Aspartate transcarbamoylase from Pseudomonas aeruginosa is a class A enzyme consisting of six copies of a 36-kDa catalytic chain and six copies of a 45-kDa polypeptide of unknown function. The 45-kDa polypeptide is homologous to dihydroorotase but lacks catalytic activity. Pseudomonas aeruginosa aspartate transcarbamoylase was overexpressed in Escherichia coli. The homogeneous His-tagged protein isolated in high yield, 30 mg/liter of culture, by affinity chromatography and crystallized. Attempts to dissociate the catalytic and pseudo-dihydroorotase (pDHO) subunits or to express catalytic subunits only were unsuccessful suggesting that the pDHO subunits are required for the proper folding and assembly of the complex. As reported previously, the enzyme was inhibited by micromolar concentrations of all nucleotide triphosphates. In the absence of effectors, the aspartate saturation curves were hyperbolic but became strongly sigmoidal in the presence of low concentrations of nucleotide triphosphates. The inhibition was unusual in that only free ATP, not MgATP, inhibits the enzyme. Moreover, kinetic and binding studies with a fluorescent ATP analog suggested that ATP induces a conformational change that interferes with the binding of carbamoyl phosphate but has little effect once carbamoyl phosphate is bound. The peculiar allosteric properties suggest that the enzyme may be a potential target for novel chemotherapeutic agents designed to combat Pseudomonas infection.

Pseudomonadaceae is a family of eu-bacteria with a wide ecological distribution that is pathogenic in animals (1, 2). Pseudomonas aeruginosa, the principle cause of morbidity and mortality in immunocompromised patients and burn victims (3–5), is an obligate pathogen. In cystic fibrosis, P. aeruginosa infection is the usual cause of death. In the design of therapeutic strategies, comparatively little attention has focused on the physiology of the organism itself, despite the unusual metabolism exhibited by Pseudomonadaceae such as uracil catabolism (6), the lack of gene repression (7, 8), and the ability to use arginine as the sole source of carbon, nitrogen, and energy (9, 10).

In other organisms, studies of de novo pyrimidine biosynthesis and salvage are actively pursued because of the relationship of these pathways to growth, development, and chemotherapy. Aspartate transcarbamoylase (ATCase,1 EC 2.1.3.2) catalyzes the formation of carbamoyl aspartate from carbamoyl phosphate and aspartate (11) in the de novo biosynthetic pathway. The structure and function of the enzyme from Escherichia coli has been extensively studied and has become the prototype of allosteric enzymes. However, ATCases are highly polymorphic differing both in structure and mode of regulation. Jones and co-workers (12) identified three distinct classes of bacterial aspartate transcarbamoylase.

E. coli ATCase, designated a class B enzyme, consists of six copies of two types of polypeptide chains, catalytic and regulatory. The 34-kDa catalytic chains associate to form catalytically active but unregulated trimers. The 17-kDa regulatory chains form dimeric subunits that bind the allosteric effectors CTP, ATP, and UTP but have no activity. Crystallographic studies (13–16) have shown that the holoenzyme is a 34 dodecamer consisting of two catalytic subunits and three regulatory subunits. The allosteric effectors bind to a common site on each regulatory chain and transmit allosteric signals to the active site located 60 Å away on the catalytic subunit. In addition to E. coli, class B ATCases are found in other members of the family Enterobacteriaceae such as Salmonella thyphimurium, Erwinia herbicola, Serratia marcescens (17), and in some hyperthermophilic archaea such as Pyrococcus abyssi (18). The class B ATCases differ in sensitivity to allosteric effectors but have the same structural organization.

The class C enzymes are much smaller, 100 kDa, and lack separate regulatory subunits. Barbson and Switzer (19) characterized the first class C ATCase from Bacillus subtilis. The enzyme was shown to be an unregulated trimer consisting of three 34-kDa catalytic chains. When the enzyme was cloned and sequenced, it was found (20) to be homologous to the E. coli ATCase catalytic chain, and subsequent x-ray studies (21) showed that it has a very similar tertiary structure.

The class A ATCases are the largest and least well understood. Early studies of Pseudomonas fluorescens ATCase (22) suggested that the molecule was dimeric; however, it was subsequently shown (23, 24) to be a dodecamer consisting of six copies of a 36-kDa catalytic chain and 45-kDa polypeptide of

1 The abbreviations used are: ATCase, aspartate transcarbamoylase; eATP, 1,N6-ethenoadenosine 5'-triphosphate; pDHO, pseudo-dihydroorotase, an inactive homolog of dihydroorotase; AMP-PNP, 5'-adenylylimidodiphosphate; MES, 4-morpholineethanesulfonic acid; PALA, N-phosphonoacetyl-l-aspartate; CPSase, carbamoyl phosphate synthetase; FSBA, 5'-fluorosulfonylebenzoyladenosine.
unknown function. When O'Donovan and associates (25, 45) cloned and sequenced the genes encoding the enzyme from *Pseudomonas putida* and *P. aeruginosa*, the surprising discovery was that, although the 36-kDa chains were clearly homologous to the catalytic chains of other well characterized ATCases, the sequence of the 45-kDa polypeptide closely resembled dihydroorotase, the enzyme that catalyzes the subsequent step in the de novo pyrimidine biosynthetic pathway. However, the enzyme complex lacks dihydroorotase activity, so the 45-kDa polypeptide has been designated a pseudo-DHOase, analogous to the homologous inactive domain found (26) in the yeast multifunctional protein encoded by the *ura2* locus.

