Aquifex aeolicus Aspartate Transcarbamoylase, an Enzyme Specialized for the Efficient Utilization of Unstable Carbamoyl Phosphate at Elevated Temperature*

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Aquifex aeolicus, an organism that flourishes at 95 °C, is one of the most thermophilic eubacteria thus far described. The A. aeolicus pyrB gene encoding aspartate transcarbamoylase (ATCase) was cloned, overexpressed in Escherichia coli, and purified by affinity chromatography to a homogeneous form that could be crystallized. Chemical cross-linking and size exclusion chromatography showed that the protein was a homotrimer of 34-kDa catalytic chains. The activity of A. aeolicus ATCase increased dramatically with increasing temperature due to an increase in K_m, with little change in the K_m, for the substrates, carbamoyl phosphate and aspartate. The K_m for both substrates was 30–40-fold lower than the corresponding values for the homologous E. coli ATCase catalytic subunit. Although rapidly degraded at high temperature, the carbamoyl phosphate generated in situ by A. aeolicus carbamoyl phosphate synthetase (CPSase) was channeled to ATCase. The transient time for carbamoyl aspartate formation was 26 s, compared with the much longer transient times observed when A. aeolicus CPSase was coupled to E. coli ATCase. Several other approaches provided strong evidence for channeling and transient complex formation between A. aeolicus ATCase and CPSase. The high affinity for substrates combined with channeling ensures the efficient transfer of carbamoyl phosphate from the active site of CPSase to that of ATCase, thus preserving it from degradation and preventing the formation of toxic cyanate.

In the pyrimidine biosynthetic pathway, carbamoyl phosphate is used as a substrate, along with aspartate, for the formation of carbamoyl aspartate in a reaction catalyzed by aspartate transcarbamoylase (ATCase); EC 2.1.3.2).

Carbamoyl phosphate + aspartate → carbamoyl aspartate + P

REACTION 1

 Whereas ATCase is a ubiquitous enzyme and the reaction catalyzed is the same in all organisms, this family of proteins is remarkably polymorphic. Bacterial ATCases have been classified into 1) Class A, 480-kDa duodecamers consisting of two catalytic trimers and six subunits that are either dihydroorotase (Thermus aquaticus (6)), the third enzyme in the pathway, or inactive dihydroorotase homologues (e.g. Proteus vulgaris (8)), these two catalytic subunits are common to all known ATCases, because the active sites are composed of residues contributed by adjacent subunits within the trimer (11).

Two possibilities can be envisioned that could account for the preservation of carbamoyl phosphate at elevated temperatures in A. aeolicus and other hyperthermophiles. The hyperthermophilic ATCase may have an unusually high affinity for carbamoyl phosphate and high k_cat, so that once formed in the reaction catalyzed by CPSase, it is rapidly converted to stable carbamoyl aspartate. Alternately, ATCase and CPSase may form a complex that allows the direct transfer or channeling of carbamoyl phosphate between active sites, thus sequestering the intermediate and preventing it from escaping into the cellular milieu. Channeling of metabolic intermediates can occur via intramolecular tunnels that serve as a conduit between proteins catalyzing sequential steps in a metabolic pathway. Structural studies have shown that these tunnels exist in several biosynthetic enzymes including tryptophan synthetase (12), carbamoyl phosphate synthetase subunits (13), the subunits of 5-phosphoryl-1-pyrophosphate amidotransferase (14), and amidazole glycerol phosphate synthase (15). In these cases, the channeling is absolute in the sense that the intermediate generated in situ is used exclusively in the biosynthetic reaction. A more common, but less well understood, channeling

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‡‡ The abbreviations used are: ATCase, aspartate transcarbamoylase; CPSase, carbamoyl phosphate synthetase; TEA, triethanolamine; PALA, N-phosphonomethyl-l-aspartate; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate.

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mechanism involves the transfer of the intermediates between enzymes that do not form a stable stoichiometric complex (16). This type of channeling is thought to occur in many metabolic pathways (17, 18), and although the physical association of the enzymes, a prerequisite for intermediate transfer, is short lived or transient, the efficiency of intermediate transfer can be quite high in these systems.

We report herein the cloning, expression, and crystallization of *A. aeolicus* ATCase and a series of criteria to assess intermediate channeling between weakly associated partners. The kinetic properties and channeling studies suggest that the enzyme is particularly well adapted for the efficient utilization of carbamoyl phosphate at elevated temperatures.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pfu Turbo DNA polymerase was obtained from Stratagene; DNA ligase was from Invitrogen; aspartam, carbamoyl phosphate, carbamoyl aspartate, antipyrine, diacetyl monoxime, triethanolamine (TEA), spectinomycin, pyridoxal phosphate, and sodium phosphate, carbamoyl aspartate, antipyrine/diacetyl monoxime reagent. Substrate saturation curves contained 1 mM aspartate, 5 mM carbamoyl phosphate, 1 ± 1 μg of the enzyme; and the velocity of the ATCase (√<sub>max</sub>) is given by the equation $\sqrt{V_{\text{max}}}$ (Eq. 2)

$$\sqrt{V_{\text{max}}} = \frac{k_{\text{i}}}{\sqrt{k_{\text{cat}}}} \cdot \frac{1}{V_{\text{e}}} \cdot \frac{1}{V_{\text{e}}^{\text{init}} + k_{\text{cat}}} $$

where $V_{\text{e}}$ is the rate of formation of carbamoyl phosphate in μmol/min measured or calculated from the $V_{\text{e}}$ and the concentration of CPSase; $\tau$ is the transient time defined by Easterby (26) as equal to $K_{\text{cat}}/V_{\text{e}}$; $K_{\text{cat}}$ is the first order rate constant for the thermal degradation of carbamoyl phosphate determined experimentally.

