In vivo formation of active aspartate transcarbamoylase from complementing fragments of the catalytic polypeptide chains

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Abstract

Despite the complexity of *Escherichia coli* aspartate transcarbamoylase (ATCase), composed of 12 polypeptide chains organized as two catalytic (C) trimers and three regulatory (R) dimers, it is possible to form active stable enzyme in vivo even with fragmented catalytic (c) chains. Based on the observation that chymotryptic digestion of the C trimers yields an active protein that can be dissociated into fragmented chains and then reconstituted in high yield, genetically engineered plasmids carrying the genes encoding each of the fragments were constructed. When the N-terminal peptide (residues 1–242) and the C-terminal peptide (residues 235–310) were expressed separately, each incomplete polypeptide chain was found in the insoluble fraction of the individual cell extracts. Mixing the two insoluble pellets in 6.5 M urea, followed by a 10-fold dilution in buffer, led to the formation of active C trimers composed of incomplete polypeptide chains with an 8-amino acid redundancy. When the two partial genes were linked into a single transcriptional unit separated by a 15-nucleotide untranslated region containing a sequence for ribosome binding, the cells produced high yields of active C trimers composed of the incomplete, partially overlapping chains. The resulting protein, purified as C trimers or as holoenzyme formed by the addition of R subunits, has a specific activity ($V_{\max}$) only slightly less than that of the wild-type C trimer and ATCase. However, K_m for aspartate exhibited by the C trimer composed of fragmented chains is more than 10-fold larger than that of the wild-type trimer. The holoenzyme formed from the C trimer containing the coexpressed peptides is devoid of cooperativity with a Hill coefficient of 1.0, as contrasted to wild-type ATCase for which the Hill coefficient is 1.7. K_m for aspartate as well as K_d for the binding of the bisubstrate analog *N*-(phosphonacetyl)-L-aspartate are significantly higher than the analogous values for wild-type ATCase. Sedimentation velocity experiments indicate that the holoenzyme containing the incomplete chains has a conformation analogous to that of the R state of wild-type ATCase.

Keywords: chain overlap; complementation; folding; oligomer formation from fragments

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Abbreviations: ATCase, aspartate transcarbamoylase; c, catalytic polypeptide chain; r, regulatory polypeptide chain; C, catalytic subunit or trimer; R, regulatory subunit or dimer; N-peptide, residues 1–242 of c chain; C-peptide, residues 235–310 of c chain; NC as subscript, complex containing N- and C-peptides, i.e., C_{NC} trimer represents a trimeric protein composed of three incomplete and partially overlapping N- and C-peptides; PC as subscript, proteolytically cleaved, i.e., C_{PC} trimer is a proteolytically cleaved trimer in which each chain is fragmented between Tyr 240 and Ala 241; WT as subscript, wild type; IPTG, isopropyl- β -thiogalactoside; PALA, *N*-(phosphonacetyl)-L-aspartate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; EIMS, electrospray ionization mass spectrometry; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HPLC, high performance liquid chromatography.

The availability of site-directed mutagenesis techniques for altering structural genes, coupled with knowledge of the tertiary and quaternary structures of the enzymes they encode, has provided an opportunity to test various models for the formation of active enzymes through non-covalent interaction between defective proteins. For oligomeric enzymes in which the active sites are located at the interfaces between chains and catalysis is dependent on the participation of amino acid residues from the adjoining chains, complementation can be achieved by constructing hybrids from appropriate inactive parental mutants. The formation both in vitro and in vivo of functional shared active sites has been demonstrated for ATC-

ase (Robey & Schachman, 1985; Wente & Schachman, 1987), mercuric reductase (Distefano et al., 1990), and ribulose biphosphate carboxylase/oxygenase (Larimer et al., 1987). Complementation also can occur if the defect in one of the parental oligomeric proteins is attributable to incorrect folding of the chains rather than the removal of an essential active-site residue. Hybridization of such chains with others that fold correctly but lack a crucial active-site residue may lead to a conformational correction of the former thereby resulting in the formation of active oligomers (Yang et al., 1988). A third mechanism for formation of active species involves an incomplete but potentially functional polypeptide either substituting for a missing region of the other chain or displacing a defective segment of the other chain. This form of complementation, by chain displacement or by fragment association, is illustrated by the results of chemical modification or limited proteolysis studies on ribonuclease (Richards, 1958; Richards & Vithayathil, 1959; Crestfield & Fruchter, 1967; Lin et al., 1970; Gutte et al., 1972; Hayashi et al., 1973), cytochrome *c* (Parr et al., 1978; Juillerat et al., 1980), and staphylococcal nuclease (Taniuchi et al., 1986). Early studies on these and other proteins have been reviewed by Wetlaufer (1981). Similar studies on fragments produced by limited proteolysis have been extended to larger proteins like alanine racemase (Galakatos & Walsh, 1987), transferrin (Williams & Moreton, 1988), and 3-phosphoglycerate kinase (Vas et al., 1990). In addition, functional oligomeric proteins like penicillin acylase (Lindsay & Pain, 1991) and *trp* repressor (Tsayco & Carey, 1992) have been reconstituted from proteolytic fragments.

