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Reconstitution of active catalytic trimer of aspartate transcarbamoylase from proteolytically cleaved polypeptide chains

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Abstract

Treatment of the catalytic (C) trimer of *Escherichia coli* aspartate transcarbamoylase (ATCase) with α-chymotrypsin by a procedure similar to that used by Chan and Enns (1978, *Can. J. Biochem. 56*, 654–658) has been shown to yield an intact, active, proteolytically cleaved trimer containing polypeptide fragments of 26,000 and 8,000 MW. V_{max} of the proteolytically cleaved trimer (C_{PC}) is 75% that of the wild-type C trimer, whereas K_m for aspartate and K_d for the bisubstrate analog, *N*-(phosphonacetyl)-L-aspartate, are increased about 7- and 15-fold, respectively. C_{PC} trimer is very stable to heat denaturation as shown by differential scanning microcalorimetry. Amino-terminal sequence analyses as well as results from electrospray ionization mass spectrometry indicate that the limited chymotryptic digestion involves the rupture of only a single peptide bond leading to the production of two fragments corresponding to residues 1–240 and 241–310. This cleavage site involving the bond between Tyr 240 and Ala 241 is in a surface loop known to be involved in intersubunit contacts between the upper and lower C trimers in ATCase when it is in the **T** conformation. Reconstituted holoenzyme comprising two C_{PC} trimers and three wild-type regulatory (R) dimers was shown by enzyme assays to be devoid of the homotropic and heterotropic allosteric properties characteristic of wild-type ATCase. Moreover, sedimentation velocity experiments demonstrate that the holoenzyme reconstituted from C_{PC} trimers is in the **R** conformation. These results indicate that the intact flexible loop containing Tyr 240 is essential for stabilizing the **T** conformation of ATCase. Following denaturation of the C_{PC} trimer in 4.7 M urea and dilution of the solution, the separate proteolytic fragments reassociate to form active trimers in about 60% yield. How this refolding of the fragments, docking, and association to form trimers are achieved is not known.

Keywords: folding and docking of polypeptides; limited proteolysis; oligomer formation from fragments

In the classical studies on ribonuclease (Richards, 1958; Richards & Vithayathil, 1959) it was shown that, despite the rupture of a single peptide bond in the enzyme upon limited digestion with subtilisin, the proteolytically cleaved enzyme was stable and active. Following that research there have been numerous studies indicating that noncovalent interactions are sufficiently strong that the tertiary and quaternary structures of some proteins persist even in the absence of intact polypeptide chains. Moreover, after denaturation and dissociation of the proteolytically cleaved proteins and isolation of the individual fragments, it has been possible to reassemble many of these proteins as active species composed of fragments that associate noncovalently into stable complexes. This has been demonstrated both for monomeric proteins such as cytochrome *c* (Taniuchi et al., 1986; Fisher & Taniuchi, 1992), staphylococcal nuclease A (Taniuchi et al., 1977), 3-phosphoglycerate kinase (Vas et al., 1990), and serum albumin (Feldhoff & Peters, 1975; Reed et al., 1976; Crouch & Kupke, 1980; Peters, 1985), as well as for oligomeric proteins such as the β_2 subunit of tryptophan

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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; C_{PC}, proteolytically cleaved trimer; WT as subscript, wild type; PALA, *N*-(phosphonacetyl)-L-aspartate; T_m, melting temperature corresponding to the maximum temperature in the endotherm obtained by differential scanning microcalorimetry; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; EIMS, electrospray ionization mass spectrometry.

synthetase (Zetina & Goldberg, 1980), lactate dehydrogenase (Opitz et al., 1987), alanine racemase (Galakatos & Walsh, 1987), penicillin acylase (Lindsay & Pain, 1991), and *trp* repressor (Tasayco & Carey, 1992). Limited proteolysis also has provided valuable information about T7 RNA polymerase (Tabor & Richardson, 1985; Ikeda & Richardson, 1987) and has been of use in separating the various enzymically active fragments of multifunctional proteins such as aspartokinase-homoserine dehydrogenase (Cohen & Dautry-Varsat, 1980), DNA polymerase (Brutlag et al., 1969; Klenow & Henningsen, 1970), and CAD, the multifunctional enzyme comprising carbamoyl phosphate synthetase, aspartate transcarbamoylase, and dihydro-orotase (Davidson et al., 1981). These studies demonstrating that intact, active proteins are obtained despite proteolytic cleavage of the chains and that limited proteolysis of multifunctional proteins yields individual folded fragments having different enzyme activities provide convincing evidence that polypeptide chains are frequently folded into domains linked by flexible, exposed loops, which are the sites of the cleavage.

Because crystallographic studies (Kim et al., 1987; Ke et al., 1988) on *Escherichia coli* ATCase (aspartate carbamoyltransferase, carbamoyl phosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2) showed that the six c chains in the enzyme are folded in clearly segmented domains (Kinemages 1, 3), it seemed of interest to determine whether limited proteolysis of the c chains could yield individual fragments capable of associating into active enzyme. Proteolysis of ATCase was performed by McClintock and Markus (1968), but their goal at that time was to determine the effects of limited proteolysis on the allosteric transition. Somewhat later, Chan and Enns (1978), in studies on the digestion of both isolated C_{WT} subunits and intact ATCase by chymotrypsin, demonstrated that it was possible to obtain cleaved C trimers with partial activity. The products of the limited proteolysis, upon analysis by electrophoresis in polyacrylamide gels containing SDS, were shown to be a major polypeptide of 26 kDa plus smaller fragments. No information was obtained about the site of the proteolytic cleavage. In view of the availability of the three-dimensional structure of the C_{WT} trimers within ATCase (Kim et al., 1987; Ke et al., 1988), showing the two separate domains and several loops in the tertiary structure of the c chains, it seemed of interest to initiate renewed proteolysis studies on the isolated C_{WT} trimers.

Research on the proteolysis of the C_{WT} trimers of ATCase could, in principle, provide valuable information not only about the stability of the trimers and the role of the individual domains but also about the assembly of the trimers from unfolded chains (Burns & Schachman, 1982a,b). The earlier studies on the stability of the trimers and their assembly leave unanswered a host of questions that need resolution. Can one or a few peptide bonds be ruptured without a loss in enzyme activity? Where is the site of the limited proteolysis? Can the fragments be separated and are they capable of reassociating into active trimers? How stable are the C_{PC} trimers? How do the individual fragments fold and dock to form a complex? At what stage are active trimers formed? Are C_{PC} trimers competent to associate with isolated regulatory (R) dimers to form ATCase-like molecules, and what are the allosteric properties of the resulting complex? This communication presents answers to some of these questions, and the results provide the basis for the manipulation of *pyrB*, the structural gene encoding the c chains of ATCase, so as to produce fragments in vivo that are capable of assembling into active enzyme (Yang & Schachman, 1993).