The regulation of the *Pseudomonas* enzyme is also unusual. In *E. coli* the flux of metabolites through the de novo pyrimidine biosynthesis is controlled by the allosteric regulation of ATCase. ATP activates the enzyme whereas CTP and UTP function as feedback inhibitors. The reciprocal regulation of the enzyme by these allosteric effectors is thought to prevent the accumulation of pyrimidines and to maintain a balance in the intracellular pools of purines and pyrimidines. In contrast, *P. aeruginosa* ATCase (22, 23) is inhibited by low concentrations of all of the nucleotide triphosphates. Although there is precedent in that the enzymes from several other bacterial species are inhibited by a broad spectrum of pyrimidines and purines (27), the rationale for this type of allosteric control of de novo pyrimidine biosynthesis has been elusive.

Mechanistic and structural studies of Pseudomonadaceae aspartate transcarbamoylase have been hampered by the difficulty in isolating sufficient quantities of the protein and by its intrinsic instability. This report describes the overexpression, purification, and characterization of *P. aeruginosa* ATCase. The extreme sensitivity of the purified recombinant protein to nucleotide triphosphates was confirmed, but the regulation was found to be unusual in several respects.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—The plasmid, pA10, that encodes both the enzyme encoding the enzyme from *P. aeruginosa* ATCase was obtained (45; GenBank™ accession number AY649949) by inserting a 3.5-kb XbaI fragment of cosmid 011125 that complemented ATCase-deficient *E. coli* strains into the *SalI* site of pUC18. The expression vector pRSETC was purchased from Invitrogen. Host *E. coli* cells were either EK1104 (F', ara, thi, *pro-lac, ΔgpyB, pyrF', rpsL*) (28), BL21 (DE3) (F' *c-MCS ompT hsdS22 F' lacZAM15 d(lacZYA-argF'80 lac-pro)*) or DH5α (F' *Δ6lacU169 endA1 recA1 hisD30 glnV50 metB1 thrA1 thi-1 supE44 gyrA96 relA1*). Routine genetic manipulations were carried out using the strain DH5α.

**Protein and DNA Methods**—Restriction digests, ligations, and other DNA methods were carried out using standard protocols (29). Protein concentration was determined by the Lowry method (30) using bovine serum albumin as the standard. SDS-gel electrophoresis was carried out on 12% polyacrylamide gels (31). In some instances, 6% non-denaturing polyacrylamide gels were run, and the ATCase activity of the bands in the gel was determined as described previously (18).

**Enzyme Assays**—Aspartate transcarbamoylase assay was assayed by the method of Prescott and Jones (12) as modified by Pastra-Landis et al. (32). Typically 50 μg of the purified protein was assayed at 37 °C for 20 min in a 1.0-mL assay mixture consisting of 5 μM carbamyl phosphate and 20 μM aspartate in 50 mM Tris-HCl, pH 8.5. Alternatively, a radioactive assay described previously (33) that uses [14C]aspartate as a substrate was used to measure aspartate transcarbamoylase activity. Unless specifically noted, the reaction was initiated by the addition of carbamoyl phosphate to the otherwise complete assay mixture. A unit of enzyme activity is defined as μmol/h/mg.

To determine whether inhibition by two nucleotides is additive, antagonistic, or synergistic, the extent of inhibition was determined in the presence of one and both effectors following the approach described by Webb (34). The activity in the presence of an inhibitor 1 is given by the expression $a = (1 - i_1)$, where $i_1$ is the fractional inhibition. In the presence of two inhibitors that function independently, inhibition should be additive, and the activity ($a_{12}$) would be the product of the activities of $a_{12} = (1 - i_1)(1 - i_2) = i_1 + i_2 - i_1i_2$. Smaller and larger values of $a_{12}$ would be indicative of antagonist or synergistic inhibition, respectively. Additive or synergistic inhibition is an indication that the inhibitors bind to different sites on the enzyme. For these experiments, one inhibitor was held constant at a concentration giving ~50% inhibition, whereas the second was varied over its effective range.

The pH activity profile was determined using a three-point buffer (35) consisting of 0.05 M MES, 0.5 μg/ml FeSO₄·7H₂O, 1.0 mM MgSO₄·7H₂O, 5 μg/ml thiamin, 0.001% tryptophan. Alternatively, the recombinant protein was obtained from BL21(DE3) *E. coli* cells transformed with pJV34, a plasmid constructed as described under “Results.” The competent host cells were prepared using the method of Huff et al. (37). The expression of the enzyme in the pJV34 transformants was under control of the T7 promoter. A 1-mL culture of exponentially growing transformed cells obtained from a single colony was used to inoculate 100 ml of YT media supplemented with 100 μg/ml ampicillin. The cells were grown at 37 °C in a rotary shaker (225 rpm) at 37 °C to an absorbance of 0.5. The expression of the protein was then induced by the addition of IPTG to a concentration of 400 μM. The cells were then grown for an additional 2 h prior to harvesting.