**Size Exclusion Chromatography**—The molecular mass of the recombinant enzyme was determined by size exclusion chromatography (27) on a 1 × 50-cm column of Sephacyr S-300 High Resolution equilibrated with 50 mM Tris-HCl, pH 8, in the presence or absence of 100 mM NaCl. 1 mL of *A. aeolicus* ATCase at a concentration of 3 mg/ml was applied to the column. The column was eluted with the same buffer at a flow rate of 0.3 ml/min, and 1.1-ml fractions were collected.

**Chemical Cross-linking**—A reaction mixture containing 35 μg of *A. aeolicus* ATCase, 10 mM dimethyl suberimidate (28), and 100 mM TEA, pH 8.5, in a final volume of 40 μl, was incubated at room temperature and quenched at the designated times by the addition of 4 μl of 1 M ammonium chloride and 0.5 ml of 1 M ammonium hydroxide. The samples (0.5 ml) were withdrawn periodically, chilled on ice, and analyzed by 12.5% SDS-PAGE gels (21). Fractions containing pure *A. aeolicus* ATCase were applied to a Nick Spin Column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 8, to eliminate NaCl and imidazole from the buffer. *A. aeolicus* CPSase was expressed in *E. coli* and purified by affinity chromatography (22).

**Enzyme Assays**—ATCase activity was measured by the colorimetric method previously described (23, 24). Asayed at 37°C, the reaction contained 1 mM aspartate, 5 mM carbamoyl phosphate, 1–11 μg of the purified enzyme, and 50 mM Tris-HCl, pH 8, in a total volume of 0.3 ml. Enzyme assay at 75°C, the concentration of aspartate and phosphate was increased to 2 mM. The incubation time was 10 min at 37°C or 1 min at 75°C. The reactions were quenched by the addition of 1 ml of the antipyryl/diacetyl monoxide reagent. Substrate saturation curves were fit to the Michaelis-Menten equation or to an equation that incorporates a term for substrate inhibition, $v = V_{\text{max}}[S]/K_{\text{m}} + [S] + [S]/K_{\text{i}}$, where $K_{\text{i}}$ is the substrate inhibition constant.

**Measurement of Carbamoyl Phosphate Stability**—The stability of carbamoyl phosphate and its degradation to cyanate was measured using previously described methods (4). Carbamoyl phosphate 14C (86,939 dpm/μmol) at a concentration of 10 mM was incubated at 75°C.

**Results**

**Identification and Sequence Analysis of *A. aeolicus* ATCase**—A Blast search of the recently sequenced *A. aeolicus* genome revealed an open reading frame that was homologous to the *E. coli* ATCase catalytic chain, whereas no gene encoding a protein homologous to the *E. coli* ATCase regulatory chain was identified.

With a molecular mass of 34 kDa, *A. aeolicus* ATCase is shorter by 20 residues than the homologous *E. coli* catalytic chain.
Fig. 1. Sequence similarity. A, the sequence of *A. aeolicus* ATCase was aligned with the sequence of the *E. coli* ATCase catalytic chain. This alignment was used in the molecular modeling described below. Identities (black boxes), conservative substitutions (gray boxes), and the active site residues (● above residue) identified in the *E. coli* ATCase structure (47, 48) are highlighted. B, the structure of *A. aeolicus* ATCase was modeled using the *E. coli* ATCase R state structure as a template as described under “Experimental Procedures.” The backbone of the model structure (green) is shown superimposed on the backbone of the template structure (yellow). The bisubstrate analog PALA (spaced-filled CPK representation) is shown bound to the active site. The diagram also shows the insertions (cyan) and deletions (red-orange) in the *A. aeolicus* structure, the hinge (red), and a potential interdomain interaction between the carbamoyl phosphate and aspartate domains (labeled K26 K138 in the *A. aeolicus* enzyme, corresponding to Lys31–Gln147 in *E. coli* ATCase).
The tertiary structure of the A. aeolicus ATCase was modeled (Fig. 1B) using the x-ray structure of the E. coli ATCase catalytic trimer as a template. Both the T-state structure (Protein Data Bank code 6AT1) and R-state (Protein Data Bank code 1D09) structure with the bound bisubstrate analog, PALA, were used as tertiary templates, and gave comparable model structures. The α-carbons of the models and of E. coli ATCase were for the most part superimposed with an root mean square deviation of 0.95 and 0.92 Å, for the R and T model structures, respectively. Major deviations in the backbone occur only in regions near major insertions and deletions (ranging from 2 to 12 residues). The active site is among the most highly conserved regions of the molecule. The backbone of the A. aeolicus protein is virtually superimposed on the template structure in this region, and all of the residues involved in substrate binding are conserved. The residues that constitute the interface between the subunits in the E. coli ATCase trimer were for the most part conserved, suggesting that the A. aeolicus ATCase is also trimeric. Thus, the tertiary fold, the oligomeric structure, and the catalytic mechanism probably resemble those of mesophilic ATCases.