Results

Limited chymotryptic digestion yields intact trimers cleaved at the bond between Tyr 240 and Ala 241

As shown by Chan and Enns (1978), limited digestion of C_{WT} trimer by α-chymotrypsin produced a partially active enzyme that was shown, after dissociation by SDS, to contain a 26-kDa fragment in addition to one or more smaller fragments. In the present work, efforts were focused on the experimental conditions required to rupture only a single peptide bond in each c chain within a C_{WT} trimer and, at the same time, to produce a population of trimers in which most of the polypeptide chains were cleaved (Kinemages 1, 2). The experimental conditions required to obtain a high efficiency of cleavage without subsequent production of small polypeptide fragments are described in Materials and methods.

The product of the limited proteolysis was found to have elution properties in anion-exchange and size-exclusion chromatography similar to those of C_{WT} trimers (data not shown). Sedimentation velocity experiments (Fig. 1A) showed that C_{PC} trimer migrated as a single sharp boundary with a sedimentation coefficient of 5.6S as compared to C_{WT} trimer with a sedimentation coefficient of 5.7S. Despite the overall similarity in size and hydrodynamic properties, C_{PC} trimers can be differentiated from C_{WT} trimers by electrophoresis in polyacrylamide gels. As seen in Figure 1B, C_{PC} migrates more slowly than C_{WT} trimer. Electrophoresis of C_{PC} trimer in polyacrylamide gels containing SDS showed two major bands (Fig. 1B) with mobilities corresponding to 26 kDa and 8 kDa, respectively, as compared to the single component of 34 kDa obtained for the intact polypeptide chain from C_{WT} trimer. The proteolytic fragments separated by SDS-PAGE were subjected to automated Edman degradation in order to determine the site of the proteolytic cleavage. N-terminal sequence analysis of the 26-kDa fragment yielded the sequence Ala-Asn-Pro-Leu-Tyr-Gln, which corresponds to residues 1–6 encoded by the gene for the c chain (Hoover et al., 1983; Schachman et al., 1984). The absence of N-terminal methionine in intact c chains was reported

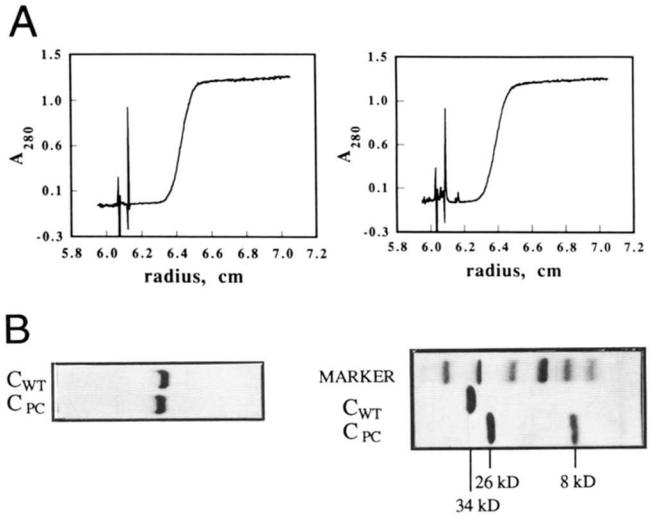

Fig. 1. Sedimentation velocity patterns and electrophoretic analysis of proteolytically cleaved C_{PC} and wild-type C_{WT} trimers. **A**: Sedimentation velocity patterns after 20 min of operation at 60,000 rpm at 21 °C. The patterns representing absorbance at 280 nm vs. distance in cm from the axis of rotation were obtained with a Beckman XL-A analytical ultracentrifuge. The patterns for both C_{PC} trimer (left) and for C_{WT} trimer (right) were obtained in the same experiment using a four-cell titanium rotor. Protein concentrations were 1.5 mg/mL for C_{PC} and C_{WT} trimer in 50 mM Tris-Cl buffer at pH 7.5, containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol. The observed sedimentation coefficients, uncorrected for temperature and the viscosity and density of the buffer, were 5.6S and 5.7S for C_{PC} and C_{WT} trimer, respectively. **B**: (Left) Patterns from electrophoresis experiment in 7% polyacrylamide gel stained by Coomassie brilliant blue R250. Upper pattern represents C_{WT} trimer and lower corresponds to C_{PC} trimer. (Right) Patterns from electrophoresis experiment in 13% polyacrylamide gel containing SDS and stained with Coomassie brilliant blue R250. The upper lane shows the molecular weight markers (left to right): 46 kDa, 30 kDa, 21 kDa, 14 kDa, 6.5 kDa, and 3.4 kDa. The middle lane represents the intact polypeptide chain (34 kDa) from C_{WT} trimer and the lower lane shows the two polypeptide fragments (26 kDa and 8 kDa) obtained from C_{PC} trimer.

previously (Konigsberg & Henderson, 1983). Analysis of the 8-kDa fragment yielded the N-terminal sequence Ala-Asn-Val-Lys-Ala, which corresponds to residues 241–245 encoded by the gene. Thus, α-chymotrypsin cleaves the peptide bond following Tyr 240 in accord with the known specificity of α-chymotrypsin.

To clarify whether additional fragments had been produced but not detected by SDS gel electrophoresis, we separated the 26-kDa and 8-kDa fragments by HPLC, and the respective molecular weights were determined by EIMS. For the 8-kDa fragment, an average molecular weight of 7,805.4 ± 2.5 was found, in agreement with that expected from the known sequence (7,807 Da). In contrast, the average molecular weight found for the 26-kDa fragment (26,521 ± 6 Da) was 9 Da larger than that expected (26,512 Da) for chains consisting of residues 1–240. Mass spectrometric analysis of intact c chains yielded an average value of 34,328 ± 11 Da, whereas the expected result for the complete chain is 34,301 Da. Hence, the mass spectrometric data provide clear evidence that no additional proteolytic cleavage had occurred other than at the bond between Tyr 240 and Ala 241.

Proteolytically cleaved C trimers are active and bind the bisubstrate ligand, PALA

Limited proteolysis of C_{WT} trimer causes only a 25% reduction in enzyme activity as seen in Table 1. In contrast, K_m for C_{PC} trimer is sevenfold larger than that for C_{WT} trimer. Similarly, the affinity of C_{PC} trimer for PALA is decreased substantially. The 7-fold increase in K_m and 16-fold increase in K_d could be attributed to the formation of the negatively charged carboxylate on Tyr 240 and the increased flexibility of the chain in the general vicinity of the active sites.

