Aquifex aeolicus Dihydroorotase

ASSOCIATION WITH ASPARTATE TRANSCARBAMOYLASE SWITCHES ON CATALYTIC ACTIVITY*

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Dihydroorotase (DHOase) catalyzes the reversible condensation of carbamoyl aspartate to form dihydroorotate in de novo pyrimidine biosynthesis. The enzyme from Aquifex aeolicus, a hyperthermophilic organism of ancient lineage, was cloned and expressed in Escherichia coli. The purified protein was found to be a 45-kDa monomer containing a single zinc ion. Although there is no other DHOase gene in the A. aeolicus genome, the recombinant protein completely lacked catalytic activity at any temperature tested. However, DHOase formed an active complex with aspartate transcarbamoylase (ATCase) from the same organism. Whereas the $k_{\text{cat}}$ of 13.8 ± 0.03 s$^{-1}$ was close to the value observed for the mammalian enzyme, the $K_m$ for dihydroorotate, 3.03 ± 0.05 mM was 433-fold higher. Gel filtration and chemical cross-linking showed that the complex exists as a 240-kDa hexamer (DHO$_2$ ATC$_3$) and a 480-kDa dodecamer (DHO$_4$ ATC$_6$) probably in rapid equilibrium. Complex formation protects both DHOase and ATCase against thermal degradation at temperatures near 100 °C where the organism grows optimally. These results lead to the reclassification of both enzymes: ATCase, previously considered a Class C homotrimer, now falls into Class A, whereas the DHOase is a Class 1B enzyme. CD spectroscopy indicated that association with ATCase does not involve a significant perturbation of the DHOase secondary structure, but the visible absorption spectrum of a Co$^{2+}$-substituted DHOase is appreciably altered upon complex formation suggesting a change in the electronic environment of the active site. The association of DHOase with ATCase probably serves as a molecular switch that ensures that free, uncomplexed DHOase in the cell remains inactive. At pH 7.4, the equilibrium ratio of carbamoyl aspartate to dihydroorotate is 17 and complex formation may drive the reaction in the biosynthetic direction.

Dihydroorotase (DHOase)$^1$ (EC 3.5.2.3) catalyzes the reversible cyclization of carbamoyl aspartate (c-Asp) to form dihydromorotate (DHO), an intermediate in de novo pyrimidine biosynthesis (1, 2).

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\text{Carbamoyl aspartate} \rightarrow \text{Dihydroorotate}
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DHOases from different species constitute a highly polymorphic family of enzymes (3). Early studies (1, 4, 5) of several bacterial DHOases showed that zinc is essential for catalytic activity. Escherichia coli DHOase, a homodimer comprised of 38-kDa subunits (1), is a monofunctional protein that does not associate with any of the other enzymes in the de novo pyrimidine biosynthetic pathway. The x-ray structure of E. coli DHOase (6), solved to a resolution of 1.7 Å, showed that the tertiary fold is reminiscent of phosphotriesterase, another member of the amidohydrolase superfamily, and that the active site consists of a binuclear metal center in which two zinc ions are bridged by carboxyllycine.

The mammalian DHOase activity is catalyzed by a domain of CAD, a multifunctional protein (7, 8) that also has carbamoyl-phosphate synthetase (CPSase) and aspartate transcarbamoylase (ATCase) activities (Fig. 1) and thus, catalyzes the first three steps in the de novo pyrimidine biosynthetic pathway. A 44-kDa catalytically active CAD fragment (9), isolated from controlled proteolytic digests, has kinetic parameters that are close to the values obtained for the DHOase activity of the parent molecule. The isolated domain exists in a concentration dependent equilibrium of catalytically active monomers and dimers and contains 1 equivalent of tightly bound zinc per monomer. The mammalian domain has the zinc binding signature motif (8, 10), HV/LHL, common to the E. coli enzyme and all other DHOases. Initial attempts to clone the mammalian DHOase domain by choosing domain junctions corresponding to the ends of E. coli DHOase, yielded an inactive protein. It was subsequently found (11–13) that an extension of at least 10 amino acids on the carboxyl end was required for catalytic activity of the recombinant protein.