Although much of the research on complementation by chain displacement or fragment association has been conducted with fragments produced by limited proteolysis, there have also been investigations on proteins in which the partial incomplete polypeptide chains are expressed independently by genetic manipulations. Each of the two domains of yeast phosphoglycerate kinase, for example, has been produced by using site-directed mutagenesis to modify the structural gene encoding the enzyme (Minard et al., 1989; Missiakas et al., 1990), and human-yeast chimeric kinases were constructed by domain interchange (Mas et al., 1986). In the case of alanine racemase of *Bacillus stearothermophilus* (Toyama et al., 1991), it has been possible to produce active enzyme in *Escherichia coli* from a modified gene so constructed as to produce two fragments of the intact polypeptide chain. The coexpression of these peptides led to the production of active dimeric enzyme containing two sets of the two polypeptide fragments.

Although it has a relatively complex structure, *E. coli* ATCase (aspartate carbamoyltransferase, carbamoyl phosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) is particularly suitable for experiments aimed at the in vivo production of polypeptide fragments that can associate

noncovalently to form active enzyme. The six *c* and six *r* polypeptide chains in the holoenzyme are organized as two C trimers and three R dimers, and the wild-type *pyrBI* operon (Schachman et al., 1984), which encodes the *c* and *r* chains, can be altered readily by appropriate mutations to produce either C trimers alone or, alternatively, only R dimers (Schachman, 1983). Moreover, both the *c* and the *r* chains have been shown by crystallographic studies (Kim et al., 1987; Ke et al., 1988) to have folded tertiary structures comprising clearly segmented domains (Kinimages 1–3). Indeed, the zinc domain of the *r* chain has been isolated by cleaving the *pyrI* gene, and its interaction with the C trimer has been studied in some detail (Zhou & Schachman, 1993). As yet, however, the analogous use of restriction enzymes to cut *pyrB* so as to produce each of the two domains of the *c* chain as soluble folded polypeptides has not been reported. Indeed, numerous attempts in this laboratory to isolate large fragments corresponding to the two domains of the *c* chain by genetic manipulation have been unsuccessful. Based on the finding that the three *c* chains in the C trimer can be cleaved by α -chymotrypsin between residues 240 and 241 and that the resulting active trimers can be dissociated in urea and then reconstituted into active enzyme (Powers et al., 1993), we used site-directed mutagenesis to produce two fragments of *pyrB* that encode the N-peptide and the C-peptide of the *c* chain of ATCase.

As shown below, it is possible to coexpress the N- and C-peptides, which associate noncovalently to form stable active complexes composed of three copies of each peptide. These C_{NC} trimers, in turn, combine with R dimers to yield ATCase_{NC} containing fragmented and partially overlapping polypeptide chains. The N- and C-peptides expressed independently are insoluble, but mixing the two extracts in 6.5 M urea, followed by dilution, leads to the assembly of active C_{NC} trimers. The characterization of the trimers produced by this interallelic complementation experiment is discussed below.

Results

Construction of gene fragments for independent expression and coexpression of N- and C-peptides

Because high levels of expression of the peptides were desired without the selection pressure operative under control by the *pyrBI* promoter, the pT7 system (Tabor & Richardson, 1985; Tabor, 1987) was used as the cloning vector. Details of construction of the gene fragments are described in Materials and methods. Several restriction sites in *pyrB* were created by site-directed mutagenesis in order to facilitate plasmid constructions. This enabled isolation of the structural gene, encompassing the codon corresponding to the N-terminus of the *c* chain to the appropriate 3'-end, for insertion within 10 bp downstream from the ribosome-binding site of the pT7-7 vector. In

the construction of the plasmid for expression of the N-peptide, termination of translation was achieved by the formation of a restriction site at the position corresponding to the desired C-terminus and insertion of an amber stop linker. Subsequent digestion with the appropriate

restriction enzyme permitted the isolation of the fragment containing only the portion encoding the N-peptide comprising residues 1–242. Figure 1A shows the starting plasmid pYY20C and the plasmid pYY21 containing the wild-type *pyrB* gene under the control of the pT7 pro-

Fig. 1. Construction of gene fragments for independent expression of N- and C-peptides. Expression of both peptides was under the control of the T7 RNA polymerase-based pT7-7 system (Tabor & Richardson, 1985; Tabor, 1987). **A:** The starting point of the construction of the two gene fragments was the 4.09-kb plasmid, pYY20C, which in turn was constructed from the 6.2-kb plasmid, pPYRB11 (Robey & Schachman, 1984), which carries the entire *pyrBI* operon (Schachman et al., 1984). This plasmid was modified by introducing the NcoI site (Eisenstein & Peterson, unpubl.) at the 5'-end of *pyrB*, which encodes the c chain of ATCase, and the XhoI site (Wente, unpubl.) at the 5'-end of the *pyrI* gene, which encodes the r chain of ATCase. The modified pPYRB11 was digested with EcoRI and BglII yielding a 1.6-kb fragment that was then ligated into the EcoRI–BamHI digest of pT7-7 to give pYY20C. The BglII/BamHI sites destroyed during ligation are shown in parentheses. Expression of intact c chains was achieved with pYY21, which was constructed by digesting pYY20C with NdeI and NcoI to excise the *pyrBI* promoter region (P_{BI}). After filling in the 5'-overhang by incubation with all four nucleoside triphosphates in the presence of Klenow fragment, the pT7 promoter and *pyrB* structural gene regions were joined by blunt-end ligation yielding pYY21. **B:** The N-terminal peptide comprising residues 1–242 was expressed by using plasmid pYY28, which was constructed from pYY20C. An SnaBI site was formed by site-directed mutagenesis just following the codon for residue 240 in pYY20C. Following digestion with SnaBI, an amber stop linker containing a stop codon at amino acid residue 243 was inserted by blunt-end ligation. This process also created an XbaI restriction site in the resulting plasmid, pYY27. Treatment of this plasmid with NdeI and NcoI led to two fragments, the larger of which (3.6-kb fragment) was purified and its 5'-overhang was filled in as above and then blunt-end ligated to give pYY28. **C:** Expression of the C-peptide was achieved by using plasmid pYY31. Site-directed mutagenesis of pYY20C was used to introduce an NdeI site at a position corresponding to amino acid residue 234. Digestion of the modified pYY20C with NdeI produced two fragments. The 2.9-kb fragment containing the T7 promoter, the origin of replication, and the 3'-end of *pyrB* was isolated and ligated to give pYY31.

