Structural similarity between ornithine and aspartate transcarbamoylases of *Escherichia coli*: Characterization of the active site and evidence for an interdomain carboxy-terminal helix in ornithine transcarbamoylase

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Abstract

Predictions of tertiary structures of proteins from their amino acid sequences are facilitated greatly when the structures of homologous proteins are known. On this basis, structural features of *Escherichia coli* ornithine transcarbamoylase (OTCase) were investigated by site-directed mutagenesis experiments based on the known tertiary structure of the catalytic (c) chain of *E. coli* aspartate transcarbamoylase (ATCase). In ATCase, each c chain is composed of two globular domains connected by two interdomain helices, one of which is near the C-terminus and is critical for the in vivo folding of the chains and their assembly into trimers. Each active site is located at the interface between two chains and requires the participation of residues from each of the adjacent chains. OTCase, a trimeric enzyme, has been proposed to be similar in structure to the ATCase trimer on the basis of sequence identity (32%), the nature of the reaction catalyzed by the enzyme, and secondary structure predictions. As shown here, analysis of OTCase and ATCase sequences revealed extensive evolutionary conservation in portions corresponding to the ATCase active site and the C-terminal helix. Truncations and substitutions within the predicted C-terminal helix of OTCase had effects on activity and thermal stability strikingly similar to those caused by analogous alterations in ATCase. Similarly, substitutions at either of two conserved residues, Ser 55 and Lys 86, in the proposed active site of OTCase had deleterious effects parallel to those caused by the analogous ATCase substitutions. Hybrid trimers comprised of chains from both these relatively inactive OTCase mutants exhibited dramatically increased activity, as predicted for shared active sites located at the chain interfaces. These results strongly support the hypothesis that the tertiary and quaternary structures of the two enzymes are similar.

Keywords: C-terminal helix; homology; shared active sites; structure prediction

Predicting the three-dimensional structure of a folded protein from its amino acid sequence remains an extremely difficult problem despite extensive research. However, methods that rely on correlations among known structures of a family of homol-

ogous proteins have been fruitful consistently, though limited in scope due to the requirement for known homologues (Chothia & Lesk, 1986). Here, we have used a homology-based approach to investigate the as yet undetermined tertiary structure of *Escherichia coli* ornithine transcarbamoylase (OTCase, carbamoyl:L-ornithine carbamoyltransferase, EC 2.1.3.3), using the known structure of *E. coli* aspartate transcarbamoylase (ATCase, carbamoyl phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) catalytic trimer as a model.

Several structures of *E. coli* ATCase, which catalyzes the first committed step in pyrimidine biosynthesis, have been solved through X-ray crystallography (for review, see Schachman, 1987; Kantrowitz & Lipscomb, 1990; Stevens et al., 1991). The holoenzyme is composed of two catalytic trimers (encoded by *pyrB*) interconnected largely by three regulatory dimers (encoded by *pyrI*). Active trimers are formed readily in the absence of regulatory chains both in vivo and in vitro (Gerhart & Schachman,

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Abbreviations: ATCase, aspartate transcarbamoylase; BSA, bovine serum albumin; c chain, ATCase catalytic polypeptide chain; CbmP, carbamoyl phosphate; CTR, C-terminal region (defined as residues 312–333 of OTCase and as residues 285–310 of ATCase); MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OTCase, ornithine transcarbamoylase; PALA *N*-phosphonacetyl-L-aspartate; PALO, δ -*N*-phosphonacetyl-L-ornithine; PMSF, phenylmethylsulfonyl fluoride.

1965; Yang et al., 1978). Each 310-residue catalytic chain is organized into two globular domains with the first domain implicated in the binding of carbamoyl phosphate and the second in the binding of aspartate (Stevens et al., 1991). The active sites of the enzyme are located at the interfaces between chains such that residues from two adjacent c chains are required for the formation of one active site (Robey & Schachman, 1985; Wentz & Schachman, 1987). *E. coli* OTCase (encoded by *argI*) is a trimer of 333-residue polypeptides and serves to catalyze the condensation of CbmP and ornithine for the sixth step in arginine biosynthesis (Piette et al., 1982; Van Vliet et al., 1984).

ATCase² and OTCase of *E. coli* have been postulated to be homologous enzymes with similar tertiary structures (Houghton et al., 1984; Houghton, 1986; Murata, 1994). This proposal is supported by several findings. (1) *argI* and *pyrB* are neighbors on the *E. coli* chromosome, suggesting the past occurrence of tandem duplication and divergence from an ancestral gene (Legrain et al., 1972; Gigot et al., 1977; Van Vliet et al., 1984). (2) The amino acid sequences of ATCase and OTCase are 25–40% identical if gaps are allowed in the alignment, as first shown by Wild and coworkers (Bencini et al., 1983; Hoover et al., 1983; Houghton et al., 1984; Murata, 1994). (3) Both enzymes require a trimeric form for activity. (4) Each catalyzes the carbamoylation of an amino acid using CbmP as the donor. (5) Several of the ATCase residues implicated in substrate interactions by crystallographic studies appear to be present at an equivalent position of OTCase (Lerner & Switzer, 1986). (6) The identity between chains is highest for the N-terminal domain, which in ATCase is involved in binding the common substrate CbmP. (7) Considerable similarity is also observed for a region of approximately 20 amino acids near the C-terminus, which in ATCase forms the critically important structural element Helix 12 (Peterson & Schachman, 1991). (8) The length discrepancy between the two enzymes may be accounted for in surface loops of varying length (Houghton et al., 1984; Murata, 1994). (9) Secondary structure predictions for each enzyme yield almost identical results (Houghton et al., 1984).