Interestingly, the bifunctional CAD homolog (Fig. 1) encoded by the yeast ura2 locus has CPSase and ATCase activities, but lacks DHOase activity (14). Sequence analysis (15) showed that the polypeptide contains a domain that is homologous to CAD DHOase, but is inactive due in part to the missing metal binding signature sequence. This type of pseudo-dihydroorotase/amidotransferase or glutaminase domain; TCEP, Tris-2-carboxyethylphosphine hydrochloride; MES, 4-morpholineethanesulfonic acid.

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$^1$ The abbreviations used are: DHOase, dihydroorotase; ATCase, aspartate transcarbamoylase; CPSase, carbamoyl-phosphate synthetase; CPS.A and CPS.B, the CPSase synthetase subunits or domains; GLN, glutamine; CPS.A and CPS.B, the CPSase synthetase subunits or domains; TCEP, Tris-2-carboxyethylphosphine hydrochloride; MES, 4-morpholineethanesulfonic acid.
The enzymes that initiate pyrimidine biosynthesis in *Aquifex aeolicus*, an extreme hyperthermophile occupying the deepest branch of the eubacterial phylogenetic tree, have an unusual structural organization (Fig. 1). The CPSase (21) is a heterotrimeric protein consisting of one copy of an amidotransferase subunit (GLN) and two synthetase subunits (CPS.A and CPS.B), whereas ATCase (22) has been isolated as an active homotrimer. The DHOase from *A. aeolicus* has been classified (3) on the basis of its size and sequence as a type I DHOase. Compared with the *E. coli* enzyme, *A. aeolicus* DHOase is 44 residues longer on the amino end, 7 residues longer on the carboxyl end, has only 13% sequence identity and contains several large insertions and deletions (Fig. 1). *A. aeolicus* DHOase has been cloned and expressed for structural studies (23). Although an analysis of its sequence shows that it has the zinc binding signature motif and that all of the other residues involved in catalysis and substrate binding are conserved, it completely lacks catalytic activity. However, we found that a stoichiometric complex of *A. aeolicus* DHOase and ATCase could be reconstituted and was fully active.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents including aspartate, carbamoyl aspartate, dihydroorotate, antipyrine, diacetyl monoxime, triethanolamine, spectinomycin, and cross-linked phosphorylase b standards were all obtained from Sigma; dimethyl suberimidate was from Pierce; Sephacyr S-300 High Resolution was from Amersham Biosciences. Gel-Bond PAG sheets were obtained from FMC BioProducts. The bacterial strain, BL21(DE3), used for expression of recombinant proteins was obtained from Stratagene.

**Expression and Isolation of the Recombinant Proteins**—The 1.3-kb *pyrC* gene encoding *A. aeolicus* DHOase was cloned into pRSET (Invitrogen), an expression vector that incorporates a His tag on the amino end of the recombinant protein. The resulting construct, pAapyrC (23), was co-transformed into the *E. coli* strain BL21(DE3), along with the helper plasmid pSJS1240 (24). The recombinant protein was purified by Ni²⁺ affinity chromatography (23). *A. aeolicus* ATCase (22) and the CPSase GLN, CPS.A, and CPS.B subunits (21) were expressed and purified as previously described.