motor. The construction of pYY28, which encodes the N-peptide, is illustrated in Figure 1B.

Expression of the C-peptide containing residues 235–310 was accomplished with pYY31, which was constructed as shown in Figure 1C by introducing an NdeI site at a codon corresponding to amino acid 234 and isolating the fragment containing the T7 promoter, the origin of replication, and the 3'-end of the *pyrB* gene.

Coexpression of the N- and C-terminal peptides in stoichiometric amounts was achieved with plasmid pYY32 in which the DNA coding for the C-terminal peptide was placed upstream of that coding for the N-terminal peptide as shown in Figure 2. These two gene fragments were arranged in that order to avoid the complications that could arise if the genes were generated sequentially with that for the N-peptide 5' to the gene encoding the C-peptide. With the latter arrangement, translation read-through or DNA recombination could lead to the formation of a wild-type sequence. This possibility was avoided by the construction of pYY32 as shown in Figure 2. The two gene fragments in pYY32 were linked by the 15-nucleotide intercistronic sequence that separates *pyrB* and *pyrI* in the *pyrBI* operon (Pauza et al., 1982) and is known to lead to the formation of stoichiometric amounts of the c and r chains for assembly into ATCase.

Coexpression of the N- and C-peptides leads to the formation of active trimers

Polyacrylamide gel electrophoresis of extracts of cells containing the coexpressed N- and C-peptides from pYY32 showed the presence of an active protein with a mobility similar to that of C_{WT} . The band representing this protein is shown in the electrophoretic patterns in Figure 3A; gels on the left were stained for protein by Coomassie blue G250 and those on the right were stained for enzyme ac-

tivity according to the procedure of Bothwell (1975). Lane 2 in each group of patterns, representing the extract from the cells coexpressing the N- and C-peptides, shows that the active species has a mobility only slightly greater than that of purified C_{WT} trimer (lane 1). The pattern obtained for purified C_{NC} trimer is shown in lane 3. For comparative purposes, the pattern for proteolytically cleaved (C_{PC}) trimer produced by limited digestion of C_{WT} trimer with α -chymotrypsin (Powers et al., 1993) is shown in lane 4. The electrophoretic patterns in Figure 3A show clearly that the active protein formed from the coexpressed N- and C-peptides must be of a size comparable to that of C_{WT} trimer and C_{PC} trimer. These results indicate that the two peptide fragments formed in vivo not only interact with each other but also that they are capable of further association noncovalently to form a complex composed of three copies of each of the two peptides.

Further evidence that the coexpressed N- and C-peptides assemble into stable oligomers comparable to C_{WT} was obtained from sedimentation velocity experiments. The pattern for C_{NC} trimer is shown in Figure 3B along with that observed for C_{WT} trimer, and the measured sedimentation coefficients were 5.8S and 6.0S, respectively. In both cases, the boundaries were relatively sharp with no evidence of low molecular weight polypeptides.

When the C_{NC} trimer was subjected to electrophoresis in polyacrylamide gels containing SDS, two bands were observed as shown in Figure 3C. One band corresponded to the larger 26-kDa N-peptide and the other represented the 8-kDa C-peptide. In contrast, the pattern for C_{WT} trimer showed a single band corresponding to intact 34-kDa c chains.

The average weight of the N-peptide determined by EIMS was $26,723.7 \pm 13.7$ Da in excellent agreement with the calculated value of 26,724 Da for a peptide composed of residues 1–242. For the C-peptide, a result of $8,530 \pm$

Fig. 2. Construction of a gene for coexpression of the N- and C-peptides of the c chain of ATCase. The two peptides from amino acid residues 235 to 310 and from 1 to 242 were expressed with pYY32, which contained not only the pT7 promoter and the coding regions for the two peptides but also a 15-nucleotide intercistronic region, which is similar to that separating *pyrB* and *pyrI* in the *pyrBI* operon (Pauza et al., 1982). The gene for the N-peptide from residues 1 to 242 was obtained by digesting pYY27 with NcoI and XbaI to yield a 700-bp fragment. After filling in and purifying this fragment, it was inserted by blunt-end ligation into pYY31 that had been linearized by digestion with XhoI and then filled in by incubation with all four nucleoside triphosphates and Klenow fragment. This construct of pYY32 places the coding region of the C-peptide in front of that for the N-peptide in order to eliminate the possibility of producing intact c chains by either read-through or recombination.

