ASPARTATE TRANSCARBAMOYLASE OF *AEROMONAS HYDROPHILA*

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This study focused on the enzyme, aspartate transcarbamoylase (ATCase) from *A. hydrophila*, a Gram-negative bacterium found in fresh water. The molecular mass of the ATCase holoenzyme from *A. hydrophila* is 310 kDa. The enzyme is likely composed of 6 catalytic polypeptides of 34 kDa each and 6 regulatory polypeptides of 17 kDa each. The velocity-substrate curve for *A. hydrophila* ATCase is sigmoidal for both aspartate and carbamoylphosphate. The Km for aspartate was the highest to date for an enteric bacterium at 97.18 mM. The Km for carbamoylphosphate was 1.18 mM. When heated to 60 ºC, the specific activity of the enzyme dropped by more than 50 %. When heated to 100 ºC, the enzyme showed no activity. The enzyme’s activity was inhibited by ATP, CTP or UTP.
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CHAPTER 1

INTRODUCTION

Pyrimidines, along with purines, carry out important roles in the cell because they are the building blocks of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). RNA and DNA are the informational macromolecules that are essential for cellular function and growth and also for the passing of genetic information from one generation to another. Since pyrimidines are essential to the cell, the demand for them by the cell is high. The pyrimidine biosynthetic pathway is responsible for the production of uridine-5’-triphosphate (UTP) and cytidine-5’-triphosphate (CTP) for RNA and dCTP and dTTP for DNA through various enzymatic reactions. De novo pyrimidine synthesis has been studied in bacteria, fungi, and mammals. The sequence of enzymatic reactions has been found to be universal in all those studied so far (O’Donovan & Neuhard, 1970; Neuhard & Kelln, 1996).

Pyrimidine Biosynthetic Pathway

The pyrimidine biosynthetic pathway is composed of nine steps with the first six involved in the production of uridine-5’-monophosphate (UMP) which is the precursor for all pyrimidine nucleotides. Fig. 1 shows the pathway in *Escherichia coli*. The first step involves the formation of carbamoylphosphate from glutamine, bicarbonate, and ATP. This reaction is catalyzed by the enzyme carbamoylphosphate synthetase (CPSase), which uses either glutamine or ammonia as the amino donor.
CPSase’s affinity for ammonia is much lower than for glutamine (Neuhard & Kelln, 1996). The second step, which forms N-carbamoyl-L-aspartate and inorganic phosphate, is the first committed step in the biosynthetic pathway. This step is catalyzed by aspartate transcarbamoylase (ATCase). The production of N-carbamoyl-L-aspartate is through a condensation reaction between carbamoylphosphate and L-aspartate in which the amino group of aspartate is carbamoylated and inorganic phosphate is released. The third step is catalyzed by dihydroorotase (DHOase). This step involves the cyclization of N-carbamoyl-L-aspartate to L-dihydroorotate with the release of water. The fourth step is the only redox reaction in de novo UMP biosynthesis. A membrane bound enzyme known as dihydroorotate dehydrogenase (DHOdehase) catalyzes the reaction. DHOdehase oxidizes dihydroorotate to orotate. In the following two steps, the first pyrimidine nucleotide is formed. First orotate phosphoribosyltransferase (OPRTase) transfers ribose 5-phosphate to orotate from α-D-5-phosphoribosyl-1-pyrophosphate (PRPP) forming orotidine 5’-monophosphate (OMP). OMP is then decarboxylated to UMP in the next step, which is catalyzed by OMP decarboxylase (OMPdecase). UMP is converted to UTP by two phosphorylation reactions. The first converting UMP to UDP using the highly specific UMP-kinase; UDP is then converted to UTP by the non-specific nucleoside diphosphate kinase (NDP). Lastly, CTP synthetase catalyzes the synthesis of CTP from UTP, ATP, and glutamine.

Genes encoding the pyrimidine biosynthetic enzymes are scattered throughout the chromosome in some organisms whilst in others are arranged in operons or clusters. In *Escherichia coli*, the *carAB* operon encodes the two subunits of CPSase. The *pyrBI*
operon encodes ATCase. The genes, *pyrC, pyrD, pyrE*, and *pyrF* encode DHOase, DHOdehase, OPRTase, and OMPdecase respectively. The last three genes are *pyrH*, which encodes UMP kinase, *ndk*, which encodes NDP kinase, and *pyrG*, which encodes CTP synthetase (Refer to Fig. 1).

**Bacterial Aspartate Transcarbamoylase**

By studying of the structure and regulation of aspartate transcarbamoylase, the catalytic mechanisms and evolution of the enzyme in different bacterial species have been worked out. In 1969, Bethell & Jones first proposed that bacterial ATCases be separated into three classes based on size, subunit composition and regulatory effects. Ultimately, the size of the ATCase holoenzyme is dependent on the type of catalytic subunit present. Fig. 2 shows the three classes of ATCases and their relationship between size and subunit composition.

