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# Structural similarity between ornithine and aspartate transcarbamoylases of *Escherichia coli*: Implications for domain switching

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## Abstract

Each catalytic (c) polypeptide chain of *Escherichia coli* aspartate transcarbamoylase (ATCase) is composed of two globular domains connected by two interdomain helices. Helix 12, near the C-terminus, extends from the second domain back through the first domain, bringing the two termini close together. This helix is of critical importance for the assembly of a stable enzyme. The trimeric *E. coli* enzyme ornithine transcarbamoylase (OTCase) is proposed to be similar in tertiary and quaternary structure to the ATCase trimer and has a predicted  $\alpha$ -helical segment near its C-terminus. In our companion paper, we have shown that this putative helix is essential for OTCase folding and assembly (Murata L, Schachman HK, 1996, *Protein Sci* 5:709–718). Here, the similarity between OTCase and the ATCase trimer, which are 32% identical in sequence, was tested further by the construction of several chimeras in which various structural elements were switched between the enzymes by genetic techniques. These elements included the two globular domains and regions containing the C-terminal helices. In contrast to results reported previously (Houghton J, O'Donovan G, Wild J, 1989, *Nature* 338:172–174), none of the chimeric proteins exhibited *in vivo* activity and all were insoluble when overexpressed. Attempts to make hybrid trimers composed of c chains from ATCase and OTCase were also unsuccessful. These results underscore the complexities of specific intrachain and interchain side-chain interactions required to maintain tertiary and quaternary structures in these enzymes.

**Keywords:** carboxy-terminal helix; chimeric protein; domain; hybrid protein

Ornithine transcarbamoylase (OTCase, carbamoyl:L-ornithine carbamoyltransferase, EC 2.1.3.3), and the catalytic trimer of aspartate transcarbamoylase (ATCase, carbamoyl:L-aspartate carbamoyltransferase, EC 2.1.3.2) of *Escherichia coli* have been proposed to be homologous enzymes of similar tertiary and quaternary structure (Gigot et al., 1977; Houghton et al., 1984; Van Vliet et al., 1984). These enzymes are 32% identical in sequence and require a trimeric form for activity (Houghton et al., 1984;

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**Abbreviations:** ATCase, aspartate transcarbamoylase;  $\beta$ ME,  $\beta$ -mercaptoethanol; c chain, ATCase catalytic polypeptide chain; c trimer, ATCase catalytic trimer; CbmP, carbamoyl phosphate; CTR, C-terminal region (defined as residues 312–333 of OTCase and as residues 285–310 of ATCase); IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OTCase, ornithine transcarbamoylase; PMSF, phenylmethylsulfonyl fluoride.

Murata & Schachman, 1996). The sequence identity is most pronounced in the N-terminal halves of the polypeptide chains, the region believed to bind their common substrate, carbamoyl phosphate (Houghton et al., 1984). ATCase catalyzes the transfer of the carbamoyl moiety to aspartate as part of the pyrimidine biosynthetic pathway; OTCase transfers the carbamoyl moiety to ornithine in the arginine biosynthetic pathway. The genes for OTCase and ATCase catalytic chains (*argI* and *pyrB*, respectively) 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).

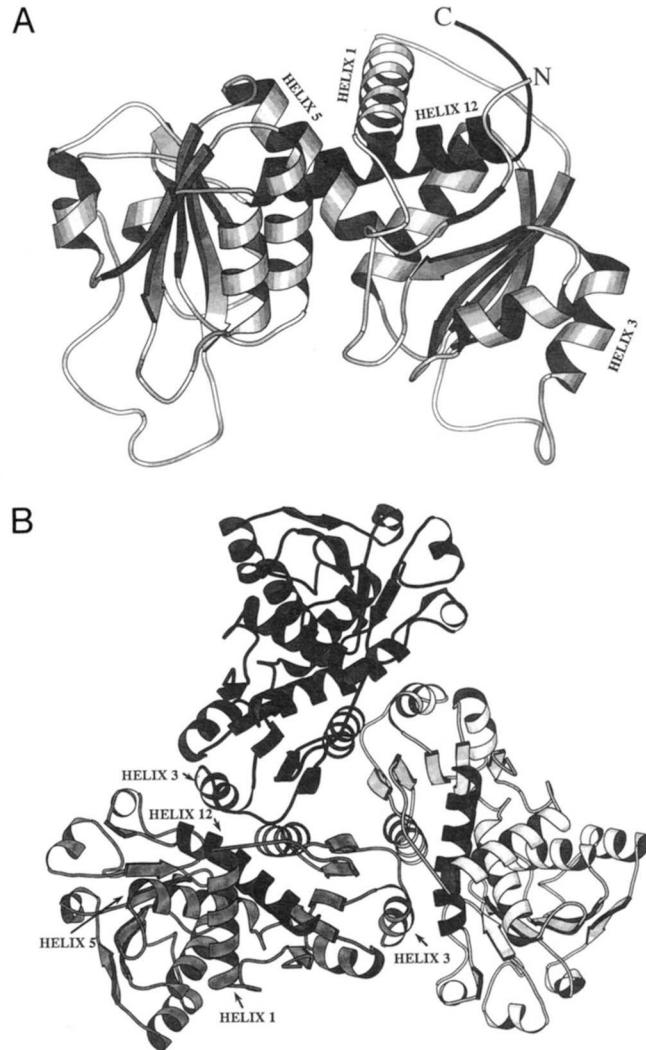
Recently, we have used structural information for the catalytic trimer of ATCase to deduce aspects of the tertiary and quaternary structure of OTCase. Through sequence comparisons and site-directed mutagenesis studies, we have found strong evidence for similarities in the two enzymes (Murata, 1994; Murata & Schachman, 1996). These include the amino acid constituents of the active sites, the location of the active sites at the chain interfaces, and the presence of a structurally important helical segment in the C-terminal regions of the chains. A striking claim

for structural correspondence extending to the level of specific side-chain interactions derives from the work of Wild and co-workers, who reported the construction of an ATCase/OTCase chimeric enzyme with ATCase activity (Houghton, 1986; Houghton et al., 1989; Wild & Wales, 1990; Wales & Wild, 1991). Subsequent to this original report of an active ATCase chimeric enzyme in which about half the catalytic polypeptide chain was derived from OTCase (Houghton et al., 1989), much has been learned about the structure of ATCase and the critical role of the C-terminal helix (Helix 12) in the assembly and stability of the enzyme (Peterson & Schachman, 1991, 1992). Because the amino acid side chains in that helix would have to make numerous contacts with residues in the CbmP domain of OTCase in the chimera, and because the sequence of Helix 12 of ATCase differs in significant ways from the presumed C-terminal helix of OTCase, we considered further investigation of this problem worthwhile. As a consequence, we constructed appropriate hybrid DNAs to encode several chimeric proteins, including the one described by Houghton et al. (1989) to examine further the exchangeability of major structural elements from the two enzymes. Our results, presented here, indicate that ATCase and OTCase, despite their striking similarities in some aspects of structure, are too different to permit construction of stable, active enzymes merely by exchanging intact domains or CTR's.