Allosteric behavior of holoenzyme containing C_{PC} trimers is reduced relative to $ATCase_{WT}$

Addition of purified R dimers to C_{PC} trimers resulted in the formation of active, stable holoenzyme in good yield. As seen in Table 1, V_{max} for holoenzyme containing C_{PC} trimers is about 56% that for $ATCase_{WT}$. Also, the concentration of aspartate required for half-maximal velocity was increased about 10-fold for the holoenzyme containing C_{PC} trimer as compared to $ATCase_{WT}$. The marked difference between the reconstituted holoenzyme containing C_{PC} trimer and $ATCase_{WT}$ is seen in Figure 2. Whereas $ATCase_{WT}$ exhibits marked cooperativity with respect to aspartate as seen by the sigmoidality in the saturation plot (Fig. 2B) or the curvature in the Eadie plot (inset of Fig. 2B), the holoenzyme composed of C_{PC} trimers yielded a hyperbolic saturation curve (Fig. 2A) and a linear Eadie

Table 1. *Catalytic and PALA-binding properties of C_{PC} trimers and reconstituted holoenzyme containing C_{PC} trimers*[a]

	Enzyme activity			PALA binding	
	V_{max}	K_m or $K_{0.5}$	n_H	K_d or $K_{0.5}$	n_H
Trimer					
C_{WT}	16	5.0	1.0	95	1.0
C_{PC}	12	37	1.0	1,600	1.0
Holoenzyme					
C_{WT}	14	5.4	1.7	110	2.0
C_{PC}	7.9	50	1.0	430	1.3

[a] Values of V_{max} for C trimer and holoenzyme are in units of μmol carbamoyl aspartate per μg of C trimer per h. The results obtained for various preparations of C trimer and holoenzyme at various times have differed significantly. For the experiments reported here, the comparisons between the wild-type and proteolytically cleaved enzymes are valid because measurements were made at the same time with identical reagents. Values of K_m or $K_{0.5}$ represent the concentration (mM) of aspartate at half maximum velocity. Binding parameters (nM) for C_{WT} trimers and holoenzyme containing C_{WT} trimers are from Newell et al. (1989).

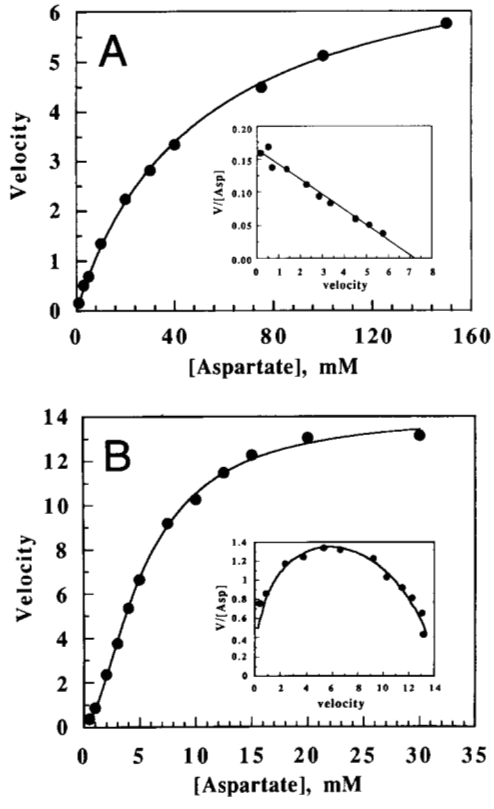

Fig. 2. Enzyme kinetics of holoenzyme containing C_{PC} trimers and $ATCase_{WT}$. Assays were performed at 30 °C in 50 mM MOPS buffer at pH 7.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA by the method of Davies et al. (1972). Enzyme activity is expressed as velocity in μmol of carbamoyl-L-aspartate formed per h per μg of trimer as a function of the concentration of aspartate and saturating [^{14}C]carbamoyl phosphate (5 mM). **A**: Data for holoenzyme containing C_{PC} trimers. The inset represents the Eadie plot of the same data as v/s (velocity/substrate) vs. v (velocity). **B**: Results for $ATCase_{WT}$. The sigmoidality of the saturation curve is emphasized by the curved Eadie plot in the inset.

plot (inset of Fig. 2A). The Hill coefficient (n_H) for the reconstituted holoenzyme containing C_{PC} trimers was 1.0 in contrast to 1.7 for $ATCase_{WT}$. In addition, the allosteric effectors ATP (2 mM) and CTP (0.5 mM) had virtually no effect on the K_m or V_{max} for the holoenzyme containing C_{PC} trimers, whereas those effectors caused marked shifts in both $K_{0.5}$ and n_H for $ATCase_{WT}$. The addition of ATP to $ATCase_{WT}$ caused changes in $K_{0.5}$ and n_H to 4.1 mM aspartate and 1.3, respectively, whereas those values for solutions containing CTP were 8.8 mM aspartate and 2.4, respectively.

Although holoenzyme containing C_{PC} trimers exhibited neither homotropic nor heterotropic effects in enzyme assays, some cooperativity was observed in the equilibrium binding of PALA. In comparison to $ATCase_{WT}$ for which n_H is 2.0 as determined by equilibrium dialysis measurements of the binding of PALA (Newell et al., 1989), the cooperativity exhibited by holoenzyme composed of C_{PC} trimers is substantially lower (n_H = 1.3). Also, it should be noted (Table 1) that $K_{0.5}$ for holoenzyme containing C_{PC} trimers is about fourfold greater than that observed with $ATCase_{WT}$.

*Holoenzyme containing C_{PC} trimers has a global conformation analogous to the **R** state of $ATCase_{WT}$*

In view of the absence of cooperativity observed in enzyme assays of the holoenzyme composed of C_{PC} trimers, it was important to determine whether the molecules had a quaternary structure analogous to the compact, low-affinity **T** state of $ATCase_{WT}$ and were not converted to the **R** state by active-site ligands or, alternatively, whether they were in the **R** conformation even in the absence of ligands. Accordingly, difference sedimentation velocity experiments were performed to determine the effect of the binding of the bisubstrate ligand, PALA (2.5 PALA per active site). For $ATCase_{WT}$ in MOPS buffer, the change in sedimentation coefficient ($\Delta s/s$) was −4.3%, in agreement with earlier results (Newell & Schachman, 1990). In contrast, $\Delta s/s$ for holoenzyme containing C_{PC} trimers was only −1.4%. When the same experiments were performed on enzymes in phosphate buffer, $\Delta s/s$ for $ATCase_{WT}$ was −3.4% (Howlett & Schachman, 1977) and −0.3% for holoenzyme composed of C_{PC} trimers. These results demonstrated clearly that the addition of PALA caused virtually no change in the global conformation of the holoenzyme containing C_{PC} trimers. However, by themselves the measurements do not indicate whether the molecules are in a conformation analogous to the **T** or **R** state of $ATCase_{WT}$. To differentiate between these two alternatives, we compared the sedimentation coefficients of $ATCase_{WT}$ and holoenzyme containing C_{PC} trimers both in the absence and in the presence of saturating amounts of PALA. The value of $\Delta s/s$ for unliganded holoenzyme containing C_{PC} trimers was −3.3% when compared to unliganded $ATCase_{WT}$, which is largely in the **T** state in the absence of active-site ligands. When PALA was added to both enzymes, thereby converting $ATCase_{WT}$ to the **R** conformation, $\Delta s/s$ was −0.6%. These results provide strong evidence that holoenzyme containing C_{PC} trimers has a conformation similar to that of the **R** state of $ATCase_{WT}$. Because virtually no change in quaternary structure occurs upon the binding of active-site ligands, very little cooperativity is observed in enzyme assays or measurements of the equilibrium binding of PALA.

