CHARACTERIZATION OF ASPARTATE TRANSCARBAMOYLASE
AND DIHYDROOROTASE IN MORAXELLA
CATARRHALIS

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Michael A. Fowler, B.S.
Denton, Texas
May, 1998
Fowler, Michael, Characterization of Aspartate Transcarbamoylase and Dihydroorotase in *Moraxella catarrhalis*. Master of Science (Biological Sciences), May 1998, 120 pp., 1 table, 33 illustrations, 58 references.

Bacterial aspartate transcarbamoylases (ATCase's) are divided into three classes that correspond to taxonomic relationships within the bacteria. The opportunistic pathogen *Moraxella catarrhalis* has undergone several reclassifications based on traditional microbiological criteria. The previously uncharacterized ATCase from *M. catarrhalis* was purified to homogeneity and its chemical properties characterized. The ATCase from *M. catarrhalis* is a class C ATCase with an apparent molecular mass of 480-520 kDa. The *M. catarrhalis* ATCase is a dodecomer composed of six 35 kDa polypeptides and six 45 kDa polypeptides. The enzyme has an unusually high pH optimum of greater than pH 10. The enzyme exhibited hyperbolic kinetic with a Km for aspartate of 2 mM. A single, separate 78 kDa dihydroorotase from *M. catarrhalis* was identified and it was not associated with ATCase. These data support the reclassification of *M. catarrhalis* out of the Neisseriaceae family.
CHARACTERIZATION OF ASPARTATE TRANSCARBAMOYLASE
AND DIHYDROOROTASE IN MORAXELLA
CATARRHALIS

THESIS

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Michael A. Fowler, B.S.
Denton, Texas
May, 1998
ACKNOWLEDGMENTS

I would like to acknowledge with great appreciation my major professor, Dr. Mark S. Shanley. It has been an honor to have been able to work under his guidance for these past years. I would also like to thank Dr. Gerard A. O'Donovan and Dr. Robert C. Benjamin for their kindness and patience. I appreciate all the people from the Shanley and O'Donovan labs for their help, especially Mr. Pat Cooke. Finally, to my wife, Heidi, and boys, Heath and Peyton, I give my sincere gratitude for extending patience, encouragement and love for the successful completion of my graduate education.
TABLE OF CONTENTS

Page

LIST OF TABLES ........................................................................................................... v

LIST OF ILLUSTRATIONS ......................................................................................... vi

Chapter

1. INTRODUCTION ................................................................................................. 1

   Pyrimidine Metabolism in Bacterial Organisms
   Enzymology of Aspartate Transcarbamoylase
   Genetic Properties of Aspartate Transcarbamoylase Forming Genes
   Characterization of Active Dihydroorotase
   *Moraxella (Branhamella) catarrhalis*

2. MATERIALS AND METHODS ............................................................................. 27

   Chemicals and Reagents
   Bacterial Strains
   Media and Growth Conditions
   Preparation of Cell Extract
   Enzyme Assay
   ATCase Enzyme Purification
   Protein Gel Electrophoresis

3. RESULTS ............................................................................................................ 41

   Preliminary investigations of *Moraxella catarrhalis* ATCase
   Determining the Molecular Weight of DHOase in *Moraxella catarrhalis*
   Aspartate Curve for *Moraxella catarrhalis* ATCase
   Protein Gel Electrophoresis

4. DISCUSSION ....................................................................................................... 103

REFERENCES .......................................................................................................... 112
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Variations of ATCase Assay</td>
<td>32</td>
</tr>
</tbody>
</table>
## LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>The <em>de novo</em> pyrimidine biosynthetic pathway in bacteria</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>ATCase catalyzed reaction</td>
<td>6</td>
</tr>
<tr>
<td>3.</td>
<td>Bacterial classifications of ATCase</td>
<td>9</td>
</tr>
<tr>
<td>4.</td>
<td>Class B ATCase holoenzyme</td>
<td>11</td>
</tr>
<tr>
<td>5.</td>
<td>Classes of genetic arrangements for the enzymes of the pyrimidine biosynthetic pathway</td>
<td>15</td>
</tr>
<tr>
<td>6.</td>
<td><em>pyrBI</em> operon and chromosomal map of <em>E. coli</em></td>
<td>17</td>
</tr>
<tr>
<td>7.</td>
<td>The attenuation mechanism of <em>E. coli</em> <em>pyrBI</em> gene expression</td>
<td>20</td>
</tr>
<tr>
<td>8.</td>
<td>Dihydroorotase catalyzed reaction</td>
<td>23</td>
</tr>
<tr>
<td>9.</td>
<td>ATCase assay results using standard ATCase assay conditions</td>
<td>43</td>
</tr>
<tr>
<td>10.</td>
<td>ATCase assay results using Tris-phosphate buffer pH range of 5.0-9.5</td>
<td>46</td>
</tr>
<tr>
<td>11.</td>
<td>ATCase assay results using Tris-phosphate buffer pH range of 8.0-10.5</td>
<td>48</td>
</tr>
<tr>
<td>12.</td>
<td>ATCase assay comparison of 1 day versus 18 day old cell pellet maintained at 4°C.</td>
<td>50</td>
</tr>
<tr>
<td>13.</td>
<td>ATCase assay comparison of using breaking buffers at pH 7.0 versus pH 8.0</td>
<td>53</td>
</tr>
<tr>
<td>14.</td>
<td>Lowry assay standard curve</td>
<td>55</td>
</tr>
<tr>
<td>15.</td>
<td>Carbamoylaspartate curve used in calculating specific activity for all ATCase assays</td>
<td>57</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>16.</td>
<td>ATCase assay showing results of 2 per cent streptomycin sulfate cut.</td>
<td>60</td>
</tr>
<tr>
<td>17.</td>
<td>Ammonium sulfate per cent saturation curve comparing the ATCase activity of the supernatant (Sup) versus the pellet (Pel) over a broad range of saturation points.</td>
<td>63</td>
</tr>
<tr>
<td>18.</td>
<td>ATCase assay results of a 25% ammonium sulfate cut followed by a 60° heat treatment.</td>
<td>66</td>
</tr>
<tr>
<td>19.</td>
<td>ATCase assay results of a 62% ammonium sulfate enzyme precipitation.</td>
<td>68</td>
</tr>
<tr>
<td>20.</td>
<td>ATCase spot assay results from <em>M. catarrhalis</em> sample run through DEAE cellulose column.</td>
<td>71</td>
</tr>
<tr>
<td>21.</td>
<td>S-300 HR Sephacryl column elution results for determining void (blue dextran) and standard elution volumes.</td>
<td>73</td>
</tr>
<tr>
<td>22.</td>
<td>ATCase spot assay results of 1 ml <em>M. catarrhalis</em> sample run through S-300 HR Sephacryl column.</td>
<td>76</td>
</tr>
<tr>
<td>23.</td>
<td>A semi-logarithmic standard curve plotting the $V_e/V_0$ versus the molecular weight of the four protein standards.</td>
<td>78</td>
</tr>
<tr>
<td>24.</td>
<td>ATCase assay results of pooled elution samples from the S-300 HR Sephacryl column.</td>
<td>80</td>
</tr>
<tr>
<td>25.</td>
<td>DHOase assay results from <em>M. catarrhalis</em> sample passed over a calibrated S-300 HR column.</td>
<td>83</td>
</tr>
<tr>
<td>26.</td>
<td>A semi-logarithmic standard curve plotting the $V_e/V_0$ of <em>M. catarrhalis</em> against the $V_e/V_0$ of the calibrating standards and their known molecular weights.</td>
<td>85</td>
</tr>
<tr>
<td>27.</td>
<td>Verification that DHOase activity in <em>M. catarrhalis</em> is proportional to the sample volume.</td>
<td>87</td>
</tr>
<tr>
<td>28.</td>
<td>ATCase assay on purified <em>M. catarrhalis</em> enzyme conducted over a range of aspartate concentrations.</td>
<td>89</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>29.</td>
<td>Michaelis Menten plot for <em>M. catarrhalis</em> ATCase</td>
<td>92</td>
</tr>
<tr>
<td>30.</td>
<td>Lineweaver Burk plot for <em>M. catarrhalis</em> ATCase</td>
<td>94</td>
</tr>
<tr>
<td>31.</td>
<td>Gradient polyacrylamide gel electrophoresis of <em>M. catarrhalis</em> ATCase</td>
<td>96</td>
</tr>
<tr>
<td>32.</td>
<td>SDS polyacrylamide gel of <em>M. catarrhalis</em> ATCase</td>
<td>99</td>
</tr>
<tr>
<td>33.</td>
<td>Subunit composition of <em>M. catarrhalis</em> ATCase</td>
<td>101</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Pyrimidine Metabolism in Bacterial Organisms

The *de novo* pyrimidine pathway is required for DNA and RNA biosynthesis in all organisms (O’Donovan & Neuhard, 1970). The pathway converts aspartate and carbamoylphosphate in nine enzymatic steps to the ribonucleoside triphosphates, UTP and CTP. Characterization of the *de novo* pyrimidine biosynthetic pathway has been most extensive for *E. coli* and the enteric bacteria. Other non-enteric bacteria where extensive studies have been carried out include members of genera *Bacillus* and *Pseudomonas*. Detailed characterization of the pathways enzymology, salvage system, genetics, and regulation make the pyrimidine biosynthetic pathway one of the most studied pathways in science.

The pyrimidine biosynthetic pathway in *E. coli* (Fig. 1) competes with the arginine metabolic pathway for its allotment of carbamoylphosphate (Pierard *et al.*, 1965; Makoff & Radford, 1978; Stalon *et al.*, 1987; Wong & Abdelal, 1990). The first enzyme in the biosynthesis of pyrimidines and arginine is carbamoylphosphate synthetase (EC 6.3.5.5; CPSase; *carAB* or *pyrA*). Carbamoylphosphate synthetase catalyzes the formation of carbamoylphosphate from the amine group of glutamine, bicarbonate, and ATP.
Fig. 1. The de novo pyrimidine biosynthetic pathway in bacteria. The abbreviations for the compounds are: OMP, orotidine-5'-monophosphate; UMP, uridine-5'-monophosphate; UDP, uridine-5'-diphosphate; UTP, uridine-5'-triphosphate; CTP, cytidine-5'-triphosphate. The gene designations are as follows: pyrA, carbamoylphosphate synthetase (CPSase, EC6.3.5.5); pyrB, aspartate transcarbamoylase (ATCase, EC2.1.3.2); pyrC, dihydroorotase (DHOase, EC3.5.2.3); pyrD, dihydroorotate dehydrogenase (DHOdehase, EC1.3.3.1); pyrE, OMP phosphoribosyltransferase (OPRTase, EC2.4.2.10); pyrF, OMP decarboxylase (OMPdecase, EC4.1.1.23); pyrH, UMP kinase (EC2.7.4.4); ndk, nucleoside diphosphate kinase (EC2.7.4.6); and pyrG, CTP synthase (EC6.3.4.2).
The next reaction is unique to pyrimidine synthesis in which aspartate transcarbamoylase (EC 2.1.3.2; ATCase; L-aspartate carbamoyltransferase; \textit{pyrB}) condenses carbamoylphosphate with the amino group of aspartate and forms N-carbamoyl-L-aspartate and inorganic phosphate. In the third step, dihydroorotate is formed when dihydroorotase (EC 3.5.2.3; DHOase; \textit{pyrC}) catalyzes the cyclizing of carbamoylaspartate with the loss of water (Baeckstrom \textit{et al.}, 1986). In the fourth step of the pathway, dihydroorotate is oxidized by dihydroorotate dehydrogenase (EC 1.3.3.1; DHOdase; \textit{pyrD}) with NAD\textsuperscript{+} as the oxidizing agent to give orotate and NADH+H\textsuperscript{+} (Larsen & Jensen, 1985). In the next reaction, orotate phosphoribosyltransferase (EC 2.4.2.10; OPRTase; \textit{pyrE}) catalyzes the condensation of orotate with 5-phosphoribosyl-1-pyrophosphate (PRPP) to give pyrophosphate and orotidine-5'-monophosphate (OMP), the first pyrimidine nucleotide (Poulsen \textit{et al.}, 1983). In the next step, uridine-5'-monophosphate (UMP) is the product of the decarboxylation of OMP by OMP decarboxylase (EC 4.1.1.23; OMPdecase; \textit{pyrF}) (Theisen \textit{et al.}, 1987). In the following step, UMP kinase (EC 2.7.4.4; \textit{pyrH}) catalyzes the phosphorylation of uridine-5'-diphosphate (UDP) by UMP with the use of ATP. Next, uridine-5'-triphosphate (UTP) is generated from the phosphorylation of UDP by nucleoside diphosphate kinase (EC 2.7.4.6; ndk) with ATP (Ginther & Ingraham, 1974). In the last step cytidine-5'-triphosphate (CTP) synthetase (EC 6.3.4.2; \textit{pyrG}) catalyzes the amination of UTP to form CTP.
The de novo pyrimidine biosynthetic pathway is regulated by two major mechanisms: allosteric regulation of enzymatic activity and transcriptional regulation of gene expression in the pathway. Allosteric control of the de novo pathway is seen in three different enzymes (Fig. 1): CPSase (Abdelal & Ingraham, 1969), ATCase (Yates & Pardee, 1956) and CTP synthetase (Long & Pardee, 1967). The eventual end product CTP, inhibits ATCase which is activated by ATP and CTP synthetase. UMP inhibits CPSase while ornithine and IMP activate CPSase in *E. coli*. ATCase in *E. coli* is commonly regarded as the model for allosteric regulation in all biological systems.