Cloning, Expression, and Purification of A. aeolicus ATCase—
The pyrB gene, encoding A. aeolicus ATCase, was amplified by PCR and inserted into the pRSETB expression vector. This vector appends a His tag to the amino end of the recombinant protein to facilitate purification. The resulting construct, pApYrB, was cotransformed with the pJS1240 into the E. coli BL21(DE3) strain (see “Experimental Procedures”). The soluble protein was expressed at high levels, 40 mg/liter of culture, and could be purified (Fig. 2A) in a single step by Ni²⁺ affinity chromatography. The specific activity of the purified protein, 1,200 μmol/h/mg at 37 °C, increased to 8,000 μmol/h/mg when assayed at 75 °C. Pyramidal crystals (Fig. 2B) of the homogenous protein were obtained by the hanging drop method, paving the way for future x-ray diffraction studies. The ATCase crystals diffract to less than 2 Å.

Subunit Structure—The molecular mass of the recombinant protein determined by SDS-polyacrylamide gel electrophoresis was 37 kDa. When the 3-kDa His tag segment is taken into consideration, the recombinant A. aeolicus ATCase had a molecular mass of 34 kDa, in good agreement with the mass calculated from the deduced amino acid sequence (33,557 Da). The size of the molecule under nondenaturing conditions was determined by size exclusion chromatography and chemical cross-linking. Chromatography on a Sephacryl S300 column in the presence of 100 mM NaCl (not shown) gave a single species with a molecular mass of 112,800 ± 3,100 Da, indicating that the A. aeolicus ATCase is a trimer. This result was confirmed by chemical cross-linking with dimethyl suberimidate. The time course of the cross-linking reaction (Fig. 2C) showed a gradual disappearance of the monomer and the sequential formation of the dimer and then the trimer. Thus, A. aeolicus ATCase is a homotrimer composed of three 34-kDa catalytic chains.

Thermostability of A. aeolicus ATCase—The stability of A. aeolicus ATCase (Fig. 3A) was measured by incubating the protein at various temperatures for 5 min and assaying the catalytic activity at 37 °C. The enzyme was found to be very stable up to a temperature of 80 °C, at which point there was a precipitous decline in activity. The temperature at which the protein was 50% inactivated was 87 °C. In another experiment (Fig. 3B), the A. aeolicus enzyme was incubated at 85 °C, and samples were periodically withdrawn for assay. There was a slow exponential inactivation of the protein with a first order rate constant of 0.020 ± 0.004 min⁻¹, corresponding to a half-life of 34.7 min. Since some of the kinetic experiments described below employed E. coli ATCase as a control, its thermostability was also measured. At 70 °C, E. coli ATCase was found to be initially quite stable. However, after 10 min, the
A. aeolicus Aspartate Transcarbamoylase

Catalytic activity began to decrease with a first order rate constant of 0.28 min\(^{-1}\) (Fig. 3B).

Carbamoyl Phosphate Stability—As a prelude to the steady state kinetic studies, the stability of carbamoyl phosphate at elevated temperature was measured. Carbamoyl phosphate degradation is not a major concern when assaying mesophilic enzymes at ambient temperature where the substrate is relatively stable (\(t = 169\) min) (4). However, previous studies indicated that at 96 °C, near the growth optimum of A. aeolicus, carbamoyl phosphate has a half-life of only 2 s (5). A temperature of 75 °C was chosen as a compromise for measuring both carbamoyl phosphate degradation and cyanate formation. Carbamoyl phosphate was found to decrease exponentially (Fig. 4A) with a first order rate constant of 0.76 ± 0.05 min\(^{-1}\), whereas the rate of formation of cyanate was 0.74 ± 0.05 min\(^{-1}\). The agreement between these values is consistent with the degradation of carbamoyl phosphate to cyanate.

The nonenzymatic reaction between carbamoyl phosphate and aspartate was measured by incubation of the two substrates at 75 °C. The spontaneous formation of carbamoyl aspartate obtained corresponded to only 3.8% of the amount formed in the enzyme catalyzed reaction under the experimental conditions (1-min assay) described below.

Steady State Kinetics—The steady state kinetic parameters of purified A. aeolicus ATCase were obtained from substrate saturation curves (not shown) for aspartate and carbamoyl phosphate measured at 37 and 75 °C. In the case of carbamoyl phosphate, due to its rapid thermal degradation, we conducted short term assays (1 min) and calculated the mean carbamoyl phosphate concentration during the assay period using the measured rate constant for the degradation of carbamoyl phosphate (Fig. 4). The saturation curves for both substrates were hyperbolic and, with the exception of the carbamoyl phosphate saturation curve measured at 37 °C, exhibited inhibition at high substrate concentrations.