#### Rationale for construction of chimeric enzymes

\alpha-螺旋连接：螺旋5（残基135-149），在CbmP域开始，在天冬酰胺域结束；螺旋12（残基285-305），在天冬酰胺域开始，并贯穿CbmP域。N端部分的螺旋12是亲水性的；它的极性面与邻近c链的螺旋3接触，而它的非极性面与自身螺旋5接触。螺旋12的C端部分是疏水性的，埋藏在CbmP域中，与螺旋1接触并形成疏水性接触。螺旋12的“尾部”（残基306-310）延伸到表面，使得N端和C端靠近（图1A）。

ATCase的CTR（残基285-310）包含螺旋12和C端“尾部”，在结构上扮演着有趣的角色。先前的研究（Peterson & Schachman, 1991, 1992）表明，螺旋12对于正确的折叠和三聚体的组装至关重要，但“尾部”残基和螺旋的最后残基是可替换的（Peterson & Schachman, 1991）。螺旋12内的局部替换导致复杂的热稳定性和链间相互作用能量变化，以及对 allosteric properties 和 intersubunit interactions 的长程效应（Peterson & Schachman, 1991, 1992）。对不同物种的ATCases进行的保守性调查揭示了螺旋12所在区域的高度保守性（Fig. 2）。这些观察结果及其在CbmP域中的位置表明螺旋12



**Fig. 1.** Ribbon representations of the  $\alpha$ -carbon backbone of ATCase c chains. The CTR (residues 285-310) is shown in black to emphasize the location of Helix 12 and the C-terminal “tail.” The locations of Helices 1, 3, and 5 are noted as well. Ribbon traces were generated with the program MOLSCRIPT (Kraulis, 1991) on a Silicon Graphics Indigo workstation based on the X-ray structure of the CTP-ligated ATCase (Kim et al., 1987) with the coordinates deposited in the Brookhaven Protein Data Bank (registry number 5AT1). **A:** A single c chain is shown with the N-terminal CbmP-binding domain on the right and the C-terminal aspartate-binding domain on the left. **B:** A c trimer is shown with the three different chains differentiated by various degrees of shading.

作为分子整体的必要结构性质，既是CbmP域的内部框架，也是将域结合在一起的纽带。事实上，这种通过末端段将域结合在一起的多域球状蛋白的常见结构特征（Thornton & Sibanda, 1983）。

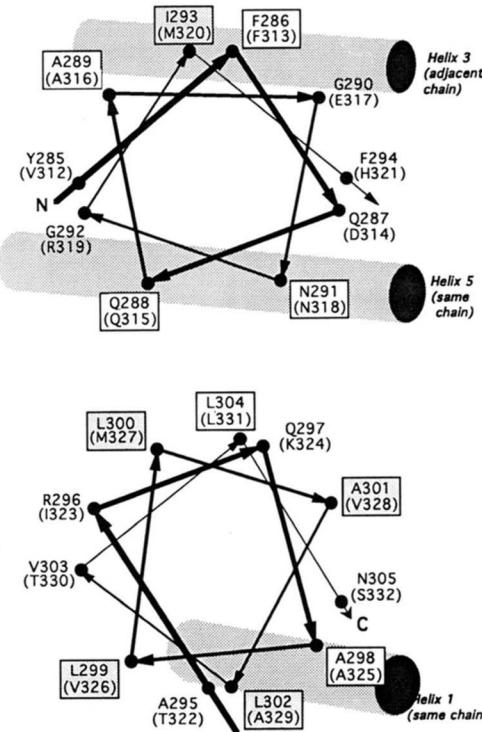
对大鼠OTCase的333残基多肽链的分析表明，C端附近存在一个可能的 $\alpha$ -螺旋段（Houghton et al., 1984）。这个段（残基312-332）在OTCases中高度保守，并且与ATCase的螺旋12具有显著的序列相似性（Fig. 2）。具体来说，螺旋12中有6个残基与这个OTCase段有保守性替换。

ATCase	HELIIX 12	TAIL...
	* * * * *	
E. coli (pyrB) (285)	Y FqQAg NGiFa RgALL AlVLn	
S. typhimurium	Y FqQAg NGiFa aqALL AlVLn	
S. marcescens	Y FqQAg NGiFa RSA.L AlVnn	
Human	Y FRQAg NGmyi RMALL AtVLG	
Hamster	Y FRQAg NGmyi RMALL AtVLG	
D. discoideum	Y FRQAg NGlyv RMSLL AlVFG	
S. cerevisiae	Y FRQmk yGLFv RMALL AmVmG	
D. melanogaster	Y FRQAg yGmyi RMALL AmVmG	
B. subtilis	i FkQmk NGvFi RMAvi qcaLq	
OTCase	* * * * *	
E. coli (argI) (312)	V FdgAE NRmHt IKAVm vatls	k (333)
E. coli (argF)	V FdgAE NRmHt IKAVm matlg	e
N. gonorrhoeae	V FdgAE NRmHt IKAVm vaalig	d
P. aeruginosa (1)	a FdgAE NRmHt IKAlm vstla	di
P. syringae	V FdgE NRmHt IKAlm letvw	p
A. nidulans	V FpEAE Nrlwa aisaL egfvv	nkgki e
A. niger	V FpEAE Nrlwa aisaL egfvv	nkgki e
P. tannophilus	V FeEgE NRlya aiAVL egfvv	nkgkl l
S. cerevisiae	V FeEAE NRlya amsai difvn	nkgnf kdlik
Mouse	V FpEAE NRkkWT ImAVm vsllt	dyspv lgkpk f
Rat	V FpEAE NRkkWT ImAVm vsllt	dyspv lgkpk f
Human	V FpEAE NRkkWT ImAVm vsllt	dyspv lgkpk F
B. subtilis	V FggAE NrlHv qKAII kailly	kges snc
P. aeruginosa (2)	a wdqAE NrlHa qKAII ellie	hahya rs
M. bovis	V wdqAE NrlHa qKAII vwlle	

**Fig. 2.** Multiple alignment of ATCase and OTCase CTR's. The location of Helix 12 and the C-terminal "tail" are noted. ATCase residues that are conserved for at least five of the nine sequences are in capitals and residues that are conserved for at least seven species in addition to *E. coli* *pyrB* are marked by an asterisk. OTCase residues that are conserved for at least 8 of the 15 sequences are in capitals and residues that are conserved for at least 12 sequences in addition to *E. coli* *argI* are marked by an asterisk. ATCase sequences are from the following species with the Swiss Databank filenames given in parentheses: *E. coli* (*pyrb\_ecoli*); *Salmonella typhimurium* (*pyrb\_salty*); *Serratia marcescens* (*pyrb\_serma*); human (*pyr1\_human*); hamster (*pyr1\_mesa*); *Dictyostelium discoideum* (*pyr1\_dicdi*); *Saccharomyces cerevisiae* (*pyr1\_yeast*); *Drosophila melanogaster* (*pyr1\_drome*); *Bacillus subtilis* (*pyrb\_bacsu*). OTCase sequences are from the following species with the Swiss Databank filenames given in parentheses: *E. coli* *argF* (*otc2\_ecoli*); *E. coli* *argI* (*otc1\_ecoli*); *Neisseria gonorrhoeae* (*otcc\_neigo*); *Pseudomonas aeruginosa* (1) (*otcc\_pseae*); *Pseudomonas syringae* (*otcp\_psesh*); *Aspergillus nidulans* (*otc\_aspn*); *Aspergillus niger* (*otc\_aspng*); *Pachysolen tannophilus* (*otc\_pacta*); *S. cerevisiae* (*otc\_yeast*); mouse (*otc\_mouse*); rat (*otc\_rat*); human (*otc\_human*); *B. subtilis* (*otc2\_bacsu*); *P. aeruginosa* (2) (*otca\_pseae*); *Mycobacterium bovis* (*otca\_myco*). Sequences were aligned using the PILEUP program of GCG (Devereux et al., 1984) and the scoring table PILEUPPEP.CMP.

have identical counterparts in the putative helix, and an additional 5 have distinct similarities. In addition, the helices share the same pattern of hydrophobicity (Fig. 3). The recent studies of OTCase (Murata, 1994; Murata & Schachman, 1996) indicate that much of the putative helix is required for production of functional enzyme *in vivo*, whereas the last residue of the putative helix (332) and the last residue of the chain (333) are dispensable. In addition, a conserved Arg residue that is present on the same face of the two helices is likely to serve an equivalent structural and catalytic role in both enzymes (Murata & Schachman, 1996).