Sequence comparisons of ATCases from nine species show extensive conservation (Lerner & Switzer, 1986; Murata, 1994). OTCases vary in quaternary structures with anabolic OTCases appearing to be trimeric, whereas most catabolic OTCases appear to be hexameric or nanomeric (Marshall & Cohen, 1972; Legrain et al., 1977). Despite these differences, OTCase sequences exhibit extensive conservation (Murata, 1994). Wild and coworkers first proposed an OTCase/ATCase sequence alignment (Bencini et al., 1983; Hoover et al., 1983; Houghton et al., 1984). Since that time, more sequences have been determined (Schachman et al., 1984; Kuo et al., 1988) and the results available from the Swiss Databank for the wild-type enzymes lead to the alignment shown in Figure 1. This alignment yields an overall sequence identity of 32% and contains 18 gaps and insertions, which appear primarily in the regions corresponding to the surface turns, loops, and coils of ATCase (Honzatko et al., 1982).

The evidence for similar structures for ATCase and OTCase indicated that a homology-based approach would be of use in investigating OTCase structure. Moreover, the comparative analysis of OTCase and ATCase sequences shown in Figure 1

OTCase	1	.SGFYHKHFLKLLDFTPAELNLLQAAKLKADKKSGKEEAKLTGKNIALIFEKSTRTR	59
ATCase	1	ANPLYQKHIIISINDLSRDDNLVLATAAKLKANPQPE...LLKHVIASCFEASTRTR	56
OTCase	60	CSFEVAAVDQGARVTYLGPSG.SQIGHK.ESIKDTARVLGRMYDGIQYRGYQGEIVETLA	117
ATCase	57	LSFETSMHRLGASVVGFSANTSLSGKKGETLADTISVISTYVDAIVMR.HPQEGAARLA	115
OTCase	118	.EY.ASPVVMN.GLTNEFHPTQLLADLLTMQEHLPKGAFTNEMTLVYAGDARNMNG.NSML	173
ATCase	116	TEFSGNVPVLNAGDGSNQHPQTLLDLFTIQE.TQGR.LDNLHVAMVGDLYGRVHSLT	173
OTCase	174	EA.AALTGLDLRLVAPQACWPEAALVTECRALAQONGNITLTEDVAKGVEGADFIY.TD	231
ATCase	174	QALAKFDGNRFYFIAPDA...LAMPQYILMDLDEKIAWSLHSSIEEVMVEDILYMR	229
OTCase	232	VWMSGAEKKEWAERIA..LLREYQVNSKMMQLTGNPEVKFLHCLPAFHDDQTTLGKMA	289
ATCase	230	VQKERLDPSE.YANVKAQFVLRASDLHN...AKANMKVLPRLPRVDEIATDVOK...	279
OTCase	290	EEFGLHGGMEVTDEVFESAASIVFDQAEINRMHTIKAVMVTLSK....	333
ATCase	280	TPHAM.....YFQQAGNGIFARQALLALVLRDLVL	310

Fig. 1. Sequence alignment of *E. coli* ATCase and OTCase. Residues of particular interest in this work are designated by asterisks. Sequences from the Swiss Databank for *pyrB* and *argI* (PYRB_ECOLI and OTC1_ECOLI, respectively) were aligned using the GAP program of GCG (Devereux et al., 1984) with the scoring table NWSGAPPE.CMP. The resultant alignment has 18 gaps, 32% identity, and 56% similarity.

led to the identification of some potentially important amino acid residues in OTCase. Two putative active site residues, Ser 55 and Lys 86, were identified, and hybridization studies using mutant proteins with substitutions at these positions provided evidence for localizing the active sites at the chain interfaces. From these studies, we propose a model for the OTCase active site analogous to that of the ATCase active site. Through mutagenesis studies involving the C-terminal region, we also find strong evidence for the existence of a C-terminal α -helix of critical importance for the structural integrity of the enzyme. Thus, OTCase appears to be similar to ATCase in its overall folding pattern.

Results and discussion

Cloning and expression of wild-type and mutant OTCases

Putative OTCase active site mutants (S55H³ and K86Q) and mutants with truncations or substitutions in the CTR (325 Δ ⁴, 327 Δ , 329 Δ , 331 Δ , R319A, and A325G) were made by oligonucleotide-directed mutagenesis on an M13mp19-derived template containing *argI* or its specifically mutagenized derivatives (Kunkel et al., 1987). The wild-type or mutant proteins were expressed in a two-plasmid T7 RNA polymerase expression system (Tabor & Richardson, 1985) in the *E. coli* chromosomal ATCase/OTCase deletion strain TB-2 (Roof et al., 1982). This expression system allowed reliable overexpression of wild-type OTCase, with significantly increased yields relative to earlier work (Kuo et al., 1982), and also allowed expression of mutant proteins in good yield in an OTCase-free background.

³ Mutant proteins are designated by the standard notation using the one-letter amino acid code. The wild-type residue is given followed by its numbered position in the polypeptide sequence and the amino acid substitution. Thus, the replacement of Ser at position 55 by His is referred to as S55H.

⁴ The truncation of the polypeptide chain is designated by " Δ ;" thus, the 325 Δ enzyme is 325 residues in length due to the introduction of a stop codon at position 326.