Of these three classes of ATCases, the class with the greatest molecular weight is class A. The representative bacteria for class A ATCases are the pseudomonads. The class A enzyme is a dodecamer, composed of six copies of a 34 kDa catalytic polypeptide and six copies of a 45 kDa polypeptide with a total molecular mass of around 480 kDa (Bergh & Evans, 1993; Shepherdson & McPhail, 1993; Schurr *et al.*, 1995). Derived amino acid sequence comparisons suggest that the 45 kDa polypeptide is a nonfunctional homolog of the next enzyme in the pathway, DHOase (Schurr *et al.*, 1995). The 45 kDa polypeptide has been found to be catalytically inactive in some organisms, but it is required for ATCase activity. It is also important for fine-tuning of the regulatory properties of the enzyme (Kumar *et al.*, 1998). Since it is inactive yet similar to
Fig. 2. Classes of bacterial ATCase. Adapted from Bergh & Evans (1993).

**Class A ATCases**

Catalytic Trimer

45 kDa

34 kDa

**Class B ATCases**

Catalytic Trimer

17 kDa

Regulatory Dimer

34 kDa

**Class C ATCases**

Catalytic Trimer

34 kDa
other known DHOases, this gene has been designated \textit{pyrC}' \cite{Schurr1995}. The catalytic and regulatory sites are located on the 34 kDa species \cite{Bergh1993}. It has been shown that these regulatory sites are located at the N-terminal region of the 34 kDa species \cite{Kumar1994}. The class A enzymes have hyperbolic substrate saturation curves and are competitively inhibited by ATP, UTP, and CTP when carbamoylphosphate was varied and aspartate kept constant at saturation. These enzymes are noncompetitively inhibited by ATP, UTP, and CTP when aspartate is varied and carbamoylphosphate kept constant at saturation \cite{Neuman1964,Bethell1969,Linscott1998}.

The enteric bacteria contain class B ATCases. The ATCase from the two most studied bacteria \textit{Escherichia coli} and \textit{Salmonella typhimurium} has been extensively studied. Class B ATCase, which is encoded by the \textit{pyrBI} operon, is composed of six catalytic polypeptides of 34 kDa each grouped into two trimers and six regulatory polypeptides of 17 kDa each grouped into three dimers creating a total molecular mass of about 310 kDa \cite{Kantrowitz1988}. Class B ATCases have sigmoidal substrate saturation curves for both aspartate \cite{Gerhart1962} and carbamoylphosphate \cite{Bethell1968}. The enzymes are inhibited by CTP and activated by ATP \cite{Gerhart1962}. UTP alone is not an inhibitor \cite{Gerhart1962} but it greatly enhances inhibition when present with CTP \cite{Wild1989}. When the holoenzyme is treated with mercurial compounds, the holoenzyme dissociates into two 100 kDa catalytic trimers with polypeptides of 34 kDa each and three 34 kDa regulatory dimers with polypeptides of 17 kDa each in size. The trimeric form of
the enzyme is still active but no longer inhibited by CTP. This proves that the regulatory and active sites are on separate polypeptides (Lipscomb, 1992).

The class with the smallest molecular weight is class C. *Bacillus subtilis* is an example of a bacterium that possesses a class C ATCase. Such enzymes are characterized by having a molecular mass of 100 kDa, insensitivity to effectors, and standard Michaelis-Menten kinetics for both the aspartate and carbamoylphosphate. Class C ATCase’s consist only of a catalytic trimer composed of 34 kDa subunits and lack any associated regulatory polypeptides. (Brabson & Switzer, 1975).

*Aeromonas hydrophila*

*Aeromonas hydrophila* is a facultative anaerobic Gram-negative rod. The organism is motile by a single polar flagellum. It is widely distributed in the environment and can be found in fresh water, sludge, and sewage. It has been considered responsible for diseases in reptiles, fish and mammals including humans (Krieg, 1984). Among the clinical manifestations associated with *A. hydrophila* are soft tissue infections, and bacteremia (Jones & Wilcox, 1995). The organism also appears to have a role as a gastro-intestinal pathogen (Janda, 1991; Jones & Wilcox, 1995). The pathogenicity of the organism has warranted much research on *A. hydrophila* especially in the organism’s viability in drinking water (Kersters et al., 1996).

Biochemical characteristics of *A. hydrophila* include oxidase positive, an optimal growth temperature of 28 ºC, and a molecular percent G+C of DNA varying between 58-62. *A. hydrophila* can use L-histidine, L-arabinose, L-arginine and salicin as carbon
sources, and hydrolyzes esculin, ferments salicin, and produces gas and acetoin from glucose (Krieg, 1984).

*A. hydrophila* has been placed in the family *Vibrionaceae* on the basis of morphological and biochemical characteristics (Farmer, 1992). Recently it has been proposed that *Aeromonas* be removed from the family *Vibrionaceae* and itself elevated to family status (Colwell *et al*., 1986; Kita-Tsukamoto *et al*. 1993; Ruimy *et al*., 1994). The relationship of *Aeromonas* to its closest relatives has not been resolved.

Although much research has been done on *A. hydrophila* in regards to its pathogenicity, little has been done regarding basic primary metabolism especially pyrimidine metabolism except in one study on ATCase (Wild *et al*., 1980). This study showed that the ATCase from *A. hydrophila* has a molecular mass of 280 kDa, which dissociates into a 100 kDa smaller form. *A. hydrophila* ATCase had sigmoidal substrate saturation kinetics with an $[S]_{0.5} = 17$ to 20 mM aspartate. The study also showed that at very low aspartate concentrations (5 mM), the enzyme was dramatically activated by ATP and CTP but at higher aspartate concentrations (20 mM), the activation was not as significant. Further, at higher aspartate concentrations, the ATCase from *A. hydrophila* is not affected by CMP, UMP, or UTP (Wild *et al*., 1980). Based on this study, *A. hydrophila* ATCase was placed in the class B, presumably encoded by a *pyrBI* operon, like *E. coli* and *S. typhimurium*.