Fig. 3. Formation of active trimers from coexpressed N- and C-peptides. **A:** Polyacrylamide gel electrophoresis patterns stained for protein by Coomassie blue G250 (on the left) and for enzyme activity (on the right). Lane 1, purified C_{WT} trimer; lane 2, cell extract containing coexpressed N- and C-peptides; lane 3, purified C_{NC} trimer; lane 4, proteolytically cleaved C trimer. **B:** Sedimentation velocity patterns obtained with purified C_{NC} trimer and C_{WT} trimer. Patterns were obtained after 25 min of operation at 60,000 rpm in a Beckman XL-A analytical ultracentrifuge. Both patterns, C_{NC} trimer on the left and C_{WT} trimer on the right, represent absorbance at 280 nm vs. distance in cm from the axis of rotation. The measured values of the sedimentation coefficient were 5.8S for C_{NC} trimer and 6.0S for C_{WT} trimer. The values are not corrected for temperature. **C:** Electrophoresis patterns in a polyacrylamide gel containing SDS. Lane 1, molecular weight markers; lane 2, the c chain from purified C_{WT} trimer; lane 3, the N- and C-peptides from purified C_{NC} trimer; lane 4, the peptide fragments produced by limited chymotryptic hydrolysis of C_{WT} trimer; lane 5, the c and r polypeptide chains from ATCase_{WT}.

5.5 Da was obtained by mass spectrometry¹ compared to the value of 8,529 Da expected for the peptide comprising residues 235–310.

C_{NC} trimers and reconstituted ATCase-like molecules containing coexpressed peptides have enzyme activities comparable to wild-type proteins

The enzyme activity of C_{NC} trimers measured by carbamoyl aspartate formation as a function of aspartate concentration (at 4 mM carbamoyl phosphate) is comparable to that of C_{WT} trimers. $V_{\max}$ for C_{NC} trimer is 21 $\mu\text{mol}/\mu\text{g}/\text{h}$ compared to 26 $\mu\text{mol}/\mu\text{g}/\text{h}$ for C_{WT} trimer. The saturation curve is shown in Figure 4A, and the Eadie plot of the same data (in the inset) demonstrates the hyperbolic relationship between enzyme activity and aspartate concentration. Figure 4B shows the analogous data for the holoenzyme (ATCase_{NC}) containing the N- and C-peptides, and the linear Eadie plot (in the inset) shows that the kinetics are Michaelian in contrast to the sigmoidal kinetics exhibited by ATCase_{WT} under the same conditions. $V_{\max}$ for ATCase_{NC} was virtually identical to that for wild-type enzyme. Table 1 summarizes the data for

both the C_{NC} trimer and the ATCase_{NC} along with results for C_{WT} trimer and ATCase_{WT}. Although the values of $V_{\max}$ for C_{NC} trimer and ATCase_{NC} are comparable to those for C_{WT} trimer and ATCase_{WT}, the values of K_m for

Table 1. Properties of C_{NC} trimer and ATCase_{NC}

Species	Enzyme kinetics ^a			Binding of PALA ^b	Sedimentation coefficient ^c	
	$V_{\max}$	K_m or $K_{0.5}$	n_H		–PALA (S)	+PALA (S)
C _{WT} trimer	26	6	1.0		6.0	
C _{NC} trimer	21	80	1.0		5.8	
ATCase _{WT}	18	8	1.7	1.0	11.5	11.1
ATCase _{NC}	17	137	1.0	6.0	11.1	11.1

^a Enzyme kinetics were performed at 30 °C with a 50 mM MOPS buffer at pH 7.0 containing 0.2 mM EDTA and 2.0 mM 2-mercaptoethanol. $V_{\max}$ is expressed as μmol carbamoyl aspartate per μg of C trimer per h. K_m or $K_{0.5}$ evaluated from the enzyme assays is expressed as mM aspartate.

^b Equilibrium dialysis measurements were performed at 23 °C with a 40 mM potassium phosphate buffer at pH 7.0 containing 0.2 mM EDTA and 2.0 mM 2-mercaptoethanol. K_d is the dissociation constant.

^c Sedimentation velocity measurements were made at about 23 °C with a Beckman Model XL-A analytical ultracentrifuge. The buffer was the same as used for the equilibrium dialysis measurements. When PALA was present, the amount was 20 mol of PALA per mol of enzyme.

¹ This value corresponded to the major component detected by mass spectrometry. About 10% of the intensity corresponded to a smaller peptide with an average mass of $7,802 \pm 4$ Da. The smaller fragment probably resulted from proteolytic cleavage at Tyr 240.

Fig. 4. Enzyme activity of purified C_{NC} trimer and ATCase_{NC}. Assays were performed at 30 °C by the method of Davies et al. (1970) with [¹⁴C]carbamoyl phosphate (4 mM) in 50 mM MOPS buffer at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Enzyme activity is expressed as velocity in μmol of carbamoyl-L-aspartate formed per h per μg of C trimer as a function of the concentration of aspartate. **A:** Data for purified C_{NC} trimer. The inset shows the Eadie plot of the same data to demonstrate that the saturation curve is hyperbolic. **B:** Results for holoenzyme formed by mixing purified C_{NC} trimer with R dimers isolated from ATCase_{WT}. The linear Eadie plot in the inset for ATCase_{NC} demonstrates that the reconstituted holoenzyme containing the fragmented c chains is devoid of the cooperativity characteristic of ATCase_{WT}.