² For the remainder of this report, "ATCase" refers to the catalytic trimer of ATCase rather than the holoenzyme, unless otherwise noted.

Wild-type and mutant proteins were purified to homogeneity, as shown by a single band on Coomassie-stained nondenaturing polyacrylamide gels, with the exception of the K86Q enzyme, for which additional minor bands were detected. Approximately 20 mg of wild-type enzyme were isolated per liter of cell culture. All mutants that were detectable by *in vivo* activity or gel electrophoresis were also purified in good yield; S55H, R319A, and 331Δ enzymes yielded about 4 mg/L of cell culture and the K86Q and A325G enzymes yielded 25 and 10 mg/L, respectively.

Properties of wild-type and mutant OTCases

On the basis of the comparison between OTCases and ATCase, we constructed two classes of OTCases mutants. In one class, putative active site residues Ser 55 and Lys 86 were modified in order to determine whether they were implicated in catalysis, as are Ser 52 and Lys 84 of ATCase (Wente & Schachman, 1987; Hsieh, 1990; Xu & Kantrowitz, 1991). In the second class, the CTR was modified to determine whether OTCases, like ATCase, had an essential C-terminal helix and some disposable C-terminal residues (Peterson & Schachman, 1991).

As shown in Table 1, the S55H and K86Q mutant proteins were much less active than the wild-type enzyme. Both mutants exhibited hyperbolic kinetics with respect to ornithine concentration and the values of $K_m(\text{ornithine})$ were only slightly greater than that of wild-type OTCases.⁵ Although the specific activity of the K86Q mutant was low (0.8% that of the wild-type enzyme), the gene for this mutant complemented arginine auxotrophy *in vivo*. In contrast, no complementation was observed with the gene encoding the S55H mutant, presumably due to the very low activity of that mutant.

The studies of the CTR of the polypeptide chain involved either the introduction of stop codons to cause deletion of a portion of the polypeptide or the introduction of individual point mutations within the CTR. The genes for the 325Δ, 327Δ, or 329Δ enzymes exhibited no complementation *in vivo*. Moreover, gel electrophoresis of extracts from cells containing genes for these truncated proteins showed no detectable amounts of the corresponding polypeptides. Hence, the loss of complementing activity can be attributed to intracellular proteolysis of the shortened polypeptide chains. Analogous experiments with the 331Δ gene did demonstrate complementing activity *in vivo*, and, moreover, the isolated enzyme exhibited a specific activity and $K_m(\text{ornithine})$ comparable to the wild-type enzyme. Replacing Arg 319 by Ala yielded a mutant enzyme with an activity about 0.02% that of wild-type OTCases. In contrast, the A325G mutant had an activity even greater than the wild-type enzyme and its $K_m(\text{ornithine})$ was increased about twofold.

Differential scanning calorimetry experiments on the wild-type and mutant OTCases yielded broad asymmetric endotherms indicative of intermediates in the melting and dissociation of the

Table 1. Kinetic parameters for wild-type and mutant OTCases^a

Enzyme	K_m^b (mM ornithine)	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$)	Relative activity (%)
Wild-type	0.18	0.29	100
S55H	0.21	0.000041	0.01
K86Q	0.33	0.0023	0.8
R319A	0.25	0.000064	0.02
A325G	0.39	0.38	131
331Δ	0.13	0.32	110

^a Enzymes were assayed at 0.1–100 $\mu\text{g}/\text{mL}$ in the presence of saturating carbamoyl phosphate (2.5–5.0 mM).

^b For the more active enzymes, the determination of K_m is somewhat compromised because data at ornithine concentrations well below K_m may not be taken strictly as initial velocity measurements. At very low ornithine concentrations, up to 25% of the substrate was consumed due to the high concentration of enzyme required to prevent loss of activity at great dilution.

trimers. The endotherms were similar to those observed for ATCase trimers (Vickers et al., 1978; Edge et al., 1985; Peterson & Schachman, 1991; Peterson et al., 1994). Table 2 provides a summary of the data for the wild-type and mutant enzymes. In some mutant proteins, such as K86Q and R319A, the thermal stability was enhanced relative to wild-type OTCases, and in others there was a slight decrease in t_m . For all of the enzymes, the ratio $\Delta H_{vH}/\Delta H_{cal}$ was greater than 3, indicating a complex melting process involving more than two states (Edge et al., 1985). These broad melting curves demonstrate that the heat denaturation involves a complex series of reactions, including some combination of the dissociation of the trimers and a possible sequential melting of the different domains in each chain, leading ultimately to the total unfolding of the chains. In view of this complexity, no attempt was made to deconvolute the thermograms.

Table 2. Calorimetric parameters for wild-type and mutant OTCases^a

Enzyme	t_m^b (°C)	ΔH_{cal}^c (kcal/mol)	ΔH_{vH}^d (kcal/mol)	$\Delta H_{cal}/\Delta H_{vH}$
Wild-type	62.5	600	140	4.3
S55H	62.3	670	90	7.4
K86Q	69.7	690	200	3.5
R319A	69.6	710	130	5.3
A325G	63.0	660	110	6.0
331Δ	58.3	480	150	3.2

^a Calorimetry was performed at pH 8.3.

^b Temperatures corresponding to peaks of thermal transitions.

^c The calorimetric enthalpy measured from the total integrated area of the endotherm. The relative error of this value is approximately 20%.