Upon initial study of *A. hydrophila* ATCase, some apparent discrepancies were noted from those that were originally reported by Wild *et al*. in 1980. First, the aspartate requirement for *A. hydrophila* ATCase holoenzyme was much greater than previously
reported. Moreover, when the enzyme was analyzed on an 8 % nondenaturing polyacrylamide gel (Laemmli, 1970) and stained for ATCase activity by the protocol of Kedzie, (1987), but using 10 times the aspartate concentration, the *A. hydrophila* ATCase appeared to migrate at a much higher molecular mass than originally reported. The objective of this thesis was to reevaluate some of the properties of *A. hydrophila* ATCase, in particular its molecular mass (and subunit composition) and its kinetics.
CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains

Aeromonas hydrophila ATCC strain 7965 (wild type) was used in all experiments. This strain was obtained from American Type Culture Collection.

Media and Growth Conditions

A. hydrophila ATCC strain 7965 cells were grown in 2 liters of autoclaved E. coli minimal medium A (Miller, 1992) with 1 mM MgSO₄, 1 µg thiamine ml⁻¹, and 0.2 % glucose, which was used as a carbon and energy source, added after autoclaving. The cells were incubated at 30 °C with shaking at 200 r.p.m. and grown to late exponential phase, which corresponds with an OD₈₀-₁.₀ on a Perkin-Elmer Lambda 3A spectrophotometer at 600 nm. The cells were then harvested in a Sorvall centrifuge (GS-3 rotor) at 10 000 g for 30 min at 4 °C. The supernatant was poured off and the pelleted cells were frozen at –20 °C until ready for use.

Preparation of Cell-free Extracts

The pelleted cells were resuspended in 2 ml ATCase breaking buffer (50 mM Tris-HCl, pH 8.0, 2 mM β-mercaptoethanol, 20 mM ZnSO₄ and 20 % glycerol) g⁻¹ of wet weight of cells used. The cells were broken in a SLM AMINCO French Pressure Cell Press (SLM Industries Inc.) at 20 000 p.s.i. Unbroken cells and cell debris were removed by centrifugation at 14 000 g for 1 h at 4 °C in a Sorvall centrifuge (SA 600 rotor).
The supernatant was collected and stored at 4 °C until ready to be used.

Purification of the *Aeromonas hydrophila* ATCase

The cell-free extract was used to purify the ATCase enzyme. All of the cell-free extract was used in the purification process except 200 µl, which was saved and stored at 4 °C. Purification of the ATCase enzyme from *A. hydrophila* began by adding ammonium sulfate to the supernatant until it was 50 % saturated. This was done because when high concentrations of salt are present, proteins tend to aggregate and precipitate out of solution. The salt concentration at which proteins precipitate is based on such factors as pH, temperature, protein purity, and the protein's isoelectric point. Ammonium sulfate was used in the purification because it is often a good way of stabilizing the protein for storage (Scopes, 1982).

Once the ammonium sulfate was added to the supernatant, the solution was stirred slowly at 4 °C overnight to insure proper saturation. The 50 % ammonium sulfate solution was then centrifuged in a Sorvall centrifuge (SA 600 rotor) at 14 000 g for 60 min at 4 °C to pellet the ATCase enzyme. The supernatant was decanted but saved and stored at 4 °C. The pellet was resuspended in 1-2 ml of ATCase breaking buffer g⁻¹ wet weight of pellet.

Next, the 50 % saturated pellet and 50 % saturated supernatant were checked for ATCase activity using an ATCase spot assay which measures the formation of carbamoylaspartate (CAA) following the procedure of Gerhart & Pardee (1962) using the modified color development method of Prescott & Jones (1969). This assay provides a quick identification for the presence of ATCase. To an assay tube 40 µl of Tribuffer (50
mM diethanolamine, 50 mM N-ethylmorpholine, and 100 mM MES (2-[N-morpholino]ethanesulfonic acid)) adjusted to pH 7.0 (Ellis & Morrison, 1982), 100 µl 100 mM aspartate, 20 µl of the sample enzyme and 640 µl of distilled water were added. The tubes were preincubated at 30 °C for 2-3 min. Then 100 µl of 50 mM carbamoylphosphate were added to each tube to start the reaction, which was carried out at 30 °C for 20 min. After 20 min, 1 ml of the color mix (2 parts antipyrine to 1 part monoxime) was added to each tube, which were then heated to 65°C for 110 min in the light. After the incubation, the absorbance of the samples were determined using a Perkin-Elmer Lambda 3A spectrophotometer at 466 nm against a reacted blank, which contained all the above-mentioned ingredients except enzyme, which was replaced with distilled water.

If the ATCase was not found in the 50 % pellet, the ammonium sulfate concentration in the 50 % supernatant was increased until it reached 75 % saturation. All the supernatant was used except 200 µl, which was saved and stored at 4 °C. The same procedures as described above were followed to determine if the ATCase enzyme salted out in the 75 % pellet. When it was determined that the ATCase enzyme did indeed salt out of the supernatant, the 75 % pellet was then resuspended in 1 ml ATCase breaking buffer g⁻¹ wet weight of pellet. The 75 % pellet solution was centrifuged in a Sorvall centrifuge (SA-600 rotor) at 14 000 g for 30 min at 4 °C to remove any denatured proteins. 200 µl of the 75 % pellet solution was saved and stored at 4 °C.