**Purification of the Recombinant Proteins**—For purification of the recombinant protein from EK1104 cells transformed with pA10, 85 g of packed cells were suspended in 100 ml of sonication buffer, 50 mM Tris, pH 8.5, 2 mM 2-mercaptoethanol, and 0.02 mM ZnCl₂ at 4 °C. The suspension was sonicated on ice and then centrifuged for 15 min at 31,000 × g to remove the cellular debris. After clarifying the extract by centrifugation at 48,000 × g for 90 min, the supernatant (128 ml) was fractionated with ammonium sulfate. Protein precipitating between 30 and 45% ammonium sulfate saturation was resuspended in 6 ml of the sonication buffer and dialyzed exhaustively against the same buffer.

The dialyzed protein was chromatographed on a 26 × 1.6-cm Sepharose Q-fast-flow column equilibrated in 50 mM Tris-HCl, pH 8.5, at a flow rate 1.0 ml/min. After washing the column overnight with 50 ml of the 0.25 M NaCl in 50 mM Tris-HCl, pH 8.5, the protein was eluted with a linear gradient of 0.25–0.6 M NaCl in the same buffer. Fractions containing ATCase activity were pooled, and glycerol was added to a final concentration of 10%. The preparation was concentrated using an Amicon Protein Concentrator under nitrogen to a final volume of 20 ml. The concentrated sample was chromatographed on a 1.9 × 30-cm Sephacryl 300HR column equilibrated and eluted with 50 mM Tris-HCl, pH 8.5, 10% glycerol at a flow rate of 12 ml/h. The active fractions were then applied to a 6 × 1.2-cm hydroxyapatite column and eluted with a 0–0.2 M gradient of potassium phosphate in the same buffer.

For purification of recombinant ATCase from pJV34 BL21(DE3) transformants, 500 mg of packed cells obtained from a 100-mL culture were washed once with 2 ml of 50 mM Tris-HCl, pH 8.5, at a flow rate 1.0 ml/min. The cells were disrupted using a Branson sonifier. The sonication protocol consisted of four 20-s bursts with a microtip set at 50% duty cycle, output 5.5, with a 1-min cooling period on ice between each burst. The cellular debris was removed by centrifugation 12,000 × g for 4 min. The resultant supernatant was immediately applied to 1 ml of ProBond™ nickel matrix (Invitrogen) that had been equilibrated with 50 mM Tris-HCl, pH 8.5, 10% glycerol, 200 mM NaCl. The column was successively washed with 2 ml of 25 mM imidazole, 2 ml of 50 mM imidazole, and 2 ml of 200 mM imidazole in the same buffer. The fractions containing ATCase were pooled and dialyzed against 50 mM Tris-HCl, pH 8.5, 10% glycerol at 4 °C and then stored at –80 °C.

**RESULTS**

**Expression and Purification of *P. aeruginosa* ATCase**—Initially, recombinant *P. aeruginosa* ATCase was isolated from *E. coli* transformed with the plasmid pA10 following the five-step protocol described under “Experimental Procedures.” The yield (Table 1) starting with 85 g of packed cells was 400 μg. The specific activity of 525 μmol/h/mg of the homogeneous protein was appreciably lower than the values observed for ATCase isolated from other bacterial sources.

Because of the low specific enzymatic activity, the low yield
of protein, and the laborious isolation procedure, the protein was recloned into a high expression system that appends a His tag affinity label to the amino end of the catalytic subunit to facilitate purification. The 2.4-kb PsI fragment of pA10 containing the entire P. aeruginosa ATCase coding sequence was inserted (Fig. 1) into the expression vector pRSETC. The resulting plasmid, pJV34, encodes the 45-kDa PDHO subunit and the 36-kDa catalytic subunit with a 3-kDa chain segment containing six histidines fused to the amino end. Following transformation into E. coli strain BL21(DE3), expression of the recombinant protein was induced with IPTG. P. aeruginosa ATCase was produced at very high levels (Fig. 2, lane Ex), but approximately two-thirds of the protein was recovered in the lane Su (Fig. 2), approximately two-thirds of the protein was recovered in the lane Ex with the precipitin, 100 mM citrate, pH 5, and 10% 6K polyethylene glycol. The protein remained in solution after removal of the denaturant, suggesting that it had refolded into a stable, soluble species, but the enzymatic activity was not recovered.

In another series of experiments, the catalytic chain coding sequence in pJV34 was deleted by digestion with XhoI and HindIII. High levels of the isolated PDHO subunit were expressed when the truncated plasmid was transformed into BL21(DE3) cells. This species was completely soluble but, as expected, had no catalytic activity.

In an attempt to reconstitute the native complex, 70 ng of the refolded catalytic subunit was titrated with increasing amounts (8–160 ng) of the soluble PDHO subunit (data not shown). Because only the catalytic chain carried the His tag affinity label, coelution of the 36- and 45-kDa subunits from the Ni²⁺ affinity column indicated that a stable complex was formed. However, the reconstituted complex also lacked catalytic activity.

