lanes 6 and 7).]
The attempt at creating a regulatory chain hybrid by way of attaching an alkaline phosphotase signal sequence to the ATCase regulatory chain was unsuccessful. The fact that the results showed regulatory chain present in the cytoplasm and not in the periplasm could be indicative of one of two things. The first of these possibilities is that the signal sequence failed to export the regulatory chain protein to the periplasm. This failure could be due to incompatibility of the regulatory chain protein with the transport mechanism, or could be due to accidental removal from pEK196 of DNA necessary to the signaling process. The second possibility is that the regulatory chain protein was exported but was degraded by proteases present in the periplasm designed to recognize and destroy molecules that do not belong in the periplasm. Without conducting this experiment in cells that have been engineered to be protease free, it is not possible to say which of these possibilities is correct.

Had this experiment gone favorably, a second plasmid would have been constructed, attaching the same signal sequence to the K60E/K94E mutant pyrI gene, and cells would have been co-transformed with both the signal sequence/wild-type plasmid, and the signal sequence/mutant regulatory chain plasmid in an attempt to promote the formation of hybrid dimers. However, as initial results were unfavorable, subsequent steps were not carried out.

*Blocking dimer formation with a synthetic polypeptide*

The synthetic pentapeptide designed to bind to the dimerization interface of the ATCase regulatory chain was allowed to equilibrate in a mixture with the ATCase regulatory chain and EDTA. Polyacrylamide gel electrophoresis was used to compare the effects of this mixture with the effects of dialyzing the regulatory chain in an EDTA
buffer without the presence of the polypeptide. Figure 24 displays the results of these dialysis experiments.

![Image of gel](image)

**Figure 24.** 15% Native-PAGE of wild-type ATCase regulatory chain protein (lane 1), wild-type ATCase regulatory chain protein dialyzed against 10 mM EDTA (lane 2), and wild-type ATCase regulatory chain protein mixed with a large molar excess of synthetic pentapeptide and dialyzed against 10mM EDTA

An equal amount of ATCase regulatory chain was loaded into all three lanes of the above gel. The lightness of the regulatory chain dimer band (bottom) in lane two as compared to the dimer band in lane 1 indicates that the EDTA may be causing the protein to cluster into large aggregates. The fact that ATCase regulatory chain will aggregate in the presence of EDTA is supported by the research of Schachman et al., who found that monomeric regulatory chains would aggregate when EDTA was added. Aggregated protein has extremely limited electrophoretic mobility due to its bulkiness, and therefore would result in a band at the very top of the gel as is seen in lane two. Lane three shows a band that travels a distance in between that of the regulatory chain dimer and the aggregated protein. It was expected that if a peptide/monomer complex formed, it would migrate farther than the regulatory chain dimer because it would have a lower molecular weight. However, on native gels electrophoretic mobility is not governed exclusively by molecular weight, therefore a more conclusive method had to be devised to interpret the effects of the pentapeptide.
This method was the sucrose density gradient experiments. These experiments work by separating proteins with different sedimentation coefficients. A protein’s sedimentation coefficient depends on its mass, its partial specific volume, and its frictional coefficient according to the equation, \( s = m(1 - \bar{v} \rho)/f \) where \( s \) is the sedimentation coefficient, \( m \) is the mass of the protein, \( \bar{v} \) is the partial specific volume of the protein, \( \rho \) is the density of the gradient, and \( f \) is the frictional coefficient of the protein, which is a measure of the protein’s shape. The assumption can be made that the shape and partial specific volumes of all proteins in use are similar enough that the only variable being dealt with is the mass. A more massive protein will sediment more rapidly than a less massive protein. Due to this property, when the protein content of the fractions collected from the gradient is measured, these data provide information about the mass (molecular weight) of the protein species. Knowledge of the molecular weight allows determination of whether the regulatory chain exists predominantly as a monomer or a dimer.

Molecular weights were not determined precisely, but were estimated based on comparison with standards of known molecular weight. These standards are carbonic anhydrase, an enzyme with a molecular weight approximately equal to that of an ATCase regulatory chain dimer, and myoglobin, an enzyme with a molecular weight approximately equal to that of an ATCase regulatory chain monomer. The fractions are collected starting from the top of the gradient and proceeding to the bottom. Therefore, the first fractions contain protein of the smallest molecular weight, and as fraction number increases, molecular weight of the protein species present increases correspondingly. The absorbance of each fraction was measured. Absorbance increases
proportionally to concentration. Therefore, determining the fractions which had the highest absorbance provided an indication of the protein molecular weight which was present at the highest concentration in each protein sample. Figure 25 is a graphical representation of this data. Primarily, it gives an indication of whether the monomer or dimer was favored in the monomer ↔ dimer equilibrium.