C_{NC} trimer and ATCase_{NC} were about 15-fold greater than those for their counterparts composed of intact c chains.

ATCase_{NC} has six active sites with a high affinity for the bisubstrate ligand, PALA

Because the enzyme kinetics demonstrated that the holoenzyme containing the fragmented c chains differed markedly from ATCase_{WT} in that the former was devoid of

the characteristic sigmoidal dependence of activity on aspartate concentration, we examined the equilibrium binding of PALA to ATCase_{NC}. As seen in the saturation curve in Figure 5 and in the linear Scatchard plot, there are six binding sites for PALA, and no cooperativity is evident in the binding of the ligand to the enzyme. The affinity of ATCase_{NC} containing the fragmented and partially overlapping c chains is reduced only slightly ($K_d = 6 \times 10^{-7}$ M) compared to ATCase_{WT} ($K_{av} = 1 \times 10^{-7}$ M).

Sedimentation velocity studies indicate that the conformation of ATCase_{NC} is analogous to that of ATCase_{WT} in the R conformation

In view of the absence of cooperativity of the holoenzyme containing the coexpressed N- and C-peptides, it was of importance to determine whether the conformation of ATCase_{NC} was analogous to either the T or R state of ATCase_{WT}. Earlier studies have shown that both ATCase_{WT} and various mutant forms that exhibit significant cooperativity undergo about a 3.5% decrease in sedimentation coefficient upon the binding of PALA at the active sites (Howlett et al., 1977; Schachman, 1988). Conversely, with most mutant forms of ATCase that are devoid of cooperativity in enzyme kinetics or PALA binding, there is virtually no change in the sedimentation coefficient ($\Delta s/s$) upon ligation with PALA. Hence sedimentation velocity experiments were performed with ATCase_{NC} in order to determine whether PALA binding resulted in a

Fig. 5. Equilibrium binding of the bisubstrate ligand PALA to ATCase_{NC}. The binding of [³H]PALA was measured by equilibrium dialysis as described in Materials and methods with solutions of ATCase_{NC} formed from mixing purified C_{NC} trimer and R subunits. Binding measurements were performed with a 40 mM potassium phosphate buffer at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Results are plotted as r , mol of bound PALA per mol of total enzyme, vs. the concentration of free PALA. In the inset is a Scatchard plot of the same data as $r/[PALA]$ vs. r where $[PALA]$ is the concentration of free PALA in μM.

change in the quaternary structure of the enzyme. The ligand-promoted change in sedimentation coefficient of ATCase_{NC} was virtually zero as shown in Table 1. Moreover, the sedimentation coefficient of the holoenzyme containing the fragmented c chains was 11.1S, in excellent agreement with the value, 11.1S, for ATCase_{WT} liganded with PALA. In contrast, unliganded ATCase_{WT} had a sedimentation coefficient of 11.5S and ($\Delta s/s$) due to PALA binding was -3.4% . Thus the quaternary structure of unliganded ATCase_{NC} is analogous to the **R** conformation of ATCase_{WT}.

Independent expression of N- and C-peptides leads to insoluble products that dissolve in urea and assemble into active trimers

When the N- and C-peptides were synthesized separately in *E. coli* strains containing plasmids pYY28 and pYY31, respectively, no enzyme activity could be detected in the extracts. Moreover, the overproduced peptides were barely detectable among the soluble proteins when the extracts were subjected to electrophoresis in polyacrylamide gels containing SDS. Instead, the peptides were found largely in the insoluble pellets obtained from the cell extracts. This was demonstrated by dissolving the pellets in 6.5 M urea and analyzing the solutions by electrophoresis in polyacrylamide gels containing SDS.

Although the individual N- and C-peptides aggregated to form insoluble material, they were capable of assembling into active trimers. The pellets from the separate extracts were suspended in a 6.5 M urea solution in 50 mM Tris-Cl buffer at pH 7.5 containing 5 mM dithiothreitol. After 1 h at 0 °C, the solutions were mixed and then diluted 10-fold with 50 mM Tris-Cl buffer at pH 7.5 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. All samples were incubated overnight at 4 °C and then subjected to electrophoresis in 6.5% polyacrylamide gels, which were stained for enzyme activity (Bothwell, 1975). The resulting electrophoresis patterns are shown in Figure 6. Whereas no active species could be detected in extracts from cells containing either the N-peptide expressed by plasmid pYY28 or the C-peptide expressed by pYY31 (lanes 4 and 5, respectively), active species were produced when those extracts were mixed and treated with 6.5 M urea followed by dilution (lane 6). The active C_{NC} trimer from an extract in which the peptides were coexpressed in cells containing plasmid pYY32 is shown in lane 7. The ability to reconstitute active C_{NC} trimers from purified C_{NC} trimers, which were dissociated into individual fragments in urea and then diluted, is illustrated in lane 8. The yield of enzyme activity in the reassembly of C_{NC} trimers was 85% that of the recovery of activity of C_{WT} trimers. The small difference in mobility between C_{NC} trimer and wild-type C trimer is illustrated by the pattern for purified C_{WT} trimer (lane 1) and that for the extract from cells containing plasmid pYY21, shown in lane 3. The