**Enzyme Assays**—The ATCase activity was assayed by measuring the time dependent formation of carbamoyl aspartate using the colorimetric procedure of Prescott and Jones (25, 26). The assay mixture consisted of 2 mM aspartate, 5 mM carbamoyl phosphate, and 50 mM Tris acetate buffer, pH 8.3, in a total volume of 0.30 ml. Following pre-
equilibrated at 75 °C for 1.5 min, the reaction was initiated by the addition of typically 4 μg of purified ATCase. After 1.5 min, the assay was quenched with 1 ml of the antipyrine/diacetyl monoxime reagent. DHOase activity was measured in the reverse direction, the formation of carbamoyl aspartate from dihydroorotate, because the equilibrium strongly favors dihydroorotate hydrolysis (27) under these conditions. The formation of carbamoyl aspartate was measured using the same colorimetric method. The assay mixture, consisting of 45 μg of DHOase and, where indicated, a stoichiometric amount of ATCase or CPSase or both, in 50 mM Tris acetate, pH 8.3, 10% glycerol, was preincubated for 1.5 min at 75 °C. The reaction was initiated by the addition of dihydroorotate (8 mM or variable) and quenched after 1.5 min by the addition of 1 ml of the diacetyl monoxime/antipyrine reagent.

**Chemical Cross-linking**—The reaction mixture consisted of 30 μg of protein in 100 mM triethanolamine, pH 8.5, 200 mM NaCl and either 1 or 10 mM dimethyl suberimidate in a final volume of 40 μl. The reaction was initiated by the addition of dimethyl suberimidate, allowed to proceed for various periods of time at 23 °C, and quenched by the addition of 10 mM of 1 M Tris-HCl, pH 8.0. The reaction mixture was analyzed by SDS-polyacrylamide gel electrophoresis on 4–20% gradient gels as described by Laemmli (28). To determine the subunit structure of the larger species, the reaction mixture was fractionated on a 3.5% polyacrylamide gel in 100 mM sodium phosphate, pH 7, 1% SDS as described by Weber and Osborn (29) and Davies and Stark (30). No stacking gel was used and “gel-bond PAG” sheets were employed while casting the gels to provide additional support. The glass plate was silanized with Rain-X®. The gel was stored overnight at 4 °C to ensure complete polymerization. The samples were preincubated at 37 °C for 5 min in sample buffer containing 100 mM sodium phosphate, pH 7, 1% SDS, 1% β-mercaptoethanol, 0.015% bromophenol blue, and 6 M urea and loaded on the gel. The electrophoresis was carried out for 5 h at a constant current of 50 mA/gel using 33 mM sodium phosphate as running buffer. Cross-linked phosphorylase b served as the molecular weight markers. Six bands were detected for the standard, ranging from monomer (97.4 kDa) to hexamer (584 kDa). The Coomassie-stained gels were quantitated by scanning with a HP ScanJet 4c and the running buffer. Cross-linked phosphorylase b was stained with silver. A 1-ml sample, 7–10 mg/ml protein, was applied and the column was eluted with the same buffer at a flow rate of 1 ml/min. Fractions were collected and analyzed by measuring the absorbance at 280 nm and by enzyme assays. The columns were calibrated with bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa), and blue dextran or with myoglobin (17 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), ovalbumin (44 kDa), bovine γ-globulin (158 kDa), thyroglobulin (670 kDa), and blue dextran.

**Gel Filtration Chromatography**—Molecular mass estimates were obtained by gel filtration chromatography on either a 1.5 × 25 or 1.5 × 120-cm Sephacryl S-300 high resolution column equilibrated with 50 mM Tris acetate buffer, pH 8.35, 200 mM sodium chloride, and 10% glycerol. A 1-ml sample, 7–10 mg/ml protein, was applied and the column was eluted with the same buffer at a flow rate of 1 ml/min.

**RESULTS**

**Characterization of A. aeolicus DHOase—** A. aeolicus DHOase was isolated from E. coli strain BL21(DE3) transformed with the plasmid pAapyrC (23). Although a significant fraction of the protein was found in inclusion bodies, the soluble protein was expressed at high levels, 100 mg/liter, and could be isolated in a single step by Ni2+ affinity chromatography. Purified DHOase was homogeneous and could be readily crystallized (23). Although the gene sequence was confirmed to be free of errors by DNA sequencing, the recombinant protein had no detectable catalytic activity (Table I). The enzyme was assayed under a variety of conditions; pH ranging from 7.5 to 9.0, substrate concentrations from 10 mM to 100 mM, temperatures from 37 to 75 °C, and 0.01–1 mM buffer concentration. Only trace catalytic activity was ever observed, e.g. a 30-min assay of 93 μg of DHOase at 60 °C gave a specific activity of 0.002 nmol/min/mg.