![Figure 25](image)

**Figure 25.** Graph depicting the absorbance at 595 nm (protein content) of fractions collected from the sucrose density gradients after 20 hour centrifugation period.

This graph illustrates that regulatory chain (green) or regulatory chain that has been dialyzed with EDTA for only a short period of time (black) migrates through the sucrose gradient a distance similar to that of the carbonic anhydrase, indicating that the regulatory chain exists here as a dimer. EDTA dialysis for only one half hour does not allow sufficient time for the EDTA to cause demetallation and monomerization of the
regulatory chain. However, when EDTA dialysis is allowed to proceed over a period of seventy-two hours (pink) monomerization does occur. Figure 25 illustrates this by showing that seventy-two hour EDTA dialysis causes the regulatory chain to migrate through the sucrose gradient a similar distance to that of myoglobin. Comparison of the line representing the sample with the polypeptide (light blue) to the sample without the polypeptide (pink), reveals that the polypeptide affects no changes that EDTA alone does not.

The results from the experiments with the pentapeptide revealed that the small polypeptide was not important in promoting the predominance of the regulatory chain monomer in equilibrium, however EDTA was important. The peptide may not have had a high enough affinity for the regulatory chain to be an effective competitor with dimerization.

The significance of EDTA is that it effectively de-metallates the regulatory chain protein. According to Schachman et al.\textsuperscript{24}, the role of zinc in the regulatory subunit is to significantly increase the interchain affinity, causing the regulatory subunit chains to associate fully to the dimer level. Dialysis of the protein in a buffer containing EDTA, a chelating agent, complexes the zinc ions, effectively removing them from the protein and creating an apo- (metal free) regulatory subunit\textsuperscript{24}. Therefore, EDTA dialysis decreases interchain affinity, and causes a shift in the regulatory chain equilibrium towards the monomer.
EDTA induced monomerization of wild-type and mutant regulatory chain subunits, followed by re-dimerization.

Wild-type and K60E/K94E mutant regulatory chain were combined and dialyzed against EDTA. The protein mixture was then removed from the EDTA mixture and dialyzed against a zinc containing buffer. The resulting protein sample was then run on a polyacrylamide gel for visualization (Figure 26). Controls were used to compare the protein sample to wild-type regulatory chain protein which had not been dialyzed in EDTA, mutant regulatory chain protein which had not been dialyzed in EDTA, wild-type regulatory chain protein which had been dialyzed in EDTA, mutant regulatory chain protein which had been dialyzed in EDTA, and a mixture of the two regulatory chain proteins without dialysis in EDTA.

![Figure 26](image)

Figure 26. 12% Native-PAGE of wild-type regulatory chain (lane 1), K60E/K94E regulatory chain (lane 2), 50:50 mixture of wild-type and K60E/K94E regulatory chain (lane 3), wild-type regulatory chain dialyzed in EDTA then in a Zn$^{2+}$ containing buffer (lane 4), K60E/K94E dialyzed in EDTA then in a Zn$^{2+}$ containing buffer (lane 5), 50:50 mixture of wild-type and K60E/K94E regulatory chain dialyzed in EDTA then in a Zn$^{2+}$ containing buffer (lane 6).

Lanes one and two of the gel reveal that the wild-type and mutant regulatory proteins have bands which are distinct from each other. The heaviest (bottom) band in lane two is the holo-regulatory subunit; the additional lighter bands above, represent the regulatory dimer having lost one or two of its zinc molecules. Lane three shows that the
mixture of wild-type and K60E/K94E regulatory dimer contains bands representative of each of these two proteins, but no additional bands which would be representative of a hybrid dimer. Lanes four and five reveal that the wild-type and K60E/K94E regulatory proteins individually subjected to the EDTA then zinc dialysis are not completely restored to the holo-regulatory subunit. In lane six, wild-type and mutant regulatory protein are dialyzed in EDTA to remove zinc and promote monomerization, and then combined in the presence of zinc to promote reformation of dimers. The bottom two bands in lane six match up with the bands resulting from the double mutant protein seen in lane two. The top band in lane six matches up with the band resulting from the wild-type protein seen in lane one. No additional bands that could represent hybrid regulatory dimer can be seen in this lane, indicating that the experiment was unsuccessful.