The holoenzyme was incubated in various concentrations of urea (0.2–2 M), and the chromatographed on the Ni²⁺ column equilibrated with the same concentration of urea. The enzyme dissociated by 2 M urea, as indicated by the elution of the 45-kDa PDHO subunits and retention of the 36-kDa catalytic chains on the column, but the catalytic activity was lost. Collectively, these results suggest that the isolated catalytic subunit must be associated with PDHO subunits to be catalytically active.

When ATCase was completely denatured in 8 M urea and then refolded by dialysis against a buffer lacking the denaturant, a complex consisting of stoichiometric amounts of the two ATCase subunits was recovered from the affinity column (Fig. 3A), but it was also inactive. Gel filtration chromatography (Fig. 3B) showed that the molecular mass of the reconstituted complex was similar to that of the native dodecamer (480 kDa), although the peak was broader, possibly an indication of the presence of higher and lower oligomers. Thus, although dissociated and denatured ATCase can be reconstituted into a complex resembling the native enzyme in size and subunit composition, it has not been possible to restore catalytic activity.

**TABLE I**

| Purification step | Protein | Activity | Specific activity | -Fold purification |
|-------------------|---------|----------|------------------|--------------------|
| pA10 clone        |         |          |                  |                    |
| 1. Cell extract   | 3374    | 11,923   | 3.53             | 1                  |
| 2. NH₄SO₄ (30–45%)| 340     | 291      | 8.6              | 2.4                |
| 3. Sepharose Q 7.5| 139     | 688      | 9.5              | 2                  |
| 4. Sephacryl 300 HR | 0.42  | 194      | 462              | 131                |
| 5. Hydroxyapatite | 0.40    | 210      | 525              | 150                |
| pJV34 clone       |         |          |                  |                    |
| 1. Cell extract   | 50      | 81,000   | 1,620            | 1                  |
| 2. Ni²⁺ affinity column | 3   | 81,000   | 27,000           | 17                 |

* The experiments were prepared from 10 liters and 100 ml of culture for pA10 and pJV34 clones, respectively.
**Steady State Kinetics**—The aspartate saturation curve of *P. aeruginosa* ATCase (Fig. 4A) was hyperbolic. As observed for the isolated *E. coli* ATCase catalytic subunit, there was no indication of cooperative substrate binding, but high concentrations of aspartate partially inhibited the enzyme (not shown). A least squares fit of the data gave a $K_m$ for aspartate of 2.6 mM and a $V_{max}$ of 12,500 μmol/h/mg (Table II). The carbamoyl phosphate saturation curve (Fig. 4B) was hyperbolic, but in this case there was no apparent substrate inhibition. The $K_m$ for carbamoyl phosphate was 0.49 mM. The pH optimum for catalysis was measured using saturating concentrations of aspartate and carbamoyl phosphate. Catalytic activity increased steeply as the pH increased from pH 7 to 8.5 and then began to decrease. A fit of the plot of $\ln(V_{max})$ against pH (not shown) to the Dixon equation gave a $pK_a$ of 8.1 for the acidic limb of the curve. The pH-rate profile of the *P. aeruginosa* enzyme closely resembles that of the *E. coli* ATCase catalytic subunit but is very different from bimodal pH dependence of the allosteric *E. coli* holoenzyme (39).

**Nucleotide Inhibition**—As reported previously (22, 23), *P. aeruginosa* ATCase was strongly inhibited by CTP, UTP, ATP, and GTP (Fig. 5A) but not the corresponding nucleotide monophosphates or nucleotide diphosphates (not shown). Measurement of the residual ATCase activity as the concentration of the nucleotide triphosphates was varied (Fig. 5B) showed the enzyme was inhibited 50% at nucleotide concentrations of about 2 μM and that all of the nucleotide triphosphates bound to the enzyme with approximately equal affinity. The apparent dissociation constant was 4 μM, a value far below the reported concentration of all of these nucleotides (40) in the *P. aeruginosa* cells.

The approach devised by Webb (34) was adopted to determine whether the ATCase inhibition produced by combinations of two nucleotides is additive, antagonistic, or synergistic. In this method, one inhibitor is held constant, whereas the other is varied over its effective range. In one series of experiments, the ATP concentration was held at 1 μM, whereas the UTP concentration ranged from 1 to 10 μM. Alternatively, the UTP concentration was held constant at 1 μM, whereas the ATP was varied from 1 to 10 μM. In both experiments at all concentrations, the observed fractional inhibition in the presence of both inhibitors ($i_{12}$) was always less than that predicted from the fractional inhibition $i_1$ and $i_2$ of the individual inhibitors acting independently (i.e. $i_{12} = i_1 + i_2 - i_1i_2$). This result, indicative of antagonistic inhibition (34), would be obtained if both nucleotides bind to the same site on the enzyme.
Effect of Nucleotide on the Steady State Kinetics—Nucleotide triphosphates dramatically altered the steady state kinetics. In the presence of 2 mM ATP, the aspartate saturation curve (Fig. 4A) became strongly sigmoidal. The concentration of aspartate required for half-saturation, [S]_{0.5}, increased from 2.6 to 14.0 mM, and the effects were even more pronounced at 4 and 6 μM ATP. ATP had little effect on V_max. These curves were fit to the Hill equation and gave Hill coefficients (Table II) that ranged from 7.5 to 10.