Proteolytic cleavage of the chains has little effect on the thermal stability of C_{PC} trimer and holoenzyme

Because the introduction of a break in the polypeptide chains of C_{PC} trimers might be expected to result in a

decreased thermal stability, it seemed of interest to examine the heat denaturation of the trimers and the holoenzyme containing C_{PC} trimers by differential scanning microcalorimetry. As in the thermal denaturation of C_{WT} trimer (Edge et al., 1988), a broad, asymmetric "melting" curve was observed with a maximum change in heat capacity at 62.5 °C. Under the same conditions, T_m for C_{WT} trimer was 61.5 °C. The addition of saturating amounts of PALA caused an increase of T_m for C_{WT} trimer to 75.6 °C, whereas the increase of T_m for C_{PC} trimer was only to 69.3 °C. Thus cleavage of the polypeptide chains did not cause much decrease in heat stability of the trimers.

Differential scanning microcalorimetry of the holoenzyme composed of C_{PC} trimers showed a single endotherm with a T_m of 67.3 °C in contrast to the two overlapping, relatively sharp transitions at 64.5 °C and 67.1 °C observed for $ATCase_{WT}$. When the enzymes were saturated with PALA, the endotherm for holoenzyme containing C_{PC} trimers showed two transitions at 62.7 °C and 71.3 °C in comparison to the transitions at 62.5 °C and 77.7 °C observed with $ATCase_{WT}$.

Active C_{PC} trimer is formed from polypeptide fragments

Because earlier studies (Burns & Schachman, 1982a,b) had demonstrated that C_{WT} trimers can be dissociated into unfolded polypeptide chains in 4.7 M urea and then reassembled in good yield (~60%) into active trimers upon dilution of the urea solutions, it was of interest to see whether the two unfolded fragments could also fold, dock, and then associate into active trimers. Accordingly, C_{WT} trimers were incubated in 4.7 M urea for 45 min at 0 °C to dissociate the protein into unfolded polypeptide fragments. Figure 3A shows a sedimentation velocity pattern for the polypeptide fragments in 4.7 M urea, and the pattern for the solution of C_{WT} trimer in 4.7 M urea is exhibited in Figure 3B. The average (uncorrected for effects of urea on the viscosity and density of the solution) sedimentation coefficient for the fragments from C_{PC} trimer is 1.0S, and the pattern clearly shows a trailing component represented, presumably, by the fragment containing amino acid residues 241–310. In contrast, the intact polypeptide chain from C_{WT} trimer had a sedimentation coefficient of 1.3S and there is no evidence of a smaller fragment.

After 45 min of incubation of the C_{PC} and C_{WT} trimers in 4.7 M urea, the solutions were diluted 10-fold into Tris buffer at 0 °C to permit reactivation and assembly of trimers. Aliquots were withdrawn at periodic intervals and assayed for enzyme activity. Figure 4A shows the kinetics of reactivation obtained from the polypeptide fragments produced by treatment of C_{PC} trimers with 4.7 M urea. About 60% of the initial enzyme activity was

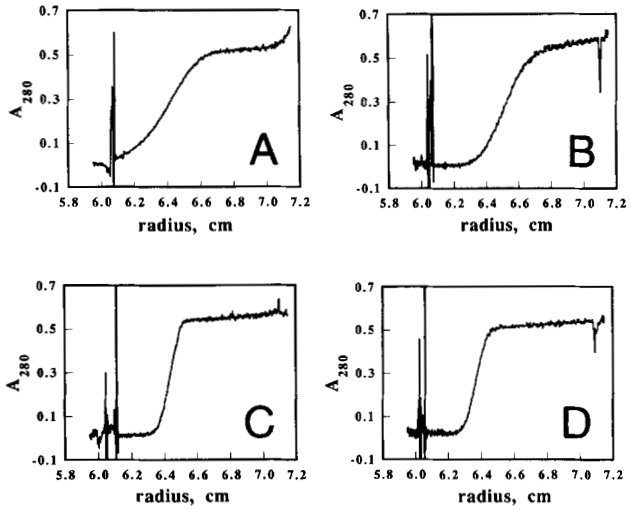

Fig. 3. Sedimentation velocity patterns of polypeptide fragments in urea and of reconstituted C_{PC} and C_{WT} trimers. Patterns representing absorbance at 280 nm vs. radial distance from the axis of rotation in cm were obtained after 20 min of operation at 60,000 rpm. Sedimentation velocity experiments were performed at 20 °C with a Beckman XL-A analytical ultracentrifuge. The buffer for all experiments was 50 mM Tris-Cl at pH 7.5, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Patterns for unfolded polypeptides from C_{PC} trimer (**A**) and C_{WT} trimer (**B**) in solutions containing 4.7 M urea. The average sedimentation coefficient, uncorrected for temperature and the viscosity and density of the urea solution, is 1.0S for the fragments from C_{PC} trimer and 1.3S for the polypeptide chains from C_{WT} trimer. Protein concentrations were 0.6 mg/mL. Patterns for reconstituted C_{PC} trimer (**C**) and C_{WT} trimer (**D**) after overnight dialysis to remove the urea. Protein concentrations were 0.6 mg/mL. The uncorrected sedimentation coefficients reconstituted for C_{PC} trimer was 5.6S and 5.6S for reassembled C_{WT} trimer.

restored. Analogous experiments were performed with C_{WT} trimer, and the kinetics of reactivation are shown in Figure 4B. In agreement with the earlier studies of Burns and Schachman (1982b), about 65% of the original enzyme activity was restored.

Although the time course of recovery of enzyme activity varied somewhat in different experiments, the reactivation from fragments was always slightly more rapid than that from intact chains. For the experiment illustrated in Figure 4, the half-times of reactivation were 23 and 38 min for the fragments and intact chains, respectively.

As a test of the reassembly of trimers, the 4.7 M urea solutions of both C_{PC} and C_{WT} trimer were dialyzed overnight against Tris buffer and the resulting solutions examined by ultracentrifugation. Figure 3C,D shows the resulting sedimentation velocity patterns. Sharp boundaries were obtained for both the reconstituted C_{PC} trimer (Fig. 3C) and reassembled C_{WT} trimer (Fig. 3D), and identical sedimentation coefficients of 5.6S were obtained. Thus the 70- and the 240-amino acid fragments are capable of folding, docking, and associating into active trimers in good yield.