The second form of control for the de novo pyrimidine biosynthetic pathway is transcriptional regulation of gene expression. In *E. coli*, transcriptional regulation has been reported to include the genes *pyrBI* (Beckwith et al., 1962); *pyrD* (Larsen & Jensen, 1985); *pyrE* and *pyrF* (Poulsen et al., 1983); and *pyrA* (*carAB*). Much research has been focused on the attenuation model of transcriptional regulation used by ATCase in *E. coli* and in *Salmonella typhimurium* (Neuhard et al., 1986; Lu et al., 1995).

**Enzymology of Aspartate Transcarbamoylase**

ATCase is the first committed step in de novo pyrimidine biosynthetic pathway (Fig. 2). When observing the enzymatic characteristics of known ATCases, differences in kinetics, regulation, size and subunit arrangement can be seen. Common to each unique structure is its allosteric properties. ATCase has a low-activity T-state and upon activation by substrate or binding of a
Fig. 2. *ATCase catalyzed reaction.* Carbamoylphosphate is derived from the transamination of oxaloacetate via the tricarboxylic acid cycle. The carbamoylphosphate is hydrolyzed releasing the free energy that drives the nucleophilic addition of the carbonyl carbon of carbamoylphosphate to the amino group of aspartate.
positive regulatory effector, ATCase undergoes a transition to a higher activity R-state. This allosteric transition from one state to the other determines the enzyme's affinity for substrates and effectors, and thus its catalytic activity (Schachman, 1988). The ATCase enzyme can be found in both eukaryotes and prokaryotes with bacterial ATCases separated into three classes according to size and regulatory properties (Bethell & Jones, 1969) (Fig. 3).

Class B ATCase enzymes are represented by *E. coli* and the Gram negative enteric bacteria. The enzyme is a dodecamer made from six copies each of two polypeptides: the *pyrB* gene encodes a 311 amino acid residue long, 33 kDa polypeptide, which forms the trimer with catalytic activity and the *pyrI* gene encodes a 153 amino acid long, 17 kDa polypeptide, which forms the regulatory dimers (Kantrowitz & Lipscomb, 1988). The dodecameric holoenzyme has a molecular mass of 310 kDa and is composed of two catalytic trimers (six catalytic polypeptides) and three regulatory dimers (six regulatory polypeptides) (Fig. 4). Crystallographic X-ray studies (Honzatko, *et al.*, 1982; Krause, *et al.*, 1987) showed that two catalytic trimers (*c₃*) are stacked above each other and are held together by the three regulatory dimers (*r₂*). The three regulatory dimers are clustered around the periphery of the molecule and maintained by a zinc atom tetrahedrally coordinated by four cysteine residues at the interface of each catalytic and regulatory polypeptide. Although the regulatory dimers have no catalytic activity, they do contain the binding sites for nucleotide effectors. *E. coli* ATCase is inhibited by CTP and is activated by ATP
Fig. 3. *Bacterial classifications of ATCase.* The Class A ATCase is the largest of the three classes with a mass weight of ~480 kDa. Class B ATCase has a molecular mass of ~310 kDa while the class C ATCase has a molecular mass of ~100 kDa.
Class A ATCases
Mr = 480 kDa

Class B ATCases
Mr = 310 kDa

Class C ATCases
Mr = 100 kDa
Fig. 4. *Class B ATCase holoenzyme.* The holoenzyme enzyme from *E. coli* is a dodecamer consisting of two catalytic trimers and three regulatory dimers \( \{2(c_3) : 3 (r_2) \} \). The catalytic trimers lie above and below the regulatory dimers in a "sandwich" arrangement.
Regulatory Subunits

Catalytic Subunits

Top View

Regulatory Subunits

Catalytic Subunits

Side View
with a sigmoidal dependence on substrate concentration at an optimum pH of 7.0. The balance of nucleotide production by pyrimidine biosynthesis with purine biosynthesis probably controlled by the level of ATP activation.

Class A represents the largest class by molecule weight and is commonly associated with the pseudomonads. Like the class B enzyme, the class A enzyme contains two different subunits. The *P. fluorescens* holoenzyme has a molecular mass of 474 kDa. The enzyme is composed of six copies of the 34 kDa catalytic polypeptide and six copies of the 45 kDa polypeptide. The six 45 kDa subunits are constructed as three dimers which attach to the two catalytic trimers (Bergh & Evans, 1993). Saturation curves for carbamoyl-phosphate and aspartate for *P. fluorescens* are hyperbolic, becoming sigmoidal when UTP was added. UTP was found to be the best inhibitor of ATCase, but ATP also strongly inhibited the enzyme while no activators where found (Neumann & Jones, 1964).

The class C ATCase is the smallest in molecular weight and includes the ATCases from the endospore forming Gram positive bacteria with *Bacillus subtilis* having been studied the most in detail. It is comprised of catalytic trimers only with a molecular mass between 100 and 140 kDa and displays hyperbolic kinetics (Brabson & Switzer, 1975; Maurizi & Switzer, 1978).

Eukaryotic ATCases belong the multifunctional polypeptide, "CAD" which is comprised of the domains of CPSase, ATCase and DHOase. The CAD protein from *Mesocricetus auratus* (Syrian hamster) was found to have
glutamine-dependent CPSase, ATCase and DHOase activity on a single 254 kDa polypeptide. The ATCase activity was associated with a 40 kDa fragment and the DHOase activity was associated with a 44 kDa fragment (Grayson & Evans, 1983; Kelly et al., 1986). UTP and PRPP control CAD at the cellular level with PRPP activating and UTP inhibiting CPSase activity (Carrey 1986). Additionally, it has been reported that phosphorylation at the two serine residues of CAD by cAMP-dependent protein kinase activates CPSase which prevails over the feedback inhibitor UTP (Carrey et al., 1985).

**Genetic Properties of Aspartate Transcarbamoylase Forming Genes**

Various studies have shown that the genetic arrangements for the enzymes in the pyrimidine biosynthetic pathway are highly diversified (Fig. 5). Such variety in genetic arrangement contributes to the diversity in gene regulation and enzyme structure between classes of enzymes.

*E. coli* ATCase is encoded from two contiguous cistrons, *pyrB* and *pyrl* (Hoover, *et al.*, 1983). The *pyrBl* bicistron is located 96.0 minutes on the *E. coli* chromosome with the *pyrBl* operon consisting in length of approximately 2.8 kbp (Fig 6). This bicistron has a control region consisting of two promoters (P1 and P2) and a regulatory region that possesses segments that code for a 44-amino acid leader polypeptide and an attenuator sequence. The P2 promoter displays a dominant role in the promotion of transcription for *pyrBl* expression with the P1 promoter contributing no more than three percent of the overall transcripts (Levin, *et al.*, 1989). Transcriptional termination occurs 23 base
Fig. 5. *Classes of genetic arrangements for the enzymes of the pyrimidine biosynthetic pathway.* Each open reading frame (ORF) is represented by a box. Boxes for fused genes and contiguous genes are drawn connected. Intergenic and non-coding intergenic regions are represented by a bar while overlapping reading frames are offset one after another.
Fig. 6. *pyrBI operon and chromosomal map of E. coli*. Found along the 96 minute region of the *E. coli* chromosome, the *pyrBI* operon is 2800 bp in length and consists of the *pyrB* gene, the *pyrl* gene and a regulatory region preceding the *pyrB* gene. The regulatory region is composed of two promoters (P₁ and P₂), a pause site and a terminator.
Escherichia coli Chromosome Map
pairs upstream of the structural genes of the operon at a rho-independent terminator. Efficient termination occurs in the presence of high levels of UTP (Turnbough, et al., 1983). The attenuation mechanism constitutes decreased expression of pyrB1 in growth environments containing cytosine or uracil compounds and derepression when the growth environment is lacking in pyrimidines (Fig. 7).

Studies on ATCase gene expression in Pseudomonas indicate that regulation for this class is quite different from that of E. coli. Pseudomonas putida for example, has a pyrB gene similar to that of E. coli which encodes the catalytic subunit of the enzyme. P. putida however does not possess dissociable regulatory and catalytic functions but instead contains a regulatory nucleotide binding site within a N-terminal extension of the pyrB-encoded subunit. The second gene in the P. putida ATCase holoenzyme complex has significant homology to DHase from other organisms, but has no DHase activity. This inactive pyrC has been designated pyrC' with a proposed function of assuring ATCase activity by conserving the dodecameric assembly of the native enzyme (Schurr et al., 1995). Additionally, unlike E. coli, Pseudomonas shows no apparent repression of gene expression when grown in pyrimidines (Isaac & Holloway, 1968; Condon, et al., 1976; Chu & West, 1990).