Based on these sequence comparisons and functional studies of mutant OTCases, we have proposed that the putative OTCase helix serves a structural role analogous to that of Helix 12 in ATCase (Murata, 1994; Murata & Schachman, 1996). According to that proposal, the helix bridges the N-terminal CbmP-binding domain and C-terminal ornithine-binding domain, is partially buried within the CbmP domain, and makes several interchain and intrachain contacts. Residue 333 in OTCase is presumed to be on the surface of the molecule and corresponds to the expendable C-terminal "tail" of ATCase (Murata, 1994; Murata &



**Fig. 3.** Comparative helical-wheel projections of Helix 12 from *E. coli* ATCase and the putative C-terminal helix of *E. coli* OTCase. The helices are divided into N-terminal (upper) and C-terminal (lower) portions for clarity, with the N-terminal ends pointing toward the viewer. The ATCase residues are given with their OTCase counterparts in parentheses. Residues that are identical for the two sequences are boxed and shaded. Major regions of contact observed in the holoenzyme structure between Helix 12 and other regions of the c trimer are illustrated schematically (Honzatko et al., 1982).

Schachman, 1996). By analogy to ATCase, the CTR of OTCase is defined as residues 312–333, which contain the putative helix and the one-residue "tail."

In the chimeric enzyme described by Houghton et al. (1989), the CbmP domain of OTCase (residues 1–162) is linked to the aspartate domain and CTR of ATCase (residues 162–310). If the chimeric protein had a structure similar to the c chain in ATCase (Fig. 1A), Helix 12 of ATCase would be required to replace the putative C-terminal helix of OTCase and satisfy the interatomic contacts with the CbmP domain of OTCase. As seen in Figure 3, there are significant differences in the sequences of these analogous regions of the enzymes. It is questionable, therefore, whether a direct substitution of helices would lead to interactions resulting in a stable enzyme unless there were compensatory conformational changes in the chimeric protein. On the basis of the work of Houghton et al. (1989) and the detailed structural information now available for ATCase (Kim et al., 1987; Ke et al., 1988; Stevens et al., 1991) as well as the knowledge that Helix 12 was essential for the formation and stability of ATCase (Peterson & Schachman, 1991), we constructed various DNA's to encode chimeric proteins containing polypeptide segments of both OTCase and ATCase. This involved linking the CbmP and aspartate (or ornithine) domains at different locations as well as the construction of tripartite chimeras so that the CTR's would, presumably, be accommodated more readily into the appropri-

ate CbmP domain. Our results show that ATCase and OTCase are too different to permit the construction of stable chimeras or hybrids despite their presumed overall similarity in tertiary and quaternary structure.

## Results

### *OTCase and ATCase chains do not associate to form hybrid trimers*

In addition to undertaking the construction of trimers from chimeric polypeptide chains containing segments from both OTCase and ATCase, we attempted to determine whether intact chains from each enzyme would assemble with one another to yield hybrid trimers. Accordingly, solutions containing both OTCase and ATCase trimers were incubated in 3 M GuHCl for periods known to cause dissociation and unfolding of the polypeptide chains, followed by dilution of the denaturant to permit reassociation of the trimers. Electrophoresis on polyacrylamide gels showed the two parental trimers with no species of intermediate mobility. It is worth noting that electrophoretic mobilities of OTCase and ATCase, though clearly different, are similar; hence, there was the risk that potential hybrid species at low concentration might have gone undetected. As a further test, similar experiments were performed on a mixture of succinylated OTCase, which has a markedly altered mobility, and native ATCase trimers. Both sets of chains reassociated with complete segregation to yield bands corresponding to trimers with very different mobilities on native polyacrylamide gels. These results show conclusively that hybrids do not form when unfolded OTCase and ATCase chains refold and associate to form trimers.

### *Design of OTCase/ATCase chimeras*

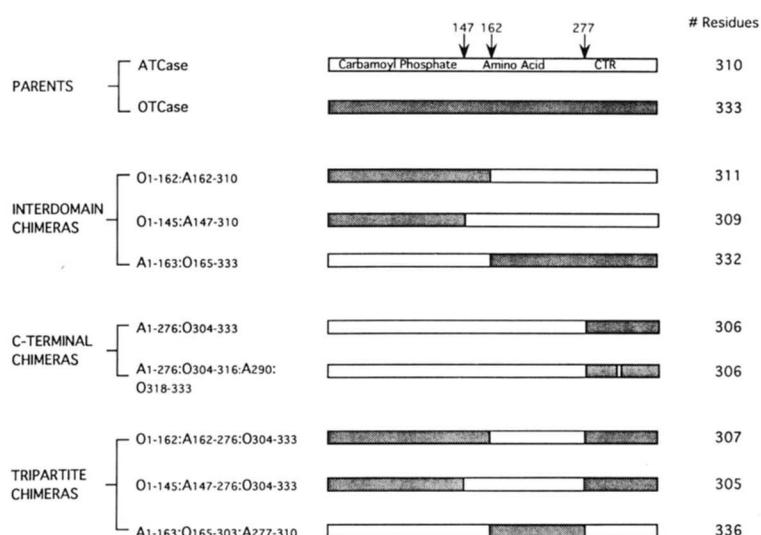
Chimeras containing various arrangements of the three major structural elements of ATCase and OTCase were made by cut-

ting and ligating the genes *pyrB* and *argI* in different combinations, as summarized in Figure 4. The junction points were designed to occur between (1) the CbmP and amino acid domains, (2) the amino acid domain and the CTR, or (3) both. Site-directed mutagenesis on an M13-derived template was used to remove or add restriction sites from *argI* to facilitate this process. The junction points, which are illustrated in Figure 5, correspond to amino acid positions 147, 162, and 277 of ATCase, which in turn correspond to positions 146, 163, and 304 of OTCase. The multiply mutated gene *argImm* contained all of the nucleotide sequence changes created in *argI* during engineering of appropriate restriction sites and served as a positive control throughout the expression and activity studies. Table 1 summarizes the plasmids used. Details of their construction are given in Murata (1994).