The \(k_{cat}\) measured at 37 °C (Table I) was comparable with the value obtained for the E. coli ATCase catalytic subunit (9.57 ± 1.0 s\(^{-1}\)) (33), whereas the \(K_m\) values for both aspartate and carbamoyl phosphate were 20 and 39 times lower, respectively. At 75 °C, the A. aeolicus ATCase \(K_m\) for aspartate and carbamoyl phosphate changed little, whereas \(k_{cat}\) increased 7.5-fold. The influence of temperature on the reaction rate was determined from a series of aspartate saturation curves obtained at different temperatures. The Arrhenius plot of \(\log V_{max}\) against 1/\(T\) (not shown) is linear, with a calculated activation energy (\(E_a\)) of 53.6 kJ/mol, a value comparable with those determined for ATCases from Pyrococcus abyssi (34), Methanocaldococcus jannaschii (33), and E. coli (35). The presence of the E. coli ATCase allosteric effectors CTP, UTP, and ATP at 5 mM had no effect on the activity of the enzyme.

The CPSase-ATCase Coupled Reaction—From the perspective of the overall synthesis of pyrimidine nucleotides in the cell, the coupled reaction in which ATCase produces carbamoyl aspartate from carbamoyl phosphate generated endogenously by CPSase is most important. The thermal degradation of carbamoyl phosphate at elevated temperatures would be expected to profoundly influence the efficiency of carbamoyl aspartate formation, since the carbamoyl phosphate generated \textit{in situ} would be partitioned between the ATCase and cyanate formed by thermal degradation.

\[
V_n = \frac{V_{max}}{1 + \frac{[S]}{K_m}}
\]

\[
\text{HCO}_3^- + \text{ATP} + \text{NH}_3 \rightarrow \text{carbamoyl phosphate} \rightarrow \text{carbamoyl aspartate}
\]

Scheme 1

An estimate of the significance of intermediate degradation was obtained by extending the Easterby treatment (26) for coupled reactions to take into consideration the two possible fates of carbamoyl phosphate. The simulations in Fig. 4B showed that if the rate of enzymatic conversion of carbamoyl phosphate to carbamoyl aspartate is comparable with its rate of thermal degradation, as is the case, then less than half of the carbamoyl phosphate would be converted to carbamoyl aspartate and the remainder would be degraded to cyanate.

Transient Time Measurements—The actual rate of formation of carbamoyl aspartate (Fig. 4C) from carbamoyl phosphate generated \textit{in situ} was determined by coupling A. aeolicus CPSase to A. aeolicus ATCase or to E. coli ATCase. For the A. aeolicus CPSase-ATCase couple, the reaction proceeded with a short transient time (\(\tau = 0.46\) min), and the rate of formation of carbamoyl aspartate approached the expected rate of 0.208 \(\mu\)mol/min at steady state. Significantly, no measurable cyanate formation could be detected in the coupled reaction. In contrast, the theoretical progress curve, calculated assuming that the intermediate is not channelled and the carbamoyl phosphate must diffuse through the bulk phase to the active site of the ATCase, exhibited a somewhat longer transient time (\(\tau = 1.08\) min). Most strikingly, the rate of carbamoyl aspartate formation at steady state, calculated from the measured kinetic