The Bacillus subtilis ATCase holoenzyme corresponds to the free catalytic trimers of E. coli encoded by pyrB, and does not have a pyrI encoded counterpart. The B. subtilis pyrB gene overlaps with the pyrC gene encoding an
Fig. 7. The attenuation mechanism of E. coli pyrBl gene expression. The model shows the relative positions of RNA polymerase and the translating ribosome when UTP levels are either low or high (Adapted from Roland et al., 1985). When the cellular UTP level is low, the RNA polymerase pauses at nucleotide -80 after the first dyad which permits the ribosome to start translation of the leader peptide. The ribosome catches up to the polymerase and the translating ribosome prevents the formation of the attenuation hairpin allowing the RNA polymerase to read through a multi-uridine region. The polymerase then proceeds into the structural genes, and the pyrBl operon is completely transcribed. Under conditions of high intracellular concentrations of UTP, RNA polymerase does not stop at the pause site of pyrBl operon and the nascent mRNA transcript forms two hairpins corresponding to the dyads in the DNA sequence. Due to the formation of the second hairpin (attenuation hairpin), transcription terminates at the uridine rich region.
LOW UTP

pyrBl Promoter

Transcription Pause site

Attenuator

RNA Polymerase

Structural Genes

Leader Transcript

Leader Polypeptide

HIGH UTP

pyrBl Promoter

No Pausing

Attenuator

RNA Polymerase

Structural Genes

Ribosome

Transcription Termination hairpin
active and independent DHOase enzyme (Lerner & Switzer, 1986; Switzer & Quinn, 1993). All six of the pyr genes in B. subtilis are found on an 11 kbp fragment with the order pyrBCADFE. Furthermore, additional regulatory studies of B. subtilis have established that the genes of this operon are transcribed as one message.

**Characterization of Active Dihydroorotase**

Dihydroorotase (pyrC) is the third enzyme in the de novo pyrimidine biosynthetic pathway (Fig. 8). In bacteria, DHOase activity results indicate that it is a monofunctional protein. However, recent studies show that some bacteria have the ATCase and DHOase protein complex similar to the CAD protein found in eukaryotic organisms (Van de Casteele et al., 1994). Through the purification and analysis of active DHOase from several organisms, classifications of DHOase based on molecular weight and regulation seem to follow that of the ATCase groupings.

The purified DHOase in E. coli, is comprised of two identical 38 kDa subunits with a zinc binding region constituting an active dimer of 76 kDa (Washabaugh & Collins, 1986). The expression of pyrC is negatively regulated by cytidine nucleotide in E. coli (Schwartz & Neuhard, 1975; Pierard et al., 1976). Unlike pyrBl which is transcriptionally regulated by the attenuation mechanism, pyrC is transcriptionally regulated by the identification of an operator region within the promoter region of pyrC by the repressor protein (Wilson et al., 1987).
Fig. 8. *Dihydroorotase catalyzed reaction*. Dihydroorotase catalyzes the reversible cyclization of $N$-carbamoyl-L-orotate to dihydro-L-orotate with the loss of a water molecule, the third step in the pyrimidine biosynthetic pathway.
Dihydro-L-ornate

\[ \text{H}_2\text{O} \]

\[ \text{N-carbamyl-L-aspartate} \]
The relative molecular mass of DHOase in *P. putida* is 82 kDa and is comprised of two identical subunits of 41 kDa each. Unlike *E. coli*, *P. putida* DHOase is noncompetitively inhibited by pyrimidine metabolism intermediate such as orotate and dihydrouracil (Ogawa *et al.*, 1995).

The DHOase in *Bacillus subtilis* was significantly larger than the active DHOase found in *E. coli* and *P. putida*. The product of the *pyrC* gene in the *B. subtilis* pyr cluster is a 46 kDa polypeptide that is rendered active as a 92 kDa dimer (Lerner *et al.*, 1987).

**Moraxella (Branhamella) catarrhalis**

*Moraxella (Branhamella) catarrhalis* is an aerobic, Gram-negative diplococcus that was known previously as *Neisseria catarrhalis* (Knapp, 1988). DNA homology experiments showed little or no genetic affinity with the *Neisseria* group and was eventually classified as *Branhamella*, a subgenus of *Moraxella* (Kingsbury, 1967; Bovre, 1979). Collectively, the *Moraxella* (Branhamella) subgenus consists of *M. (B.) catarrhalis, M. (B.) caviae, M. (B.) cuniculi, and M. (B.) ovis* (Bovre & Hagen, 1981). Additionally, 16S Ribosomal DNA sequence analysis has determined that *M. catarrhalis* no longer belongs in the *Neisseria* group in the beta subdivision of the Proteobacteria, but is now classified as a member of the *Acinetobacter* subgroup under the *Pseudomonas* and relatives group in the gamma subdivision (Enright *et al.*, 1994).

In recent years, *M. catarrhalis* has attracted much attention as a human pathogen. It causes a number of infections including bronchitis, pneumonia,
conjunctivitis and otitis media but little is known about its virulence factors.

Some strains of *M. catarrhalis* are β-lactamase-producing for which no genetic mechanism of β-lactams is known. Biochemical characteristics such as nutritional requirements for *M. catarrhalis* are poorly understood (Catlin, 1990). However, known characteristics include oxidase and catalase positive, an optimum growth temperature at 33-35°C, a molecular percent G+C of DNA at 40-43, and natural competence. Research on the *de novo* pyrimidine biosynthetic pathway for *M. catarrhalis* has been limited, though it has been documented that the effector CTP is a strong inhibitor of *M. catarrhalis* while ATP or GTP have no stimulating effect (Jyssum, 1992).

The main objective of this thesis is to show how ATCase and DHOase from *M. catarrhalis* can be categorized into one of the previously defined ATCase classes. The sizes of the active forms of ATCase and DHOase in *M. catarrhalis* have been determined along with the characterization of optimum conditions, purification, and kinetics of ATCase in *M. catarrhalis*. These results substantiate the recent reclassification of *M. catarrhalis* into the *Pseudomonas* and relatives group in the *gamma* subdivision of the Proteobacteria.
CHAPTER 2

MATERIALS AND METHODS

Chemicals and Reagents

N-carbamoyl-L-aspartate, L-aspartic acid, carbamoylphosphate, L-
dihydroorotic acid, 1,5-dimethyl-2-phenyl-3-pyrazone (antipyrine), 2,3
butanedione monoxime, potassium phosphate, zinc acetate, bovine serum
albumin fraction V, sodium bicarbonate, streptomycin sulfate, imidazole, DEAE
cellulose, blue dextran 2000, apoferritin, carbonic anhydrase, and alcohol
dehydrogenase were purchased from Sigma Chemical Company (St. Louis,
Missouri). Tris [hydroxymethyl] aminomethane, β-mercaptoethanol (BME), Folin
Phenol Reagent, potassium tartrate, cupric sulfate, ammonium sulfate,
ethylendiaminetetraacetec acid (EDTA), sodium dodecyl sulfate (SDS),
acrylamide, bis-acrylamide, bromophenol blue, lead nitrate, and potassium
hydroxide were purchased from Fisher Scientific Company (Fair Lawn, New
Jersey). Acetic acid and phosphoric acid were purchased from T. J. Baker, Inc.
(Phillipsburg, New Jersey). Hydrochloric acid, glycine, sulfuric acid, sodium
hydroxide, and glycerol were purchased from EM Science (Gibbstown, New
Jersey). N, N, N', N' tetramethylethylene diamine (TEMED), ammonium
persulfate, and coomassie blue were purchased from Bio-Rad (Richmond,
California). Sephacryl S-300 HR media was purchased from Pharmacia (Pittscataway, New Jersey). Brain heart infusion agar media and brain heart infusion broth media were purchased from Difco (Detroit, Michigan). Distilled deionized water was used in all experiments.

Bacterial Strains

*Moraxella catarrhalis* American Type Culture Collection strain 25238 (wild type) was used for all experiments. The strain was stored in a 50% glycerol solution at -80°C.

Media and Growth Conditions

Bacteria from -80°C stock were streaked onto a plate containing Difco Brain Heart Infusion Agar medium. The streaked plates were placed in a 37°C incubator for 24 hours. Difco Brain Heart Infusion broth was prepared by adding one liter of water to 35 grams powdered medium in a 1.8 L Fernbach flask. Ten liters were sealed with aluminum foil, then sterilized in an autoclave. After autoclaving, each flask was allowed to cool to room temperature and then inoculated using individual colonies from a streak plate. Flasks were shaken at 200 rpm on a reciprocating incubator shaker in a controlled environment of 37°C. The growth of cells was followed by monitoring the change in Klett Units on a Klett-Summerson colorimeter equipped with a #54 filter. Uninoculated medium was used as a blank. Cells were harvested during mid to late exponential phase at approximately 100 Klett Units (one Klett Unit equals...
approximately $5 \times 10^6$ cells per ml. Cells were harvested in a Sorvall centrifuge using a GSA rotor spun at 10,000 xg for 10 minutes at 4°C. Pelleted cells were washed with one hundredth volume of fresh media, repelleted and stored at 4°C for no longer than 24 hours.

**Preparation of Cell Extracts**

The pellet was resuspended in 0.04M KH$_2$PO$_4$, pH 7.0 or pH 7.5, since pH 7.5 was determined as the optimum pH after initial investigations. The resuspended buffer also contained 0.02 mM zinc acetate and 1 mM dithiothreitol. A 2 ml volume of buffer per gram weight of cells was used to resuspend cells. Cells were broken in a French pressure cell (SLM Aminco, Urbana, Illinois) at 1,000-1,200 pounds per square inch while maintained at a 4°C temperature. Unbroken cells and cell debris were removed by centrifugation in a Sorvall centrifuge using a SA600 rotor at a speed of 10,000 xg for 10 minutes at 4°C. The resulting supernatant was collected and recentrifuged at 18,000 xg for one hour at 4°C. The clarified cell free extract was collected and dialyzed in a Pyrex beaker against one liter of 0.04M KH$_2$PO$_4$, pH 7.5 containing 0.02 mM zinc acetate for two hours. The resulting crude extract was removed from the dialysis tubing and stored in a 50 ml screw top conical tube at 4°C.
Enzyme Assay

The enzyme activity of ATCase was determined by the measuring the formation of carbamoylaspartate according to the method of Gerhart and Pardee (1962) using the modified method of Prescott and Jones (1969). Different assay conditions were employed to characterize optimum parameters of the enzyme that were unique to the ATCase for *M. catarrhalis*. An ATCase spot assay was used for quick identification of ATCase activity. This spot assay is not an end point assay and is used to assay only for the presence and relative amount of the enzyme. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as the standard. DHOase activity was determined by measuring the rate of conversion of dihydroorotate to carbamoylaspartate (Beckwith *et al.*, 1962; Schwartz & Neuhard, 1975).