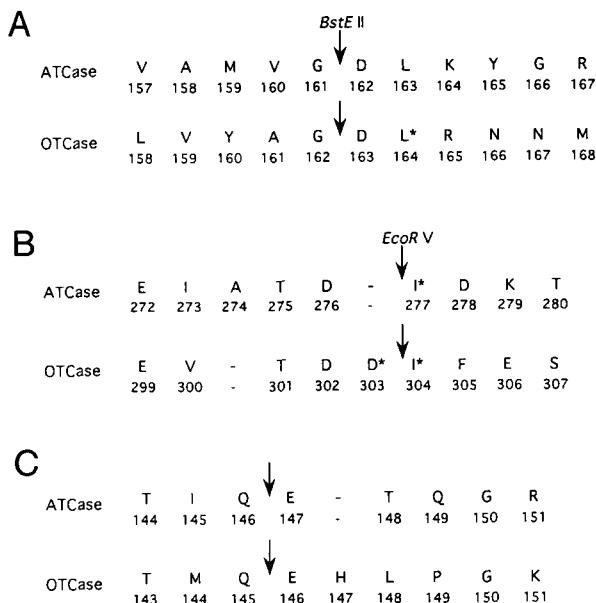
### *Construction of interdomain chimeras*

The chimeric gene described by Houghton (1986) and Houghton et al. (1989) is equivalent to our *OAA162* and encodes the protein  $O_{1-162}:A_{162-310}$ <sup>2</sup> (Fig. 6A). In separate constructs, this gene was placed under control of the *argI* promoter (plasmid pLM5D), as in the above-mentioned work, or under control of the T7 RNA polymerase promoter pT7 (plasmid pT7-OAA162), as described here. An additional gene construct (*OAA162+R*)

<sup>2</sup> Chimeric proteins are designated by listing the origin of the segment, A for ATCase and O for OTCase, and the position and identity of the polypeptide fragment in the newly constructed polypeptide chain. Thus, the protein described as  $O_{1-162}:A_{162-310}$  contains the polypeptide segment 1–162 of OTCase joined at its C-terminus to the N-terminus of the segment 162–310 of ATCase. The corresponding gene is designated by italics in a shorthand notation indicating the origin of the gene segments encoding the three major structural elements of the polypeptide chain and the location of the interdomain junction (ATCase numbering). Thus,  $O_{1-162}:A_{162-310}$ , which derives its first element (the CbmP domain) from OTCase and its second and third elements (the amino acid domain and the CTR) from the ATCase c chain, is encoded by *OAA162*.



**Fig. 4.** Schematic representation of ATCase/OTCase chimeras discussed in this report. The junction separating the CbmP and amino acid domains is at position 147 or 162 of ATCase. The junction separating the amino acid domain and the CTR is at position 277 of ATCase. The gene for  $O_{1-162}:A_{162-310}$  was constructed in two forms. One form (*OAA162+R*) contains the entire coding sequence for ATCase regulatory chains (*pyrB*) joined to *OAA162* so that the genes form one transcriptional unit, as in the *pyrBI* operon. The other form (*OAA162*) contains a truncated nonfunctional *pyrB*.



**Fig. 5.** Map of ATCase/OTCase junctions in chimeric proteins. The exact junctions and surrounding sequences are shown. Changes in amino acid sequence arising from oligonucleotide-directed mutagenesis are marked by an asterisk. Sequence alignments for A and C are from Murata and Schachman (1996); for B, the alignment is from Houghton et al. (1984). **A:** Junctions between the CbmP and amino acid domains were made by exploiting the *BstE* II restriction site, which occurs in *pyrB* at codon 162. In order to create a *BstE* II site in *argI* at the analogous position (163), an alanine (GCG) to leucine (CTG) mutation was made at codon 164. In addition, a silent mutation was made at codon 264 of *argI* to remove the *BstE* II restriction site already present. Chimeric genes *OAO162* and *OAA162*, and *OAA162+R* were made by cutting, swapping, and ligating the genes at their *BstE* II restriction sites. This site was also used in construction of tripartite chimeras *OAO162* and *AOA162*. **B:** No convenient restriction sites were present to facilitate separation of the CTR's from the amino acid domains. In creating new restriction sites through site-directed mutagenesis, we were limited to those requiring no more than two base changes; we created an *EcoR V* site at equivalent positions in both genes (in *pyrB* at amino acid position 277 and in *argI* at position 304). The required nucleotide sequence changes resulted in changes of ATCase Val 277 (GTT) to isoleucine (ATC) and of OTCase Glu 303 (GAG) to aspartate (GAT) and Val 304 (GTC) to isoleucine (ATC). The *EcoR V* restriction site was used in construction of chimeric genes *AAO*, *OAO162*, and *AOA162*. An additional round of mutagenesis was performed on *AAO* to change Glu 317 (OTCase numbering) back to glycine, the residue at the equivalent position (290) of ATCase, resulting in fusion *AAO\**. **C:** Two of the chimeric genes (*OAO162* and *OAA162*) were subjected to further mutagenesis to move the junction between domains from position 162 to 147. This mutagenesis was carried out on M13mp19-derived templates carrying *OAO162* or *OAA162*, and involved use of a 78-base oligonucleotide designed to loop out the unwanted codons derived from *argI* (146–162) and replace them with the corresponding residues from *pyrB* (147–161) (Murata, 1994). The resulting genes were *OAO147* or *OAA147*, respectively.

in each of these systems included the complete *pyrI* gene encoding the ATCase regulatory chains as one transcriptional unit with  $O_{1-162}:A_{162-310}$  (plasmids pLM4C and pT7-OAA162+R). Gene *AOO162* encodes the complementary chimeric protein  $A_{1-163}:O_{165-333}$ . Because the interdomain junction at position 162 for  $O_{1-162}:A_{162-310}$  is buried within the amino acid domain of ATCase (Honzatko et al., 1982), we constructed another gene, *OAA147*, which encodes a protein with a junction near the C-terminus of Helix 5 between the two domains ( $O_{1-145}:A_{147-310}$ ) (Fig. 6B).

### Construction of CTR chimeras

The CTR chimeras were designed to replace a C-terminal portion of ATCase with that of OTCase. The choice of a junction site at the CTR was limited by technical restrictions requiring specific nucleotide sequence characteristics. The site chosen, ATCase 277, resulted in replacement of the entire CTR plus the additional eight residues directly preceding the CTR (Fig. 6C). The CTR swap resulting in protein  $A_{1-276}:O_{304-333}$ , encoded by *AAO*, results in some nonconservative amino acid changes in the critical Helix 12 region (Fig. 3). One of these changes (the replacement of OTCase Glu 317 for ATCase Gly 290) was reverted to the “native” residue (Gly) in the protein encoded by *AAO276\** ( $A_{1-276}:O_{304-316}:A_{290}:O_{318-333}$ ).

### Construction of tripartite chimeras

In the tripartite chimeras, the CTR and CbmP domain have the same origin (Fig. 6D). Gene *OAO162* encodes the chimeric protein  $O_{1-162}:A_{162-276}:O_{304-333}$ . Gene *AOA162* encodes the reverse fusion protein  $A_{1-163}:O_{165-303}:A_{277-310}$ . Mutagenesis of *OAO162* resulted in movement of the junction point from residue 162 to residue 147 to create *OAO147* encoding  $O_{1-145}:A_{147-276}:O_{304-333}$ .

### ATCase/OTCase chimeras exhibit no activity in vivo

None of the chimeric genes conferred arginine or uracil prototrophy on the *E. coli* ATCase/OTCase deletion strain TB-2 under any of the conditions tested in the two different expression systems (Table 1). Of the pBR322-derived plasmids carrying *OAA162*, *OAA162+R*, or *argImm* under control of the *argI* promoter (pLM5D, pLM4C, and pAI4, respectively), only plasmid pAI4 enabled growth of TB-2 in arginine-free media. The prototrophy conferred by pAI4 demonstrated that the promoter was functional and that all the sequence changes invoked to create chimeras could be tolerated in the parent protein. All of the chimeric genes, carried on derivatives of plasmid pT7-7, were tested in a two-plasmid T7 RNA polymerase expression system. Only the control plasmids carrying the wild-type *pyrBI* operon (pYY20A) or the *argImm* gene described above (pT7-LM6) enabled growth in uracil- and arginine-free media, respectively.