^d The van't Hoff enthalpy in kcal/mol, equivalent to the enthalpy expected for a two-state thermal denaturation process. The relative error of this value is approximately 10%.

⁵ There was a marked decrease in the specific activity of various OTCases preparations as the concentration was lowered beyond 50 ng/mL (Murata, 1994). Similar behavior was noted previously for ATCase trimers (Porter et al., 1969). In the case of OTCases, the addition of BSA (50 $\mu\text{g}/\text{mL}$) provided no protection. This apparent loss of activity at great dilution was not evident in the studies of Kuo and coworkers, who used a calorimetric assay and reported specific activities (Kuo et al., 1982, 1985) comparable to those in Table 1.

Although OTCase was comparable to ATCase trimers in terms of heat stability, it was clear that OTCase is much more resistant to denaturation by urea than is ATCase. Incubation of mixtures of OTCase and succinylated OTCase in 3 M urea for three weeks did not yield any hybrids (Murata, 1994). In contrast, analogous experiments with ATCase trimers have shown that they readily produce a hybrid set, even in the absence of denaturant, due to the dissociation of the trimers into monomers and their random reassociation (Yang & Schachman, 1987; Peterson & Schachman, 1991).

Active sites are located at the interface between chains and require the participation of Lys 86 and Ser 55 from adjacent chains

Many residues known to be critically important for substrate interactions (binding and/or catalysis) in *E. coli* ATCase are also present in *E. coli* OTCase and are conserved for OTCases in general, as summarized in Table 3. This is illustrated by the strictly conserved ATCase residue Ser 52, which has an identical counterpart, Ser 55, that is also conserved in all OTCases. In ATCase, replacement of Ser 52 with His, Gly, Ala, or Cys reduces enzyme activity dramatically, whereas the Thr substitution has a very small effect on the enzyme (Wente & Schachman, 1987; Hsieh, 1990). These and other results have led to the conclusion that Ser 52 is important in catalysis, probably serving to assist productive binding of CbmP (Hsieh, 1990; Xu & Kantrowitz, 1991). In order to determine whether Ser 55 of OTCase serves a simi-

lar role, we replaced Ser 55 with His. This substitution resulted in a large decrease in activity of the enzyme to about 10^{-3} that of wild-type OTCase (Table 1). $K_m(\text{ornithine})$ was not affected. This result parallels that observed for the S52H mutation in ATCase (Wente & Schachman, 1987). It also correlates with the inability of S55H OTCase to complement arginine auxotrophy in vivo despite its successful overexpression and confirms that this residue is crucial for catalysis.

Lys 84 of ATCase is also strictly conserved and implicated in substrate interactions. Replacement of this residue by Gln or Arg reduces the activity of the trimer by factors of 11,000 and 2,000, respectively (Robey et al., 1986; Wente & Schachman, 1987). A Lys is present in *E. coli* OTCase at position 86, but is conserved for only 5 of 15 OTCases (Table 3). Other residues observed at this position include Asn (for seven of the sequences), and Gly (for two of the sequences). Because this Lys is only partially conserved for OTCase, its requirement for enzyme activity seemed less certain. Nonetheless, we chose to study this residue because the partial conservation of Lys 86 suggested that it may serve some important function and, in ATCase, the Ser 52 and Lys 84 in each active site are located in different polypeptide chains. Replacing Lys 86 by Gln did indeed lead to a marked reduction in activity to about 1% that of wild-type OTCase (Table 1). Although this reduction in activity is not as large as that for the S55H mutation, it is sufficient to indicate that Lys 86 is implicated in catalysis. Examination of OTCase sequence alignments (Murata, 1994) reveals that, although this Lys is not strictly conserved, each sequence has a polar or positively charged residue at position 85 and/or 86 (*E. coli* numbering), suggesting that a residue capable of participating in a hydrogen bond is needed in this region. The Gln substituted at position 86 may be able to participate in a hydrogen bond formerly formed by Lys 86. Alternatively, His 85 may have a sufficiently high pK_a in the K86Q mutant to be positively charged under assay conditions and, as such, may partially compensate for the removal of the positively charged Lys.

Hybridization studies performed with ATCase active site mutants S52H and K84Q have demonstrated that the active sites are located at the interfaces between adjacent polypeptide chains in the trimers; Ser 52 and Lys 84 from adjoining chains are required for formation of a functional active site (Wente & Schachman, 1987; Wente, 1988). If ATCase and OTCase have similar tertiary and quaternary structures, then OTCase would be expected to have shared active sites as well. Because the results indicated that OTCase Ser 55 and Lys 86 are the likely equivalents of ATCase Ser 52 and Lys 84, we performed hybridization experiments to determine if they are participants in a shared active site (Table 4). If so, formation of the four-membered hybrid set from S55H and K86Q parent trimers should result in a dramatic increase in activity. This increase would be due to the formation of one "wild-type" active site in each hybrid resulting from the complementary juxtaposition of the unaffected parts of the polypeptide chains at the interfaces between the S55H and K86Q chains. In contrast, if each active site is wholly contained within one polypeptide chain, then formation of hybrids should not lead to any increase in enzyme activity.

