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Peptide–protein interaction markedly alters the functional properties of the catalytic subunit of aspartate transcarbamoylase

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Abstract

Interaction of a 70-amino acid zinc-binding polypeptide from the regulatory chain of aspartate transcarbamoylase (ATCase) with the catalytic (C) subunit leads to dramatic changes in enzyme activity and affinity for ligand binding at the active sites. The complex between the polypeptide (zinc domain) and wild-type C trimer exhibits hyperbolic kinetics in contrast to the sigmoidal kinetics observed with the intact holoenzyme. Moreover, the Scatchard plot for binding *N*-(phosphonacetyl)-L-aspartate (PALA) to the complex is linear with a K_d corresponding to that evaluated for the holoenzyme converted to the relaxed (**R**) state. Additional evidence that the binding of the zinc domain to the C trimer converts it to the **R** state was attained with a mutant form of ATCase in which Lys 164 in the catalytic chain is replaced by Glu. As shown previously (Newell, J.O. & Schachman, H.K., 1990, *Biophys. Chem. 37*, 183–196), this mutant holoenzyme, which exists in the **R** conformation even in the absence of active site ligands, has a 50-fold greater affinity for PALA than the free C subunit. Adding the zinc domain to the C trimer containing the Lys 164 → Glu substitution leads to a 50-fold enhancement in the affinity for the bisubstrate analog yielding a value of K_d equal to that for the holoenzyme. A different mutant ATCase containing the Gln 231 to Ile replacement was shown (Peterson, C.B., Burman, D.L., & Schachman, H.K., 1992, *Biochemistry 31*, 8508–8515) to be much less active as a holoenzyme than as the free C trimer. For this mutant holoenzyme, the addition of substrates does not cause its conversion to the **R** state. However, the addition of the zinc domain to the Gln 231 → Ile C trimer leads to a marked increase in enzyme activity, and PALA binding data indicate that the complex resembles the **R** state of the holoenzyme. This interaction leading to a more active conformation serves as a model of intergenic complementation in which peptide binding to a protein causes a conformational correction at a site remote from the interacting surfaces resulting in activation of the protein. This linkage was also demonstrated by difference spectroscopy using a chromophore covalently bound at the active site, which served as a spectral probe for a local conformational change. The binding of ligands at the active sites was shown also to lead to a strengthening of the interaction between the zinc domain and the C trimer.

Keywords: allosteric transition; communication; conformational change; interchain interaction; intergenic complementation; linkage; zinc domain

Allosteric interactions are known to be crucial in the regulation of enzyme activity (Monod et al., 1965). Hence, the structural basis of the allosteric transition of an enzyme from a low affinity (**T**) state to a higher affinity (**R**) conformation has been the focus of extensive studies. It is generally accepted that ligand or substrate binding at one site in an oligomeric allosteric protein promotes global conformational changes affecting the energy of interaction between subunits and the affinity of ligand binding at other sites, and that the allosteric transition is mediated by changes in intersubunit interactions. As a consequence, it is of interest to demonstrate not only how individual intersubunit interactions affect the function of active sites, but also how active site ligands change the free energy of intersubunit interactions. The regulatory enzyme aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase, carbamoyl phosphate: L-aspartate carbamoyltransferase, EC 2.1.3.2), which catalyzes the first committed reaction in the biosynthesis of pyrimidines in *Escherichia coli*, is known to undergo pronounced conformational

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changes upon binding substrate analogs that promote the allosteric transition of the enzyme from the low affinity **T** state to the higher affinity **R** state (Gerhart & Schachman, 1968; Howlett et al., 1977; Kim et al., 1987; Krause et al., 1987; Kantrowitz & Lipscomb, 1988; Ke et al., 1988; Schachman, 1988). Hence ATCase has become a model enzyme for analyzing ligand-promoted changes in quaternary structure and function in terms of interchain interactions.