### ATCase/OTCase chimeras are insoluble when overexpressed

Each of the chimeric proteins was overexpressed in high yield in the T7 RNA polymerase lysogenic strain BL21(DE3)pLysS. A comparison of the soluble and insoluble cellular fractions by SDS-PAGE (data not shown) indicated that the chimeric polypeptide was present only in the insoluble fraction. In each case, a major band of the predicted molecular weight was present in the insoluble fraction, but no band was evident in the cellular extract. In some expression systems, a decrease in temperature has been shown to aid the production of soluble proteins (Schein & Noteborn, 1988; Schein, 1989; Tabor, 1990). However, in this case, lower growth temperature (21 °C) did not result in soluble chimeric proteins in either rich or minimal media.

Attempts to renature insoluble proteins  $A_{1-276}:O_{304-316}:A_{290}:O_{318-333}$ ,  $O_{1-162}:A_{162-310}$ ,  $A_{1-163}:O_{165-303}:A_{277-310}$ , and  $O_{1-145}:A_{147-276}:O_{304-333}$  by dilution from 7 M urea to various renaturing

**Table 1.** Characteristics of plasmids used in this report

						Uracil	Arginine	Prototrophy <sup>a</sup>
pAI1 <sup>b,c</sup>	pBR322	<i>argI</i>	<i>argI</i>	OTCase		—	+	
pAI4 <sup>c</sup>	pBR322	<i>argImm</i> <sup>d</sup>	<i>argI</i>	mmOTCase <sup>d</sup>		—	+	
pLM7C <sup>c</sup>	pT7-7	<i>argI</i>	<i>pT7</i>	OTCase		—	+	
pT7-LM6 <sup>c</sup>	pT7-7	<i>argImm</i> <sup>d</sup>	<i>pT7</i>	mmOTCase <sup>d</sup>		—	+	
pLM5D <sup>c</sup>	pBR322	<i>OAA162</i>	<i>argI</i>	$O_{1-162}:A_{162-310}$		—	—	
pLM4C <sup>c</sup>	pBR322	<i>OAA162 + pyrI</i> <sup>e</sup>	<i>argI</i>	$O_{1-162}:A_{162-310} + R^e$		—	—	
pT7-OAA162 <sup>c</sup>	pT7-7	<i>OAA162</i>	<i>pT7</i>	$O_{1-162}:A_{162-310}$		—	—	
pT7-OAA162+R <sup>c</sup>	pT7-7	<i>OAA162 + pyrI</i> <sup>e</sup>	<i>pT7</i>	$O_{1-162}:A_{162-310} + R^e$		—	—	
pT7-OAA147	pT7-7	<i>OAA147</i>	<i>pT7</i>	$O_{1-145}:A_{147-310}$		—	—	
pT7-OAO162	pT7-7	<i>OAO162</i>	<i>pT7</i>	$O_{1-162}:A_{162-276};O_{304-333}$		—	—	
pT7-OAO147	pT7-7	<i>OAO147</i>	<i>pT7</i>	$O_{1-145}:A_{147-276};O_{304-333}$		—	—	
pT7-AOA162	pT7-7	<i>AOA162</i>	<i>pT7</i>	$A_{1-163}:O_{165-303}:A_{277-310}$		—	—	
pT7-AOO162	pT7-7	<i>AOO162</i>	<i>pT7</i>	$A_{1-163}:O_{165-333}$		—	—	
pT7-AAO	pT7-7	<i>AAO</i>	<i>pT7</i>	$A_{1-276}:O_{304-333}$		—	—	
pT7-AAO*	pT7-7	<i>AAO*</i>	<i>pT7</i>	$A_{1-276}:O_{304-316}:A_{290}:O_{318-333}$		—	—	
pYY20A <sup>c</sup>	pT7-7	<i>pyrBI</i>	<i>pT7</i>	ATCase	+	—	—	
pAIB205 <sup>f</sup>	pUC8	<i>OAA162</i>	<i>argI</i>	$O_{1-162}:A_{162-310}$		—	—	

<sup>a</sup> Results of auxotrophy tests as given in the text. —, no detectable growth after 36 h; +, growth to high density within 12 h.

<sup>b</sup> Piette et al. (1982).

<sup>c</sup> Detailed structure of plasmid and/or insert given in Murata (1994).

<sup>d</sup> Multiply mutated OTCase encoded by *argImm* contains all sequence changes invoked in constructing chimeras.

<sup>e</sup> The ATCase regulatory chains encoded by *pyrI* are included on the same transcriptional unit as *OAA162*.

<sup>f</sup> Houghton (1986).

buffers were unsuccessful. Renaturation of some proteins has been shown to be aided by molecular chaperones, but attempts to assist the refolding of these proteins by the presence of GroE and ATP under conditions similar to those successful for the renaturation of rhodanese were to no avail (data not shown) (Menendoza et al., 1991).

## Discussion

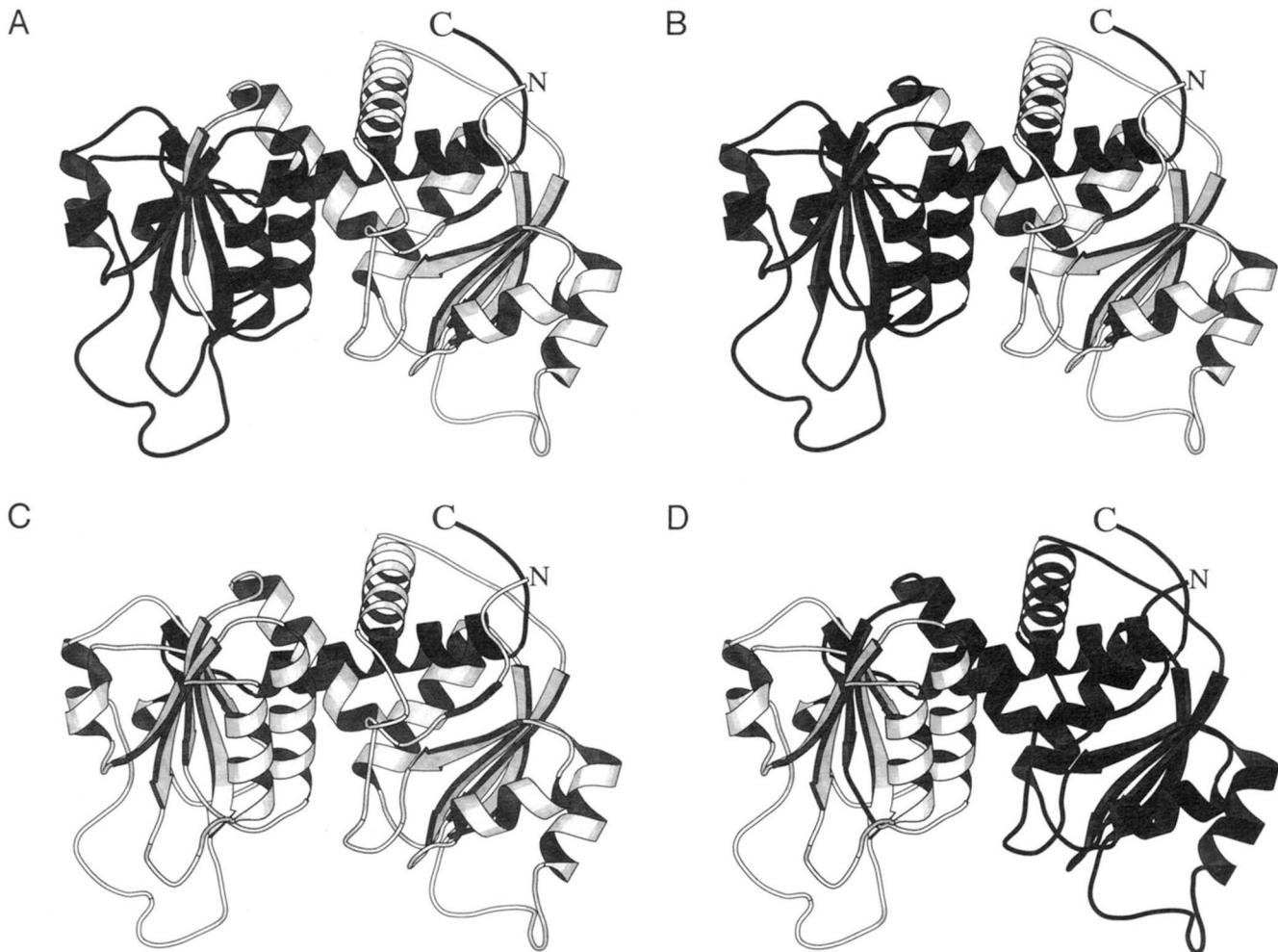
The previously posed argument for extensive similarity between the tertiary structures of OTCase and ATCase trimers is quite convincing (Houghton et al., 1984) and has been strengthened further by the site-directed mutagenesis experiments described in the companion paper (Murata & Schachman, 1996). However, the experiments described here demonstrate that this similarity is insufficient to allow switching of entire polypeptide chains or of significant portions of polypeptide chains between the two enzymes.