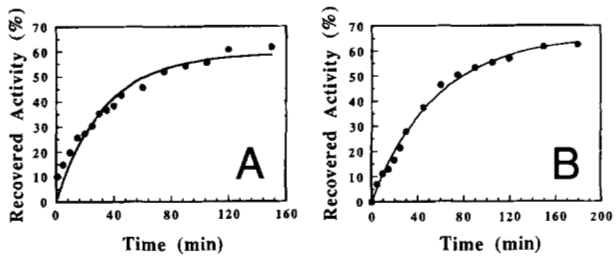

Fig. 4. Kinetics of reactivation following dilution of urea denatured and dissociated C_{PC} and C_{WT} trimers. Both C_{PC} and C_{WT} trimers at 2 mg/mL were incubated at 0 °C for 45 min in 50 mM Tris-Cl buffer at pH 7.5, containing 4.7 M urea, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA. Following this period of dissociation and denaturation, the samples were diluted 10-fold into the standard Tris-Cl buffer at 0 °C. At various intervals, 50-μL aliquots were removed and transferred to an assay solution (450 μL) for measurement of enzyme activity. Assays were performed by the method of Davies et al. (1970) in solutions containing 5 mM [^{14}C]carbamoyl phosphate and 200 mM aspartate for the reconstituted C_{PC} trimer and 50 mM aspartate for the reassembled C_{WT} trimer. **A:** Reactivation from polypeptide fragments from C_{PC} trimer. **B:** Reactivation from intact chains from C_{WT} trimer.

Discussion

Properties of C_{PC} trimer

Limited chymotryptic digestion of the C subunit of ATCase produces a trimeric protein in which each of the polypeptide chains is hydrolyzed at the bond between Tyr 240 and Ala 241 (Kinemages 1, 2). Despite the proteolytic cleavage, there is no dissociation of the complex comprising three of the larger fragments containing residues 1–240 and three of the smaller polypeptides comprising residues 241–310. N-terminal sequence analyses coupled with EIMS not only led to the identification of the site of cleavage of the chain but also showed that proteolysis does not occur elsewhere in the chain.

The catalytic and ligand-binding properties of C_{PC} are altered only slightly in comparison to those of C_{WT} trimers. Although K_m for aspartate is significantly higher for C_{PC} trimers (40 mM) than for C_{WT} trimer (5 mM), V_{max} is reduced by only 25%. In addition, C_{PC} trimer still exhibits a strong affinity for the bisubstrate analog PALA; K_d is about 1,600 nM compared to 95 nM for C_{WT} trimer. These moderate changes in catalytic and ligand-binding properties as a result of the proteolysis can be understood in terms of the proximity of the cleavage positions to the active sites. Although the loop containing Tyr 240 is not itself part of the active site, the loop follows closely residues (Arg 229 and Gln 231) that are either part of the active site or may play a role in domain movement during catalysis (Kinemage 3; Ke et al., 1988; Middleton & Kantrowitz, 1988; Middleton et al., 1989; Stebbins et al., 1990; Peterson et al., 1992). The altered enzyme kinetics and affinity for PALA may reflect distortion of the active sites caused by the flexibility resulting from the frayed ends and the introduction of new charged groups.

Despite the rupture of a single bond in each of the chains, the overall frictional properties of the complex are not altered significantly as shown by the sedimentation coefficient of 5.6S for C_{PC} trimer, which is very similar to that of C_{WT} trimer (5.7S). Moreover, the thermal stability as measured by differential scanning microcalorimetry was not altered as a result of the proteolytic cleavage; T_m for C_{PC} trimer is 62.5 °C compared to 61.5 °C for C_{WT} trimer. Finally, the C_{PC} trimers combine with high efficiency with R dimers to form stable ATCase-like molecules with a sedimentation coefficient similar to that of $ATCase_{WT}$. Hence the surface regions of the C trimers, which are involved in interchain interactions with r chains, are fully functional.

Loss of allosteric properties in holoenzyme containing cleaved c chains

Although the reconstituted holoenzyme containing two C_{PC} trimers and three wild-type R dimers is very active (V_{max} about 56% that of $ATCase_{WT}$), the dependence of enzyme activity as a function of aspartate concentration is hyperbolic as contrasted to the sigmoidal dependence exhibited by the wild-type enzyme. The striking differences in behavior are shown by the linear Eadie plot, on the one hand (Fig. 2A), as compared to the curved plot for $ATCase_{WT}$ (Fig. 2B). Why does the holoenzyme containing C_{PC} trimers lack cooperativity? Is it in a conformation like the **T** state of $ATCase_{WT}$? Can the binding of active-site ligands like substrates or PALA not promote the conversion to an **R** conformation? Or alternatively, is the reconstituted holoenzyme already in the **R** conformation even when unliganded?

The results from the difference sedimentation velocity experiments indicate that the holoenzyme containing C_{PC} trimers has a quaternary structure similar to that of the **R** conformation of $ATCase_{WT}$. When PALA was added to the cleaved holoenzyme in MOPS buffer, the change in sedimentation coefficient, $\Delta s/s$, was only -1.4% compared to -4.3% for $ATCase_{WT}$. In analogous experiments for the enzymes in phosphate buffer, which itself is known to promote a slight shift in the T ⇌ R equilibrium toward the **R** conformation (Newell & Schachman, 1990), $\Delta s/s$ was -0.3% for the holoenzyme containing C_{PC} trimers as contrasted to -3.4% for $ATCase_{WT}$. Moreover, a direct comparison of the two enzymes in phosphate buffer, both in the absence of PALA and in its presence, provided additional evidence that the quaternary structure of the unliganded holoenzyme containing C_{PC} trimers was similar to that of liganded $ATCase_{WT}$. Because the cleaved holoenzyme has a conformation analogous to the **R** state even in the absence of active-site ligands and no change occurs upon binding ligands, it is to be expected that no cooperativity would be detected in enzyme kinetics. Some cooperativity was detected in the binding of PALA to the

holoenzyme containing C_{PC} trimers, as shown by the Hill coefficient of 1.3, but this cooperativity was much less than that of $ATCase_{WT}$ under similar conditions (n_H = 2.0). This difference in cooperativity exhibited in enzyme kinetics, on the one hand, and equilibrium binding of PALA, on the other, has been observed previously (Eisenstein et al., 1990) with a mutant form of ATCase. These seemingly contradictory results were readily resolved when it was shown that carbamoyl phosphate itself causes a shift in the T ⇌ R equilibrium as measured by the decrease in sedimentation coefficient of various mutant forms of ATCase upon the addition of carbamoyl phosphate (Eisenstein et al., 1990; Newell & Schachman, 1990; Peterson et al., 1992). On the basis of these observations with mutant forms of ATCase, we conclude that unliganded, cleaved holoenzyme is largely but not completely in the **R** conformation and that the addition of carbamoyl phosphate shifts the T ⇌ R equilibrium completely to the **R** state.