Metal ion analysis indicated that the protein contained 0.8 mol eq of zinc (23), not two as observed in the E. coli DHOase x-ray structure (6). Prior to metal analysis, DHOase was exhaustively dialyzed against EDTA, so it is possible that a second loosely bound metal ion was lost during dialysis. However, the DHOase activity was not recovered upon the addition of 0.2 mM zinc chloride. This result indicated that other metals, including Co2+, Ni2+, Mn2+, Mg2+, Cd2+, and Pb2+ represented less than 0.01 mol/mmol of DHOase. The addition of 0.2 mM Cu2+, Ni2+, Mn2+, and Ca2+ had no significant effect, although a slight activation was observed at high concentrations (10 mM) of some of the divalent cations (Table II). Monovalent cations are known to be essential for the catalytic activity of N-methylhydantoin amidohydrolase (33), a closely related enzyme. However, K+, Cs+, and NH4+ had no effect on the DHOase catalytic activity (Table II).

Unexpectedly, the inclusion of 1:1 stoichiometric amounts of

### Table I

| Protein       | Activity*      | μmol/min |
|---------------|----------------|---------|
| DHO           | None           | 1.58    |
| DHO, BSA      | None           | 0.012   |
| DHO, GLN, CPS,A, CPS,B, ATC | 0.44           |
| DHO, GLN, CPS,A, CPS,B, ATC, β-mercaptoethanol | <0.02        |

* 45 μg of A. aeolicus DHOase, with or without the addition of a stoichiometric amount of the other proteins and 10 mM β-mercaptoethanol was assayed at 75 °C as described under “Experimental Procedures.”

### Table II

| Protein Cation* | DHOase activity | % |
|----------------|-----------------|---|
| DHO-ATC        | None            | 100 |
| DHO            | None            | 0.79 |

* The concentrations of Zn2+, Co2+, Pb2+, Ni2+, Fe2+, Ca2+, Mg2+, and Mn2+ were 10 mM, whereas the effect of K+, Cs+, and NH4+ was measured at both 10 and 100 mM.
purified *A. aeolicus* ATCase and the CPSase subunits, GLN, CPS.A, and CPS.B, to the assay restored the catalytic activity of the DHOase (Table I). It was possible that the other proteins may have nonspecifically stabilized the DHOase structure. However, catalytic activity was not recovered when the assay was conducted in the presence of bovine serum albumin, suggesting that one or more of the *A. aeolicus* enzymes form a specific complex with DHOase. Like the *E. coli* enzyme (1), the DHOase activity of the putative complex was inhibited by β-mercaptoethanol.

As described below, *A. aeolicus* DHOase was stable at temperatures near 100 °C. However, previous studies (21) showed that the GLN-CPS.A-CPS.B complex was denatured above 80 °C, so that a temperature of 75 °C was chosen for routine assays. The substrate, dihydorotate was also verified to be stable at temperatures up to 100 °C (data not shown). A dihydorotate saturation curve (not shown) carried out at 75 °C in the presence of stoichiometric amounts of GLN, CPS.A, CPS.B, and ATCase was hyperbolic. A non-linear least squares fit of the saturation curve to the Michaelis-Menten equation gave a *Km* of 3.03 ± 0.05 μM and a *Vmax* of 17.1 ± 0.04 μmol/min/mg of DHOase, corresponding to a *kcat* for the putative DHO synthesizing complex of 13.8 ± 0.03 s⁻¹.