The inability to form hybrid trimers containing both OTCase and ATCase polypeptide chains is not unexpected because the amino acid domains in the respective chains are so different in sequence as to make them incompatible for mixed association into trimers.

Switching of smaller structural units between OTCase and ATCase also fails to produce viable proteins. Here, several chimeras of ATCase and OTCase were produced by recombinant DNA techniques and all were inactive and insoluble. The chimeric proteins fell into three major classes summarized in Figure 4. First, interdomain fusions linked the CbmP domain of one enzyme to the amino acid domain and CTR of the other enzyme. The junction between domains was made either at the site within the amino acid domain (ATCase 162) that was reported by Houghton (1986) and Houghton et al. (1989) to result in a

functional enzyme, or, alternatively, at a site located on Helix 5 (ATCase 147), between the two domains (Fig. 6A,B). Second, C-terminal fusions joined the CTR of OTCase to the CbmP and aspartate domains of ATCase (Fig. 6C). In one of these chimeras, site-directed mutagenesis was used to change Glu 317 (OTCase numbering) to Gly in an attempt to fine-tune the contacts between the OTCase CTR and the ATCase CbmP domain. Third, tripartite fusions combined the CbmP domain and CTR of one enzyme with the amino acid domain of the other. These seemed most likely to yield viable proteins, because the presumably closely interacting CbmP domains and CTR's originated from the same enzymes (Fig. 6D). However, none of the engineered chimeric proteins exhibited activity *in vivo* and all were insoluble when overexpressed.

The insolubility of the designed fusion proteins indicates that the differences between the parent enzymes are too great for the chimeras to maintain the specific interactions required for formation of correct tertiary and quaternary structure. These results are explained easily by the complex multidomain and multisubunit structure of the ATCase trimer (and inferred for OTCase), and the differences between the primary structures of the two enzymes. For example, folding of  $O_{1-162}:A_{162-310}$ ,  $O_{1-145}:A_{147-310}$ ,  $A_{1-163}:O_{165-333}$ , or  $A_{1-276}:O_{304-333}$  would require that the CTR derived from one enzyme make several interchain and intrachain contacts with the CbmP domain derived from the other enzyme. Although the CTR's of OTCase and ATCase are similar, there are some significant differences between them (Figs. 2, 3), any one of which might be sufficient to destroy a chimera's ability to fold. For example, an examination of the ATCase crystal structure reveals that one of these residues, Gly 290, is buried in the interchain interface and makes close contact with Thr 97 of the adjacent chain (Honzatko et al., 1982). In  $A_{1-276}:O_{304-333}$ , Glu 317 of OTCase is introduced as



**Fig. 6.** Approximate representations of structures that might be assumed by productively folded ATCase/OTCase chimeras. The structure of a c chain in the *E. coli* ATCase holoenzyme was used as a model for the chimeric proteins (Kim et al., 1987). The portions arising from the two different parent proteins are differentiated by shading as white or black. The  $\alpha$ -carbon ribbon traces were generated with the program MOLSCRIPT (Kraulis, 1991) on a Silicon Graphics Indigo workstation. **A:** Hypothetical structure of interdomain chimeras ( $O_{1-162}:A_{162-310}$  or  $A_{1-163}:O_{165-333}$ ) with the junction at ATCase position 162, within the amino acid domain. **B:** Hypothetical structure of interdomain chimera  $O_{1-145}:A_{147-310}$  with the junction at ATCase position 147, near the C-terminal end of Helix 5. **C:** Hypothetical structure of CTR chimera  $A_{1-276}:O_{304-333}$  with the junction at ATCase position 277, eight residues prior to Helix 12. **D:** Hypothetical structure of tripartite chimeras ( $A_{1-163}:O_{165-303}:A_{277-310}$  or  $O_{1-162}:A_{162-276}:O_{304-333}$ ) with the junctions at residues 162 and 277 of ATCase.

one of the members of this contact pair. Because the placement of a large charged residue in this small hydrophobic pocket seemed likely to be severely destabilizing, this residue was replaced by Gly, yielding  $A_{1-276}:O_{304-316}:A_{290}:O_{318-333}$ . However, this single change was insufficient to allow productive folding.

Chimeric polypeptide chains in which the CbmP domain could retain more of its native contacts would be expected to be more likely to fold and assemble into soluble trimers. Thus, tripartite chimeras ( $O_{1-162}:A_{162-276}:O_{304-333}$ ,  $O_{1-145}:A_{147-276}:O_{304-333}$ , and  $A_{1-163}:O_{165-303}:A_{277-310}$ ) were made in which the CbmP domains and CTR's originated from the same enzyme (Fig. 6D). However, retention of the CTR's with their correct CbmP domains was not sufficient to allow productive folding. Apparently, additional interactions at the domain interfaces, both within and between chains, must be maintained.

The sites chosen as junction points in the construction of chimeras are of crucial importance. In the absence of an OTCase

structure, it is impossible to predict with certainty the structural correspondence between apparently analogous residues on the two different proteins in order to select ideal sites. Flexible hinge regions or surface loops within regions of high identity would seem the most likely to tolerate a splice site. The interdomain junction site (Asp 162) originally chosen by Houghton (1986) and Houghton et al. (1989) seemed an unpromising choice, because it is buried within the amino acid domain and resides within an unconserved region of sequence. It should be noted, however, that Asp 162 itself is conserved for both enzymes and is known to be important in ATCase stability and activity (Newton et al., 1992). When all of the interdomain and tripartite fusion polypeptides utilizing this site were found to be insoluble, this junction was moved to position 147, which is in the conserved region of the interdomain Helix 5 of ATCase (Fig. 6B). Because Helix 5 connects the two domains, it was thought that it might be somewhat flexible and therefore tolerate the splice

site. However, both the chimeras utilizing this splice site were insoluble as well. The junction used in this work for swapping CTR's (ATCase 277) switches eight residues in addition to the desired CTR; no further studies as to the significance of this splice site have been made, however.