Why does the rupture of the peptide bond between Tyr 240 and Ala 241 cause the destabilization of the **T** state of the holoenzyme relative to the **R** conformation? Crystallographic studies of the CTP-liganded **T** conformation of ATCase (Kinemage 3; Kim et al., 1987) showed that side chains in the loop comprising residues 230–245 in each c chain in one C trimer interact with side chains of the c chain in the apposing C trimer in the holoenzyme. In the **T** conformation of $ATCase_{WT}$ this interaction involves the carboxylate group of Glu 239 from one chain (c1) interacting with the ϵ-amino group of Lys 164 and the phenolic hydroxyl group of Tyr 165 in the apposing c chain (c4). When the enzyme is converted to the **R** conformation upon the addition of PALA, this intersubunit interaction is disrupted, the two C trimers are separated from each other by about 10 Å along the threefold symmetry axis (Ke et al., 1988), whereupon Glu 239 interacts with the Lys 164 of the same c chain. These crystallographic studies thus confirm and extend the ligand-promoted "swelling" of ATCase demonstrated by the sedimentation velocity measurements (Gerhart & Schachman, 1968; Howlett & Schachman, 1977; Schachman, 1988). These hydrodynamic studies also showed that the conformational state is highly sensitive to amino acid substitutions for Lys 164 and Glu 239 (Newell & Schachman, 1990). A mutant form of ATCase in which Lys 164 was replaced by Glu was shown to be in the **R** conformation even in the absence of ligands. Replacement of Glu 239, in contrast, by a Gln or Lys residue produced holoenzymes that are partially or largely in the **T** state in the absence of active-site ligands, but whose conformational equilibrium is readily shifted toward the **R** state by the addition of ligands such as carbamoyl phosphate or inorganic phosphate (Ladjimi & Kantrowitz, 1988; Gouaux et al., 1989; Newell & Schachman, 1990; Stevens & Lipscomb, 1990; Tauc et al., 1990). In view of the evidence that certain amino acid substitutions in the loop region comprising residues 230–245 tend to destabilize the **T** conformation of unliganded enzyme relative to the **R** state, it might be expected that the T ⇌ R equilibrium would be altered due to the introduction of the charged amino acid carboxylate groups along with the additional flexibility resulting from the proteolytic cleavage of the polypeptide chain between residues 240 and 241. In this regard it is of interest that a genetically altered ATCase, in which the c polypeptide chains not only are not intact but also the fragments have an overlap of eight residues (from 235 to 242), exhibits no cooperativity in PALA binding as well as in enzyme kinetics (Yang & Schachman, 1993). Apparently for this molecule, the T ⇌ R equilibrium is shifted virtually completely toward the **R** conformation.

Assembly of active C_{PC} trimers from proteolytic fragments

The observation that the proteolytically cleaved trimers remain intact and enzymically active as well as stable at relatively high temperature indicates that noncovalent interactions between the fragments suffice to maintain the tertiary and quaternary structures characteristic of C_{WT} trimers. Even more convincing evidence of the intrinsic stability of C_{PC} trimers stems from the successful assembly of C_{PC} trimers from separate unfolded fragments produced in 4.7 M urea. Presumably during the assembly process, the fragments separately fold into tertiary structures sufficiently similar to their three-dimensional structures within C_{WT} trimers for mutual recognition and docking to form cleaved monomers, which then associate into C_{PC} trimers. As yet we have no information about the properties of the individual 8-kDa and 26-kDa fragments and can only speculate that the dilution of the 4.7 M urea solution of the two fragments permits some folding and association before the individual fragments aggregate nonspecifically.

Although the kinetic pathway of assembly of C_{PC} trimer must differ from that for the reconstitution of C_{WT} trimers from unfolded, intact polypeptide chains (Burns & Schachman, 1982b), the similarity in the time course for regeneration of active trimers indicates that the overall processes are not too different. Preliminary analysis of the rate of formation of active species from the fragments (Fig. 4A) suggests a first order rate-limiting step. Clearly, additional experiments are needed with different ratios and concentrations of the two fragments in order to justify further speculation about the mechanism of assembly.

That the urea-denatured 8-kDa and 26-kDa fragments reassemble into folded, active, stable trimers seems remarkable when one examines the folding pattern of an individual c chain in a C trimer of ATCase (Kim et al., 1987; Ke et al., 1988). Figure 5A is a ribbon diagram illustrating the folded chains of C_{WT} trimer in ATCase with the bulk of each chain in gray and the part encompassing residues 285–310 in black. An individual c chain is shown in Figure 5B and Kinemage 2 to illustrate the folding of the polypeptide chain into clearly demarcated N- and C-