Fig. 3. Thermostability of A. aeolicus ATCase. A, purified A. aeolicus ATCase was incubated at the indicated temperatures at a concentration of 0.13 mg/ml in 50 mM Tris-HCl, pH 8, for 5 min. The samples were then chilled on ice, and the residual enzymatic activity was assayed at 37 °C. B, A. aeolicus (○) and E. coli (○) ATCase in the same buffer were incubated at 85 and 70 °C, respectively, and samples were withdrawn at the indicated time intervals, cooled, and assayed at 37 °C. The first order rate constant for thermal inactivation of the A. aeolicus enzyme was calculated from a least squares fit of the curve to a monoeponential equation.
The rate of decomposition was measured by incubating [14C]carbamoyl phosphate (7 µCi/µmol) at a concentration of 10 mM in 50 mM Tris-HCl, pH 8, at 70 °C. Samples were withdrawn at the indicated times and reacted with NH₄Cl to convert the cyanate formed by carbamoyl phosphate to urea. The samples were processed as described under "Experimental Procedures" and counted. The first order rate constants for carbamoyl phosphate degradation were determined by least squares fit of the respective curves to a monoexponential equation. A. aeolicus Aspartate Transcarbamoylase coupled reaction was calculated using a modified Easterby equation that incorporates a term for the degradation of carbamoyl phosphate. For the simulations, values of Vo of 20 µmol/min and τ = 1 min were assumed. The calculated curves show the rate of formation of carbamoyl aspartate from carbamoyl phosphate generated by CPSase in the absence of degradation (Vo, solid line), the rate of carbamoyl aspartate formation assuming the carbamoyl phosphate is degraded with the observed first order rate constant of 0.74 min⁻¹ (dashed line), and the rate of formation of cyanate from carbamoyl phosphate degradation (dashed and dotted line). C. transient time measurement. The time-dependent formation of carbamoyl aspartate in the coupled CPSase-ATCase reaction was measured at 70 °C. The reaction mixture consisted of 50 mM 14C-labeled sodium bicarbonate, 30 mM ATP, 32 mM MgCl₂, 200 mM NH₄Cl, 2 mM aspartate, in 50 mM Tris-HCl, pH 8, and 15 µg of A. aeolicus CPSase in a total volume of 0.5 ml. For the reaction in which A. aeolicus CPSase was coupled to A. aeolicus ATCase, 4.2 µg of ATCase was used. The formation of carbamoyl aspartate (solid line) was measured as described under "Experimental Procedures." The experimental transient time (τ) for the A. aeolicus CPSase-ATCase (26 s) was determined by extrapolating the steady state rate of product formation to the x axis. The concentration of the intermediate (i), was determined from the y intercept (intercept = i) to be 38 µM. The rate of product formation was also calculated using the extended Easterby treatment (see "Experimental Procedures") that assumes no channeling and that intermediate is released from the complex and diffuses to the active site of the second enzyme. The expected rate of formation of carbamoyl aspartate (dashed line) was calculated from the measured steady state rate of the reaction (V = 205 nmol/min), the measured Kᵢ and Vₒ of ATCase for carbamoyl phosphate (Table I), and the rate of degradation of carbamoyl phosphate (kᵢ = 0.74 min⁻¹). The theoretical curve gave a value for τ of 1.08 min. For the reaction of A. aeolicus CPSase coupled to E. coli ATCase (C), the reaction mixture contained 7.1 µg of ATCase. The steady state rate of carbamoyl aspartate was not reached during the course of the experiment, so an accurate value for τ could not be obtained. When these data were fit to the modified Easterby equation (solid line), fixing the known values for Vₒ = 205 nmol/min and kᵢ = 0.74 min⁻¹, a τ of 5.92 ± 0.21 min was obtained for the A. aeolicus CPSase/E. coli ATCase coupled reaction.
transient time. The transient time was measured, as described in the legend to Fig. 4, at seven molar ratios of ATCase to CPSase (monomer/monomer)2 ranging from 0.33:1 to 10:1. A, the progress curves for carbamoyl aspartate formation at a molar ratio of ATCase to CPSase of 0.33 (○) and 3.0 (●). The other curves are not shown for clarity, but the transient time was obtained for all seven curves by fitting the steady state rate of carbamoyl aspartate formation to the equation for a straight line and determining the x intercept, B, the reciprocal transient time (1/τ, s-1) versus the molar ratio of ATCase to CPSase (○). The saturation curve was fit (solid line) to the equation 1/τ = 1/τm + (r°)(1/τm)(K°+ r°)), where τm and τr are the minimum and maximum values for τ, n is the Hill coefficient, r is the molar ratio of ATCase to CPSase, and K is the molar ratio for half-maximal increase in the reciprocal transient time.

Competition of Active and Inactive ATCase in Complex Formation—An ingenious approach devised by Geck and Kirsch (36) was adopted here to investigate the channeling of carbamoyl phosphate between CPSase and ATCase. If a transient complex is formed between the donor (CPSase) and the acceptor (ATCase) enzymes, the addition of an excess of inactive ATCase should displace the active species from the complex and abolish channeling.

Pyridoxal 5’ phosphate reacts with the catalytic subunit of E. coli ATCase, forming a Schiff base with Lys-37 (37), an active site residue essential for the binding of both carbamoyl phosphate and aspartate. Reduction of the Schiff base with sodium borohydride results in the formation of an inactive N-ε-pyridoxal-1-lysine derivative with a stoichiometry of 3 mol/mol of catalytic trimer. Lys-37 (E. coli numbering), a residue conserved in all known ATCases (11, 38), corresponds to Lys-75 in A. aeolicus ATCase.

Purified A. aeolicus ATCase was titrated with increasing amounts of pyridoxal phosphate, reduced to the secondary amine by reaction with sodium borohydride and then dialyzed to remove excess reactants. The loss of ATCase activity (Fig. 6A) was biphasic, and the initial linear part of the curve extrapolated to 0.9 mol of pyridoxal phosphate/mol of ATCase monomer. Thus, as observed for other ATCases, pyridoxal phosphate specifically reacts with and inactivates A. aeolicus ATCase. A completely inactive ATCase pyridoxal amine derivative was prepared as described under “Experimental Procedures.” The transient time was measured with increasing amounts of the inactivated enzyme added to a mixture (monomer/monomer)2 of CPSase and active ATCase (Fig. 6B). To promote efficient competition, the molar ratio of active ATCase to CPSase was set at 0.7:1.0 so that the transient time was 26 s in the absence of inactive ATCase. As the ratio of inactive to active ATCase increased, there was a pronounced increase in the transient time. Again, the curve was sigmoidal with a Hill coefficient of 2.9 ± 0.1. The half-maximal increase in transient time occurred at a molar ratio of active to inactive ATCase of 1:1.3, suggesting that the two proteins have comparable affinity for the CPSase. The maximum transient time, 65 s, is close to the limiting value observed at very low molar ratios of ATCase to CPSase (Fig. 6B). This result suggests that the inactive ATCase competes with the active ATCase in the complex formation and disrupts channeling.

Effect of PALA on the Coupled Reaction—The bisubstrate ATCase analog PALA is a competitive inhibitor of carbamoyl phosphate in the reaction catalyzed by E. coli ATCase (39). The Kᵢ for PALA for A. aeolicus ATCase at 70 °C was determined from carbamoyl phosphate saturation curves (Fig. 7A) obtained for four concentrations of PALA. A replot of the apparent Kᵢ/Vₘₐₓ for PALA against the PALA concentration (inset) gave a Kᵢ value of 3.9 × 10⁻⁷ M. In contrast to the E. coli enzyme and other allosteric ATCases, no increase of activity was observed in the presence of low concentrations of PALA, confirming the lack of cooperative substrate binding (40). As in other ATCases, ATP and the nonhydrolyzable ATP analog AMP-PNP were found to be weak competitive inhibitors of A. aeolicus ATCase (Kᵢ = 2.5 × 10⁻⁷ M), presumably because the phosphate moiety binds to the carbamoyl phosphate site (data not shown).