None of the chimeric polypeptides described above, with various combinations of the CbmP and amino acid domains as well as the CTR's, fold and assemble into soluble, stable, active trimers. Moreover, as shown in Table 1, none of them could confer pyrimidine prototrophy on a *pyrB* deletion strain TB-2. These observations are in marked contrast with the claim by Houghton (1986) and Houghton et al. (1989) that the plasmid pAIB205 that carried the gene identical to *OAA162* did have complementation activity in TB-2. It is worth noting that their plasmid pAIB205 differed in this respect from the plasmid constructed originally for their studies (pAIB201), which carried the chimeric gene, but could not complement pyrimidine auxotrophy in strain TB-2 (Houghton, 1986). However, growth of cells containing pAIB201 under extreme selective pressure for at least 36 h, according to Houghton (1986), gave rise to the new plasmid, pAIB205, which did have complementation activity. Because the promoter region and the chimeric gene in plasmids pAIB205 and pAIB201 were reported to be identical (Houghton, 1986) and are identical to those on our plasmid pLM5D, it is difficult to account for the claim that only pAIB205 has complementation activity.<sup>3</sup> It was suggested that the new activity might have arisen from an increase in the level of expression of the chimeric gene due to a change on the plasmid external to the gene itself (Houghton, 1986). However, this explanation seems unlikely because, in our studies, *OAA162* was expressed at very high levels from plasmid pT7-OAA162 and still did not complement pyrimidine auxotrophy in TB-2.

Although the reasons for these discrepancies are not known, they might be explained by the presence of an unstable ATCase mutant in the preparations of Houghton et al. According to Houghton (1986) and Houghton et al. (1989), gel electrophoresis of extracts transformed with pAIB205 showed a protein with ATCase activity and the same mobility as trimers from ATCase. Also, kinetic analysis of the enzyme activity of crude extracts yielded a  $K_m$  for aspartate equal to that of ATCase trimers, although the activity was reported to be less than that of ATCase. In addition, this protein, according to Houghton (1986) and Wild and Wales (1990), readily combined with the regulatory subunits of ATCase to form a complex that exhibited the cooperativity characteristic of the wild-type ATCase holoenzyme and some regulation by CTP, but not ATP. Attempts to purify the presumed chimeric protein resulted in the loss of activity, and there have been no further studies reported subsequent to the initial claims.

Domains have been proposed to represent independent structural, functional, and evolutionary units (Wetlaufer, 1973; Rich-

ardson, 1981); indeed, switching of domains between proteins has resulted in functional chimeras in several instances in which the parent proteins are nearly identical or have negligible inter-domain interfaces (Schneider et al., 1981; Mas et al., 1986; Major et al., 1989; Moe et al., 1989; Armstrong, 1990; Wong et al., 1991). Engineering of an active OTCase/ATCase chimera by simple domain switching would be a surprising result, however, given the complex structures of the parent enzymes. The extensive interactions at the interfaces between domains and between chains of ATCase (and OTCase, by inference) would be difficult to maintain in a chimera because of the significant differences in amino acid sequence. Thus, although these enzymes almost certainly share highly similar tertiary structures, our inability to create viable hybrid trimers or native-like chimeras is evidence that the similarity does not extend to the level of specific side-chain interactions. These results underscore the importance of specific side-chain interactions even in the context of globally similar structure. In the case of these two transcarbamoylases, design of a functional chimera will probably require the determination of OTCase structure at high resolution.

## Materials and methods

### Determination of conditions for dissociation of OTCase

Conditions for dissociation of wild-type OTCase and the unfolding of the chains in response to GuHCl were determined by UV difference spectroscopy. The GuHCl-promoted change in  $A_{293-286\text{nm}}$  was measured using a Cary 118 double-beam spectrophotometer and 1.0-mL tandem quartz cells. Wild-type OTCase was dialyzed extensively against 50 mM MOPS, pH 7.5, 0.2 mM EDTA, 2 mM  $\beta$ ME (MOPS buffer) and concentrated to 0.4 mg/mL. The cells were scanned from 320 to 220 nm at room temperature at 0.5 nm/s to obtain a baseline. The sample cell was then inverted several times to mix the contents and scanned repeatedly until no further change was observed. The final concentration of GuHCl in the mixed sample ranged from 0.5 to 3.8 M. Measurement of the change in  $A_{293-286\text{nm}}$  for each spectrum showed that complete unfolding of OTCase occurred by 3 min in 3 M GuHCl. Because ATCase trimers are more prone to dissociation than OTCase, these conditions were also sufficient for denaturation of ATCase trimers (Yang & Schachman, 1987; Murata & Schachman, 1996).

### Preparation of hybrid trimers

Purified wild-type ATCase trimer was kindly provided by Pinghui Zhang in this laboratory. Equimolar mixtures of OTCase and ATCase trimer at approximately 0.2 mg/mL each were incubated in the presence of 3 M GuHCl in MOPS buffer at room temperature for 20 min. After a rapid 1:20 dilution into cold MOPS buffer, samples were incubated at 4 °C overnight, concentrated to their original volumes by ultrafiltration, and analyzed for species of intermediate mobility on a nondenaturing 7% polyacrylamide gel stained with Coomassie brilliant blue. In other experiments that served as controls for the procedure, OTCase and ATCase trimer were modified by reaction with succinic anhydride as described elsewhere (Murata, 1994), and then each modified enzyme was mixed with its unlabeled counterpart and subjected to the procedures outlined above. As a separate control, succinylated OTCase was also mixed with native ATCase trimers and treated similarly.

<sup>3</sup> A preparation of pAIB205 was obtained at the conclusion of these studies (courtesy of J. Wild) and was found to be clonally heterogeneous, containing at least two species. One of these plasmids contained most of the 3' half of *pyrB* joined to the pUC8 vector by an unidentified DNA sequence. The other contained the designed chimeric gene. By sequencing this gene, we have confirmed that it is identical in sequence to our *OAA162*. (Schachman et al., 1984; Kuo et al., 1988; Murata, 1994). Unlike Houghton et al. (1989), we found that pAIB205 did not confer pyrimidine prototrophy on TB-2 cells. Tests for auxotrophy by methods described here showed no evidence of growth of TB-2 cells in the absence of uracil supplementation after 48 h.

### Construction of chimeric genes

ATCase/OTCase chimeras were created by cutting the genes *pyrB* and *argI* with restriction enzymes and ligating the fragments in various combinations. The junction points were designed to occur between (1) the CbmP and amino acid domains, (2) the amino acid domain and the C-terminal helix, or (3) both. In some cases, oligonucleotide-directed mutagenesis on a U-labeled M13 template (M13mp19*argI* or its derivatives [Murata & Schachman, 1996]) carrying the desired mutant or wild-type *argI* gene was used to create or remove restriction sites so that unique restriction sites existed at the desired junction points (Kunkel et al., 1987). Details of the constructions are given in Figure 5 and Murata (1994).