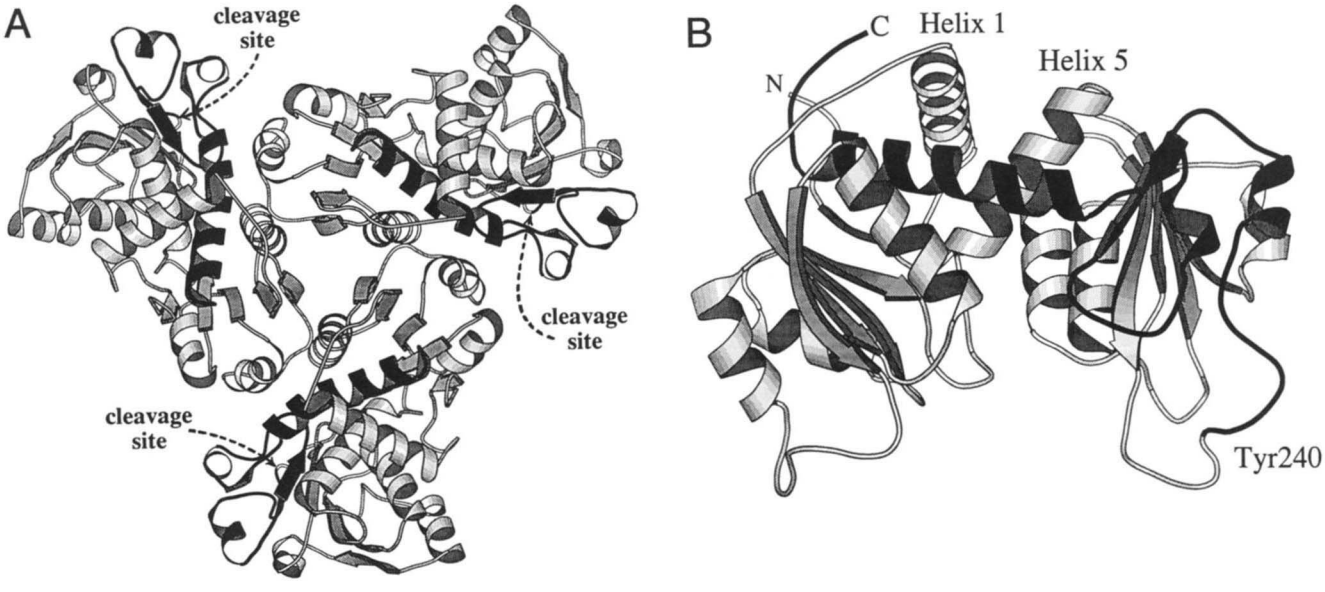

Fig. 5. Structures of C_{WT} trimer, the folded c chain within C_{WT} trimer and the fragments produced by proteolytic cleavage by α-chymotrypsin. **A:** Quaternary structure of C_{WT} trimer within $ATCase_{WT}$. The orientation is from the outside of the ATCase molecule along the threefold axis of symmetry. The region of each polypeptide chain from residue 281 to 310 is shown in black. **B:** Tertiary structure of a single c chain within a C_{WT} trimer of ATCase. The section of the polypeptide chain comprising residues 241–310 is in black. The N-terminal domain comprising residues 1–134 is on the left and the C-terminal domain consisting of residues 149–284 on the right. The two domains are linked covalently by an intervening helix, comprising residues 135–149, and designated as Helix 5. In addition, Helix 12, consisting of residues 285–305, crosses from the C-terminal domain and threads through the N-terminal domain where it interacts with many residues in that domain. The site of the proteolytic cleavage at Tyr 240 is located on a loop comprising residues 235–245 that extends away from the C-terminal domain. The structures were generated with the program Molscript (Kraulis, 1991) on a Silicon Graphics Indigo workstation based on the X-ray structure of the CTP-liganded ATCase (Kim et al., 1987) with the coordinates deposited in the Brookhaven Data Bank (Registry Number 5AT1).

terminal domains deduced from the crystallographic studies on intact CTP-liganded ATCase (Kim et al., 1987). The 8-kDa fragment corresponds to a patch of mixed secondary and tertiary structure on the surface of the C-terminal domain followed by a long helical segment comprising residues 285–305 (Helix 12), which crosses over into the N-terminal domain. Following that helix are five additional residues (306–310) with the C-terminus in relative proximity of the N-terminal amino acid. Helix 12 is partially buried within the N-terminal domain, covered in part by a flap comprising residues 1–35. The region encompassing residues 1–35 forms a horseshoe-like structure that substantially covers one side of Helix 12, crossing the middle of the helix at residues 25–35. The other side of Helix 12 is bordered by the remainder of the N-terminal domain (residues 36–149). Some residues in Helix 12 contribute to the hydrophobic interior of the N-terminal domain.

Achieving the final folded structure is likely to be dependent on numerous and intricate interactions among amino acid side chains in Helix 12 and other residues in the N-terminal domain (Kinemage 2). The importance of Helix 12 to the folding and stability of the c chains and their incorporation into C trimers and holoenzyme was shown by Peterson and Schachman (1991). Genetic manipulations that lead to truncation of the c chains within the helix yield auxotrophic strains requiring pyrimidines for growth. No ATCase could be detected in cells when stop codons were introduced at positions 291 or 299. Nor could polypeptide chains analogous to the c chains be observed in cell extracts. It appears, therefore, that Helix 12 is needed for the folding of the c chains and their association into stable C_{WT} trimers. In addition, studies on mutant forms of ATCase, involving amino acid substitutions for residues in Helix 12, showed that both the thermal stability and catalytic properties are sensitive to changes in that segment (Peterson & Schachman, 1991; Peterson et al., 1992). Because a substantial amount of catalytic activity (~60%) is recovered in the assembly of C_{PC} trimers from the fragments in urea, it appears that the critical contacts are established between Helix 12, a part of the 8-kDa peptide, and the N-terminal domain within the 26-kDa fragment. How this occurs is not known.

Materials and methods

Chemical and analytical electrophoresis

Dilithium carbamoyl phosphate was from Boehringer Mannheim, dilithium [^{14}C]carbamoyl phosphate from

New England Nuclear, and aspartate from Cal Biochem. PALA and [^{3}H]PALA were generous gifts from J.O. Newell and D.W. Markby. Neohydrin for preparing free C trimers and R dimers from ATCase was from K&K Labs. MOPS was from Serva. Ultrapure urea was from Schwartz/Mannheim Biotech. Polyacrylamide gel electrophoresis for native proteins was performed by the method of Jovin et al. (1964) and electrophoresis in gels containing SDS by the method of Laemmli (1970).

Protein preparation

Wild-type ATCase was purified from *E. coli* strain HS1061 as described by Gerhart and Holoubek (1967), with the exception that the final size exclusion step was omitted. C trimer and R dimer were isolated from holoenzyme as described by Yang et al. (1978). Protein concentrations were determined using extinction coefficients (280 nm) of 0.59 cm^2/mg for holoenzyme, 0.72 cm^2/mg for C trimer, and 0.32 cm^2/mg for R dimer. The extinction coefficient of C_{PC} trimer is the same as that of C_{WT} trimer (data not shown).

C_{PC} trimer was prepared by incubating C_{WT} trimer (0.5 mg/mL) with α-chymotrypsin (Sigma Chemical Company; 5.6 μg/mL) at room temperature in a reaction buffer containing 100 mM Tris and 5 mM $MgCl_2$ adjusted to pH 9.0 with HCl. At higher protein concentrations the efficiency of cleavage at the desired site was decreased, and also small molecular weight degradation products were formed in significant amounts. Aliquots of α-chymotrypsin from a frozen stock solution (10 mg/mL in 0.1 mM HCl) were diluted into reaction buffer just prior to use. The reaction was stopped after 90 min by adding PMSF (from Sigma), 100 mM in isopropanol, to a final concentration of 0.2 mM, and the mixture was loaded on a column of DE-52 anion-exchange resin equilibrated in 50 mM Tris-Cl at pH 8.0. The yield of cleaved chain is greater than 95%. Following clearance of residual PMSF and α-chymotrypsin with two column volumes of equilibration buffer, C_{PC} trimer was eluted with 50 mM Tris-Cl buffer at pH 8.0, containing 500 mM NaCl and 2 mM 2-mercaptoethanol. Trimer fractions were combined, concentrated by ultrafiltration (Amicon YM-30), and dialyzed against 50 mM Tris-Cl, pH 7.5, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA.