Fig. 6. Formation of active C_{NC} trimer from separate N- and C-peptides. Electrophoresis patterns from 6.5% polyacrylamide gels that were stained for enzyme activity. Lane 1, purified C_{WT} trimer; lane 2, extract of BL21 (DE3) pLysS containing plasmid pT7-7; lane 3, extract of cells containing plasmid pYY21, which produces C_{WT} trimer; lane 4, N-peptide produced in cells containing plasmid pYY28; lane 5, C-peptide produced in cells containing plasmid pYY31; lane 6, active C_{NC} trimers formed by mixing cell extracts containing the N-peptide and C-peptide, incubating them in 6.5 M urea, and then diluting the mixture to permit association of the peptides and C_{NC} trimer formation; lane 7, active C_{NC} trimers formed in cells in which the N- and C-peptides were coexpressed by plasmid pYY32; lane 8, purified C_{NC} trimers, which were dissociated into individual fragments in 6.5 M urea and then reassembled into active trimers. The C_{NC} trimers were purified from extracts of cells containing plasmid pYY32, which coexpresses the N- and C-peptides.

extract containing vector pT7-7 as a negative control is shown in lane 2.

Discussion

Through the use of site-directed mutagenesis of *pyrB*, which encodes the c chain of ATCase, we have constructed gene fragments encoding an N-terminal peptide comprising residues 1–242 and a C-terminal peptide composed of residues 235–310. Incorporating the respective DNA fragments separately into plasmids under the control of T7 RNA polymerase (Tabor & Richardson, 1985), as shown in Figure 1B,C, led to high-level production of the two peptides. Although the bulk of the peptides expressed separately in cells was found among the insoluble debris when extracts were prepared, their presence was readily demonstrated when the insoluble material was dissolved in 6.5 M urea and subjected to electrophoresis in polyacrylamide gels containing SDS. Moreover, when the urea-solubilized material from the two different extracts was mixed and then diluted 10-fold, substantial levels of enzyme activity were obtained. The enzymatically active species (C_{NC} trimer) formed in this way had an electrophoretic mobility similar to (although clearly different from) that of wild-type C trimers (cf. lane 6 and lane 1 in Fig. 6).

Although active C_{NC} trimers containing incomplete and partially overlapping polypeptide chains could be formed by mixing extracts from cells producing the N-

and C-peptides separately, a more efficient assembly appeared to result when the peptides were coexpressed in the same cell. The construction of the tandem DNA sequences in plasmid pYY32 encoding the C-peptide (residues 235–310) and N-peptide (residues 1–242) is illustrated in Figure 2. Extracts from cells containing plasmid pYY32 showed an active species (Fig. 3A) with a mobility close to that of C_{WT} trimer. Moreover, purification of the coexpressed C_{NC} trimer yielded a protein with a sedimentation coefficient of 5.8S, only slightly lower than that (6.0S) of purified C_{WT} trimer (Fig. 3B). These results demonstrate that the product formed by the assembly of the coexpressed N- and C-peptides has almost the same electrophoretic mobility and sedimentation coefficient as the trimer containing intact c chains. Although the results of the sedimentation velocity experiments are only preliminary, it seems likely that the value of the sedimentation coefficient of C_{NC} trimer is significantly less than that for C_{WT} trimer. This difference is almost certainly attributable to a larger frictional coefficient for the C_{NC} trimer containing the fragmented and partially overlapping c chains.

The specific activity of purified C_{NC} trimer is virtually identical to that of C_{WT} trimer (Table 1). In contrast, K_m for the trimer composed of fragmented chains is significantly higher (80 mM aspartate) than that for the wild-type subunit (6 mM aspartate). Some of this decreased affinity for aspartate may be attributable to the two negatively charged residues, Asp 236 and Glu 239, in the redundant region of the N- and C-peptides. In this regard, it is of interest that the affinity $ATCase_{NC}$ for PALA is only slightly lower than that for $ATCase_{WT}$ (K_d of 6×10^{-7} M for $ATCase_{NC}$ compared to 1×10^{-7} M for $ATCase_{WT}$). The possible role of the overlapping negatively charged amino acid residues in decreasing the affinity for aspartate is worth investigating further by alternative constructions of the gene fragments so as to eliminate the redundancy.

As seen in Figure 4B, the holoenzyme composed of the fragmented and partially overlapping c chains is devoid of the cooperativity characteristic of $ATCase_{WT}$. The linear Eadie plot of the kinetic data as well as the linear Scatchard plot for the equilibrium binding of PALA demonstrate clearly the absence of homotropic effects. Is the absence of cooperativity attributable to the inability of aspartate or PALA to promote the characteristic T $\rightarrow$ R conversion? Or alternatively, is $ATCase_{NC}$ already in the R conformation even when unliganded? The results of the sedimentation velocity experiments summarized in Table 1 show clearly that unliganded $ATCase_{NC}$ has a swollen structure characteristic of PALA-liganded $ATCase_{WT}$. Because it is already in the R state ($s = 11.1S$), the addition of PALA to $ATCase_{NC}$ has virtually no effect. In contrast, with $ATCase_{WT}$ under the same experimental conditions the sedimentation coefficient decreases from 11.5S to 11.1S upon the addition of a saturating amount of PALA, i.e., $\Delta s/s = -3.4\%$.