Mixing S55H and K86Q trimers yielded 0.4% the activity of an equivalent amount of wild-type enzyme. This result represents the average of the two mutant enzymes, as expected. When, however, the trimers in the mixture are subjected to dissociation by the addition of GuHCl and then allowed to reform

Table 3. Conservation of residues implicated in substrate interactions for ATCase and OTCase

ATCase residue ^a	Frequency in ATCases ^b	Conserved for OTCase? ^c	OTCase residue ^d	Frequency in OTCases ^c
S52 ^f	9/9	Yes	S55	15/15
R54 ^g	9/9	Yes	R57	15/15
T55 ^h	9/9	Yes	T58	15/15
K84 ⁱ	9/9	Yes	K86	5/15
R105 ^j	9/9	Yes	R106	15/15
H134 ^k	9/9	Yes	H133	15/15
R229 ^l	8/9	No	D231	15/15
Q231 ^m	8/9	No	W233	13/15

^a Identity and position of residue implicated in substrate interactions in *E. coli* ATCase.

^b Proportion of ATCase sequences containing this residue at the equivalent position (Murata, 1994).

^c Presence of this residue at the equivalent position in *E. coli* OTCase (Fig. 1).

^d Identity and position of residue at the equivalent position in *E. coli* OTCase (Fig. 1).

^e Proportion of OTCase sequences containing this residue at the equivalent position (Murata, 1994).

^f Hsieh (1990); Xu and Kantrowitz (1991).

^g Stebbins et al. (1989); Stebbins et al. (1992).

^h Waldrop et al. (1992a).

ⁱ Kempe and Stark (1975); Robey et al. (1986); Wente & Schachman (1987).

^j Stebbins et al. (1989).

^k Kleanthous et al. (1988); Waldrop et al. (1992b).

^l Middleton et al. (1989).

^m Stebbins et al. (1990); Peterson et al. (1992).

Table 4. Kinetic parameters for mixtures and hybrid sets of S55H and K86Q OTCases

	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$)	K_m^a (mM ornithine)	Relative activity (% observed)	Relative activity (% predicted) ^b
Wild-type	0.29	0.18	100	
S55H	0.000041	0.21	0.01	
K86Q	0.0023	0.33	0.8	
S55H/K86Q hybrid set	0.038	0.19	13	25.2
S55H/K86Q mixture	0.0012	0.34	0.4	0.4

^a Given the limited accuracy of these values for K_m (see Table 1), they may be considered to be approximately equivalent.

^b Relative activity predicted for the hypothetical case of shared active sites and random reassociation of chains (see the Discussion). Upon formation of the four-membered hybrid set, the regenerated parent homotrimers S55H and K86Q will retain their original activities of 0.01% and 0.8%, respectively. The hybrid heterotrimer containing one K86Q and two S55H chains will contain one "wild-type" active site and one S55H active site of negligible activity (0.003%), for a total activity of 33.3%. The heterotrimer containing two K86Q and one S55H chain will contain one "wild-type" active site (33.3% activity) and one K86Q active site (0.27%), for a total activity of 33.6%. Formation of the hybrid set in a binomial distribution will result in a total activity for the hybrid set of $[1(0.0001) + 3(0.333) + 3(0.336) + 1(0.008)]/8 = 25.2\%$ of the activity of the wild-type enzyme with three active sites per trimer.

at random, the hybrid set, which was formed from S55H and K86Q trimers, exhibited 13% of the activity of wild-type OTCase. This 32-fold increase in activity over that of the mixture of the two parental mutants constitutes striking evidence for shared active sites. This measured activity for the hybrid set is one-half the value expected for random association of the chains forming perfect shared sites at the interfaces between chains (Table 4).

As seen in Figure 2, the predicted increase in specific activity generated upon formation of the hybrid set between S55H and K86Q chains in the case of shared active sites is directly dependent on the ratio of the parental trimers. Because each hybrid would have one active site per trimer and the optimal formation of hybrid species occurs when an equal number of each type of chain is present and association of chains to form trimers is random, the maximum activity is expected at a 1:1 ratio of parental trimers. Although the yield of recovered activity did not equal that expected for a binomial distribution of species with the two

hybrids each having one wild-type-like active site, the results for the various mixtures show clearly that the maximum relative activity of 13% occurs at a 1:1 ratio of parental trimers (Fig. 2).

Model for substrate binding to OTCase

Crystallographic studies of the ATCase holoenzyme (Honzatko et al., 1982; Kim et al., 1987; Krause et al., 1987; Ke et al., 1988) have provided evidence for the participation of numerous amino acid side chains in the binding of the bisubstrate analogue, PALA. Among these, as illustrated schematically in Figure 3A, are Ser 52, Thr 55, Lys 84, Arg 104, His 134, Arg 229, and Gln 231. On the basis of the site-directed mutagenesis experiments described here, and the conservation of residues in ATCase and OTCase (Table 3), we can ascribe roles for analogous residues in OTCase in the binding of the bisubstrate analogue PALO (Fig. 3B). Those residues thought to be implicated in the binding of the phosphonate part of PALA and PALO are conserved in both ATCase and OTCase.

Whereas Ser 55 in OTCase appears to fulfill the same critical role as Ser 52 in ATCase, Lys 86 seems to be less important in OTCase and is certainly not indispensable. Their respective roles may differ because of the nature of the substrates. In ATCase, Lys 84 is thought to make important contacts with the phosphonate oxygen and both the α - and β -carboxylates of aspartate (Krause et al., 1987). A reduced role for Lys 86 in OTCase could be anticipated because of its bonding to only the phosphonate oxygen and the single carboxylate of the ornithine moiety of PALO.