Junctions between the CbmP and amino acid domains were made by exploiting the *Bs*/E II restriction site, which occurs in *pyrB* at codon 162, as described previously (Houghton, 1986; Houghton et al., 1989). An A164L mutation in OTCase was made that enabled cutting of the genes at ATCase 162 (OTCase 163). Cutting, swapping, and ligation of the genes at this site was used in construction of chimeric proteins A<sub>1-163</sub>:O<sub>165-333</sub>, O<sub>1-162</sub>:A<sub>162-310</sub>, O<sub>1-162</sub>:A<sub>162-276</sub>:O<sub>304-333</sub>, and A<sub>1-163</sub>:O<sub>165-303</sub>:A<sub>277-310</sub>. An additional construction (*OAA162+R*) was made in which *pyrI* immediately followed *OAA162* so that the genes for the fusion protein and the ATCase regulatory chains could be coexpressed as one transcriptional unit. Junctions between the amino acid domains and CTR's were created by placing an *Eco*R V restriction site in *pyrB* at position 277 and in *argI* at position 304. These nucleotide sequence changes resulted in amino acid sequence changes for both chains (ATCase V277I and OTCase E303D, V304I). Cutting, swapping, and ligation of the genes at this site was used in construction of chimeric genes *AAO*, *OAO162*, and *AOA162*. An additional round of mutagenesis was performed on *AAO*, to change OTCase Glu 317 back to glycine, the residue at the equivalent position (290) of ATCase, resulting in *AAO\**. A round of deletion-insertion mutagenesis was also performed on *OAO162* and *OAA162* to move the junction from ATCase position 162 to 147, resulting in O<sub>1-145</sub>:A<sub>147-276</sub>:O<sub>304-333</sub> and O<sub>1-145</sub>:A<sub>147-310</sub>, respectively. Complete details of the constructions are given in Murata (1994).

The chimeric genes were subcloned into two types of vectors for expression (Table I). First, genes *OAA162* and *OAA162+R* were subcloned into plasmid pAI1 (Piette et al., 1982) to form plasmids pLM5D and pLM4C, respectively. In these plasmids, the chimeric genes were expressed from the *argI* promoter. Second, all the chimeric genes were subcloned into plasmids pLM7C or pYY20A. In these pT7-7-derived plasmids, the chimeric genes were expressed from the T7 RNA polymerase promoter. Subcloning of genes containing the N-terminal coding sequence of *argI* required additional rounds of mutagenesis to create a unique *Nde* I site immediately upstream of *argI*, as described previously (Murata & Schachman, 1996).

For use as a control, a multiply mutated OTCase gene (*argImm*) was also constructed. This gene contained all of the nucleotide sequence changes created in engineering the chimeric genes: a new *Nde* I site immediately prior to the start codon, silent mutations at amino acid positions 66 and 264, and amino acid changes V164L, E303D, and V304I. *ArgImm* was subcloned into pAI1 and pT7-7 to create plasmids pAI4 and pT7-LM6, respectively. The sequence of each gene construct and its promoter region was confirmed by double-stranded sequencing using the

Sequenase 2.0 kit (United States Biochemical) and the structure of each plasmid was confirmed by restriction analysis.

### Auxotrophy tests and expression of chimeric proteins

The pBR322-derived plasmids carrying *OAA162*, *OAA162+R*, or *argImm*, under control of the *argI* promoter (pLM5D, pLM4C, and pAI4, respectively), were transformed into *E. coli* strain TB-2 ( $\Delta argF$ ,  $\Delta argI-pyrB$ ), which is a uracil and arginine auxotroph (Roof et al., 1982). Each of these plasmids was tested for ability to confer arginine or pyrimidine prototrophy on TB-2. Single colonies were used to inoculate M9 media (supplemented with 50 mg/L ampicillin, 60 mg/L kanamycin, and 0.0001% B<sub>1</sub>) with or without 0.0003% uracil and/or 0.02% casamino acids. Cultures were shaken at 37 °C for 36–72 h and inspected visually for growth.

The pT7-7-derived plasmids were transformed into TB-2 cells carrying plasmid pGP1-2 (Tabor & Richardson, 1985; Tabor, 1990) and into *E. coli* strain BL21(DE3)pLysS (Studier et al., 1990). In these plasmids, the gene is expressed from the T7 RNA polymerase promoter (p<sub>T7</sub>) by T7 RNA polymerase. In TB-2[pGP1-2], the polymerase gene is carried on plasmid pGP1-2, whereas in BL21(DE3)pLysS, it is carried on the chromosome behind an inducible *lac* promoter. Isolates of TB-2[pGP1-2] carrying the plasmid of interest were tested for arginine prototrophy and pyrimidine prototrophy as described above. Transformants of BL21(DE3)pLysS were used for overexpression of protein for further study. Overexpression was induced in Luria Broth (supplemented with 50 mg/L ampicillin and 75 mg/L chloramphenicol) or minimal media (M9 supplemented with ampicillin, chloramphenicol, and B<sub>1</sub> as above) at 21 °C or 37 °C by addition of IPTG to 0.4 mM.

### Isolation and analysis of chimeric proteins

Cell pellets from induced BL21(DE3)pLysS cultures were washed and resuspended in 100 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1 mM PMSF, 0.005% DNase, 0.005% RNase (resuspension buffer). Resuspended pellets were subjected to two freeze-thaw cycles from –20 °C to 37 °C, and centrifuged. The soluble (supernatant) and insoluble (pellet) fractions were compared on SDS polyacrylamide gels stained with Coomassie brilliant blue. Attempts were made to renature insoluble chimeric proteins A<sub>1-163</sub>:O<sub>165-303</sub>:A<sub>277-310</sub>, O<sub>1-145</sub>:A<sub>147-276</sub>:O<sub>304-333</sub>, O<sub>1-162</sub>:A<sub>162-310</sub>, and A<sub>1-276</sub>:O<sub>304-316</sub>:A<sub>290</sub>:O<sub>318-333</sub>. Pellets containing these proteins were washed with resuspension buffer containing 1 mg/mL deoxycholic acid and were then solubilized in 7 M urea. Subsequently, the proteins were diluted into 100 mM Tris-Cl buffers at various pHs with various additives, including 1 M NaCl, 1 M NaSCN, and 1 M urea. After incubation in these buffers, the solutions were centrifuged and the soluble and insoluble fractions were analyzed by SDS-PAGE. Proteins were visualized with Coomassie brilliant blue.

In addition, an attempt was made to assist refolding of A<sub>1-163</sub>:O<sub>165-303</sub>:A<sub>277-310</sub>, O<sub>1-145</sub>:A<sub>147-276</sub>:O<sub>304-333</sub>, O<sub>1-162</sub>:A<sub>162-310</sub>, and A<sub>1-276</sub>:O<sub>304-316</sub>:A<sub>290</sub>:O<sub>318-333</sub> with molecular chaperones GroEL and GroES. In an adaptation of the procedure proven successful for renaturation of rhodanese (Mendoza et al., 1991), after solubilization in 7 M urea as above, proteins were diluted 1:50 into 50 mM Tris-Cl, pH 7.8, 15 mM MgCl<sub>2</sub>, 15 mM KCl, 2 mM ATP, 2 mM βME containing 0.4 μM GroEL 14-mer and

0.1  $\mu\text{M}$  GroES 7-mer. The final concentration of chimeric protein was approximately 4  $\mu\text{g}/\text{mL}$ . After a 90-min incubation at room temperature, solutions were centrifuged and the soluble fractions were analyzed by nondenaturing gel electrophoresis. Proteins were visualized by silver staining (Morrissey, 1981).

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