Titration with the bisubstrate analog, N-phosphonacetyl-L-aspartate (PALA), was used to determine whether the enzyme exhibits true cooperative substrate binding. When the E. coli holoenzyme is assayed using subsaturating concentrations of aspartate and carbamoyl phosphate, PALA appreciably activates (10–20-fold) at low concentrations and then inhibits the enzyme as the concentration of PALA approaches saturation. This unusual response has been interpreted (41) to be a consequence of the T to R transition that the enzyme undergoes upon substrate binding. The binding of PALA to one site on the enzyme is sufficient to shift the equilibrium to the high affinity, active R state, whereas the activity is lost as PALA binds to the remaining active sites on the enzyme. Initial activation followed by inhibition by carbamoyl phosphate analog, phosphonoacetate, was also observed for the E. coli enzyme. However, in assays (not shown) conducted using 5 mM carbamoyl phosphate and 1 mM aspartate, the P. aeruginosa enzyme was inhibited at all concentrations of PALA. Similarly, phosphonoacetate inhibited at all concentrations tested when the assay contained 20 mM aspartate and 40 μM, carbamoyl phosphate. Neither compound activated the P. aeruginosa ATCase, a result consistent with the hyperbolic saturation curves observed in the absence of nucleotides.

In contrast, when 2 mM ATP was present, the response to PALA (Fig. 6) was found to be qualitatively similar to that exhibited by the native E. coli enzyme. The activity increased with increasing inhibitor concentrations up to a peak corresponding to a 2.4-fold activation at 1 nM PALA. The activity then began to decrease with increasing PALA concentration. These results indicated that the sigmoidal saturation kinetics observed in the presence of ATP can be attributed in part to cooperative interactions between the catalytic sites. However, the PALA activation was much less than that observed for...
E. coli ATCase despite the very high Hill coefficients (Table II), suggesting that other factors are involved. As discussed below, conformational changes induced by nucleotide binding are only slowly reversed. This hysteretic phenomenon is likely to be primarily responsible for the sigmoidal aspartate saturation curves.

Order of Addition of Substrates and Effectors—Unexpectedly, ATP had no effect on the carbamoyl phosphate saturation curves (Fig. 4B). The carbamoyl phosphate concentration in the assays for the aspartate saturation curve was 5 mM, whereas the aspartate concentrations used for the carbamoyl phosphate saturation curve was 20 mM. At the common point in these two plots (Fig. 4, A and B), 20 mM aspartate and 5 mM carbamoyl phosphate, the velocity was very different although the composition of the assay mixture was identical. The only difference between the assays for these two experiments was the order of addition of the substrate. The aspartate and carbamoyl phosphate saturation curves were initiated by the addition of carbamoyl phosphate and aspartate, respectively, to the complete assay mixture. ATP did not inhibit the enzyme when the assays for the carbamoyl phosphate saturation curve (Fig. 4B) were initiated by the addition of aspartate. If, instead, the carbamoyl phosphate assays were initiated with carbamoyl phosphate and aspartate, respectively, to the complete assay mixture. ATP did not inhibit the enzyme when the assays were initiated by the additional of carbamoyl phosphate. The addition of MgCl₂ to the enzyme and exhibited normal saturation kinetics (not shown). The dissociation constant for the eATP-ATCase complex was 2.5 μM, close to the value obtained from the ATP inhibition kinetics indicating that ATP and eATP bind to the enzyme with comparable affinity. In the absence of Mg²⁺, eATP inhibited the enzyme activity to the same extent as that observed in the presence of MgCl₂. A plot of the residual enzyme activity (Fig. 5) showed that inhibition closely paralleled the concentration of free ATP calculated from the concentration of MgCl₂ and Mg²⁺ in the assay mixture.

Binding of an ATP Analog—The binding of ATP fluorescent analog, 1, N⁶-ethenoadenosine 5'-triphosphate (eATP), to P. aeruginosa ATCase was measured by fluorescence spectrophotometry. The fluorescence intensity increased with increasing concentrations of eATP added to the enzyme and exhibited normal saturation kinetics (not shown). The dissociation constant for the eATP-ATCase complex was 4 μM, close to the value obtained from the ATP inhibition kinetics indicating that ATP and eATP bind to the enzyme with comparable affinity. In the absence of Mg²⁺, eATP inhibited the enzyme activity to the same extent as that observed in the presence of MgCl₂. A plot of the residual enzyme activity (Fig. 5) showed that inhibition closely paralleled the concentration of free ATP calculated from the concentration of MgCl₂ and Mg²⁺ in the assay mixture.

**DISCUSSION**

P. aeruginosa ATCase is a representative of the class A aspartate transcarbamoylase first identified by Jones and colleagues (22). The class B and C ATCases have been well characterized, but progress on the class enzyme has been hampered by the lack of adequate amounts of the purified protein. The level of expression of the protein in P. aeruginosa cells is low, and the purification procedure involves many steps, and the protein is relatively unstable so that the purified enzyme has low specific enzyme activity. We report here the cloning and overexpression of P. aeruginosa enzyme. The enzyme carries a His tag appended to the amino end of the catalytic subunit that allows the homogeneous protein to be recovered in a single step on a Ni²⁺ affinity column. The observation that the purified enzyme had a specific activity that was 51-fold higher than the protein isolated from PA10 transformants and 11-fold higher that the protein directly purified (23) from P. aeruginosa cells underscores the efficacy of a rapid isolation procedure.