Other residues that bind to the phosphonate part of PALA, such as Thr 55, Arg 105, and His 134, are highly conserved and have their counterparts in Thr 58, Arg 106, and His 133 in OTCase. Presumably, they are implicated in binding CbmP in both enzymes. In contrast, none of the residues with assigned roles in the binding of aspartate at the active sites of ATCase are conserved in OTCase. Instead of Arg 229, which is conserved in most ATCases and may be involved in an interaction with the β -carboxylate of aspartate (Middleton et al., 1989), there is an Asp at the equivalent position (231) in OTCase that is conserved

Fig. 2. Effect of initial ratio of parent trimers on specific activity of the hybrid set. The initial ratio of S55H to K86Q trimers was varied from 10:1 to 1:10. Hybrid sets were assayed in the presence of 2.5 mM CbmP and 6.0 mM ornithine at pH 8.0. Data points represent averages of initial velocity measurements. The curve represents a fit of the data to a second-order polynomial.

Fig. 3. Models for binding of bisubstrate analogues in the active sites of *E. coli* ATCase and OTCase. Residues believed to be important in catalysis and/or substrate binding are shown with their proposed interactions. The portions of the bisubstrate analogues corresponding to the amino acid substrates are shaded. **A:** Binding of PALA to the active site of ATCase. This scheme is derived from crystallographic studies as well as other experimental results (Gouaux et al., 1987; Krause et al., 1987; Gouaux & Lipscomb, 1988; Kuo et al., 1988; Allewell, 1989; Schachman, 1993). **B:** Our proposal for the interaction of PALO (Hoogenraad, 1978) with the active site of OTCase, derived from the sequence analyses and mutagenesis and hybridization studies described here.

in all OTCases and may interact with the α -amino group of ornithine.

Role of the CTR in OTCase

Each 310-residue c chain within the ATCase holoenzyme has been shown by X-ray diffraction studies (Honzatko et al., 1982) to be folded into two globular domains that are linked by two interdomain α -helices (Fig. 4). The second of these helices, Helix 12, comprises residues 285–305 and extends from the aspartate domain through the CbmP domain, where it is partially buried. The remainder of the c chain (residues 306–310) emerges onto the surface of the CbmP domain as a flexible pentapeptide “tail” that brings the C-terminus of the chain near the N-terminus. The results of previous studies of the ATCase CTR (residues 285–310) are summarized in Table 5. These studies demonstrated that Helix 12 plays an indispensable role in the assembly and stability of the enzyme, but that the “tail” residues and the last residue of the helix are expendable (Peterson & Schachman, 1991, 1992; J. Quan & C.B. Peterson, unpubl. results). There is extensive conservation in this region of the polypeptide chain (Peterson & Schachman, 1991; Murata, 1994), thereby providing additional evidence for the important role of

Fig. 4. Ribbon representation of the α -carbon backbone of an ATCase c chain. The N-terminal CbmP-binding domain is shown on the right and the C-terminal aspartate-binding domain is shown on the left. The CTR (residues 285–310) is shown in black to emphasize the location of Helix 12 and the C-terminal “tail.” The ribbon trace was generated with the program MOLSCRIPT (Kraulis, 1991) on a Silicon Graphics Indigo workstation based on the X-ray structure of the CTP-ligated ATCase holoenzyme (Kim et al., 1987) with the coordinates deposited in the Brookhaven Protein Data Bank (registry number 5AT1).

Helix 12 in all ATCases. A similar conservation of amino acid residues in the CTR's of OTCase has been noted (Houghton et al., 1984; Murata, 1994). Moreover, an analysis of the region from residue 312 to 332, in terms of the propensities of the amino acids for different conformations, indicated that this region would be helical (Houghton et al., 1984). This postulated C-terminal helix in OTCase not only has striking similarities in composition to Helix 12 in ATCase (Fig. 1), but also both would appear to be amphipathic for their N-terminal portion and hydrophobic for their C-terminal region (Murata, 1994).

According to the alignment of the sequences in Figure 1, residues 332–333 in OTCase correspond to the expendable C-terminal residues 305–310 in ATCase. By truncating the OTCase CTR at positions 325, 327, and 329 (Table 5), we have confirmed that the postulated C-terminal helix is crucial for the folding and assembly of this enzyme as well. Loss of the “tail” residue (333) or the last residue of the postulated helix (332) results in a stable active enzyme with kinetic and thermal stability characteristics similar to the wild-type enzyme. These results, summarized in Table 5, precisely parallel the results for truncations of ATCase and support the alignment shown in Figure 1.

Several amino acid substitutions at conserved positions in the CTR of ATCase have been shown to have diverse effects on the stability and activity of the C trimer (Peterson & Schachman, 1991, 1992). As seen in Table 5, analogous substitutions at conserved positions in the CTR of OTCase have similar effects. Ala 298 of ATCase is conserved for 8 of 9 sequences, and the equivalent residue of OTCase, Ala 325, is conserved for 12 of 15 sequences. For both enzymes, replacement of each Ala by Gly causes slight increases in t_m , while not significantly affecting K_m or V_{max} . The increase in thermal stability is particularly notable because Ala is a strong helix former, whereas Gly tends to break helices (Chou & Fasman, 1978).