The remaining 75 % pellet solution was placed in a treated dialysis tube. The dialysis tube was boiled for at least 30 min in 10 mM sodium bicarbonate in 1 mM
EDTA solution. It was then rinsed thoroughly in distilled water (Richmond et al., 1985). The 75 % pellet solution was dialyzed against ATCase column buffer (1 mM Tris pH 8.2, 1 mM EDTA pH 8.0, 0.02 mM zinc acetate, and 150 mM potassium acetate) at 4 °C for 2 h. After 2 h, the buffer was changed, and the dialysis was continued overnight. Dialysis was performed to change the buffer solution of the protein and to remove the high amount of ammonium sulfate in the solution (McPhie, 1971).

After dialysis was completed, the dialysate was passed through a Q Sepharose anion exchange column (Pharmacia Biotech) with a sodium chloride concentration from 0 M to 1 M in ATCase column buffer. The column had a flow rate of 1.5 ml per min. Seventy-one 2-ml fractions were collected off the column. The fractions were assayed for ATCase activity by the method described above to determine which fraction(s) contained the ATCase enzyme. The fractions that contained the ATCase enzyme were then combined and 200 µl was saved and stored at 4 °C.

The fractions were then concentrated in a Centriprep 30 concentrator (Amicon) to a concentration of about 750 µl. A total of 250 µl of the concentrated mix was saved and stored at 4 °C while the remaining 500 µl was loaded onto a Sephacryl S-300 HR size exclusion column (Pharmacia Biotech) and was eluted with ATCase column buffer. Ninety 1 ml fractions were collected and assayed to determine which fraction(s) contained the ATCase enzyme. These fractions were analyzed on a 15 % SDS polyacrylamide gel (Laemmli, 1970) to determine the purity of the enzyme.
Protein Gel Electrophoresis

The molecular mass of the ATCase holoenzyme of *A. hydrophila* was determined by analyzing a sample of the prepared cell-free extract on a 4-20% nondenaturing gradient gel (Bio-Rad) and staining the gel for ATCase activity (Kedzie, 1987). To do so the gel was placed in a container with 125 ml of 50 mM ice-cold histidine pH 7.0 for 5 min to equilibrate the gel. 2.5 ml of 5.0 M aspartate (final concentration of 100 mM aspartate) and 5 ml of 0.1 M carbamoylphosphate (final concentration of 4.0 mM carbamoylphosphate) were added to the solution containing the gel, which was shaken for 10 min. The gel was then rinsed with 3 changes of ice-cold distilled water and placed in another 125 ml of 50 mM ice-cold histidine pH 7.0 with 3 mM lead nitrate added. The addition of the lead nitrate caused the orthophosphate trapped in the gel to precipitate. After 10 min the gel was rinsed with 3 changes of ice-cold distilled water. The white lead-phosphate precipitate corresponded to the ATCase enzyme. Purified *E. coli* and *P. aeruginosa* ATCases were also analyzed on the gradient gel to be used as size markers.

The subunit composition was determined by analyzing the partially purified enzyme sample collected from the Sephacryl S-300 HR size column on an 8% nondenaturing polyacrylamide gel (Laemmli, 1970) that was stained for ATCase activity as described above. The bands corresponding to the *A. hydrophila* ATCase were cut out of the gel. An electroelution was performed on the extracted gel pieces to elute the protein after it was separated by gel electrophoresis. The gel pieces were placed in a small (1.0 cm diameter) dialysis tube, which was treated as described above. Then 200 µl of electrophoresis buffer [3.02 g of Tris, 14.4 g of glycine, and distilled water to 1000
ml at pH 8.3 (Bollag & Edelstein, 1991)] was added to the dialysis tube, which was placed in an electrophoresis cell that was filled with the electrophoresis buffer. The electroelution was performed by applying 150 V at 4 °C for 4-5 h, changing the buffer in the cell, and then applying 100 V at 4 °C for 9 h. The proteins, which had dissolved into the buffer solution following the electroelution, were transferred to a Microcon-30 concentrator (Amicon) and centrifuged at 1500 g at 4 °C for 10 min. The sample volume was checked and the centrifugation was repeated until the desired volume, typically 40 µl, was obtained. From the electroeluted sample 20 µl was analyzed on a 15 % SDS polyacrylamide gel along with purified samples of E. coli ATCase, and a commercially made standard marker. The gel was stained with Coomassie Brilliant Blue (Sigma). The bands corresponding to the polypeptide size were faint in color so the gel was then silver stained, with a GELCODE silver staining kit (Pierce), to determine the polypeptide size of the A. hydrophila ATCase. The silver staining procedure was used because it is sensitive in the nanomolar range.