The results of this project suggest that the K94E/K60E mutant and wild-type regulatory chain are incompatible for dimerization to form hybrid regulatory subunit. The engineered mutations may have altered the protein in a way that affected the rate or manner of protein folding. If the wild-type and mutant proteins do not fold into proper conformation at the same rate as each other, they will be less likely to dimerize heterogeneously. The mutations may have made the regulatory chain much less stable or more prone to aggregation than the wild-type protein. If the mutant protein is unstable, it would be far less favorable for the wild-type protein to dimerize with a mutant chain as opposed to another stable wild-type chain. If the mutations caused the regulatory chain to aggregate, these proteins would not be available for dimerization to form a hybrid. For continued work on the regulatory chain hybrid project, it would be advantageous to design a new mutant which would compromise nucleotide binding in a manner similar to
the K60E/K94E mutant, but may be more compatible for dimerization with the wild-type protein.
CHAPTER 4

Kinetic Characterization of ATCase mutant D141A: Results and Discussion
To determine the effects of a mutation made to an enzyme, a series of kinetic assays is performed. The results of these assays can be compared to responses of wild-type enzyme to the same assays, in order to establish the mutant’s deviation from the norm. This information can then be used to deduce the importance of the residue being studied in the wild-type enzyme.

For a complete characterization of a mutant enzyme, assays should be performed which measure the enzyme’s response to increasing concentrations of its two substrates, L-aspartate and carbamoyl phosphate, as well as its three nucleotide effectors, CTP, ATP, and CTP and UTP together. This project reports progress towards a complete characterization. L-aspartate, CTP, and ATP saturation curves have been obtained.

**Asparate Saturation Curve**

![Asparate Saturation Curve](image)

**Figure 27.** Influence of L-aspartate on D141A enzyme. Colorimetric assays were performed at 25° in 0.1 M tris-acetate buffer at pH 8.3 in the presence of increasing concentrations of aspartate. Specific activity is measured in m mols of N-carbamoyl-L-aspartate formed per hour per mg of ATCase. The maximum velocity of the enzyme is 1.24 mmol/mg. The half-saturation ([S] 0.5) of the enzyme is 11.54 mM Asp.
As can be seen from the aspartate saturation curve, the specific activity of D141A is 1.24 mmol/ mg. This value is significantly lower than that of wild-type ATCase, which is typically around 18.0 mmol/mg\(^2\). This is a clear indication that this residue is critical to functioning of ATCase. The half-saturation point, the concentration of aspartate required for the enzyme to reach half of its maximum velocity, is calculated to be 11.5 mM aspartate. This value is similar to that of wild-type ATCase.

![CTP Saturation Curve](#)

**Figure 28.** Influence of CTP on D141A enzyme. Colorimetric assay was performed at 25° in 0.1M tris-acetate buffer at pH 8.3 in the presence of increasing concentrations of CTP. Aspartate concentration was held constant at one half of the half saturation value.

The activity of the mutant enzyme is inhibited by CTP to about 70% of its original level of activity. Wild-type ATCase typically has only about 30% residual activity in the presence of CTP\(^2\). CTP inhibition therefore, is partially compromised. The D141A mutant is inhibited by CTP to a much smaller extent than wild-type enzyme.
is. As with the aspartate saturation curve, it is clear here that mutating residue 141 of the catalytic chain has a significant effect on the overall functioning of the enzyme.

![ATP Saturation Curve](image)

**Figure 29.** Influence of CTP on D141A enzyme. Colorimetric assay was performed at 25° in 0.1M tris-acetate buffer at pH 8.3 in the presence of increasing concentrations of ATP. Aspartate concentration was held constant at one half of the half saturation value.

The mutant was activated about 500% by ATP. The behavior of the mutant seen here, in response to activation by ATP, is approximately similar to the behavior of the wild-type enzyme.

Without more extensive and thorough characterization of the mutant it is difficult to hypothesize the specific nature of ATCase’s dependence on residue 141. It is however already clear that mutating the residue causes significant changes in the enzymes response to increasing concentrations of aspartate or of CTP.
CHAPTER 5

References