Table 5. Effects of mutations in the CTR's of OTCCase and the ATCase catalytic trimer

ATCase ^a			OTCCase		
Position	Mutation	Effect ^b	Position ^c	Mutation	Effect ^b
284	STOP	No enzyme	311		N/D
291	STOP	No enzyme	318		N/D
296 ^d	R → A	0.5%; +3 °C	319 ^d	R → A	0.02%; +7 °C
298	A → G	100%; +5 °C	325	A → G	131%; +0.5 °C
299	STOP	No enzyme	326	STOP	No enzyme
301		N/D	328	STOP	No enzyme
303 ^e	STOP	No enzyme	330	STOP	No enzyme
305 ^e	STOP	Active; stable ^f	332	STOP	110%; -4 °C
306 ^e	STOP	93%; -2 °C	333		N/D

^a Peterson and Schachman (1991, 1992).

^b The effect of the mutation is listed as "no enzyme" if the mutant gene fails to complement auxotrophy and no enzyme is detectable in cell extracts; for enzymes that are produced, the relative specific activity and Δt_m compared to the wild-type enzyme are given.

^c The equivalent position in the ATCase/OTCCase sequence alignment shown in Figure 1.

^d These residues are not at equivalent positions in primary sequence, but are proposed to be functionally equivalent (see text).

^e J. Quan and C.B. Peterson (unpubl. results).

^f Results from auxotrophy tests and gel electrophoresis.

A conserved Arg is present in the CTR's of both enzymes. In ATCase, Arg 296 is conserved for eight of nine sequences (Murata, 1994) and, in OTCCase, Arg 319 is strictly conserved. These arginines are not at equivalent positions in the sequence alignment shown in Figure 1, but are four residues apart. This spacing places them on the same face of their respective helices, however, which may enable them to play the same functional role. The presence of these charged residues within the predominantly hydrophobic CTR further suggests their involvement in a critical function. For both enzymes, replacement of the conserved Arg by Ala causes a marked decrease in V_{max} , little change in K_m , and an increase in t_m (Table 5). Thus, the function of these arginines is obviously not one of stabilization. In fact, the R319A mutation is similar to the K86Q mutation in that both increase t_m significantly (+7 °C) and decrease enzymatic activity dramatically. Also, these arginines are not likely to participate directly in catalysis because Arg 296 of ATCase is not near the active site. It has been proposed, however, that Arg 296 of ATCase binds a water molecule that assists in catalysis (Krause et al., 1987); this function could certainly account for the inactivating effect of the alanine substitution in ATCase and could apply to Arg 319 of OTCCase as well.

The results of these studies constitute strong evidence that residues 312–332 of OTCCase form a helical segment that is structurally analogous to Helix 12 of ATCase. This postulated α -helix in OTCCase could help to link the two domains in the enzyme and play an indirect role in catalysis. As discussed in more detail in the companion paper (Murata & Schachman, 1996), this helix appears to be partially buried within the CbmP domain, and probably makes several interchain and intrachain contacts.

Many of the details of OTCCase structure have been inferred through this comparative approach using ATCase as a model. In this way, we have been able to define the roles of some specific residues, localize the active sites at the chain interfaces, and assign a structural role for the CTR. The strikingly consistent similarities between OTCCase and ATCase observed in these ex-

periments are entirely in accord with the hypothesis of similar tertiary and quaternary structures for the two enzymes.⁶

Materials and methods

Cloning and mutagenesis of OTCCase

The 2.6-kb *Hind* III–*Eco* R I fragment of plasmid pA11 (Piette et al., 1982) was subcloned into M13mp19 to form M13mp19argI. This fragment contains *argI*, as well as several hundred flanking nucleotides of lambda DNA. Single-stranded sequencing (Sanger et al., 1977) of the resultant 9.8-kb construct confirmed that the sequence of *argI* exactly matched that of Kuo et al. (1988) and also revealed part of the lambda-derived region. M13mp19argI was then used as template for oligonucleotide-directed mutagenesis to place a silent mutation at amino acid 66, which removed the internal *Nde* I site, and to place an *Nde* I site immediately prior to *argI*. The double-stranded *Nde* I–*Eco* R I fragment was then subcloned immediately downstream of the T7 RNA polymerase promoter on the expression plasmid pT7-7 (Tabor & Richardson, 1985) to form pLM7C. The complete sequence of the gene and promoter region was confirmed by double-stranded sequencing using the Sequenase 2.0 kit (United States Biochemical), and the plasmid construction was confirmed by restriction analysis. Various mutants were constructed and their sequences determined in a similar way (Murata, 1994).

Expression

Wild-type and mutant OTCases were expressed in *E. coli* strain TB-2 ($\Delta argF$, $\Delta argI$ -pyrB) (Roof et al., 1982) using a two-

⁶ Subsequent to our submission of this paper, Villeret et al. (1995) reported on the crystal structure of a mutant form of catabolic OTCCase. Their observations of the two domains, an interdomain C-terminal α -helix, and the location and nature of the active sites are in complete agreement with the conclusions presented here.

plasmid T7 RNA polymerase overexpression system in which plasmid pGP1-2 carries the gene for T7 RNA polymerase. In this system, the gene of interest is expressed from the T7 RNA polymerase promoter on a pT7-7-derived expression plasmid, and the T7 RNA polymerase gene is expressed from a heat-inducible promoter on plasmid pGP1-2 (Tabor & Richardson, 1985; Tabor, 1990). For expression of wild-type, R319A, A325G, and 332Δ OTCases, Luria Broth with 50 mg/mL ampicillin and kanamycin was inoculated with a 1/50 volume of a saturated culture of TB-2[pGP1-2] carrying the desired expression plasmid, and shaken at 37 °C for 15–25 h. Specific induction of the T7 RNA polymerase by early growth at 30 °C followed by heat shock at 42 °C did not increase expression levels over constant growth at 37 °C. Expression of S55H and K86Q OTCases was essentially as described above except that minimal media (M9CA supplemented with 50 mg/L ampicillin, 60 mg/L kanamycin, 0.0001% B₁, and 0.0003% uracil) was used for the final growth stage.