The standard ATCase assay was initially performed as follows with modifications noted in Table 1. The assay mix consisted of 0.2 ml of 0.05 M K-aspartate pH 7.0 (5 mM aspartate final concentration), 0.08 ml of 1 M Tris-phosphate buffer pH 7.0 (40 mM KH$_2$PO$_4$ final concentration), cell free extract diluted with 0.04 M KH$_2$PO$_4$, pH 7.0, 1.32 ml H$_2$O, and 0.2 ml of 8 mg/ml in H$_2$O carbamoylphosphate (5 mM CP final concentration) for total of 2 ml. Pre-incubation reaction tubes (no CP added) were placed in a 30°C water bath for 3 minutes. Stop tubes were prepared (1 tube per time point, 3 tubes per assay) by adding 0.5 ml of distilled deionized water. After initial studies, tris-phosphate
buffer was used for all subsequent enzyme assays and all kinetic characteristics reported here (Table 1). The reaction was started with addition of 0.2 ml of carbamoyl phosphate. At 10 minutes, 20 minutes, and 30 minutes, 0.5 ml samples of the reaction mixture were transferred into stop tubes containing 0.5 ml H₂O and immediately, 1.0 ml of color mix was added (color mix ingredients: two parts of 0.5 grams antipyrine in 100 ml of 50% sulfuric acid and one part 0.4 diacetyl monoxime in 50 ml of 5% acetic acid mixed immediately before use in a brown bottle and kept on ice). Each tube was immediately mixed well with a vortex mixer and placed in a ice-water bath until all time points had been collected. The tubes were than capped with marbles, placed in a 60°C water bath and exposed to light for 110 minutes. The tubes were removed from the water bath and placed in a room temperature water bath for 3 minutes. Each tube was read at 466 nm using a Beckman (Du-40) spectrophotometer against a reacted buffer blank (reacted buffer blank contains all mixture ingredients except CFX for which water is substituted). Additionally, a carbamoylaspartate curve was performed in which 1 ml of color mix was added to tubes containing 0.1 ml of 10 mM CAA in 0.9 ml 0.04 M KH₂PO₄, 0.05 ml of 10 mM CAA in 0.95 ml 0.04 M KH₂PO₄, 0.02 ml of 10 mM CAA in 0.98 ml 0.04 M KH₂PO₄, and 0 ml of 10 mM CAA in 1.0 ml 0.04 M KH₂PO₄. Each tube was mixed, incubated in the light for 110 minutes in a 60°C water bath, cooled for 3 minutes, and read at 466 nm using a Beckman (Du-40) spectrophotometer against a reacted blank.
<table>
<thead>
<tr>
<th>Assay Mix Reagents</th>
<th>Standard Assay</th>
<th>pH Curve Assay #1</th>
<th>pH Curve Assay #2</th>
<th>Enzyme Stability in pellet</th>
<th>Cell Free Extract pH Stability</th>
<th>ATCase Purification</th>
<th>Velocity-Substrate Curve for Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-aspartate</td>
<td>5 mM pH 7.0</td>
<td>5 mM pH 7.0</td>
<td>5 mM pH 7.0</td>
<td>5 mM pH 7.0</td>
<td>5 mM pH 7.0, 8.0</td>
<td>5 mM pH 7.5</td>
<td>1, 2, 3, 4, 5, 7, 10, 13, 16, and 20 mM pH 7.5</td>
</tr>
<tr>
<td>Tris-phosphate Buffer</td>
<td>40 mM pH 7.0 (K-phosphate buffer)</td>
<td>100 mM pH 5.0 - 9.5</td>
<td>100 mM pH 8.0 - 10.5</td>
<td>100 mM pH 7.0 - 9.5</td>
<td>100 mM pH 7.0 - 9.5</td>
<td>100 mM pH 9.0</td>
<td>50 mM pH 9.0</td>
</tr>
<tr>
<td>Cell Free Extract Diluted</td>
<td>Diluted 1:1, 1:2, 1:10, 1:100 w/ 40 mM K-phosphate pH 7.0</td>
<td>Diluted 1:10 w/40 mM K-phosphate pH 7.0</td>
<td>Diluted 1:50 w/40 mM K-phosphate pH 7.0</td>
<td>Diluted 1:37.5 w/40 mM K-phosphate 7.0</td>
<td>Diluted 1:50 w/40 mM K-phosphate pH 7.0, 8.0</td>
<td>Diluted 1:10, 1:25, 1:50 w/40 mM K-phosphate pH 7.5</td>
<td>Diluted 1:100 w/40 mM K-aspartate pH 7.5</td>
</tr>
<tr>
<td>Carbamoyl-Phosphate</td>
<td>5 mM</td>
<td>5 mM</td>
<td>5 mM</td>
<td>5 mM</td>
<td>5 mM</td>
<td>5 mM</td>
<td>5 mM</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.32 ml</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>Total Volume</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>
Spot assays were performed in the following manner. Forty ml of water were added to a 250 ml Erlenmeyer flask with 10 ml 0.5 M Tris-phosphate buffer, pH 9.0, 5 ml carbamoyl phosphate, and 5 ml potassium aspartate, pH 7.5 for a total volume of 60 ml. One ml of assay mix was then added per tube on ice. To each tube was added 10 μl of sample, followed by mixing, and then the tubes were placed in a 30°C water bath for 15 minutes. The tubes were removed and placed on ice for 5 minutes. One ml of color mix was placed in each tube on ice and mixed. The tubes were placed in a 60°C water bath for varying times until visible color developed and then cooled in a room temperature water bath for 3 minutes. Samples from each tube were read at 466 nm against a water blank.

The Lowry protein assay was performed as follows. Standards for the Lowry assay consisted of 0-100 μg bovine serum albumin per ml (BSA) per tube made from standard where one μg equals one μl of BSA. Tubes were allocated BSA in 10 μl (0.1 mg/ml) increments from 0 to 100 μl and topped off with H₂O so that each tube had a total volume of 0.2 ml. Samples were prepared by adding 1, 3, and 5 μl of sample to individual tubes and topping each off with H₂O so that each tube had a total volume of 0.2 ml. A 0.8 ml amount of Alkaline Copper Reagent (made by mixing 0.5 mls of 2% Na/K tartrate with 0.5 mls of 1% CuSO₄•5 H₂O and adding 49 ml of 2% NaCO₃ in 0.1
N NaOH) was added to all tubes, mixed, and left to stand at room temperature for 10 minutes. After standing, 0.1 ml of 1N Folin Phenol Reagent (diluted 1:1 with H₂O for 1N) was added to all of the above tubes, mixed and left to stand at room temperature for 30 minutes. Samples from each tube were read at 660 nm using a Beckman Du-40 spectrophotometer.

The DHOase assay was conducted in the following way. On ice, 790 µl H₂O, 100 µl Tris buffer, pH 9.0, and 10 µl of sample enzyme were added per individual tube. The tubes were placed in a 30°C water bath for 3 minutes. 100 µl of 20 mM dihydroorotate in 0.1 M phosphate buffer was added to each tube followed by 7 minutes of incubation in a 30°C water bath. After removal from the water bath the tubes were place on ice. One ml of freshly prepared color mix was added to each tube, followed by mixing, covering with a marble, and incubation in the light for 110 minutes in a 60°C water bath. After incubation was complete the tubes were cooled in a room temperature water bath for 3 minutes and read at 466 nm using a Beckman Du-40 spectrophotometer against a reacted blank.

**ATCase Enzyme Purification**

Enzyme purification was conducted using modifications of the Gerhart and Holoubek method (1967). Purification of enzyme from cell free extract included a streptomycin sulfate cut, an ammonium sulfate cut, a heat step, ammonium sulfate precipitation, DEAE cellulose anion exchange column, and a
Sephacryl S-300 HR column size exclusion chromatography. All steps of purification were individually characterized in order to establish optimum results.

A streptomycin sulfate cut was conducted on 10 ml of CFX (0.04 M KH$_2$PO$_4$, pH 7.5, 0.02 mM zinc acetate). The CFX was placed in a 30 ml Corex centrifuge tube, treated with 2% streptomycin sulfate, and mixed by shaking for 15 minutes at 4°C. It was then placed in a Sorvall centrifuge and spun down in an SA600 rotor for 10 minutes at 10,000 xg at 4°C. The supernatant was poured into a 15 ml screw top conical tube and supernatant volume measured was yielding approximately 8 ml. Then supernatant was decanted into a clear 30 ml Corex tube. An ammonium sulfate cut was performed by adding 2.25 ml of 4.5 M ammonium sulfate in 0.125 M Tris base, pH 9.2 to the supernatant thus creating an ammonium sulfate concentration of 25% saturation. The 2.25 ml of ammonium sulfate was added slowly on ice and mixed by shaking for 15 minutes at 4°C. The suspension was then placed into a 60°C gyrotory water bath shaker (New Brunswick Scientific) and swirled for 4 minutes. It was next placed in a Sorvall centrifuge and spun down in a SA600 rotor at 10,000 xg for 10 minutes at 4°C. The supernatant was pored into a 15 ml screw top conical tube, and measured yielding 9 ml, then pored into a clear 30 ml Corex tube. The percent saturation was adjusted to 62% by adding 1.99 grams of ammonium sulfate crystals slowly on ice to the 9 ml of extract and shaking for 1
hour at 4°C. After shaking, the precipitate was placed in a Sorvall centrifuge and spun down in a SA600 rotor at 10,000 xg for 30 minutes at 4°C. The supernatant was poured off and the pellet was resuspended in 10 ml of 0.05 M KIB, pH 8.5 (0.05 M KCl, 0.01 M Imidazole, 0.02 mM zinc acetate, 0.02 M BME, 2x10^{-4} M EDTA, pH 8.5) (During the characterization of each step, both the supernatant and pellet were assayed for ATCase activity and compared against the ATCase activity of uncut CFX).

A DEAE cellulose column was used for anion exchange purification. The DEAE media was equilibrated using 0.05 KIB, pH 8.5. After constant stirring over a 24 hour period, the fines were removed, and the media was packed into a 25 cm column. A salt gradient was established by connecting two bottles with a hose containing a release valve. One bottle contained 150 ml of 1 M KIB, pH 8.5 while the other bottle contained 250 ml of 0.05 M KIB, pH 8.5. The latter contained a stir bar and was continuously stirred. It also was connected by hose to the top of the column. At the bottom of the column was a flow valve connected to a hose that ran to the fraction tubes. Collection started by opening the flow valve and adding the 10 ml of partially purified sample into the top of the column. Applying the hose to the top of the column allowed flow to occur from the 0.05 KIB bottle. When 100 ml of the 0.05 KIB has been used, the gradient was started by opening the release valve and allowing 1 M KIB to flow into the 0.05 KIB bottle. Eluted samples were collected in fraction tubes using a
Gilson microfactionator until both KIB bottles were empty. After collection was complete, a spot assay on the fraction tubes was performed and 42 ml of extract (tubes with 0.128 or higher absorbance) were pooled for dialysis. The pooled extract was dialyzed against 433.33 ml of 4.5 M AS in 0.125 M Tris-HCl, pH 9.2, 21 ml 1 M Tris-HCl, pH 7.0, 0.75 ml of 0.02 M zinc acetate, 15 ml 1 M BME, 0.3 ml 0.5 M EDTA, pH 8.0, 237.62 ml H₂O, and 42 ml of extract for a total of 750 ml. Dialysis was performed for 24 hours in a 1 liter Pyrex beaker at 4°C and resulted in a 66.8% saturation. The dialyzed extract was transferred to a 30 ml Corex tube, placed in a Sorvall centrifuge, and spun down in a SA600 rotor at 10,000 xg for 30 minutes at 4°C. The pellet was resuspended in 1 ml of ATCase buffer, pH 7.0 (ATCase buffer consists of 0.04 M KH₂PO₄, 0.02 mM zinc acetate, 1 mM BME, pH 7.0).

A calibrated Sephacryl S-300 HR column was used for size exclusion purification. The media was packed into a 35 cm Pharmacia column and eluted with ATCase buffer for 24 hours. Coolant at 4°C was circulated around the column and a flow rate of 30 ml per hour was established. Calibration was constituted by individually passing 1 ml of each standard through the column and reading the collected fractions at 280 nm. Blue dextran (MW 2,000 kDa) at 10 mg/ml was run first and the void volume was calculated. The remaining standards run were 10 mg/ml Apoferritin (MW 433 kDa), E. coli ATCase (MW 310 kDa), Alcohol Dehydrogenase (MW 150 kDa), and carbonic anhydrase
(MW 29 kDa). A standard curve was determined using the formula $V_e/V_0$ ($V_e$ is the elution volume for standards, $V_0$ is the void volume of the blue dextran). The 1 ml of sample extract was run through the column and collected fractions were examined for ATCase activity using the spot assay method. After determining the $V_e/V_0$ value for the sample extract, its molecular weight was determined from the standard curve.