ATCase is composed of two trimeric C subunits and three dimeric regulatory (R) subunits (Gerhart & Schachman, 1965; Weber, 1968; Wiley & Lipscomb, 1968; Rosenbusch & Weber, 1971; Cohlberg et al., 1972). Whereas isolated enzymatically active C trimers exhibit hyperbolic kinetics, the holoenzyme exhibits a sigmoidal dependence of enzyme activity on the concentration of the substrates carbamoyl phosphate and aspartate (Gerhart & Pardee, 1962; Bethell et al., 1968). Moreover, the holoenzyme is activated by ATP and inhibited by CTP. There are five different types of interchain interactions implicated in the assembly of the holoenzyme from the six catalytic (c) and six regulatory (r) polypeptide chains (Kim et al., 1987; Krause et al., 1987; Ke et al., 1988). Within each C trimer are the c:c interactions: c1c2, c2c3, and c3c1. Similarly, within each R dimer are the r:r interactions: r1r6, r2r4, and r3r5. Linking the two types of subunits are the principal c:r interactions: c1r1, c2r2, and c3r3, etc. In addition, there are contacts c1r4, c2r5, c3r6, etc., which exist in the **T** conformation of ATCase, but not when it is converted to the **R** state. Finally, the X-ray evidence shows that c chains in apposing C trimers are in slight contact in the **T** state to give c1c4, c2c5, and c3c6 interactions. When the enzyme is converted from the **T** to the **R** conformation, these interactions are virtually abolished. Hence the c1r1, c2r2, c3r3, etc. interactions are important in stabilizing the **R** conformation along with the various c:c interactions within the trimers, whereas the additional interactions involving, for example, c1r4 and c1c4, are required for maintaining the **T** state. Despite intensive efforts, it has been difficult to determine effects of individual interchain interactions on the energetics of the allosteric transition and the functional properties of the active sites because of the multiplicity, strength, and interdependence of the protein–protein contacts in ATCase. This difficulty has been circumvented by studying a stable complex containing only one catalytic (C) trimer and three zinc-containing 70-amino acid polypeptide fragments of the regulatory chain of ATCase (Markby et al., 1991). In effect, this complex represents one half of the ATCase molecule (as shown in Kinemage 1), and the interchain interactions within it correspond to the c:c interfaces represented by c1c2, c2c3, and c3c1 along with the c:r interfaces designated as c1r1, c2r2, and c3r3.

The 70-amino acid zinc-binding polypeptide from the r chain of ATCase, which lacks the dimerization domain, forms a stable complex with the C subunit leading to markedly altered enzyme activity (Markby et al., 1991). Studies of enzyme kinetics indicate that the zinc domain–C trimer complex is an analog of intact ATCase in the **R** state. However, interpretations of the steady-state enzyme kinetics in terms of molecular models are complicated by the inability to distinguish between changes in substrate affinity and catalytic turnover caused by the peptide–protein interaction. In addition, it is difficult to obtain accurate kinetic data at high substrate concentrations due to pronounced substrate inhibition of mutant enzymes where activity and substrate affinity are very low. These problems were circumvented by equilibrium dialysis measurements of the binding of the bisubstrate analog PALA in order to test the proposed analogy between the zinc domain–C trimer complex and the **R** state of holoenzyme.

Depending upon the presence or absence of specific metabolites, ATCase can exist in either the low affinity **T** state or the **R** conformation with higher affinity, and the interconversion between the two conformations is readily achieved and mediated by interchain interactions. Certain mutant forms of ATCase have been shown to be predominantly populated in either the **R** state or the **T** state under certain conditions. For example, the mutant ATCase containing the Lys 164 → Glu substitution in the c chains exists in the **R** state even in the absence of active site ligands (Newell & Schachman, 1990). In contrast, the mutant ATCase containing the Gln 231 → Ile substitution in the c chains exists in the **T** state even in the presence of carbamoyl phosphate and aspartate (Peterson et al., 1992). How would these mutant C trimers be affected by interactions with the zinc domain? Can the complexes be considered as analogs of either the **T** or the **R** state of the holoenzyme? In order for the complex to be considered as an **R** state analog, it must display the catalytic activity and affinity for ligands characteristic of the holoenzyme in the **R** conformation.

An ultimate understanding of an allosteric interaction relies on a description of both the structural and energetic aspects of the communication between sites, which is mediated by the change in intersubunit interactions. The zinc domain–C trimer complex offers an excellent opportunity to study the communication between active sites and intersubunit interfaces due to the simplicity of protein–protein interactions in the complex. Evidence provided in this paper suggests that there is a conformational and energetic linkage between zinc domain binding at the interface with c chains and PALA binding at the active site. This observation should contribute to further understanding of the molecular mechanism underlying the allosteric transition.

Results

PALA binding to Zn domain–C subunit complexes

As shown by Markby et al. (1991), the addition of zinc domain to wild-type C trimer causes an alteration in the

catalytic activity which is manifested by a decrease in both K_m and V_{max}. The enzyme kinetics of the complex were analogous to those of the holoenzyme in the presence of excess substrates, i.e., the **R** state. Additional evidence in support of the view that the complex is an **R** state analog is shown in Figure 1A. Equilibrium dialysis measurements of the binding of PALA to the holoenzyme, to the free C subunit, and to the complex between C trimer and zinc domain are presented as Scatchard plots. Both the C subunit and the complex yield linear plots indicative of the absence of cooperativity, but the slopes of the lines differ considerably with the affinity of the complex for PALA being substantially greater than that of the free C subunit. In contrast, the Scatchard plot for the holoenzyme exhibits marked curvature characteristic of cooperativity in binding. The results are summarized in Table 1. It is of interest that the slope for the complex is very similar to that of the holoenzyme at a high extent of saturation, in support of the view that the complex is an analog of the **R** state of the holoenzyme.