Purification

For purification of wild-type OTCase, the cell pack from 4–6 L of culture was suspended in 80 mL 100 mM KPO₄, pH 7.5, 0.2 mM EDTA, 0.2 mM PMSF, 0.05% lysozyme, 0.005% RNase, 0.005% DNase, passed twice through a French pressure cell at 7,000–8,000 psi, and centrifuged to remove cell debris. Cell-free extracts were then heated to 70 °C for 10 min with gentle agitation and cooled quickly on ice. After removal of the precipitate by centrifugation, the supernatant fraction was fractionated with (NH₄)₂SO₄, with retention of the precipitate formed between 40% and 65% saturation, and the pellet was dialyzed extensively against 50 mM MES, pH 5.5, 50 mM KCl, 0.2 mM EDTA, 2 mM βME, and applied to a 25 × 160-mm Blue Sepharose CL-6B column (Pharmacia) that was eluted with a linear salt and pH gradient to 50 mM MOPS, pH 7.5, 250 mM KCl, 0.2 mM EDTA, 2 mM βME. The enzyme was then precipitated with (NH₄)₂SO₄ at 85% saturation, and the pellet was resuspended in 8 mL 50 mM MOPS, pH 7.8, 0.2 mM EDTA, 2 mM βME, and further purified on a 26-mm × 72-cm Sephacryl S-200 or S-300 column (Pharmacia). Mutant OTCases were purified essentially as described above with minor modifications. Protein concentrations were calculated using an extinction coefficient at 280 nm of 0.96 OD per mg/mL of protein per centimeter of pathlength (Kuo et al., 1988). For all quantitative studies, the protein was dialyzed extensively against two or more changes of buffer and passed through a 0.2-μm filter.

Auxotrophy tests

Expression plasmids carrying the wild-type or mutant genes were tested for their ability to confer arginine prototrophy on TB-2[pGP1-2]. Single colonies of TB-2[pGP1-2] containing the expression plasmid of interest were used to inoculate M9 media (supplemented with ampicillin, kanamycin, B₁, and uracil, as described above) with or without casamino acids. Cultures were shaken at 37 °C for 18–45 h and inspected visually for growth.

Enzyme assays

The activity of the purified enzymes, mixtures, and hybrid sets was measured by a modification of the [¹⁴C]-CbmP method de-

veloped to measure ATCase activity (Davies et al., 1970). Ornithine stock solutions were prepared from ornithine-HCl (Serva) and brought to pH 8.0 with KOH. Preliminary studies performed at saturating substrate concentrations (2.5 or 5 mM CbmP and 3 mM ornithine) indicated that specific activity decreased dramatically at enzyme concentrations below 50 ng/mL. Consequently, assays were performed at 0.1–100 μg/mL OTCase. For saturation curves, ornithine was varied from 0.047 to 6.0 mM. Reactions were allowed to proceed for 2–10 min at 22 °C in 50 mM Tris-acetate, pH 8.0, 0.5 mM EDTA, 2 mM βME (kinetics buffer) before quenching with acetic acid. The resultant data were fit to the Michaelis–Menten equation by nonlinear least-squares analysis using Kaleidograph (Synergy Software).

Differential scanning microcalorimetry

Differential scanning microcalorimetry was performed on a Microcal MC-2 instrument interfaced with an IBM-XT computer. The instrument was operated at a scan rate of 43–48 °C/h in the temperature range 30–90 °C. Wild-type and mutant OTCases were scanned at pH 8.3, at concentrations of approximately 1.2–1.6 mg/mL in 40 mM borate, 0.2 mM EDTA, 5 mM βME. Exotherms, indicative of aggregation, were not observed under these conditions. Enthalpies were calculated by the DA-2 software provided by Microcal; calorimetric enthalpies were determined by integrating the area of the endotherm defined by empirical selection of the beginning and end points of the deviation from linearity. Assignment of the baseline was performed assuming multiple independent overlapping transitions with no ΔC_p.

Formation of mixtures and hybrid sets

Wild-type, S55H, and K86Q OTCases were dialyzed extensively against kinetics buffer and concentrated to ≈1 mg/mL. For formation of hybrid sets, enzymes were mixed in various binary combinations (S55H + K86 Q) in desired proportions (ranging from 1:10 to 10:1) to a final enzyme concentration of ≈0.6 mg/mL in the presence of 3 M GuHCl. After incubation at room temperature for 20 min, the denatured enzyme solutions were diluted rapidly 1:10 into cold kinetics buffer and incubated at 0 °C for 1–4 h. Precipitates were removed by centrifugation at 10,000 × g for 15 min. Recovered solutions were concentrated to 0.15–0.30 mg/mL, incubated at 4 °C overnight, and filtered and assayed for enzyme activity. Analogous experiments were performed on mixtures of the two mutant enzymes that were not subjected to dissociation and denaturation by GuHCl.

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