As yet we have no quantitative data indicating the amounts of N- and C-peptides recovered in extracts when they are coexpressed in the same cell as compared to the yields when the peptides are expressed individually in different cells. There may be, for example, more extensive proteolysis both in vivo and in vitro in the cellular extracts when the individual peptides are expressed separately and cannot, therefore, assemble into trimers. Preliminary studies indicate that purified C_{NC} trimers can be dissociated into unfolded fragments in concentrated urea solutions and that dilution leads to the reassembly of active trimers in high yield. Indeed, the reconstitution from fragments is almost as efficient as that observed with intact chains. This result does not, of course, rule out the possible role in vivo of chaperonins when the N- and C-peptides are coexpressed in the same cell and active C_{NC} trimers are formed. As in the assembly of C_{PC} trimers from the proteolytically cleaved chains (Powers et al., 1993), we have no information about the sequence of individual steps in the assembly of C_{NC} trimers from the N- and C-peptides. Do the fragments first fold into partially stable "native-like" secondary and tertiary structures that permit docking and then association into stable trimers? Analysis of such a process requires devising experimental conditions, other than concentrated urea solutions, for obtaining the fragments in soluble form.

In this study the genetic manipulation of *pyrB* to produce the N- and C-peptides was based on the demonstration (Powers et al., 1993) that the proteolytic fragments comprising residues 1–240 and 241–310 could reassemble efficiently into active C_{PC} trimers. The results from the genetic constructions show that assembly is also efficient even though there is a 8-amino acid overlap in the two fragments. Can there be deletions as well as overlaps? Will other pairs of fragments prove competent for assembly? Must the chain fragments involve a loop region in the structure such as that in the segment including residues 230–245? These are some of the questions that can be answered by further genetic studies of interallelic complementation involving incomplete polypeptide chains.

Materials and methods

Construction of gene fragments for independent expression of N- and C-peptides

The starting point for expression of the c chain and only a fragment of the r chain of *ATCase* was the 4.09-kb plasmid, pYY20C, which was constructed from a modified form of pPYRB11 (Robey & Schachman, 1984). This modification involved the introduction of an *NcoI* site at the 5'-end of *pyrB* (Eisenstein & Peterson, unpubl.) along with an *XhoI* site at the 5'-end of *pyrI* (Wente, unpubl.). The modified pPYRB11 was digested with *EcoRI* and *BglII* to form a 1.6-kb fragment containing the *pyrBI* promoter, the entire *pyrB* gene, the modified intercistronic

region of the *pyrBI* operon (Pauza et al., 1982), and a part of the *pyrI* gene. This 1.6-kb fragment was then ligated into the EcoRI-BamHI digest of pT7-7 (Tabor, 1987) to yield pYY20C (Fig. 1A), which, with further modification (see below), was used for expression of incomplete c chains. For expression of intact c chains in the T7 RNA polymerase-based system, pYY20 was digested with NdeI and NcoI, in order to excise the *pyrBI* promoter, and the fragment containing the pT7 promoter and *pyrB* gene was filled in and ligated to yield pYY21.

Expression of the N-terminal peptide comprising residues 1–242 was achieved with plasmid pYY28, which was produced as shown in Figure 1B. This was accomplished by first constructing plasmid pYY27 by site-directed mutagenesis of pYY20C at the codon corresponding to amino acid 241 to form an SnaBI site, followed by the insertion of an amber stop linker by blunt-end ligation into the SnaBI site. This led to the introduction of a stop codon at amino acid 243 and an XbaI restriction site. Deletion of the *pyrBI* promoter (P_{BI}) was achieved by digestion of pYY27 with NdeI and NcoI, which produced two fragments. The 3.6-kb fragment was purified, and its 5'-overhang was filled in by incubation with Klenow fragment in the presence of all four nucleoside triphosphates. This filled-in 3.6-kb fragment was cyclized by blunt-end ligation to give pYY28.

The expression of the C-terminal peptide containing residues 235–310 was achieved with pYY31, which was constructed as shown in Figure 1C by introducing an NdeI site at a codon corresponding to amino acid 234. Two fragments were produced upon digestion with NdeI, and the 2.9-kb fragment containing the T7 promoter, the origin of replication, and the 3'-end of the *pyrB* gene was isolated and cyclized by ligation.

Construction of gene for coexpression of N- and C-peptides

Coexpression of the N- and C-terminal peptides in stoichiometric amounts was achieved with plasmid pYY32 in which the DNA coding for the C-terminal peptide was placed upstream of that coding for the N-terminal peptide. Construction of pYY32 is illustrated in Figure 2. Separating the two partial genes was the 15-nucleotide sequence that exists between *pyrB* and *pyrI* in the *pyrBI* operon (Pauza et al., 1982). The gene for N-peptide was obtained by digesting pYY27 (Fig. 1B) with NcoI and XbaI (which cuts at a nucleotide corresponding to amino acid residue 242), and the 700-bp fragment, encoding the first 242 residues in the c chain, was filled in and purified. This was then inserted by blunt-end ligation into a linear XhoI digest of pYY31 (Fig. 1C), which had been filled in by incubation with all four nucleoside triphosphates and the Klenow fragment. This construct, pYY32, shown in Figure 2, contains the nucleotide sequence corresponding to residues 235–310, a 15-base intercistronic sequence sim-

ilar to that in *pyrBI*, a sequence encoding amino acid residues 1–242, and a partial sequence from *pyrI*.