Holoenzyme containing C_{PC} trimers was prepared from C_{PC} trimers and intact R dimers. In a typical protocol, purified R dimer (16 mg in 4.6 mL of 10 mM Tris-Cl, pH 8.7 containing 100 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM zinc acetate) was added gradually with stirring at room temperature to a solution of C_{PC} trimer (20 mg in 12 mL of 40 mM potassium phosphate, pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA). The resultant mixture was concentrated to 3 mL, using an Amicon concentrator, and loaded onto a Sephacryl S-300 column (90 × 2.5 cm) equilibrated in 50 mM potassium MOPS buffer at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA. Purified holoenzyme containing C_{PC} trimers was obtained in about 50% yield (16 mg).

N-terminal sequence analysis

The 26- and 8-kDa peptides from C_{PC} trimer were separated on a 12.5% polyacrylamide gel containing SDS and electroblotted onto a polyvinylidene difluoride membrane as described by Matsudaira (1987), except that electroblotting was carried out for 60 min at 15 W, constant power. Following direct visualization of the transferred bands by Coomassie brilliant blue staining, the membrane was rinsed in water for 5 min and air dried. The stained bands were then excised from the membrane with a clean razor and stored at −20 °C. Automated Edman degradation was performed on an Applied Biosystems Model 470A gas-phase sequenator at the Microchemical Facility at the University of California at Berkeley. The excised bands were loaded directly into the sequenator cup for sequencing.

Mass spectrometry of the polypeptide fragments

The molecular masses of the 26-kDa and 8-kDa fragments were determined by EIMS at the Mass Spectrometry Facility of the University of California at San Francisco, using a VG BIO-Q Mass Spectrometer (VG Biotech Ltd., UK; 3,000-Da mass range for singly charged ions). Prior to EIMS analysis, C_{PC} trimer (60 μg) was chromatographed on a Vydak C-18 HPLC column (250 × 4.6 mm). Following a 5-min pregradient step, a linear gradient from 0 to 70% CH_3CN in 0.1% trifluoroacetic acid/water was used over 65 min with a flow rate of 1 mL/min. The fragments, which eluted with retention times of about 45 min (8-kDa fragment) and 53 min (26-kDa fragment), were collected and dried in a Speed Vac concentrator (Savant Instruments, Inc.). For EIMS analysis, each sample was introduced into the ion source in a 1:1 (v/v) mixture of acetonitrile/water containing 1.0% acetic acid, at an estimated sample concentration of ~20 pmol/μL.

Enzyme activity assays and PALA binding

Enzyme activities were measured using the method of Davies et al. (1970) utilizing ^{14}C-labeled carbamoyl phosphate. Assays were performed at 30 °C in a 50 mM MOPS buffer at pH 7.0, containing 0.2 mM EDTA, saturating carbamoyl phosphate (5 mM), and various concentrations of aspartate. Data were fitted to the Hill and Michaelis-Menten equations, as appropriate, using the program Kaleidagraph (Synergy Software). PALA-binding affinities were determined by equilibrium dialysis at 23 °C in 40 mM potassium phosphate buffer at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA, as described by Newell et al. (1989).

Sedimentation velocity measurements

Difference sedimentation velocity experiments were performed with a Beckman-Spinco Model E Ultracentrifuge, using Schlieren optics as described earlier (Howlett & Schachman, 1977; Eisenstein et al., 1990). Proteins at about 3 mg/mL in either 50 mM MOPS buffer at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA or 40 mM potassium phosphate buffer at pH 7.0, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA, were placed in paired single sector cells one of which had an upper 1° positive wedged window to displace the image and thereby permit simultaneous recording of the Schlieren patterns of the two samples. This technique was used to determine the effect of PALA on the holoenzyme containing C_{PC} trimers and on $ATCase_{WT}$ by measuring the difference in sedimentation coefficient for the enzyme in the presence of PALA (2.5 molecules of PALA per active site) as compared to the enzyme in the absence of PALA. Results are given as $\Delta s/s$ in %. Also, the difference sedimentation velocity method was used to compare the holoenzyme composed of C_{PC} trimers with $ATCase_{WT}$.

Sedimentation velocity experiments were conducted on C_{PC} and C_{WT} trimers as well as on the unfolded polypeptides produced when C_{PC} and C_{WT} trimers were incubated in 4.7 M urea in 50 mM Tris-Cl buffer at pH 7.5, containing 2.0 mM 2-mercaptoethanol and 0.2 mM EDTA. These experiments were performed with a Beckman XL-A analytical ultracentrifuge equipped with absorption optics, using a four-cell titanium rotor. Calculated sedimentation coefficients were not corrected for temperature or the viscosity and density of the solutions. Similar sedimentation velocity experiments were performed on the reconstituted C_{PC} and C_{WT} trimers.

Sedimentation velocity studies of urea-denatured and reassembled nicked and intact C trimers were performed at 60,000 rpm and 21 °C using a Beckman XL-A analytical ultracentrifuge. Samples consisted of trimer (0.5–0.75 mg/mL) in 50 mM Tris-Cl, pH 7.5, containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol, in the presence or absence of 4.7 M urea. Following centrifugation, urea-denatured samples were dialyzed overnight against urea-free buffer and then loaded again in the ultracentrifuge to assess reassembly. Successful reassembly of these samples was verified independently by native gel electrophoresis as described above. Centrifugation data were collected and analyzed using the programs VBETA11 and VELGAM2 (Beckman Instruments).

Differential scanning microcalorimetry

Differential scanning microcalorimetry was performed using a Microcal MC-2 calorimeter interfaced with an IBM-XT computer for data collection and analysis. Scans were performed at a rate of 53 °C per h in the range of 30–90 °C. A potassium borate buffer (40 mM) containing 5 mM 2-mercaptoethanol and 0.2 mM EDTA was used to avoid protein aggregation observed at lower pH (R.E. Silversmith & H.K.S., unpubl.). Protein concentrations were 3 mg/mL in all experiments.

Reassembly of C_{PC} and C_{WT} trimer following denaturation in 4.7 M urea

Urea denaturation and reassembly of the C_{PC} and C_{WT} trimers were performed essentially as described by Burns and Schachman (1982b). C_{PC} or C_{WT} trimer (~2 mg/mL in standard Tris buffer: 50 mM Tris-Cl, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.5, 0 °C) was mixed with an equal volume of 9.4 M urea in standard Tris buffer, and the resulting 4.7 M urea solution was maintained at 0 °C for 45 min. Reassembly of the trimer was initiated by 10-fold dilution of the mixture into standard Tris buffer equilibrated at 0 °C. At various times, 50 μL aliquots were removed from the reassembly solution and transferred to an assay solution (450 μL) for immediate assay. Assays were performed by the method of Davies et al. (1970), with a constant concentration of L-aspartate (50 mM for C_{WT} trimer, and 200 mM for C_{PC} trimer). The assay of each aliquot was limited to 1 min in duration to minimize reassembly during the assay. Typically, 60–80% of enzyme activity was recovered for both C_{PC} and C_{WT} trimer.

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