Specific Activity of the Enzyme

The enzyme activity of ATCase from A. hydrophila was measured by performing an ATCase assay, as described above, on the samples collected from each purification step, namely: the cell-free extract, the 50 % ammonium sulfate pellet, the 50 % ammonium sulfate supernatant, the 75 % ammonium sulfate pellet, the Q Sepharose anion exchange column, the Sephacryl S-300 HR size exclusion column, and the electroeluted sample. One unit of enzyme specific activity is the number of nmol of carbamoylaspartate (CAA) produced min⁻¹ (mg of protein)⁻¹. The protein was estimated
by the methods of Lowry *et al.* (1951) and Bradford (1976) using bovine serum albumin (BSA) and lysozyme, with both methods, to create standard curves.

For each standard curve 10 µg increments of BSA or lysozyme, ranging from 0-100 µg, were added to assay tubes, which were brought up to 200 µl with distilled water. For the Lowry protein assay 0.8 ml of the alkaline copper reagent (0.5 ml 2 % sodium/potassium tartrate and 0.5 ml 1 % CuSO$_4$· 5 H$_2$O with 49 ml 2 % Na$_2$CO$_3$ in 0.1 N NaOH) was added to each tube and then let stand for 10 min. Then 0.1 ml of the Folin Reagent (Sigma) was added to each tube and mixed by vortexing. The tubes were allowed to stand for 30 min before their absorbance was measured on a Perkin-Elmer Lambda 3A spectrophotometer at 660 nm.

For the Bradford protein assay 0.8 ml of the Bradford Reagent (Sigma) was added to each tube with mixing and incubated in the dark for 10 min. The absorbencies of the samples were then read on a Perkin-Elmer Lambda 3A spectrophotometer at 565 nm. The protein concentration was calculated for each of the above-mentioned samples from the purification of the enzyme using both the Lowry protein assay and the Bradford protein assay. From each sample 5 µl, 10 µl, and 20 µl were each used with enough distilled water to have a volume of 200 µl to estimate protein concentration. The protein from the cell-free extract, 50 % pellet, and 75 % pellet were also measured using a 1 to 10 dilution of the samples.

A CAA standard curve was also made. Assay tubes, which contained 50 µl of 10 mM CAA, 20 µl of 10 mM CAA, 10 µl of 10 mM CAA, and 0 µl of 10 mM CAA, plus 40 µl of Tribuffer, were brought up to a volume of 1000 µl with distilled water. These
tubes were then incubated at 30 °C for 20 min. After the tubes were incubated, 1 ml of the color mix from the ATCase assay described above was added to each tube. The tubes were then further incubated at 65 °C for 110 min in the light and the absorbance was measured using a Perkin-Elmer Lambda 3A Spectrophotometer at 466 nm against a reacted blank. The CAA standard curve can be seen in Fig. 6.

Determination of Optimal pH

ATCase assays to measure the amount of CAA produced were performed with the partially purified *A. hydrophila* ATCase by the method described above. This method was modified to determine the optimal pH of the enzyme. Each tube contained an aspartate and carbamoylphosphate concentration that corresponded to the apparent Km for *A. hydrophila* ATCase. The pH was determined by varying the pH of the Tribuffer solution and the pH of the aspartate solution from pH 5.5 to 10.0 in 0.5 pH intervals. After the assay, the absorbance of each tube was measured on a Perkin-Elmer Lambda 3A Spectrophotometer against a reacted blank to determine at which pH the *A. hydrophila* ATCase produced the most CAA.

Determination of the Heat Stability of the Enzyme

The prepared cell-free extract for *A. hydrophila* was heated to 50 °C, 60 °C, and 85 °C for 10, 20, 40 and 60 min for each temperature. The heated samples were then assayed for ATCase activity, along with a sample of *A. hydrophila* cell-free extract that was not heated and a sample that was heated to 100 °C, by the method described above. The heated samples, and non-heated samples of *E. coli*, *P. aeruginosa*, and *A. hydrophila* cell-free extract, were analyzed on an 8 % nondenaturing polyacrylamide gel and stained.
for ATCase activity (Kedzie, 1987). This was done to determine if upon heating, the enzyme dissociated into a smaller form but still remained active (e.g. from 310 kDa to 100 kDa as for *E. coli* ATCase). The time and temperature at which 50% of the specific activity is destroyed was then ascertained. The specific activity was ascertained by the protein concentration determined by Lowry *et al.*, 1951 and Bradford, 1976 and the amount of CAA made as determined by the CAA standard curve described earlier.

**Determination of Apparent Km**

An ATCase assay was performed with the partially purified *A. hydrophila* ATCase enzyme, as described above, to determine the apparent Km for both substrates, aspartate and carbamoylphosphate. This assay was performed by varying the concentrations of one substrate while keeping the other at saturating concentrations. Each assay was performed at the optimal pH for the *A. hydrophila* ATCase as determined by the pH assay described earlier. The concentration of aspartate was varied from 0 mM to 200 mM, at 10 mM increments, while keeping the carbamoylphosphate concentration at 5.0 mM. The concentration of carbamoylphosphate was varied from 0 mM to 5.0 mM, at 0.5 mM increments, while keeping the aspartate concentration at 200 mM. The absorbance of the enzyme, at each condition tested, was measured on a Perkin-Elmer Lambda 3A spectrophotometer at 466 nm against a reacted blank. A velocity-substrate curve was produced by plotting the absorbance at 466 nm against the concentration of the substrate. The apparent Km and saturation kinetics of the enzyme were determined from the saturation curve.
Effector Response