PALA inhibition of the CPS-ATC coupled reaction (Fig. 7B) was also measured. The concentration of PALA required for 50% inhibition of the rate of carbamoyl aspartate formation was unexpectedly high (1 mM), considering that the concentration of carbamoyl phosphate estimated from the presteady state progress curves was very low (38 μM). The concentration of carbamoyl phosphate that would be required to give the observed PALA inhibition curve can be calculated assuming the measured kinetic constants. A least squares fit of these data to the equation for competitive inhibition (see legend to Fig. 7B), using the measured values for the Kᵢ of PALA and ATP, the Kᵢ for carbamoyl phosphate, and the Vₘₐₓ for ATCase, gave a value of the carbamoyl phosphate concentration of 1.6 ± 0.3 mM. The most plausible interpretation of these results is that

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**Table I**

| Temperature | Kᵢ aspartate | Kᵢ carbamoyl P | Vₘₐₓ | kᵢat | kᵢcat/Kᵢa |
|------------|-------------|---------------|------|------|----------|
| °C         | μM          | μM            | μmol/hmg | s⁻¹ | s⁻¹ μmol⁻¹ |
| 37         | 1.00 ± 0.30 | 0.55 ± 0.03   | 1189 ± 203 | 5.60 ± 0.16 | 10.6     |
| 75         | 1.12 ± 0.21 | 1.26 ± 0.21   | 8003 ± 720 | 41.7 ± 2.6 | 47.4     |

**Fig. 5.** Effect of increasing concentration of ATCase on the transient time. The transient time was measured, as described in the legend to Fig. 4, at seven molar ratios of ATCase to CPSase (monomer/monomer)² ranging from 0.33:1 to 10:1. A, the progress curves for carbamoyl aspartate formation at a molar ratio of ATCase to CPSase of 0.33 (○) and 3.0 (●). The other curves are not shown for clarity, but the transient time was obtained for all seven curves by fitting the steady state rate of carbamoyl aspartate formation to the equation for a straight line and determining the x intercept. B, the reciprocal transient time (1/τ, s⁻¹) versus the molar ratio of ATCase to CPSase (●). The saturation curve was fit (solid line) to the equation 1/τ = 1/τₘ + (r°)(1/τₘ)(K°+ r°)), where τₘ and τᵡ are the minimum and maximum values for τ, n is the Hill coefficient, r is the molar ratio of ATCase to CPSase, and K is the molar ratio for half-maximal increase in the reciprocal transient time.

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2 The ATCase monomer is defined as the 34-kDa polypeptide chain. A. aeolicus CPSase is unusual in that the synthetase subunit catalyzing the formation of carbamoyl phosphate from NH₄⁺, bicarbonate, and ATP consists of two homologous 60-kDa polypeptides, CPS.A and CPS.B (22). The CPSase monomer is defined as the 120-kDa complex consisting of one copy of CPS.A and one copy of CPS.B.
the effective concentration of carbamoyl phosphate in the vicinity of the active site must be ~42-fold higher (1.6 mM/0.038 mM) than the overall concentration in the reaction mixture.

An alternative possibility is that CPSase and ATCase form a complex in which the access of PALA to the active site is physically restricted. To test this hypothesis, the effect of PALA on the ATCase reaction, using exogenous carbamoyl phosphate and aspartate, was measured in the presence and absence of A. aeolicus CPSase. CPSase had little effect on the inhibition of the ATCase reaction by PALA (Fig. 7C). It is also possible that the presence of CPSase substrates might induce conformational changes in the CPSase-ATCase complex that reduce access of PALA to the active site. However, when the PALA inhibition of ATCase was measured in the presence of the CPSase substrates, NH₃, bicarbonate, and the ATP analog AMP-PNP (Fig. 7C), the reduction in velocity in the absence of PALA and the 10-fold decrease in the PALA $K_i$ could be fully accounted for by competitive inhibition of ATCase by AMP-PNP. Thus, the relatively ineffective inhibition by PALA can probably be attributed to a high local concentration of the intermediate rather than by restricted access of PALA to the ATCase active site.