**Identification of the Complex**—Titrations were carried out (Fig. 2A) to determine which of the other proteins interact with DHOase to confer catalytic activity. A fixed concentration of DHOase was titrated with an equimolar mixture of the CPSase subunits, GLN, CPS.A, CPS.B, and ATCase. The DHOase activity linearly increased and then leveled off as the DHOase became saturated. The end point corresponded to a molar ratio of 1 to 1.2, suggesting that a stoichiometric complex was formed. A similar titration of the DHOase with ATCase alone also showed a recovery of DHOase activity. The end point corresponded to a 1 to 1.2 complex of DHOase and ATCase, but in this case, the increase in activity was somewhat more gradual suggesting that the CPSase subunits may enhance the stability of the ATCase-DHOase complex. In contrast, the presence of the CPSase subunits alone had no effect on the DHOase catalytic activity. This experiment indicated that ATCase alone specifically activates DHOase.

The isolated recombinant ATCase is fully active and forms transient complexes with the GLN-CPS.A-CPS.B (22). To determine whether DHOase or CPSase influences the function of the *A. aeolicus* ATCase, a similar series of titrations (Fig. 2B) were carried out holding the ATCase concentration constant. Neither DHOase, CPSase, nor an equimolar mixture of the two enzymes had any significant effect on the ATCase activity.

**Oligomeric Structure of *A. aeolicus* DHOase**—Under denaturing conditions, DHOase had an estimated molecular mass of 49 kDa, in good agreement with the calculated mass deduced from the amino acid sequence when the molecular mass of the His tag is taken into consideration. Gel filtration chromatography of purified DHOase on a calibrated Sephacryl S-300 column showed that the protein eluted as a single symmetrical peak (Fig. 3A) with a calculated molecular mass of 53 kDa. The fractions constituting the peak were found to be catalytically active in the presence of excess purified *A. aeolicus* ATCase added to the assay, but had no activity when the ATCase was omitted from the assay mixture. Thus, in contrast to the homodimeric *E. coli* enzyme (1), the *A. aeolicus* DHOase is a monomer.

This interpretation was confirmed by chemical cross-linking of the protein with dimethyl suberimidate (Fig. 3B). The monomer was the only species observed throughout the course of the reaction. No higher oligomeric structures of DHOase were observed even upon incubation with 10 mM dimethyl suberimidate.
The Sephacryl S-300 column was calibrated with standard proteins and the elution volume of the DHOase/H18528ATCase complex gave a molecular mass of 386 kDa. Because ATCase forms stable trimers, we anticipated based on its composition, that the oligomeric structure would correspond to either a 258-kDa hetero-hexamer, \((\text{ATCase})_3(\text{DHOase})_3\) or possibly a 516-kDa duodecamer, \(((\text{ATCase})_3(\text{DHOase})_3)_2\). Interestingly, the observed molecular mass was midway between that of the hexameric and duodecameric forms, suggesting that both complexes were present in rapid equilibrium.

**Oligomeric Structure of the DHOase/ATCase Complex**—Chemical cross-linking was used to further explore the size of the DHOase/ATCase complex. The cross-linked proteins were analyzed by SDS-gel electrophoresis on 4–20% polyacrylamide gradient gels (Fig. 5A). As the reaction proceeded, there was a gradual and simultaneous decrease in the DHOase and ATCase monomers accompanied by the accumulation of two high molecular mass species in the range of 250–550 kDa. The relative amount of the two high molecular weight species showed that the lower molecular weight oligomer appeared first and was gradually converted to the larger species. No ATCase dimers or trimers, as observed in Fig. 3B, were present indicating that the interactions between the DHOase and ATCase are as strong or stronger than the interactions between the ATCase monomers in the trimer.