UTP, CTP, and ATP at 1mM and 5mM concentrations were added to the standard ATCase assay mixture. The activities of the enzyme with the effectors present were compared to the activity of the enzyme with no effectors added. Each effector was added to the assay tubes under the same conditions as the saturation curves described above for both substrates. After the assay, the absorbance was measured on a Perkin-Elmer Lambda 3A spectrophotometer at 466 nm against a reacted blank. The absorbance was plotted for each effector response along with the saturation curve for each substrate.
CHAPTER 3

RESULTS AND DISCUSSION

Protein Gel Electrophoresis

In the present study, initial results from *A. hydrophila* cell-free extracts on non-denaturing gel electrophoresis produced conflicting findings from those originally reported by Wild *et al.*, (1980). At first, the results of gel electrophoresis were unsuccessful because the enzyme appeared to have no ATCase activity. Many different approaches were taken from varying the pH to varying the method by which the cells were grown and broken in order to create the cell free extract. Finally, it was determined that the aspartate requirement for ATCase activity was much greater than reported in the original protocol for ATCase activity staining by Kedzie, (1987). When the gel was stained for ATCase activity using 20 mM of aspartate no activity was seen. The aspartate concentration used in the staining procedure was then increased to 200 mM, which is 10 times the suggested requirement. It was only then that ATCase activity could be seen on the gel. The molecular mass of the resultant protein was greater than originally reported in the paper by Wild *et al.*, (1980). The ATCase from *A. hydrophila*, when stained for ATCase activity using 200 mM aspartate, showed two bands with ATCase activity. The upper band appeared to migrate alongside that of *P. aeruginosa* ATCase enzyme, at 480 kDa, while the lower band migrated alongside the *E. coli* ATCase, at 310 kDa. Fig. 3 shows the 8% non-denaturing polyacrylamide gel stained for *A. hydrophila* activity.
Fig. 3. 8 % Nondenaturing gel electrophoresis of *A. hydrophila* ATCase

Lane designations are as follows: 1 *P. aeruginosa* ATCase, 2-7 *A. hydrophila* ATCase, and 8 *E. coli* ATCase. This gel was stained for ATCase activity at 200 mM aspartate and 5 mM carbamoylphosphate. As can be seen on the gel, the lanes with *A. hydrophila* show two bands, one that migrates at the same size as *P. aeruginosa* (480 kDa), the other at the same size as *E. coli* (310 kDa).
To confirm the molecular mass of the ATCase holoenzyme from *A. hydrophila*, the cell-free extract was analyzed on a 4-20 % nondenaturing gradient gel as described in the Methods and stained for ATCase activity as seen in Fig. 4. The results from the gradient gel showed that the ATCase from *A. hydrophila* migrated alongside the *E. coli* ATCase and not the *P. aeruginosa* ATCase as seen previously on the 8 % nondenaturing polyacrylamide gel electrophoresis originally showed. Based on this, it was determined that the ATCase from *A. hydrophila* has a holoenzyme molecular mass of about 310 kDa like that of *E. coli*. Moreover, the gradient gel for the *A. hydrophila* ATCase did not show the two bands that the 8 % nondenaturing polyacrylamide gel electrophoresis showed.

The subunit composition of the ATCase from *A. hydrophila* further substantiated the results of the gradient gel. In order to rule out the possibility that the upper band, which appeared on the 8 % nondenaturing polyacrylamide gel, was not a different ATCase in the same organism, both bands were electroeluted to see if they were different in regards to subunit composition. Both samples were analyzed on a 15 % SDS denaturing gel along with a purified *E. coli* ATCase holoenzyme sample and a commercially made marker. Fig. 5 shows the results of the 15% SDS denaturing gel. Both electroeluted samples gave a 34 kDa band on the SDS gel which is identical in size with the catalytic polypeptide of *E. coli* ATCase. However, no 17 kDa band, representative of the regulatory polypeptide of a class B ATCase was observed. Since *A. hydrophila* ATCase holoenzyme is 310 kDa, similar to *E. coli*, it would be expected that the polypeptide composition would be similar, with *A. hydrophila* having six 34 kDa
Fig. 4. 4-20% Nondenaturing gradient gel of *A. hydrophila* ATCase holoenzyme.

Lane designations are as follows: 1 *P. aeruginosa* ATCase, 2 *E. coli* DH5α ATCase, 3 *E. coli* purified ATCase holoenzyme, 4 *E. coli* ATCase trimer, 5 *A. hydrophila* ATCase.

This gel was stained for ATCase activity as described above at 100 mM aspartate and 5 mM carbamoylphosphate. The gel shows that the molecular mass of the ATCase from *A. hydrophila* is the same as that from *E. coli*, which is 310 kDa.
Lane designations are as follows: 1 Broad Range Marker (BioRad), 2 *E. coli* purified ATCase enzyme, 3 *E. coli* ATCase from cell extract, 4 *A. hydrophila* ATCase electroeluted lower band, 5 *A. hydrophila* ATCase electroeluted upper band. This gel was first stained with Coomassie Brilliant Blue, then silver stained with a GELCODE silver staining kit from Pierce. Based on the sizes of the marker, *A. hydrophila* ATCase has a polypeptide size of 34 kDa, similar in size to the *E. coli* ATCase catalytic polypeptide.
polypeptides that compose the two catalytic trimers, and six 17 kDa polypeptides that compose the three regulatory dimers. One explanation for seeing only a 34 kDa band on the SDS gel would be if the regulatory dimmers, which would be 34 kDa in size, did not dissociate when the sample was analyzed on the gel. This may well be the case in 200 mM aspartate. Another possibility is that the ATCase from A. hydrophila does not have a 17 kDa regulatory polypeptide. Further study on the gene that encodes the ATCase from A. hydrophila would have to be performed to answer these questions.