The complete conservation in P. aeruginosa ATCase of all of the residues implicated in catalysis in the E. coli ATCase catalytic subunit, as well as an identical pH dependence, suggests that the two enzymes share a common catalytic mechanism. E. coli ATCase, a class B enzyme, can be dissociated by
The components of the assay mixture were added to a final concentration of 0.05 μg/ml P. aeruginosa ATCase, 20 mM aspartate, 5 mM carbamoyl phosphate, and 6 μATP.

| Order of addition | Activity (µmol/h/mg) |
|-------------------|----------------------|
| 1                  | Enzyme Aspartate Carbamoyl phosphate | 14,470 |
| 2                  | Enzyme Carbamoyl phosphate Aspartate | 15,200 |
| 3                  | Enzyme Aspartate ATP | 814 |
| 4                  | Enzyme Carbamoyl phosphate ATP | 13,900 |
| 5                  | Enzyme Aspartate Carbamoyl phosphate ATP | 14,800 |
| 6                  | Enzyme ATP Aspartate Carbamoyl phosphate | 740 |
| 7                  | Enzyme Aspartate ATP Carbamoyl phosphate | 14,200 |

*a In this experiment, the enzyme was incubated with ATP for 5 min prior to the addition of carbamoyl phosphate.

catalytic subunit, once folded, required the pDHO subunit for activity. The insoluble protein was solubilized in 8 M urea and refolded into soluble trimers but did not recover catalytic activity. The addition of the pDHO polypeptide to the refolded catalytic trimer resulted in the formation of a stable complex but did not restore catalytic activity. These results suggest that in the absence the catalytic chains are unable to assume the tertiary structure required for activity. This conclusion remains tentative as it is possible that conditions could be found that result in proper folding of the isolated catalytic subunit in catalytically active form, but nevertheless it is true that coexpression of the two subunits facilitates the formation of native complex.

P. aeruginosa ATCase is exquisitely sensitive to micromolar concentration of all the nucleotide triphosphates. The intracellular concentrations of ATP, CTP, UTP, and GTP have been found (40) to be 4.4, 0.8, 2.1, and 3.0 mM respectively, in P. putida cells, so it is difficult to imagine circumstances under which ATCase can be catalytically active. The regulatory logic of the system was also puzzling. Inhibition by purine nucleotides, as well as pyrimidine nucleotides, while not unprecedented, is difficult to rationalize. In E. coli ATCase, for example, ATP is an allosteric activator that signals the availability of purine nucleotides and coordinates the flux through the pyrimidine and purine pathways. Also the Hill coefficients of the P. aeruginosa enzyme measured in the presence of ATP are
much higher than those of *E. coli* ATCase. The observation that PALA activates the enzyme at low concentration confirms that weak cooperative transitions between active sites do occur, but these alone cannot account for the strongly sigmoidal saturation curves.

The unusual kinetics can be explained by two additional observations. First, the inhibition is abolished by Mg$^{2+}$ and the extent of inhibition exhibits a convincing inverse correlation with the concentration of the MgATP complex. Thus, it is likely that only free ATP can bind to the enzyme. Neither the total magnesium ion concentration nor the concentration of free nucleotide triphosphates in the cell are known. However, it is possible that these nucleotides exist primarily as the Mg$^{2+}$ complex, in which case inhibition of ATCase inhibition would be attenuated. Thus, the Mg$^{2+}$ effectively buffers the nucleotides and the activity of the enzyme *in vivo* probably depends on the precise balance of free nucleotides and their metal ion complexes.

Second, the inhibition is observed only when the reaction is initiated with carbamoyl phosphate. No inhibition is observed if the enzyme is first preincubated with this substrate, a result that suggests that the nucleotides may bind near the active site. In support of this interpretation, chemical modification with the ATP analog, FSBA, showed (23) that the nucleotides bind to the catalytic subunit. Moreover, deletion mutants lacking the first 34 residues at the amino end of the *P. putida* catalytic chain have been found (25) to be insensitive to nucleotides, indicating that this region of the molecule participates in feedback inhibition. Much of this chain segment may well be important, although 8 of these residues have been substituted in the construct described here, with the complete retention of nucleotide sensitivity. The lack of discrimination between nucleotide bases and the failure of mono- and diphosphate nucleotides to inhibit the enzyme might suggest that the γ-phosphate moiety of the triphosphates may compete with carbamoyl phosphate in binding at the active site. Nucleotide triphosphates, pyrophosphate, and other phosphate compounds have been shown to be competitive inhibitors of carbamoyl phosphate binding to the *E. coli* enzyme. However, the observation that the inhibition constants are in the micromolar range, not in the millimolar range as observed for the *E. coli* enzyme (44), and that ATP inhibition is non-competitive, not competitive, with respect to carbamoyl phosphate argues against this interpretation. Thus, it is likely that there is a high affinity nucleotide-binding site on the catalytic chain distinct from the active site.