**DISCUSSION**

Although A. aeolicus occupies the deepest branch of the eubacterial phylogenetic tree, the polypeptide encoded by the pyrB gene closely resembles the catalytic chain of ATCases from other organisms in size and sequence. The protein is a trimer composed of 34-kDa subunits. No homotropic or heterotropic interactions were found in A. aeolicus ATCase, an observation that is consistent with the absence of regulatory subunits. The $K_m$ values for both carbamoyl phosphate and aspartate are appreciably lower than the corresponding values for the E. coli ATCase catalytic subunit. Molecular modeling showed that the active site of A. aeolicus ATCase is very similar to that of the E. coli enzyme and that there are no apparent new interactions that could explain the high affinity of the hyperthermophilic protein for its substrates. Studies of E. coli ATCase have shown that the catalytic subunit exists in two conformations: an open, low affinity conformation (T) and a closed high affinity conformation (R) that is induced when both substrates are bound. This conformational change, an essential element of the catalytic cycle, involves repositioning of the first critical loops that bring active site residues into the correct juxtaposition. There is also an accompanying domain closure (42) that involves a movement of ~8° about the hinge region (Fig. 1B) connecting the two domains. A possible explanation for the enhanced substrate affinity is that the R-T equilibrium is shifted toward the high affinity R-state in the A. aeolicus enzyme. Modeling studies suggest that both R and T conformations are equally accessible to the A. aeolicus enzyme and that all of the interactions that are known to stabilize the R-state are present. In contrast, one of the two T-state-specific interactions probably cannot occur. In E. coli ATCase, there is an interdomain hydrogen bond between Lys$^{31}$ and Gln$^{147}$ that may contribute to the stability of the T-state. This lysine (Lys$^{26}$; A. aeolicus numbering) is present in the A. aeolicus enzyme (Fig. 1B), but the glutamine is replaced by a second lysine (Lys$^{138}$). The disruption of this hydrogen bond or the repulsive interaction between these two positively charged residues may destabilize the T-state in the A. aeolicus enzyme and account for the low $K_m$ values. X-ray studies, now under way, should clarify this issue. Regardless of the molecular mechanism, the high affinity of the A. aeolicus enzyme for its substrates would promote the efficient utilization of carbamoyl phosphate. For example, the apparent second order rate constant ($k_{cat}/K_m$) is 20-fold higher than the E. coli catalytic subunit at 37 °C and increases another 5-fold at 75 °C.

The efficient transfer of carbamoyl phosphate between its site of synthesis on CPSase and the active site of ATCase is a crucial consideration in an organism that grows optimally at 95 °C. At this temperature, carbamoyl phosphate must be sequestered within the complex, since it is rapidly degraded to toxic cyanate. Theoretical calculations suggest that, at 75 °C, if carbamoyl phosphate was released from CPSase and had to
Fig. 7. PALA Inhibition of A. aeolicus ATCase. A, the $K_i$ for PALA inhibition of the ATCase reaction at 70 °C was determined by measuring the carbamoyl phosphate saturation curves in the presence of 1 µM (●), 3 µM (○), 6 µM (△), and 10 µM (□) PALA. A replot of the data (inset) gave a $K_i$ of $3.9 \times 10^{-7}$ M. B, the effect of PALA on the rate of the coupled reaction catalyzed by a stoichiometric mixture of A. aeolicus CPSase and ATCase (●) was measured. The reaction mixture contained 50 mM sodium bicarbonate, 200 mM ammonium chloride, 10 mM ATP, 12 mM MgCl$_2$, and 2 mM aspartate and the indicated concentrations of PALA. The data were fit to the equation for a competitive inhibition, $v = V_{max}[S]/([S] + K_m(1 + [I]/K_i))$, where [I] is the concentration of PALA, and $K_i$ is the inhibition constant. A least squares fit of the data (solid line) to this equation gave a carbamoyl phosphate concentration of 1.6 ± 0.3 mM, the concentration required to give the observed inhibition curve. However, carbamoyl phosphate does not accumulate during the coupled reaction, and the actual concentration, estimated from the data in Fig. 4, was only 38 µM. The theoretical PALA inhibition curve calculated using 38 µM and the same values for all of the other parameters is shown (dashed line) for comparison. C, the effect of PALA on the ATCase reaction assayed with exogenous carbamoyl phosphate (1.5 mM), and aspartate (2.0 mM) was measured in the absence of CPSase (●) and in the presence of a stoichiometric amount of CPSase without the CPSase substrates (○) and with 50 mM sodium bicarbonate, 200 mM ammonium chloride, 10 mM AMP-PNP, and 12 mM MgCl$_2$ (■). The nonhydrolyzable ATP analog was used to preclude endogenous carbamoyl phosphate formation by CPSase.
diffuse through the aqueous milieu to the active site of ATCase, more that half of the carbamoyl phosphate generated in situ would be degraded, and there would be a proportional decrease in the steady state rate of carbamoyl aspartate formation. This is an especially serious concern, since a search of the *A. aeolicus* genome did not reveal a gene encoding a protein homologous to the cyanase in *E. coli* (43) and other bacteria. However, the experimentally determined transient time for the coupled reaction was low, the steady state rate of formation of carbamoyl aspartate corresponded to the rate of carbamoyl phosphate synthesis, and no cyanate was produced. Intermediate channeling is thought to require the physical association of the participating enzymes to allow direct transfer of the intermediates between active sites. In contrast, *E. coli* ATCase would not be expected to form the prerequisite complex with *A. aeolicus* CPSase. When *A. aeolicus* CPSase was coupled to the *E. coli* enzyme, the transient time was much longer, the rate of conversion of endogenous carbamoyl phosphate to carbamoyl aspartate corresponded to the rate of carbamoyl aspartate formation from aspartate and exogenous carbamoyl phosphate synthesized, and no cyanate was produced. The effect of PALA on the coupled reaction also provided strong evidence for channeling and the formation of a transient complex between limiting concentration of ATCase and CPSase. When the ratio of ATCase/CPSase far exceeded the concentration (42-fold) in the aqueous milieu, the transient time was quite slow, and most of the intermediate was degraded to cyanate, indicating that channeling did not occur in the heterologous system.