Expression of fragments

Peptides and complete c chains were overexpressed in *E. coli* strain BL21 (DE3) pLysS, which contains the gene for T7 RNA polymerase on the *E. coli* chromosome under the control of the *lac* promoter (Studier et al., 1990). This strain, kindly furnished by Dr. F.W. Studier of Brookhaven National Laboratory, contains a chloramphenicol-resistant plasmid pLysS carrying the gene for T7 lysozyme, an inhibitor of T7 RNA polymerase. The cloning vector pT7-7 (Tabor, 1987) was a gift from Dr. Stanley Tabor of Harvard Medical School. Plasmids constructed to express the various peptides and complete chains were transformed to BL21(DE3)pLysS, and the transformed cells were inoculated in LB medium plus 25 μ g/mL of ampicillin and 20 μ g/mL chloramphenicol. Cells were grown at 37 °C for 3–4 h until the desired cell density was attained, and then IPTG was added to a final concentration of 0.4 mM. After continued incubation for 2–3 h to allow expression of the target genes, the cells were collected by centrifugation.

Preparation of cell extracts involved collection of cells from 1-L cultures by centrifugation followed by resuspension in 10 mL of 0.1 M Tris-Cl at pH 8.5, containing 5 mM MgCl₂. Freezing and thawing without the addition of lysozyme was used to prepare extracts to which DNaseI was added in the presence of 1 mM PMSF to lower the viscosity of the suspension and inhibit protease activity.

Site-directed mutagenesis

Restriction endonucleases were obtained from New England Biolabs or BRL and used according to the supplier's specifications. Site-directed mutagenesis was performed by the method of Kunkel (1985).

Purification of C_{NC} trimer from coexpressed N- and C-peptides

Two methods were employed to obtain purified C_{NC} trimers for characterization in terms of electrophoretic mobility, sedimentation coefficient, enzyme activity, and equilibrium binding of the bisubstrate analog PALA. The first procedure involved applying 5 mL of extract from 1 L of cell pack, prepared as described above, onto a G150 Sephadex column (1.5 $\times$ 100 cm) and then onto a DEAE-cellulose column (Whatman DE52, 1.5 $\times$ 20 cm). Samples were eluted in 50 mM Tris-Cl at pH 7.5, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA with a linear 0–0.6 N KCl gradient. The pooled fractions were purified further through S-Sepharose 300 (1.5 $\times$ 100 cm) in 40 mM potassium phosphate buffer (pH 7.0) containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. The

final step involved a Sepharose Q column (0.9×20 cm) with the same buffer conditions as used for the DE52 column. The alternative procedure for obtaining purified C_{NC} trimer involved adding purified wild-type R subunit in sufficient quantity to convert the C_{NC} trimer in the crude extract to $ATCase_{NC}$. The resulting holoenzyme was purified by the standard procedure used for purifying $ATCase_{WT}$. The holoenzyme, $ATCase_{NC}$, was then treated with neohydricin to obtain C_{NC} trimer by procedures described earlier (Yang et al., 1978).

Mass spectrometry of the fragments from coexpressed N- and C-peptides

The masses of the N- and C-peptides were determined by EIMS at the Mass Spectrometry Facility of the University of California at San Francisco. Following purification of C_{NC} trimer from coexpressed N- and C-peptides, the fragments were separated on a Vydak C-18 HPLC column as described in the previous paper (Powers et al., 1993).

Assays of enzyme activity

Measurements of enzyme activity at 30°C were performed by the method of Davies et al. (1970) using ^{14}C -labeled carbamoyl phosphate purchased from New England Nuclear. The buffer was 50 mM MOPS at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Nonlinear least-squares analysis was used to evaluate $V_{\max}$ and K_m .

Equilibrium binding of PALA

Equilibrium dialysis with ^3H -labeled PALA was used to measure the binding of PALA to $ATCase_{NC}$ at 23°C according to the procedure of Newell et al. (1989). The buffer was 40 mM potassium phosphate at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA.

Polyacrylamide gel electrophoresis

Intact C_{WT} and C_{NC} trimers were subjected to electrophoresis (Gabriel, 1971) in 6.5% polyacrylamide gels that were stained for total protein using Coomassie brilliant blue G250 or for enzyme activity by the method of Bothwell (1975). Electrophoresis of dissociated and denatured proteins was performed with polyacrylamide gels containing SDS according to the method of Laemmli (1970) followed by staining with Coomassie brilliant blue R250.

Sedimentation velocity measurements

A Beckman Model XL-A analytical ultracentrifuge equipped with absorption optics was used for determination of sedimentation coefficients. Protein concentrations were about 1 mg/mL in 40 mM potassium phosphate

buffer at pH 7.0, containing 2.0 mM 2-mercaptoethanol and 0.2 mM EDTA. Experiments were conducted at 20 – 23°C , and the reported sedimentation coefficients are not corrected to the standard conditions of 20°C and a solvent with the density and viscosity of H_2O . A rotor containing three double-sector cells and a counterbalance reference cell was used to permit comparison of one sample relative to another. Patterns representing plots of absorbance at 280 nm vs. distance from the axis of rotation were analyzed with various computer programs provided by Beckman Instruments, Inc.

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