The interaction of ATP with the enzyme was investigated in steady state kinetic studies and binding studies using εATP, a fluorescent analog that binds with comparable affinity and elicits the same inactivation as ATP. Once bound the nucleotide triphosphate is tightly associated with the enzyme and is not in rapid equilibrium with carbamoyl phosphate. As the nucleotide is slowly displaced from the enzyme by high concentrations of carbamoyl phosphate, the activity is gradually restored. This explanation accounts for the extreme sigmoidicity of the aspartate saturation curves. Conversely, when carbamoyl phosphate is bound to the enzyme, the nucleotide binds weakly and cannot inhibit the enzyme. The on constant for the association of nucleotides with the enzyme, as well as the off constant, must be slow, because when both the nucleotide and carbamoyl phosphate are simultaneously present, the nucleotide does not inhibit the enzyme.

Many eubacteria, including *P. aeruginosa*, have a single CPSase (46) that provides carbamoyl phosphate for both pyrimidine and arginine biosynthesis. In addition (47), *P. aeruginosa* has an arginine deiminase pathway (Fig. 9) that allows the organism to use arginine as an energy source. The latter pathway is considered to be particularly important during anaerobic growth (48). Carbamoyl phosphate, produced by the concerted action of arginine deiminase and catabolic ornithine decarboxylase, is converted to ATP, CO$_2$, and NH$_3$ by carbamate kinase. Thus, there are two routes to carbamoyl phosphate synthesis and three fates for the intermediate once formed. Consequently, the regulation of carbamoyl phosphate is complex and not well understood. When the cells grow in a medium rich in nucleotides and a plentiful source of energy-producing metabolites, the intracellular concentration of NTPs is high. It may be that inhibition of ATCase occurs if the concentration of free NTPs exceeds that of available Mg$^{2+}$, thus blocking further *de novo* synthesis of pyrimidines. The elevated ATP levels also inhibit two enzymes of the arginine deiminase pathway (46, 47). It is interesting that Mg$^{2+}$ ion activates arginine deaminase by decreasing the $K_v$ value for arginine 12-fold (49); thus limiting Mg$^{2+}$ when NTPs are abundant would down-regulate the arginine deiminase pathway as well as the pyrimidine biosynthesis. Arginine biosynthesis can proceed unless the level of citrulline is too low to sustain further arginine synthesis and activate CPSase. When the energy charge is low, inhibition of the arginine deiminase pathway is relieved allowing the organism to extract energy from arginine catabolism. Partial UMP inhibition of CPSase and limiting ATP would be expected to decrease the synthesis of carbamoyl phosphate for pyrimidine biosynthesis. However, carbamoyl phosphate is also an intermediate in the arginine deiminase pathway and in principle might be expected to enter the pyrimidine pathway especially because the $K_m$ value for carbamoyl phosphate of ATCase (0.4 mM) reported here is 6-fold lower than that of carbamate kinase (5 mM at 2 mM ADP) (9). This crossover apparently does not occur in the cell because carB mutants, deficient in CPSase, require uracil for growth (50). This observation suggests that the carbamoyl phosphate produced by arginine catabolism is unavailable for pyrimidine biosynthesis. Thus, there must be other as yet undiscovered mechanisms that control carbamoyl phosphate entry into the pyrimidine and arginine biosynthetic pathways or sequester the intermediate formed by the arginine deiminase pathway.

In summary, using the construct described here, *P. aeruginosa* ATCase can be rapidly purified to homogeneity in quantities that make possible, for the first time, structure-function
studies of any class A ATCase. The pPhO subunit is needed for optimal folding of the ATCase and perhaps to stabilize the optimal conformation once the enzyme has folded. Kinetic studies demonstrated that free nucleotides, but not the Mg\(^{2+}\) complex, bind to the enzyme and that carbamoyl phosphate antagonizes nucleotide binding. These observations provide an explanation for the unusual inhibition by a low concentrations of a broad range of nucleotide triphosphates, although the physiological role remains to be established. However, the effect of metabolites on the catalytic activity of Pseudomonas ATCase is distinctly different from that reported for ATCase from other prokaryotic and all eukaryotic organisms, and thus this enzyme may represent a potential target for drug design. For example, a cell-permeable nucleotide analog that inhibits P. aeruginosa ATCase in the presence of Mg\(^{2+}\) would be expected to abolish selectively pyrimidine biosynthesis in the microorganism.