Specific Activity of the Enzyme

Both Lowry and Bradford protein assays were performed on the cell extract and on each of the samples saved during the purification of the enzyme. Values for protein concentration were then calculated from the standard curves created from the Lowry assay and the Bradford assay using bovine serum albumin and lysozyme with both methods. The average protein concentration of the cell extract was 28.8 mg of protein ml⁻¹.

The activity of ATCase (amount of carbamoylaspartate (CAA) produced min⁻¹ (mg of protein)⁻¹) was determined by comparing the absorbance measurements of the assay to the standard carbamoylaspartate curve. Fig. 6 shows the standard curve for CAA. The specific activity was measured as the nmol of CAA produced min⁻¹ (mg of protein)⁻¹. The specific activity was measured for all steps during the purification of the enzyme. The specific activity of the enzyme increased 170-fold from that of the cell extract to the electroeluted sample.
Fig. 6. Carbamoylaspartate standard curve. This curve was used to calculate the specific activity for all ATCase assays performed.
Determining the Optimal pH

Based on the activity of the enzyme at various pHs, it was determined that the optimal pH of *A. hydrophila* ATCase was pH 7.0.

Determination of Heat Stability of Enzyme

*A. hydrophila* ATCase displayed some amount of activity at all temperature and time intervals tested except when the enzyme was heated to 100 °C. Fig. 7 shows the activity of the enzyme at the different times and temperatures. Heating the enzyme to 60 °C decreased its activity by more than 50 % when compared to the activity of the enzyme held at room temperature. Heating the enzyme to 85 °C produced very little activity and heating the enzyme to 100 °C produced no activity.

When the samples were analyzed on the 8 % nondenaturing polyacrylamide gel and stained for ATCase activity as described above, activity was detected for some of the temperatures and time intervals. Fig. 8(a) and 8(b) shows the ATCase activity of the enzyme following electrophoretic separation on an 8 % nondenaturing gel. A band showing ATCase activity from *A. hydrophila* was detected when the enzyme was heated to 50 degrees for all time intervals. The heated ATCase from *A. hydrophila* was 310 kDa in size. No activity was seen on the gel at temperatures of heating 60 °C or greater. The gel also showed that the enzyme did not dissociate upon heating, into a smaller, yet still active, ATCase.

Determination of the Apparent Km

Each of the assays to determine the apparent Km for both aspartate and carbamoylphosphate were done in triplicate and the data points represent an average of
Fig. 7. Residual ATCase activity after various heat treatments. This graph depicts *A. hydrophila* ATCase holoenzyme activity measured by the absorbance at 466 nm, plotted against the times of heating.
Fig. 8(a). 8 % Nondenaturing polyacrylamide gel of *A. hydrophila* after heat treatment. This is one of the two gels that show ATCase activity after heat treatment. This gel was stained for ATCase activity as described above with 100 mM of aspartate and 5 mM of carbamoylphosphate.

Gel 1 lane designation is as follows: 1 *P. aeruginosa* ATCase, 2 *E. coli* DH5α ATCase, 3 *A. hydrophila* ATCase at room temperature, ATCase from *A. hydrophila* heated as follows: 4 50 ºC for 10 min, 5 50 ºC for 20 min, 6 50 ºC for 40 min, 7 50 ºC for 60 min, 8 60 ºC for 10 min, 9 60 ºC for 20 min.
Fig. 8(b). 8 % Nondenaturing polyacrylamide gel of *A. hydrophila* after heat treatment.

This is the other gel that shows ATCase activity from heat treated *A. hydrophila*. This gel was stained for ATCase activity as described above with 100 mM aspartate and 5 mM carbamoylphosphate.

Gel 2 lane designations are as follows: 1 *P. aeruginosa* ATCase, 2 *E. coli* DH5α ATCase, 3 *A. hydrophila* ATCase at room temperature, ATCase from *A. hydrophila* heated as follows: 4 60 °C for 40 min, 5 60 °C for 60 min, 6 85 °C for 10 min, 7 85 °C for 20 min, 8 85 °C for 40 min, 9 85 °C for 60 min.
the three readings. The velocity-substrate curves for both substrates appeared sigmoidal as verified and tested for cooperativity as described by Koshland et al., (1966). Specifically, the substrate concentration at 0.9 Vmax, was divided by the substrate concentration at 0.1 Vmax. If this number deviated significantly from 81 then the enzyme displayed cooperativity. If the ratio was significantly less then 81, the enzyme showed positive cooperativity. If the ratio was greater than 81, the enzyme showed negative cooperativity. At 4.3, the velocity-substrate curve is clearly sigmoidal.

The velocity-substrate curve for aspartate for A. hydrophila ATCase was sigmoidal showing positive cooperativity. Fig. 9 shows the velocity-substrate curve for aspartate. Based on these data, the enzyme has an apparent $K_{m_{\text{asp}}}$ of 97.18 mM and a $V_{\text{max}_{\text{asp}}}$ of 3.05 nmol CAA min$^{-1}$, that was reached at 200 mM aspartate. The [S] at 0.9 Vmax divided by the [S] at 0.1 Vmax was 4.3.