**Protein Gel Electrophoresis**

Protein gel analysis was provided by results obtained from the electrophoresis of a 4-20% gradient polyacrylamide gel and a denaturing 10% polyacrylamide SDS gel. Protein bands were exposed using an ATCase activity stain and Coomassie stain for the gradient gel and a Coomassie stain for the SDS gel.

The 4-20% gradient polyacrylamide gel was a 6" x 6" Bio-Rad ready gel that was assembled in a Bio-Rad mini-Protean II cell gel electrophoresis apparatus. The apparatus was filled with running buffer (4.5 g Tris base, 21.6 g glycine per 1.5 liters H$_2$O, pH 8.3), de-aerated for 5 minutes using a water jet aspirator) so that the wells of the gel were completely submerged. Each well was loaded with a 30 μl of sample (24 μl elution sample mixed with 6 μl of a 5X 10% glycerol and 0.001% bromophenol blue solution) using a 50 μl Hamilton syringe. The gel was run for 8 hours at 50 volts at 4°C in order for the samples to establish equilibrium within the gradient. The gel was removed from the
apparatus and an ATCase activity stain was performed as follows. The gel was placed into a Pyrex dish containing 100 ml of cold (4°C) 50 mM imidazole, HCl, pH 7.5 for 5 minutes. Five ml of 1.0 M aspartate and 10 ml of 0.1 M carbamoylphosphate were added to the dish followed by a waiting period of 10 minutes. The solution was poured off and rinsed 3 more times to remove reactants. The enzymatic release of orthophosphate trapped in the gel was precipitated by immersing the gel in 100 ml of cold (4°C) 3 mM lead nitrate, 50 mM Imidazole, HCl, pH 7.5 (Bothwell, 1975). After 10 minutes, the lead nitrate was removed with three changes of ice water and the results were recorded. The gel was then transferred to a Tupperware tray where 100 ml of 0.05% coomassie blue in 7% acetic acid was added and gently shaken for 12 hours. The gel was then destained for 12 hours with 2 changes of 7% acetic acid while being shaken gently. The gel was rinsed with water and fixed to Whatman paper using a Bio-Rad (Model 483) slab dryer apparatus set at 80°C for 3 hours.

A denaturing 10% SDS polyacrylamide gel was assembled in a Hoefman Scientific (SE 600 series) vertical slab gel unit. The plates were prepared by cleaning, placing spacers, taping together plates and applying clips to the edge of the plates. In a 100 ml Pyrex beaker, 1.1 grams of Tris base was added to 27 ml of H₂O. The solutions pH was adjusted to 8.9 and transferred to a vacuum flask. Added to the solution was 2.33 g acrylamide, 0.063 g bis-acrylamide,
0.014 g ammonium persulfate, and 0.03 g SDS. After mixing, the solution was deaerated for 5 minutes with a water jet aspirator. Ten μl of TEMED was gently mixed into the solution and gel was poured using a 25 ml glass pipett. The poured gel was allowed to polymerize for 3 hours. The solidified gel was placed into the gel apparatus and filled with running buffer (4.5 g Tris base, 21.6 g glycine, 1.5 g SDS in 27 mls H$_2$O, pH 8.3) so that the loading well were completely immersed. Each sample was dissolved in 0.0625 M Tris-HCl, pH 6.5, 25 SDS, 10% glycerol, 5% MBE, and .001% bromophenol blue. Samples were heated in boiling water for 1.5 minutes before application. A thirty μl of sample was loaded into each well using a 50 μl Hamilton syringe. The gel was run for 4 hours at 100 volts at room temperature. After running, the gel was removed from the apparatus and placed in a Tupperware tray. One hundred ml of 0.05% Coomassie Blue in 7% acetic acid was added to the tray and gently shaken for 12 hours. The gel was then rinsed and destained with two changes of 7% acetic acid again gently shaken for 12 hours. The gel was rinsed with water and fixed to Whatman paper using a gel drying apparatus set to 70°C for 3 hours.
CHAPTER 3

RESULTS

Preliminary investigations of *Moraxella catarrhalis* ATCase

Since the enzyme from *Moraxella catarrhalis* had not been previously examined, a number of studies were undertaken initially to determine the properties of the enzyme with respect to its level of expression, its stability and its optimal assay conditions. In the absence of any previous studies with ATCase from *M. catarrhalis*, published conditions used for the *E. coli* enzyme were used as a starting point for further enzymatic determinations and refinements. Time course enzyme assays were performed to determine if the enzyme were stable through the 30 minute course of the reaction, as well as stable under the conditions of the assay (pH 7.0 and 30°C). At the same time these initial assays provided an estimate of the level of expression of the ATCase in *M. catarrhalis* in repressing conditions, given the fact that rich medium was used for all growth experiments (Material and Methods). In addition, the pH optimum, level of substrate saturation, and the best conditions for storage stability were determined before subsequent studies were performed. Of course, the linearity of the enzyme assay with time was measured in every case on every sample assayed through out this study (except spot assays). Such initial enzyme assays and studies of the
M. catarrhalis ATCase were performed on cell extracts in order to define the optimal parameters of the enzyme. After ATCase assays were conducted using the established conditions for the assay of E. coli ATCase, adjustments in pH, phosphate buffer composition and concentration, and protein were made to optimize the assay activity for M. catarrhalis and to ensure that subsequent assays were in the linear range of the assay, as well as within the limits of the assay. ATCase assays were also carried out to examine the effect on enzyme activity as a result of variations in cell pellet storage and breaking buffer pH (versus assay pH). Lowry protein assays were used to determine the total protein concentration in the cell free extracts and thus calculate the specific activity of ATCase, as a reflection of the level of gene expression as well as purity.

The initial assays was performed using E. coli ATCase conditions and covered a range of dilution values (Fig. 9). The results showed that the cells expressed sufficient ATCase at levels that encourage future study and attempts at purification. Indeed, when these first assays were performed, the cell extracts required dilution to obtain accurate measurements of the enzyme activity. There was significant activity at a 1:10 dilution and measurable activity at a 1:100 dilution (Fig. 9, note that the range of activities dictates a large scale for the y-axis).

After these range finding assays, a constant dilution value of 1:10 was used and the assay performed when the assay buffer was varied over a pH range of 5.0 to 9.5. In this case the buffer used was a Tris-phosphate buffer that is capable of buffering well over this range because of the multiple pKa’s for the buffer system components. The effect of varying the pH of the assay is depicted
Fig. 9.  ATCase assay results using standard ATCase assay conditions.
Several dilutions of the enzyme from crude cell extracts were measured to
determine a suitable range of enzyme concentration that gave measurable
results without saturating the assay conditions. A dilution of 1:10 was chosen
for assays from this particular preparation.
ATCase Assay Using Standard Conditions

Abs @ 466 nm

Time (minutes)

- 1: 1 dilution
- 1: 2 dilution
- 1: 10 dilution
- 1: 100 dilution
in Figure 10. The data showed that the ATCase from *M. catarrhalis* possessed an optimum pH of 9.0 to 9.5. Since the optimum activity observed occurred at the end of the pH activity curve, a second pH study was undertaken to see what effects resulted from even higher pH values. Further examination showed that activity increased with increased pH up to pH 10.5 (Fig. 11); however, all further assays used a pH 9.0 buffer at a final concentration of 100 mM in the assay mix. Though the value of pH 9.0 is not optimum for the enzyme it represents a compromise between assay sensitivity and relevance to actual cellular conditions.

Cell growth, cell harvesting, cell breakage and enzyme assay are all time consuming processes. It would be more convenient to perform some of the tasks on different days, if possible. Large quantities of cells required for purification can be grown and collected on different days and pooled only if enzyme is stable in unbroken cells. Cells were examined to distinguish the difference in activity of cells broken immediately after harvesting versus the activity of cells broken after being stored as centrifuge pellets for longer periods up to 18 days subsequent to harvesting. Pelleted cells kept at 4°C for 18 days before being broken had 41% less activity than cells broken on the day of cell harvest (Fig. 12). Since the enzyme deteriorated upon storage, cell extracts were made immediately after harvesting for all subsequent experiments. Cell pellets were immediately washed with buffer, resuspended in buffer and broken by explosive decompression in an SLM-Aminco French pressure cell. This method of breakage resulted in almost complete breakage of the cells (few viable cells after breakage) and preserved enzyme activity. Since the enzyme had a high pH optimum, the pH of the breaking buffer became a concern, with
Fig. 10. *ATCase assay results using Tris-phosphate buffer pH range of 5.0-9.5.*

The graph depicts the change in absorbance throughout the assay for the enzyme at different pH values. An increase in the value and slope indicate an increase in relative specific activity. Except for the pH, conditions were kept constant for all samples. An $A_{466}$ of 0.5 corresponds to a value of 100 nmole carbamoyl aspartate produced.
ATCase Assay/pH Curve
(All dilutions 1:10)

Abs @ 466 nm

Time (minutes)
Fig. 11. ATCase assay using Tris-phosphate buffer pH range of 8.0-10.5. The graph depicts the change in absorbance throughout the assay for the enzyme at different pH values. An increase in the value and slope indicates an increase in relative specific activity. Except for the pH, conditions were kept constant for all samples. An A_{466} of 0.5 corresponds to a value of 100 nmole carbamoylaspartate produced.
ATCase Assay/pH Curve
(All dilutions 1:10)
Fig. 12. *ATCase assay comparison of 1 day versus 18 days old cell pellet maintained in 4°C environment.* Samples were diluted 1:37.5 immediately before assay and assayed under identical conditions of pH, substrate concentration and temperature.
ATCase Assay/Pellet After 1 vs. 18 Days
(All dilutions 1:37.5)

Abs @ 466 nm

Time (minutes)

pH 9.0/1 day
pH 9.0/18 days
the understanding that the pH for stability does not necessarily correspond to the optimum pH for assay. Cells were broken in pH 7.0 buffer and pH 8.0 buffer, and then were assayed and compared to distinguish which pH presented the most stable environment. The pH of the assay in each case remained the same. ATCase activity showed that cells broken and assayed in pH 7.0 buffer had 23 per cent less activity than did the cells broken and assayed in pH 8.0 buffer (Fig. 13). It was determined from these results that cell free extract would be prepared from breaking buffer with pH 7.5.

A Lowry protein assay was conducted on each cell free extract, as well as on subsequent partially purified preparations of the enzyme. The specific activity could be calculated in those instances where relative measurements were insufficient (level of enzyme expression and level of enzyme activity). In cases where the relative activity was measured, specific activities were not always calculated, though protein was measured at every step. A standard BSA curve was constructed that measured protein over a range of 0 to 100 µg of protein with a sensitivity of approximately 5 µg of protein per tube and an accuracy of ± 2 µg of protein per sample (Fig. 14). Values for protein concentration were thus calculated from standard curves using bovine serum albumin as standard. A typical protein concentration for cell free extract was 22.5 mg protein per ml of sample for the sample used in the previous figures (Fig 9-13).