If the association of ATCase and CPSase is transient, complex formation would be expected to increase with increasing concentrations of the proteins. In accord with this prediction, kinetic measurements showed that the transient time significantly decreased with an increasing ratio of ATCase/CPSase. Significantly, the plot of 1/τ versus the ratio of ATCase/CPSase exhibited saturation, indicating that all of the CPSase was present as a stoichiometric complex at high concentrations of ATCase. At this point, the measured transient time was quite low, 3.6 s, but could still be detected, indicating that channeling is not absolute as would be expected if there were a closed, intramolecular tunnel connecting the active sites of the enzymes. Although complex formation is transient, the molar ratio of ATCase/CPSase at half-saturation was 1.5, suggesting a reasonably high affinity between the proteins. ATCase is a stable trimer, and the addition of successive CPSase monomers appears to be cooperative, judging from the sigmoidal saturation curve (Hill coefficient n = 2.9).

Further evidence for the formation of a functional complex was obtained from experiments that showed that an inactive variant of ATCase could effectively compete with the wild type ATCase in complex formation. The most plausible interpretation of this result is that the active ATCase is displaced by the formation of a complex between limiting concentration of CPSase and excess inactive ATCase. Although the total concentration of active ATCase remained unchanged, the ATCase associated with CPSase was inactive and could not catalyze carbamoyl aspartate formation. Thus, channeling could not occur, and carbamoyl aspartate formation required that carbamoyl phosphate be released from the complex and diffuse to the active site of catalytically competent, uncomplexed ATCase molecules.

The effect of PALA on the coupled reaction also provided strong evidence for channeling and the formation of a transient CPSase-ATCase complex. Whereas PALA was a potent inhibitor of carbamoyl aspartate formation from aspartate and exogenous carbamoyl phosphate, it was a relatively ineffective inhibitor of the coupled reaction, the formation of carbamoyl aspartate from carbamoyl phosphate generated in situ by CPSase. Similar results have been observed for the coupled reaction catalyzed by the mammalian multifunctional protein CAD (44), by the yeast bifunctional complex (CPSase-ATCase (45), and by mixtures of *P. abyssi* ATCase and CPSase (46). This study provides the first indication that the inefficiency of PALA inhibition is not due to restricted access of the inhibitor to the ATCase active site in the CPSase-ATCase complex. The most probable explanation is that the local “concentration” of carbamoyl phosphate near its site of synthesis within the complex far exceeded the concentration (42-fold) in the aqueous phase so that it could compete much more effectively with PALA for the ATCase active site.

In summary, *Aquifex aeolicus* ATCase is an unregulated homotrimeric enzyme. The structural organization, sequence, and catalytic mechanism closely resemble those of other eubacterial ATCases. However, the enzyme appears to have undergone adaptations that include structural changes conferring thermal stability, increased affinity for substrates, and intermediate channeling that ensure efficient utilization of the unstable carbamoyl phosphate at the elevated temperatures where this organism thrives. The effect of PALA on the coupled reaction also provided strong evidence for channeling and the formation of a transient CPSase-ATCase complex. Whereas PALA was a potent inhibitor of carbamoyl aspartate formation from aspartate and exogenous carbamoyl phosphate, it was a relatively ineffective inhibitor of the coupled reaction, the formation of carbamoyl aspartate from carbamoyl phosphate generated in situ by CPSase. Similar results have been observed for the coupled reaction catalyzed by the mammalian multifunctional protein CAD (44), by the yeast bifunctional complex (CPSase-ATCase (45), and by mixtures of *P. abyssi* ATCase and CPSase (46). This study provides the first indication that the inefficiency of PALA inhibition is not due to restricted access of the inhibitor to the ATCase active site in the CPSase-ATCase complex. The most probable explanation is that the local “concentration” of carbamoyl phosphate near its site of synthesis within the complex far exceeded the concentration (42-fold) in the aqueous phase so that it could compete much more effectively with PALA for the ATCase active site. The effect of PALA on the coupled reaction also provided strong evidence for channeling and the formation of a transient CPSase-ATCase complex. Whereas PALA was a potent inhibitor of carbamoyl aspartate formation from aspartate and exogenous carbamoyl phosphate, it was a relatively ineffective inhibitor of the coupled reaction, the formation of carbamoyl aspartate from carbamoyl phosphate generated in situ by CPSase. Similar results have been observed for the coupled reaction catalyzed by the mammalian multifunctional protein CAD (44), by the yeast bifunctional complex (CPSase-ATCase (45), and by mixtures of *P. abyssi* ATCase and CPSase (46). This study provides the first indication that the inefficiency of PALA inhibition is not due to restricted access of the inhibitor to the ATCase active site in the CPSase-ATCase complex. The most probable explanation is that the local “concentration” of carbamoyl phosphate near its site of synthesis within the complex far exceeded the concentration (42-fold) in the aqueous phase so that it could compete much more effectively with PALA for the ATCase active site.
A. aeolicus Aspartate Transcarbamoylase

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