As it was difficult to accurately estimate the molecular mass of the higher oligomers using this gel electrophoresis system, the reaction mixtures were also analyzed on 3.5% SDS gels (Fig. 5B) calibrated with a mixture of cross-linked phosphorylase b multimers ranging in mass from the 97-kDa monomer to the 584-kDa hexamer. Using this system, the molecular mass of the two higher oligomers was found (Fig. 5C) to be 277 and 517 kDa in good agreement with the calculated mass of the
Thermostability of the DHOase/ATCase Complex—The thermostability of the complex and its components was assessed by incubating the proteins for 10 min at temperatures ranging from 30 to 99 °C, cooling the reaction mixture and then assaying the DHOase and ATCase activities at 37 °C (Fig. 6). As reported previously (22), preincubation of ATCase at elevated temperatures above 60 °C results in a slight increase in enzyme activity that was followed by a precipitous drop above 85 °C. The DHOase activity of the isolated subunit was stable at higher temperatures, but decreased by 90% at 99 °C. In contrast, both activities of the DHOase/ATCase complex remained stable even at 99 °C, indicating that the association of the subunits stabilizes both components.

Activation of DHOase—It was possible that the isolated DHOase domain was improperly folded or unstable. In this event, the activation of the protein upon association with ATCase may result from a stabilization of the active conformation. CD spectrometry of the isolated A. aeolicus DHOase and ATCase (Fig. 7A) indicated that both subunits had a well-defined secondary structure. The CD spectrum of the DHOase/ATCase complex was intermediate between that of the isolated (ATCase)_3(DHOase)_3 hexamer and the ((ATC)_3(DHOase)_3)_2 duodecamer.

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DHOase is monomeric. Whereas most monofunctional DHO-ATCase complexes are dimeric, the enzymes from the parasitic protozoa, *Plasmodium berghei* and *Crithidia fasciculata* (34), were also found to be monomers. The 49-kDa recombinant protein contained a single zinc ion and had no catalytic activity despite the fact that an exhaustive search of the *A. aeolicus* genome revealed no other gene encoding a DHOase that could participate in the *de novo* pyrimidine biosynthetic pathway. Incubating purified DHOase with zinc and other metal ions did not restore enzymatic activity. However, we report here that *A. aeolicus* DHOase forms a 1:1 stoichiometric complex with ATCase. The complex was found to exist as an equilibrium mixture of (ATCase)₃(DHOase)₂ hexamers and (ATC)₃(DHOase)₃₂ duodecamers. Significantly, the DHOase component, as well as the ATCase, in the complex was catalytically active.

The active sites of all known ATCases are comprised of residues from adjacent subunits in the catalytic trimer and thus, ATCase monomers are invariably inactive (35). In the scheme (Fig. 8) depicting the assembly of the DHOase-ATCase complex, inactive ATCase monomers associate to form an active trimer. The inactive DHOase monomers then associate with the ATCase trimer to form a heterohexameric complex that has both catalytic activities. Functional studies (22) showed that *A. aeolicus* DHOase forms a 1:1 stoichiometric complex with ATCase. The complex was found to exist as an equilibrium mixture of (ATCase)₃(DHOase)₂ hexamers and (ATC)₃(DHOase)₃₂ duodecamers. Significantly, the DHOase component, as well as the ATCase, in the complex was catalytically active.

**DISCUSSION**

*A. aeolicus* DHOase was cloned and expressed in *E. coli* and could be purified to homogeneity in a single step (23). Gel filtration and chemical cross-linking showed that the isolated DHOase is monomeric. Whereas most monofunctional DHO-
ATCase activity. In contrast, the DHOase subunit has no effect on the first three steps of the pathway. One strategy for driving the carbamoyl aspartate to dihydroorotate is 17 (16). Thus, the consequences. At physiological pH, the equilibrium ratio of carbamoyl aspartate to dihydroorotate is 17 (16). Thus, the active sites in the DHOase complex from Streptomyces griseus and Mycobacterium tuberculosis (38). Thus far, the enzymes from 22 organisms have been assigned as type IA enzymes although only a few have been biochemically characterized, so it is possible that other enzymes in this subgroup will also have to be reclassified. The results of this study also requires reclassification of A. aeolicus ATCase. ATCases have been designated as Class A, B, or C depending on their size, subunit composition, and regulatory properties (39). The A. aeolicus ATCase has been considered a Class C enzyme, an unregulated trimer like the B. subtilis ATCase. The finding that it associates to form a 1:1 duodecameric complex with DHOase indicates that it should be reclassified with the 450–500-kDa Class A enzymes.