The activity of ATCase (actual amount of carbamoylaspartate produced) was determined by comparing the absorption readings of the assay to a standard carbamoylaspartate curve (Fig. 15). Thus some values are reported as nmole product formed per unit time. Specific activity was measured
Fig. 13. ATCase assay comparison of using breaking buffers at pH 7.0 versus pH 8.0. The same culture was divided into equal volumes (500 ml each) and treated identically except that the pH of the breaking buffer was either pH 7.0 or pH 8.0.
ATCase Assay/pH 7.0 vs. 8.0
(All dilutions 1:50)
Fig. 14. *Lowry assay standard curve.* Bovine serum albumin was used as the standard. A typical sample is plotted to illustrate determination of protein concentration (see text). In this case a 3 µl sample was assayed with a resulting concentration of 22.5 mg protein per ml.
Lowry Assay

Abs @ 660 nm

mg BSA/Tube

Std Plots
Fig. 15. Carbamoylaspartate curve used in calculating specific activity for all ATCase assays. A typical standard curve for carbamoylaspartate is shown. The standard was treated identically to sample thus the zero concentration tube as served as a buffer and assay blank. The buffer and assay mix components give some background color, thus the curve does not go through zero. This data can be plotted as is or the value of the blank subtracted from each value to get a line that intersects the origin. There was very little day to day deviation (less than 5%) in the carbamoylaspartate curves.
throughout the enzyme's purification process as a means of determining the percent yield and purity of the enzyme.

**Enzyme Purification**

In order to measure the properties of the enzyme, a purification was carried out. The enzyme was subjected to a variety of purification procedures. The enzyme purification was loosely based on classical methods for the purification of ATCase from *E. coli*. Most significantly a heat step was attempted. This also gave some idea of the stability of the enzyme. Each purification process required evaluation of percent yield, fold purification, feasibility and characterization of optimum conditions. Ultimately, purification of the ATCase enzyme of *M. catarrhalis* consisted of a streptomycin sulfate cut, an ammonium sulfate cut, heat treatment, a DEAE cellulose column, and a S-300 HR Sephacryl column. A typical purification is described.

10 liters of cells were collected, washed, resuspended in breaking buffer, broken and centrifuged to remove cell debris. A two per cent streptomycin sulfate cut was conducted on 10 ml of cell extract in order to remove much of the ribosomal debris. After centrifugation, the resulting 8 ml of supernatant was assayed and compared against the activity of the resuspended pellet. Results showed a substantial retention of enzyme in the supernatant (Fig. 16). In addition to precipitating little of the ATCase activity, this step resulted in removal of significant amounts of contaminating protein, presumably ribosomal proteins based on the known mechanism of streptomycin sulfate cuts.

Salting out of an enzyme is a standard purification technique in almost every protein purification scheme. Ammonium sulfate precipitation is also used to concentrated the enzyme from large volumes of sample. Ammonium sulfate
Fig. 16. ATCase assay showing results of 2 per cent streptomycin sulfate cut. The streptomycin sulfate pellet (SSP) was resuspended and brought to volume equal to that of the supernatant. SSP, the streptomycin sulfate supernatant (SSS), and crude extract (CFX) standard were assayed at a 1:50 dilution. Most of the original activity was recovered in the supernatant.
ATCase Assay of Streptomycin Sulfate Cut

Abs @ 466 nm

Time (minutes)
cuts precipitate proteins by neutralizing the charge on the surface of the molecule so that it is no longer interacts with and is solvated by the water molecules. Initially proteins are more soluble at low concentrations of the sale (salting in), but can no longer be dissolved in solution (salting out) as the salt concentration increases. Every protein has its own optimum ammonium sulfate concentration for salting out based on the properties of that particular protein. The concentration of ammonium sulfate that would not precipitate the enzyme was desired, so that a cut could be performed as a purification step such that some proteins salted out, while ATCase remained in solution. The concentration of ammonium sulfate that would complete precipitate the enzyme was also desired so that the enzyme could be cut from crude extracts and concentrated from dilute, partially purified enzyme preparations. A range of ammonium sulfate cuts were performed to establish the saturation point and precipitant point of the sample enzyme (Fig. 17). After centrifugation, the supernatant was removed and the pellet was resuspended in a volume equal to the supernatant. *M. catarrhalis* ATCase began precipitating at a significant rate at an ammonium sulfate concentration of 40 per cent saturation. The enzyme was precipitated at an ammonium sulfate concentration of 60 per cent saturation. From the results it was determined that 25 per cent would be the saturation point and 62 per cent would be used when precipitating enzyme.

The enzyme was stable in 25 per cent ammonium sulfate and did not show measurable levels of precipitation. ATCase from *E. coli* is reportedly stabilized during heat treatment by high levels of ammonium sulfate. The heat step was thus performed with the enzyme preparations at an ammonium sulfate concentration of 25 per cent saturation. The streptomycin sulfate supernatant
Fig. 17. Ammonium sulfate percent saturation curve comparing the ATCase activity of the supernatant (Sup) versus the pellet (Pel) over a broad range of saturation points. Each line on the plot represents the relative activity in the sample plotted against the ammonium sulfate concentration expressed as the per cent saturation. Relative, activity measurements in this plot are measured as the absorbance increase over a 20 minute time period (the difference between the 10 and 30 minute time readings in the assay).
Ammonium Sulfate Percent Saturation Curve

- Sup Total Activity
- Pel Total Activity

Total Activity (Change 466 nm/20 min) vs. % Saturation
was prepared with 25% ammonium sulfate and heat treated at 60°C for 4 minutes. The resulting supernatant and resuspended pellet were assayed for ATCase activity (Fig. 18). Heat treatment at 60°C resulted in minimal loss of enzyme activity. A significant and visible amount of protein precipitated and was removed by centrifugation. This pellet was resuspended and assayed for ATCase activity. Negligible amounts of ATCase were measurable, probably residual ATCase in the fluid wetting the pellet before resuspension. Small amounts of ATCase were lost in the heat treatment (approximately 10 per cent), probably due to thermal denaturation of enzyme quaternary and tertiary structure. This small loss of activity was acceptable given the effective removal of contaminating protein in this step.

The heat treated supernatant was then precipitated at 62% ammonium sulfate to concentrate the enzyme for subsequent purification steps. Again, the resulting supernatant and resuspended pellet were assayed for ATCase activity (Fig. 19). In this step as well, a small amount of enzyme was lost but the overall yield was considered sufficient.

The heat step, streptomycin sulfate cut and ammonium sulfate cut expended all the common batch methods for solution protein purification. Several chromatographic techniques were examined and a number of determinations made to define optimum conditions. Ion exchange chromatography over a DEAE anion exchange column was first conducted on the precipitated pellet resuspended in KIB pH 7.5. When this sample was added to the column, it failed to bind to the resin as shown by spot assay results and no gradient was run to elute the sample. The column packing was removed, washed to remove bound proteins and recharged. The pH of the KIB
Fig. 18. ATCase assay results of a 25% ammonium sulfate cut followed by a 60°C heat treatment. In this case the actual amount of enzyme remained little changed from the untreated sample. The difference in absorbance represents a changing blank. The more purified sample has a lower blank that the untreated sample with more protein and the same amount of enzyme activity. The amount of enzyme activity is determined by the rate or slope of the line, not the absolute values. The heat treated pellet (HTP), the heat treated supernatant (HTS), and crude extract (CFX) standard were assayed at a 1:50 dilution.
Ammonium Sulfate Cut With Heat Treatment

Abs @ 466 nm

Time (minutes)
Fig. 19. ATCase assay results of a 62 per cent ammonium sulfate enzyme precipitation. Precipitation of ATCase activity was complete after treatment with ammonium sulfate at 62 per cent saturation. The precipitated spun supernatant (PSS), the precipitated spun pellet (PSP), and crude extract (CFX) standard were assayed at a 1:50 dilution.
Ammonium Sulfate Precipitation

Abs @ 466 nm vs Time (minutes)

- PSS/1:50
- PSP/1:50
- CFX STANDARD
buffer was raised to 8.5 to increase the overall net negative charge on the protein and enhance binding to the column. In this case, the ATCase activity was bound to the column. A small amount of enzyme was eluted in the void (Fig. 20) and the column washes. This could be enzyme that was present in excess relative to the binding capacity of the column, enzyme that did not equilibrate because the flow was too fast, or another form of the enzyme (for example a subunit deficient form). Activity was washed from the column with a salt gradient. Spot assays of ATCase activity now showed that the enzyme came off the column well within the gradient (Fig. 20). Fraction tubes with a significant spot assay activity absorption reading (an $A_{488}$ of 0.128 and higher for the example shown) were pooled together. Significant dilution of the enzyme occurred at this step and the original sample for the data shown had a resulting total volume of 42 ml. The pooled fractions was precipitated by dialysis against a large volume (1-2 liter) of a 62% ammonium sulfate solution. The precipitated protein was recovered from the dialysis bag and resuspended in 1 ml ATCase buffer.

A S-300 HR Sephacryl size exclusion column was constructed in order to further purify the enzyme, as well as to determine the enzyme's relative molecular weight. An initial elution, using blue dextran, determined the column's void volume ($V_0$) while additional elution's were used to determine the elution volume ($V_e$) of the protein standards (Fig. 21). The protein samples used included purified *E. coli* ATCase (graciously provided by Pat Cooke), alcohol dehydrogenase, apoferritin and carbonic anhydrase. In each case the standards gave a single peak that was symmetrical. The 1 ml sample of *M. catarrhalis* ATCase, resuspended in ATCase buffer, was then run through the
Fig. 20. *ATCase* spot assay results from *M. catarrhalis* sample run through *DEAE cellulose column*. The first peak (small peak) is ATCase enzyme that eluted from the column in the wash prior to the gradient. The second peak representing the majority of the ATCase activity was enzyme that bound to the column and was eluted by the gradient.
ATCase Spot Assay Of DEAE Cellulose Column Extract
Fig. 21. *S*-300 HR Sephacryl column elution results for determining void volume (blue dextran) and standard elution volumes. Samples were applied to a Pharmacia column (2.5 cm X 35 cm) and 1 ml fractions were collected. For all standards elution was measured by the proteins' absorbance at 280 nm.
S-300 HR Column Calibration

- Blue Dextran
- Apoferritin/Carbonic Anhydrase
- E. coli ATCase
- Alcohol Dehydrogenase

Abs @ 280 nm

Milliliters Eluted
calibrated column and its $V_e$ was determined using the ATCase spot assay method (Fig. 22). *M. catarrhalis* ATCase eluted over a narrow peak spanning approximately 20 fractions. A shoulder was observed, but its relative contribution to the total activity was not significant and could represent a loading artifact. The tube with maximal activity was chosen for determination of the $V_e$ for *M. catarrhalis* ATCase.

A semi-logarithmic standard curve was constructing by dividing the $V_e$ of each eluted sample by the $V_0$. The standard curve indicated that the $V_e/V_0$ for the *M. catarrhalis* sample corresponded to a molecular mass of 490 to 520 kDa. (Fig. 23).

Elution tubes with ATCase spot assay absorption readings of 0.094 or more were pooled together as three groups. Tubes before $V_e$ (6 ml), tubes after $V_e$ (10 ml), and the tubes at $V_e$ (3 ml) constituted the three groups. Each group was then concentrated 60% and assayed for ATCase activity (Fig. 24).