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REFERENCES

1. Palmer, D., and Bender, C. (1993) Appl. Environ. Microbiol. 59, 1619–1626
2. Zhang, Y., Rowley, K., and Patil, S. (1993) J. Bacteriol. 175, 6451–6458
3. Preston, M., Kernack, K., and Berk, R. (1993) Infect. Immun. 61, 2713–2716
4. Hobden, J., Hill, J., Engel, L., and Ocallaghan, R. (1993) Antimicrob. Agents Chemother. 37, 1856–1859
5. Tredget, E., Shankowsky, H., Joffe, A., Inkson, T., Volpel, K., Paramchyn, W., Kibsey, P., Alton, J., and Burke, J. (1992) Clin. Infect. Dis. 15, 941–949
6. Xu, G., and West, T. (1992) J. Gen. Microbiol. 138, 2459–2463
7. Condon, S., Collins, J., and O’Donovan, G. (1976) J. Gen. Microbiol. 92, 375–383
8. Isaac, J., and Holloway, B. (1968) J. Bacteriol. 96, 1732–1741
9. Abdelal, A. T., Bibb, W. F., and Nainan, O. (1982) J. Bacteriol. 151, 1411–1419
10. Abdelal, A., Bussey, L., and Vickers, L. (1983) Eur. J. Biochem. 129, 697–702
11. Jones, M., Spector, L., and Lipmann, F. (1955) J. Am. Chem. Soc. 77, 819–820
12. Prescott, L. M., and Jones, M. E. (1969) Anal. Biochem. 32, 408–419
13. Hozzakio, R. B., Crawford, J. L., Monaco, H. L., Ladner, R. C., Eward, B. F., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C., and Lipscomb, W. N. (1982) J. Mol. Biol. 160, 219–263
14. Ke, H. M., Hozzakio, R. B., and Lipscomb, W. N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4037–4040
15. Krause, K. L., Volz, K. W., and Lipscomb, W. N. (1987) J. Mol. Biol. 193, 527–533
16. Lipscomb, W. N. (1994) Adv. Enzymol. Relat. Areas Mol. Biol. 68, 67–151
17. Wild, J. R., and Wales, M. E. (1990) Annu. Rev. Microbiol. 44, 193–218
18. Purcaro, C., Herve, G., Ladjimi, M. M., and Cinin, R. (1997) J. Bacteriol. 179, 4143–4157
19. Barson, J. S., and Switzer, R. L. (1975) J. Biol. Chem. 250, 8664–8669
20. Lerner, C. G., and Switzer, R. L. (1986) J. Biol. Chem. 261, 11156–11165
21. Stevens, R. C., Reinisch, K. M., and Lipscomb, W. N. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6087–6091
22. Adair, L. B., and Jones, M. E. (1972) J. Biol. Chem. 247, 2308–2315
23. Bergh, S. T., and Evans, D. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9818–9822
24. Shepherdson, M., and McPhail, D. (1993) FEMS Microbiol. Lett. 114, 201–206
25. Schurr, M. J., Vickery, J. F., Kamar, A. P., Campbell, A. L., Cumin, R., Benjamin, R. C., Shanley, M. S., and O'Donovan, G. A. (1995) J. Bacteriol. 177, 1751–1759
26. Souciet, J. L., Nayg, M., Legouar, M., Lacroute, F., and Potier, S. (1989) Gene (Amst.) 79, 59–70
27. Wales, M. E., Madison, L. L., Glaser, S. S., and Wild, J. R. (1999) J. Mol. Biol. 294, 1387–1400
28. Nowlan, S. F., and Kantrowitz, E. R. (1985) J. Biol. Chem. 260, 14712–14716
29. Maniatiat, T., Fritsch, E. R., and Sambrook, I. (1992) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Pastra-Landis, S. C., Foote, J., and Kantrowitz, E. R. (1981) Anal. Biochem. 118, 358–363
33. Perbal, B., and Herve, G. (1972) J. Mol. Biol. 57, 511–529
34. Webb, J. (1963) Enzyme and Metabolic Inhibitors, Vol. I, pp. 507–510, Academic Press, New York
35. Leger, D., and Herve, G. (1988) Biochemistry 27, 4293–4298
36. Perrin, D. D., and Sharma, V. S. (1966) Biochim. Biophys. Acta 127, 35–41
37. Huffman, J. P., Grant, B. J., Penning, C. A., and Sullivan, K. F. (1990) BioTechniques 9, 570–577
38. Jancarik, J., and Kim, S. H. (1991) J. Appl. Crystallogr. 24, 409–411
39. Weitzman P. D. J., and Wilson, I. B. (1966) J. Biol. Chem. 241, 5481–5488
40. Dutta, G., and O'Donovan, G. (1987) J. Chromatogr. 385, 119–124
41. Foote, J., and Schachman, H. (1985) J. Mol. Biol. 186, 175–184
42. Grayson, D. R., and Evans, D. R. (1983) J. Biol. Chem. 258, 4123–4129
43. Maley, J. A., and Davidson, J. N. (1988) Mol. Genet. 213, 278–284
44. Issaly, I., Pairet, M., Taie, P., Thiry, L., and Herve, G. (1982) Biochemistry 21, 1612–1623
45. Vickery, J. F. (1993) Isolation and Characterization of the Operon Containing Aspartate Transcarbamoylase and Dihydroorotase from Pseudomonas aeruginosa. Ph.D. thesis, University of North Texas, Denton, TX
46. Stalon, V., Ramos, F., Piard, A., and Wiame, J. M. (1972) Eur. J. Biochem. 29, 25–35
47. Mercenier, A., Simon, J. P., Vander Vauwen, C., Haas, D., and Stalon, V. (1980) J. Bacteriol. 144, 159–163
48. Shoesmith, J. G., Sherris, J. C. (1960) J. Gen. Microbiol. 22, 44–47
49. Bauer, H., Luethi, E., Stalon, V., Mercenier, A., and Haas, D. (1989) Eur. J. Biochem. 179, 53–60
50. Haas D., Holloway B. W., Schambock A., and Leisinger T. (1977) Mol. Genet. 154, 7–22