One functional advantage of the association of DHOase and ATCase is that it confers stability upon both component enzymes at the elevated temperatures at which A. aeolicus flourishes. The most intriguing observation is that the association of the A. aeolicus DHOase with the ATCase serves as a molecular switch that activates the former enzyme probably by inducing an active conformation of the enzyme. Activation is not a consequence of a major reorganization of the secondary structure that could be detected by CD spectroscopy. However, the visible absorption spectrum of Co2+/DHOase is clearly perturbed upon complex formation suggesting a change in the electronic environment of the metal ion, perhaps in coordination number or the nature of the metal binding ligands. This interpretation is supported by the 1.6-A x-ray structure determination of isolated A. aeolicus DHOase showing that the active site is occluded by a loop that spans the substrate binding cleft and interacts with the active site zinc (23). In contrast, access to the active site of E. coli DHOase is unimpeded (6). A plausible hypothesis is that the association with ATCase displaces the loop rendering the active DHOase active site accessible. This sort of functional interaction between DHOase and ATCase has not been previously observed and it is not known whether a similar switch mechanism occurs in the 450–500-kDa duodecameric complex of other type IC enzymes (38).

Interestingly, there is evidence that the association of the inactive pseudo-DHOase homolog with ATCase in P. putida (20) and P. aeruginosa (19, 20) may be necessary to preserve the ATCase activity. In contrast, the DHOase subunit has no effect on the ATCase activity of the A. aeolicus complex.

The observation that DHOase is active only when associated in a complex with ATCase may have important physiological consequences. At physiological pH, the equilibrium ratio of carbamoyl aspartate to dihydroorotate is 17 (16). Thus, the intermediate, carbamoyl aspartate, is the major product of the first three steps of the pathway. One strategy for driving the reaction in the biosynthetic direction, probably operative in E. coli and other prokaryotes, is to functionally couple the active, uncomplexed DHOase to the next enzyme in the reaction sequence, dihydroorotate dehydrogenase. A. aeolicus may employ an alternative strategy. If the active sites in the ATCase-DHOase complex are in close proximity, the transfer of the intermediate would be facilitated and the reaction would be driven forward, because the effective “concentration” of the carbamoyl aspartate near the DHOase active site would be much higher than could be achieved if the intermediate is released from the complex and transferred to the ATCase active site by simple diffusion. Moreover, a slight excess of uncomplexed, active DHOase in the cell would convert much of the dihydroorotate formed by the complex back to carbamoyl aspartate. The degradative reaction could not occur if free, uncomplexed DHOase is inactive. In this regard, it is interesting that the k_diss = 13.8 ± 0.3 s⁻¹ for the A. aeolicus enzyme is close to the value reported (40) for mammalian CAD, 6.1 ± 0.4 s⁻¹. However, with a Kₐ for dihydroorotate of 3.03 mM, the apparent affinity of A. aeolicus DHOase for this substrate is 433-fold lower than the mammalian enzyme (Kₐ = 7.38 ± 0.4 μM). The low affinity of the free DHOase for the substrate provides an additional means of ensuring that the free enzyme is inactive. The association of ATCase and DHOase in the complex with the active sites in close proximity could compensate for weak substrate binding.

In summary, although A. aeolicus DHOase has a single zinc ion and a well defined tertiary structure, it lacks catalytic activity. Association with A. aeolicus ATCase induces changes in the metal ion site and fully activates the enzyme. The functional consequences of the association of ATCase and DHOase into active hexameric or duodecameric complexes were shown for the first time to protect the constituent enzyme activities from thermal degradation, to promote or stabilize the active conformation of DHOase, and perhaps to facilitate the transfer of the intermediate between catalytic sites.

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