**Determination of the Molecular Weight of DHOase in *M. catarrhalis***

Bacterial ATCases with large molecular weights like the *M. catarrhalis* enzyme (class C ATCases) have associated with them an inactive form of dihydroorotase. Unlike their eukaryotic counterparts, DHOase activity is not present in a multifunctional enzyme. However recent reports indicate that some bacterial ATCases do indeed have both ATCase and DHOase activity present in a single protein complex. Thus the possibility that *M. catarrhalis* ATCase had associated with it an active DHOases was investigated. Crude extracts from freshly grown, harvested and broken cultures of *M. catarrhalis* were passed over the calibrated S-300 HR column in order to determine whether DHOase co-eluted with ATCase activity indicating some sort of enzyme complex was
Fig. 22. ATCase spot assay results of 1 ml M. catarrhalis sample run through S-300 HR Sephacryl column. A 1 ml sample of partially purified M. catarrhalis ATCase was passed over the calibrated 2.5 cm X 35 cm Sephacryl S-300 HR column. Fractions of 1 ml were collected and ATCase was detected by spot assay.
ATCase Spot Assay Of S-300 HR Column Extract
Fig. 23. A semi-logarithmic standard curve plotting the $V_e/V_0$ versus the molecular weight of the four protein standards. The $V_e/V_0$ of the *M. catarrhalis* sample when compared to the standard curve, indicates a molecular mass of 520 kDa for the *M. catarrhalis* ATCase.
Semi-logarithmic Standard Curve
Of S-300 HR Column

- M. catarrhalis ATCase (520 kDa)
- Apoferritin (443 kDa)
- E. coli ATCase (310 kDa)
- Alcohol Dehydrogenase (150 kDa)
- Carbonic Anhydrase (29 kDa)
Fig. 24.  ATCase assay results of pooled elution samples from the S-300 HR Sephacryl column. Fractions were collected and pooled after Sephacryl S-300 HR chromatography. Three separate pooled samples were prepared that corresponded to the fractions about the peak value, fractions on the shoulder ahead of the peak and fractions on the activity shoulder behind the peak.
ATCase Assay Of Pooled Samples
From S-300 HR Column

Abs @ 466 nm

Time (minutes)
formed. Column fractions were collected from a calibrated column and assayed for DHOase activity (Fig. 25). DHOase activity was observed in low but measurable quantities, sufficient to get accurate elution values for the enzyme. The Ve/Vo was compared to a semi-logarithmic standard curve as before that was ascertained from the S-300 HR column. Results proved that the DHOase of *M. catarrhalis* was not active within the ATCase holoenzyme complex and that it was active as an enzyme with an apparent molecular weight of 78 kDa (Fig. 26).

Due to the low activity of the DHOase, it was necessary to verify the data and that the absorbance values performed in the spot assay corresponded to enzymatic activity expected. Enzymatic conversion of the substrate dihydroorotate to carbamoylaspartate (the enzyme is assayed in the reverse reaction) must therefore be proportional with respect to time, and amount of enzyme assayed and exhibit saturation. To ensure that the color development observed in the spot assay corresponded to DHOase enzymatic activity, samples of 10 µl, 20 µl, and 40 µl were tested to determine if the activity was proportion to the change in sample volume. In addition, the enzyme activity for each and every sample was measured at different time points to ensure linearity with respect to time. Results showed that the activity of 20 µl samples was double that of the 10 µl sample as was the activity of the 40 µl sample double that of the 20 µl sample (Fig. 27).

**Aspartate Curve for *M. catarrhalis* ATCase**

The purified enzyme from *M. catarrhalis* was examined to determine its enzymatic and catalytic properties. The enzyme was assayed under conditions of varying substrate in order to determine the saturation kinetics of the enzyme. Data from the enzyme assay are depicted in Fig 28. The data are re-plotted in
Fig. 25. DHOase assay results from *M. catarrhalis* sample passed over a calibrated S-300 HR column.
DHOase Assay Of S-300 HR Column Extract

Abs @ 466 nm

Milliliters Eluted

DHOase Activity
Fig. 26. A semi-logarithmic standard curve plotting the $V_e/V_o$ of *M. catarrhalis* against the $V_e/V_o$ of the calibrating standards and their known molecular weights. The results indicate that there is only one form of active DHOase and that the active form of DHOase from *M. catarrhalis* is a 78 kDa protein.
Semi-logarithmic Standard Curve
Of S-300 HR Column

Ve/Vo

log Molecular Mass (kDaltons)

Apolipoprotein (443 kDa)
E. coli ATCase (310 kDa)
Alcohol Dehydrogenase (150 kDa)
M. catarrhalis DHOase (78 kDa)
Carbonic Anhydrase (29 kDa)
Fig. 27. Verification that DHOase activity in M. catarrhalis is proportional to the sample volume.
DHOase Activity Comparison

Abs @ 466 nm

Sample

- 10 ul Sample
- 20 ul Sample
- 40 ul Sample
Fig. 28. ATCase assay on purified *M. catarrhalis* enzyme conducted over a range of aspartate concentrations.
Aspartate Curve (1:100 Dilution)

Abs @ 466 nm

mM Aspartate

- 10 Minutes
- 20 Minutes
- 30 Minutes
traditional Michaelis Menten and Lineweaver Burk plots in Fig. 29 and 30, respectively. The enzyme showed traditional Michaelis-Menten (hyperbolic) kinetic response to the substrate aspartate. No sigmoidicity indicative of cooperative response to substrate, was observed in the Michaelis Menten plot and the Lineweaver Burk double reciprocal plot gave a straight line with no deflections. The enzyme showed half maximal velocity at approximately 2 mM aspartate on the Michaelis Menten plot. The Lineweaver Burk plot gave an intercept of -0.5 corresponding to a Km of 2.0 mM for the substrate aspartate.

**Protein Gel Electrophoresis**

Protein gel electrophoresis was conducted on pooled samples from the S-300 HR column in order to assess purity and obtain an estimate of molecular weight by a different analytical method. A non-denaturing gradient polyacrylamide protein gel was run with standards and stained specifically for ATCase activity. After the gel was stained for enzyme activity, the gel was also stained with coomassie blue stain to determine the holoenzyme's size relative to the standards and assess the purity of the pooled ATCase fractions from the size exclusion column (Fig. 31).

The gradient polyacrylamide gel was run using purified *P. aeruginosa* ATCase as the standard since its molecular weight is accurately known from the amino acid sequence deduced from DNA sequence studies. An activity stain showed a large smeared band for the ATCase from *M. catarrhalis* that corresponded to an apparent molecular weight of 470 kDa when measured from the leading edge of the band. The gel was then stained with Coomassie Blue in order to visualize the protein molecular weight standards. The results showed two bands that co-migrated to a point on the gel corresponding to
Fig. 29. *Michaelis Menten plot for M. catarrhalis ATCase.* The saturation kinetics of the enzyme gave a hyperbolic response with respect to the substrate aspartate.
Michaelis Menten Aspartate Saturation Curve
for Moraxella catarrhalis ATCase
Fig. 30. *Lineweaver Burk plot for M. catarrhalis ATCase.* A double reciprocal plot was constructed from the data in Fig. 29. The y intercept gave a Km value for aspartate of 2 mM.
Fig 31. *Gradient polyacrylamide gel electrophoresis of M. catarrhalis ATCase.*

A 4 to 20 per cent gradient gel was run on the purified enzyme from *M. catarrhalis* (lane 1). Purified ATCase from *P. aeruginosa* was used as the standard (lane 2).
Gradient Polyacrylamide Gel

- M. catarrhalis ATCase
- P. aeruginosa ATCase (484 kDa)
molecular masses of 470 kDa and 520 kDa. Since the activity detected on the
gel was smeared over both bands it is possible that both represent ATCase.

An SDS protein gel was run to determine subunit sizes. The SDS
polyacrylamide gel was run on the purified ATCase from *M. catarrhalis* along
with a protein molecular weight ladder set of standards. The gel was stained
with Coomassie Blue and two bands (Fig. 32) were observed in the lane
corresponding to the pool fractions of the peak activity from the size exclusion
column. The fractions corresponding to the peak shoulders showed additional
protein bands and thus were not combined with the purified ATCase. Still the
predominant bands in the other fractions migrated to an identical position as
that for the most highly purified *M. catarrhalis* ATCase. The two subunit bands
(Fig 32) migrated to positions corresponding to subunit sizes of ~45 kDa and ~
35 kDa when compared with standards (Fig 33).
Fig. 32. *SDS polyacrylamide gel of M. catarrhalis ATCase.* A 10 per cent SDS gel was run on the pooled fractions of peak activity (lane 1) from the size exclusion chromatography column. The standards (lane 2) correspond to a molecular mass range of 14 to 97 kDa.
SDS Polyacrylamide Gel

M. catarrhalis ATCase

14 to 97 kDa Ladder
Fig 33. *Subunit composition of M. catarrhalis ATCase.* The standards gave a linear response when the distance migrated was plotted against the log molecular weight of the standards. The two bands corresponding to the *M. catarrhalis ATCase* migrated 6.5 and 7.4 cm. This gave subunit molecular mass of ~35 and ~45 kDa.
SDS Standard Curve

Distance Migrated (cm)

log Molecular Mass (kDa/tons)
CHAPTER 4

DISCUSSION

At the time these studies were initiated, little was known or was published of the enzyme from *Moraxella catarrhalis*. In reference to the *M. catarrhalis* enzyme (Jyssum, 1992), the only information reported was the enzyme's response to nucleotide effectors, and this in a single table only. There was no mention of the enzyme's molecular weight, subunit structure or kinetic response to substrate. In Jyssum's study, the classification of *M. catarrhalis* was in flux, having just been renamed *Branhamella catarrhalis* from *Neisseria catarrhalis*. The transfer of the species to the genus *Moraxella* had been proposed (Henricksen & Bøvre, 1968). But before inclusion of the organism in the genus *Moraxella*, it was transferred to the genus *Branhamella* (Catlin, 1970), then reclassified as a subgenus of *Moraxella* (Bøvre, 1979). Thus, the organism used in this study had been previously classified as a "false neisseriae", moved to a new genus *Branhamella*, later reclassified as a subgenus of *Moraxella*, and finally classified as *Moraxella catarrhalis* (Bøvre & Hagen, 1981). Its reclassification and association with several diverse groups of bacteria, made *M. catarrhalis* a candidate for studies of its ATCase. In other words, ATCase could be used to establish the taxonomic position of the organism.

The organism is very fastidious and requires complex media that would be considered repressing with respect to the enzymes of pyrimidine metabolism, since the media contained sources of exogenous pyrimidines. The
relative high levels of ATCase found in crude cell extract were encouraging given the conditions for growth. No experiments were performed to investigate levels of expression with respect to increasing amounts of exogenously fed pyrimidines (repression) or removal and limitation of pyrimidines (de-repression) since suitable media for these experiments were not available. Still, a working hypothesis based on the available data suggests that the enzyme is not regulated at the level of gene expression in *M. catarrhalis*. It is possible that exogenously fed pyrimidines are not taken up and metabolized. Further study of pyrimidine salvage in this organism is thus warranted. The specific activity of the enzyme was comparable to levels of ATCase found in *E. coli* grown on minimal medium without added pyrimidines. The specific activity was much higher than the levels found in *E. coli* grown on comparable rich, repressing medium (Foltermann, et al., 1983) and the specific activity in *M catarrhalis* is much lower that found in derepressed mutant strains or recombinant strains of *E. coli* carrying multiple copies of the *pyrBI* genes (Nowlan & Kantrowitz, 1985).