The velocity-substrate curve for carbamoylphosphate was also sigmoidal. Fig. 10 shows the velocity-substrate curve for carbamoylphosphate. The ATCase enzyme from A. hydrophila had an apparent $K_{m_{\text{CP}}}$ of 1.18 mM and has a $V_{\text{max}_{\text{CP}}}$ of 2.9 nmol CAA min$^{-1}$ that was reached at 5.0 mM carbamoylphosphate. The [S] at 0.9 Vmax divided by the [S] at 0.1 Vmax was 35.

Effect Response

In all cases in which effectors were added to the ATCase assay, the activity of ATCase from A. hydrophila was diminished. Table 1 shows the effector response, as a measure of percent activity, of A. hydrophila ATCase for both aspartate and carbamoylphosphate. Of the three effectors used, ATP, CTP, and UTP, CTP was the best
Fig. 9. Aspartate saturation curve for *A. hydrophila* ATCase holoenzyme. This graph shows the activity of *A. hydrophila* ATCase at varying aspartate concentrations, 0-200 mM, while the carbamoylphosphate concentration was held constant at 5 mM. Based on this curve the apparent $K_m$ is 97.18 mM and the $V_{max}$ is 3.05 nmol CAA min$^{-1}$ which was reached at an aspartate concentration of 200 mM.
Fig. 10. Carbamoylphosphate saturation curve for *A. hydrophila* ATCase holoenzyme.

This graph shows the activity of *A. hydrophila* ATCase at varying carbamoylphosphate concentrations, 0-5 mM while the aspartate concentration was held constant at 200 mM. Based on this curve the apparent $K_m_{CP}$ is 1.18 mM and the $V_{max}$ is 2.9 nmol CAA min$^{-1}$ which was reached at a carbamoylphosphate concentration of 5 mM.
inhibitor with UTP the poorest. No significant difference was seen between the addition of 5 mM of an effector and 1 mM of an effector.

Table 1. Percent activity of *A. hydrophila* ATCase in the presence of ATP, CTP, or UTP at 1 mM and 5 mM concentrations of effector.

<table>
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<th>Substrate</th>
<th>No Effector</th>
<th>ATP 1 mM</th>
<th>ATP 5 mM</th>
<th>CTP 1 mM</th>
<th>CTP 5 mM</th>
<th>UTP 1 mM</th>
<th>UTP 5 mM</th>
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</thead>
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<td>Aspartate</td>
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<td>70</td>
<td>65</td>
<td>64</td>
<td>85</td>
<td>86</td>
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<td>Carbamoylphosphate</td>
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<td>70</td>
<td>66</td>
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CHAPTER 4

CONCLUSION

The study of pyrimidine metabolism in *A. hydrophila* has uncovered some interesting things about the organism. Though the information from this study does not change the earlier placement of *A. hydrophila* ATCase as a class B bacterial ATCase, some differences do occur between *A. hydrophila* and *E. coli*, the model class B organism.

The size of the ATCase holoenzyme from *A. hydrophila* is similar to the ATCase holoenzyme of *E. coli*, which would give the enzyme a molecular mass of 310 kDa. This size is slightly greater than the originally reported 280 kDa by Wild *et al.*, (1980). It was also determined that the polypeptide molecular mass of *A. hydrophila* is 34 kDa similar to the molecular mass of the catalytic polypeptide of *E. coli*. Even though the 17 kDa polypeptide was not seen on the 15 % SDS polyacrylamide gel, based on this study, it is probable that the *A. hydrophila* ATCase holoenzyme is composed of 6 catalytic polypeptides of 34 kDa each and 6 regulatory polypeptides of 17 kDa each. Therefore, based on size *A. hydrophila* would be considered a class B bacterial ATCase.

The kinetics of ATCase in *A. hydrophila* would also seem to place the enzyme in the class B bacterial ATCase. Both aspartate and carbamoylphosphate velocity substrate curves were sigmoidal with positive cooperativity, which also is similar to *E. coli* (Gerhart & Pardee, 1962; Bethell *et al*., 1968). Another major difference noted was the
A. hydrophila had an apparent $K_{\text{m,asp}}$ of 97.18 mM which was much greater than the $[S]_{0.5} = 17-20$ mM found by Wild et al., (1980). Though the aspartate requirements between class B ATCase organisms vary, as shown by Wild et al., (1980), the aspartate requirement for A. hydrophila appears to be the highest found so far for an enteric bacterium. The reason for the high aspartate requirement is not known at this time. Although the enzyme displayed sigmoidal kinetics, the response to effectors was different than that from E. coli. A. hydrophila ATCase activity was diminished by the presence each effector, as opposed to E. coli, which is inhibited by CTP and activated by ATP (Gerhart & Pardee, 1962). This is different from the results of Wild et al., (1980), which reported that A. hydrophila activity was increased by the presence of ATP or CTP.

Lastly it was shown that A. hydrophila remains active upon heating of the enzyme. The enzyme was stable when heated at 50 ºC and slightly stable when heated to 60 ºC. Though some differences occur, based on the findings, A. hydrophila should still be considered to have a class B bacterial ATCase.
REFERENCES