The enzyme's properties in crude cell extracts were examined. The enzyme was fairly stable, but activity decreased after storage of whole cells. Thus all studies were performed with enzyme that had been released from cells immediately and then stored as cell extract or as (partially) purified enzyme preparation. The most remarkable property of the enzyme was the pH optimum. The enzyme was active over a broad range of pH but was most active above pH 9.5. This is in sharp contrast to the *E. coli* ATCase. ATCase from *E. coli* has a pH optimum of 6.8 at low aspartate levels, rising to pH 8.2 at high levels of aspartate (Gerhart and Pardee, 1964). In addition, class B bacterial ATCases
demonstrate a pH dependence of the activation by ATP (Thiry and Hervé, 1978). This high pH value is clearly non-physiological. The increase in pH causes de-protonation of both the enzyme and the substrates. This high pH corresponds roughly to the pKa for the amino group of the substrate aspartate. It is possible that a positively charged (or partially charged) residue on the enzyme at the active site normally interferes with substrate binding. De-protonation of the amino group of the substrate aspartate would eliminate the charge repulsion and enhance substrate binding, dramatically increasing enzyme activity as observed. Of course it is impossible to tell from the experiments reported here if the effect is due to de-protonation of the substrates or of some critical residue on the enzyme. It is also possible that the change in pH induces a conformational change in the enzyme that results in stimulation of the enzyme activity. Clearly the enzyme from *M. catarrhalis* suffers from “catalytic constraint” since at normal, neutral pH the enzyme is not operating at its maximal velocity. Later studies of enzyme activity involving estimates of Km and response to effectors were done at high pH, but not so high as to be unrealistic with respect to conditions inside the cell. In addition kinetic characterizations were performed with purified enzyme.

ATCase from *M. catarrhalis* was purified by adapting several well-established techniques used for the purification of bacterial ATCases. Initial treatment of the cell free extract with streptomycin sulfate and ammonium sulfate gave significant increases in purity with practically no loss of activity. The enzyme preparation was subjected to a heat step in the purification. In this purification technique, the solution is heated to high, non-physiological temperatures (60°C). The proteins in solution all have differing thermal
stabilities. Some proteins are denatured by this process, unfold and precipitate out of solution as their hydrophobic interior is exposed to solvent. The precipitate in this step was very heavy and flocculant. That a protein survives such drastic treatment is testament to its thermal stability. Typically bacterial ATCases are very thermostable proteins, the subunit interactions in the oligomer providing significant and stable bonding during the heating. The enzyme's thermal stability has been attributed to the reinforcing interactions between the subunits of the oligomeric holoenzyme. Though simple proteins (e.g. RNase and any protein from a thermophile) can be heat stable, the stability of the *M. catarrhalis* enzyme through the heat treatment indicated that the enzyme might be a large molecular weight enzyme (Class B or Class C) with multiple subunits that interact to stabilize the tertiary and quaternary structure.

One step in the purification supported the case for a large molecular weight class ATCase for this species. After purification by ion exchange chromatography, the enzyme was further purified by size exclusion chromatography. The enzyme was purified over a column that had been earlier calibrated with molecular weight standards. The column was both a preparative and an analytical column. In addition to fractionating the enzyme, an estimate of the molecular weight was obtained (Fig. 23). The enzyme eluted at a volume (Ve) that corresponded to a molecular mass of 520 kDa. This value was obtained by extrapolation instead of interpolation, Thus the value is subject to argument since the value that was obtained lay outside the range of the standards. No molecular weight standards in this range were available. In addition, this molecular weight range (greater than 500,000) is on the outer limit of the column material's ability to effectively sieve the protein. Values obtained
for very large molecular weight proteins are subject to greater experimental error since a relative small difference in the elution volume, $V_e$, corresponds to very large differences in the molecular weight. Thus the value of 520 kDa must be considered an approximate molecular weight for the *M. catarrhalis* ATCase. Still, the enzyme must be larger than apoferritin (MW 443 kDa) since it was eluted from the Sephacryl S-300 HR column before the apoferritin.

When the pooled fractions from the ATCase activity peak were run on gradient polyacrylamide gels, a single band of ATCase activity was detected. The band was necessarily very broad since the activity stain stains for phosphate released during the reaction. The phosphate ion is a (relatively) small molecular and migrates rapidly through the gel. The temperature during the process is kept as low as possible (4°C) to prevent diffusion, but the temperature can not be so cold that enzyme activity decreases to undetectable levels. The protein bands themselves are immobilized in the gradient gel. The purified enzyme preparation showed two bands (Fig. 31), one corresponding to an apparent molecular mass of 470 kDa and one of 520 kDa. In a gradient polyacrylamide gel, native proteins move to a position in the gel where the pore size prevents further migration which is proportional to their apparent molecular weight. These gels are run to completion and thus information is obtained about both the purity (number of bands) and size (position of the bands) of the protein preparation. Bands with similar molecular weight are not unexpected since the preparation had just been fractionated by size exclusion chromatography. The bands could be two distinct proteins. Based on the results from the SDS denaturing gels, it is more likely that the two bands both represent *M. catarrhalis* ATCase.
When the fractions of purified *M. catarrhalis* ATCase were run on denaturing gels again two bands were visible (Fig. 32). When compared with standards (Fig. 33), the smaller band had an apparent molecular mass of approximately 35 kDa. The larger band had an apparent molecular mass of approximately 45 kDa. If six of each subunit are combined to form the *M. catarrhalis* ATCase, the holoenzyme would have a molecular weight of (6 X 35) plus (6 X 45) to give a total molecular weight of 480 kDa. This is approximately equal to the values obtained from the size exclusion chromatography and gradient gel electrophoresis, especially considering the experimental error inherent in measuring the large molecular weight proteins. The two bands could correspond to two forms of the enzyme, one that is deficient in a single subunit. The values obtained from the subunit molecular weights do not perfectly coincide with the values obtained for the native enzyme (480 kDa versus 470 - 520 kDa) since these two methods only measure an apparent molecular weight relative to the standards chosen and subject to the limitations characteristic of each procedure.

*M. catarrhalis* ATCase exhibits hyperbolic kinetics (Fig. 29). Class B ATCases, typified by the well characterized *E. coli* enzyme, show sigmoidal kinetics due to cooperative interactions between subunits in response to increasing amounts of substrate. Even though the *M. catarrhalis* ATCase is a multisubunit enzyme, it does not demonstrate cooperativity, just like the other class A bacterial ATCases that have similar subunit structures and subunit molecular weights. This property, along with its subunit structure and molecular weight place the *M. catarrhalis* ATCase with the class A bacterial ATCases, typified by the *Pseudomonas fluorescens* ATCase (Bergh & Evans, 1993). The
molecular weight, based on the primary sequence deduced from the DNA sequence, of the *P. aeruginosa* ATCase subunits are 36.4 and 44.2 kDa (Schurr, et al. 1995). The *M. catarrhalis* ATCase had a Km for aspartate of 2.0 mM.

If the *M. catarrhalis* ATCase is a class A ATCase, presumably the 35 kDa polypeptide corresponds to the catalytic subunit, while the 45 kDa subunit polypeptide is homologous with the protein DHOase. Though the large polypeptide in the *P. aeruginosa* ATCase is homologous to DHOase, it does not possess catalytic activity. Recently, two bacteria have been identified with class A ATCases that have active DHOase associated in the holoenzyme complex. *Deinococcus radiophilus* and *Thermus aquaticus* (Van de Casteele et al., 1997; Shepardson et al., 1997) both have ATCase activity and DHOase activity in the same protein complex. More recently it has been reported that ATCase in the streptomycetes is also a protein complex with both functional ATCase and DHOase together (Hughes, 1998). In order to determine if the *M. catarrhalis* ATCase possessed DHOase activity, DHOase activity was measured in enzyme preparations. The two activities were present in crude extracts, but did not co-purify. When the DHOase activity in crude extracts was fractionated by size exclusion chromatography, no overlap of ATCase and DHOase activity was observed in the fractions. The *M. catarrhalis* DHOase had an apparent molecular weight of 78 kDa., far smaller than the 480-520 kDa. *M. catarrhalis* ATCase. Most bacterial DHOase's are dimers with native molecular masses of between 78 and 90 kDa.

Given the properties of the *M. catarrhalis* ATCase, it is clearly distinct from ATCases found in the true Neisseriaceae family. These enzymes were all
class B enzymes, more typical of those found in the enterics. Regulatory and enzymatic properties could be disrupted by heat or mercurials, and the enzyme from *N. meningitidis* had a typical class B molecular mass of 295 kDa when determined by size exclusion chromatography over a calibrated column. Thus this work has shown that the *M. catarrhalis* ATCase is a class A bacterial ATCase and that the transfer of *M. catarrhalis* out of the genus *Neisseria* is further justified based on the molecular properties of the enzyme reported here. As in previous studies (Wild et al., 1980; Foltermann, et al., 1981), morphological and biochemical definition of bacterial classes is supported by molecular data.

**Experimental Accomplishments and Conclusions**

1. The enzyme ATCase from *M. catarrhalis* is expressed in rich medium at levels sufficient to support a relatively straightforward purification of the enzyme.

2. *M. catarrhalis* ATCase has a high pH optimum, the enzyme being active at pH 9.5 and higher.

3. Properties of the *M. catarrhalis* ATCase were defined that direct the design of experimental procedures for its purification. The enzyme is stable at 60°C for five minutes, soluble at 25 per cent saturation ammonium sulfate concentration, precipitated at 60 per cent saturation ammonium sulfate concentration, bound to DEAE cellulose at pH 8.5 in 50 mM salt and eluted completely from DEAE cellulose with a linear 50 to 500 mM salt gradient.
4. The enzyme exhibits hyperbolic saturation with the substrate aspartate and no cooperativity (no allosteric response to increasing concentration of the substrate) was detected.

5. The purified enzyme from *M. catarrhalis* has a Km of 2 mM aspartate.

6. The purified enzyme does not have associated with it an active DHOase. *M. catarrhalis* cells expressed a single active DHOase in these studies with an apparent molecular weight of 78 kDa, and that was not associated with the *M. catarrhalis* ATCase.

7. ATCase from *M. catarrhalis* has an approximate molecular mass of 480-520 kDa. The enzyme is a dodecamer composed of six 35 kDa subunits and six 45 kDa subunits.

8. The *M. catarrhalis* ATCase is a class A bacterial ATCase since this subunit molecular weight, oligomeric architecture, high pH optimum and Michaelis Menten kinetic response to aspartate is common to all bacterial class A ATCases.

9. Based on the properties of its ATCase, *M. catarrhalis* 'reclassification out of the *Neisseria* is clearly justified. *M. catarrhalis* is more closely aligned with the pseudomonads.
REFERENCES


Carrey, E. A. (1986). Nucleotide ligands protect the inter-domain regions of the multifunctional polypeptide CAD against limited proteolysis, and also stabilize the thermolabile part-reactions of the carbamoyl-phosphate synthase II domains within the CAD polypeptide. *Biochemical Journal* 236, 327-335.

Chu, C. P. & West, T. P. (1990). Pyrimidine biosynthetic pathway of 

*Pseudomonas fluorescens.* Journal of General Microbiology 136, 875-880.

and pyrimidine biosynthesis in *Pseudomonas putida.* Journal of General 
Microbiology 92, 375-383.

Phylogenetic relationships between some members of the genera *Neisseria,*  
*Acinetobacter,* *Moraxella,* and *Kingella* based on partial 16S ribosomal DNA 

Gerhart, J. C. & Pardee, A. B. (1962). The enzymology of control by feedback 

transcarbamylase of *Escherichia coli* and separation of its protein subunits. 
Journal of Biological Chemistry 242, 2886-2892.

*Salmonella typhimurium.* Journal of Biological Chemistry 249, 3406-3411.

Grayson D. R. & Evans, D. R. (1983). The isolation and characterization of the 
aspartate transcarbamylase domain of the multifunctional protein, CAD. 
Journal of Biological Chemistry 258, 4123-4129.

Honzatko, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Edwards, B. F. P.,  
Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C. & Lipscomb, W. N.


inactive dihydroorotase for assembly into the dodecameric holoenzyme.


MSB8: Modes of expression in *E. coli* and properties of their products.