Table 1. *Parameters for PALA binding to various C subunits, holoenzymes, and zinc domain–C subunit complexes*

	K_d (nM)	n_H
Wild type[a]		
C subunit	100	–[b]
Holoenzyme	105[c]	2.0
Zinc domain–C subunit	60	–
Lys 164 → Glu		
C subunit	24,000	–
Holoenzyme	460	–
Zinc domain–C subunit	480	–
Gln 231 → Ile		
C subunit	2,900	–
Holoenzyme	960[c]	1.6
Zinc domain–C subunit	500	–

[a] Values are averaged from different experiments.
[b] Dash indicates no cooperativity detected in Scatchard plot.
[c] For the holoenzymes that exhibit cooperativity the average dissociation constants ($K_{0.5}$) are given.

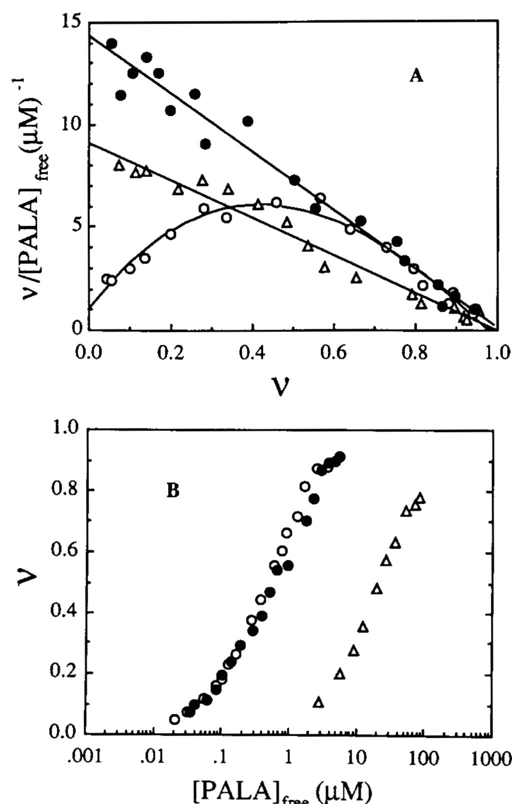


Fig. 1. Effects of zinc domain on PALA binding affinities of wild-type C trimer and mutant C trimer containing the Lys 164 → Glu replacement. Experiments were performed in 40 mM potassium phosphate, pH 7.0, containing 2 mM 2-mercaptoethanol. **A:** Scatchard plots of the PALA binding data of wild-type ATCase. △, C subunit alone; ●, zinc domain–C subunit complex; ○, holoenzyme. **B:** Saturation curves of PALA binding to Lys 164 → Glu ATCase. Data represent fractional saturation of the total number of binding sites as a function of free ligand plotted in terms of the logarithm of the concentration. △, C subunit alone; ●, zinc domain–C subunit complex; ○, holoenzyme.

More striking changes of the effect of the zinc domain on PALA binding were obtained with the mutant C trimer containing the Lys 164 → Glu substitution. As shown by Newell and Schachman (1990), the C subunit has a much lower affinity for PALA than the holoenzyme, which was found by sedimentation velocity experiments to be in the **R** conformation even in the absence of substrates. Figure 1B illustrates the 50-fold enhancement in the affinity for PALA when free mutant C trimer is incorporated into a complex with zinc domain. The value of K_d is changed from 24,000 nM to 480 nM upon complex formation. Moreover, the value of 480 nM for K_d of the complex is very similar to that (460 nM) obtained earlier for the mutant holoenzyme. The Scatchard plots for the binding of PALA to free C subunit, the holoenzyme and the complex were all linear; and the values of K_d are summarized in Table 1.

Effect of Zn domain on enzymatic activity of C trimer containing the Gln 231 → Ile substitution

The mutant form of ATCase containing the Gln 231 → Ile substitution in the c chains provided another model to test the proposal that the zinc domain–C trimer complex is an analog of the **R** state of the holoenzyme. As shown by Peterson et al. (1992), this mutant holoenzyme is much less active than the free mutant C trimer. Their studies demonstrate that, unlike wild-type ATCase, the mutant enzyme persists in the **T** state even in the presence of relatively high concentrations of substrates. Only the bind-

ing of the bisubstrate ligand was capable of overcoming the free energy difference between the **T** and **R** conformations to yield much more active holoenzyme. Hence it is relevant to inquire whether the addition of zinc domain could enhance the activity of the free C subunit containing the Gln 231 → Ile replacement. Figure 2A shows the results of assays of enzyme activity at varying concentrations of aspartate. The C subunit is much more active (V_{max}) than the holoenzyme. Moreover, the addition of zinc domain to the C trimer enhances the enzyme activity at low substrate concentrations with only a slight effect on V_{max}. That the effect of the zinc domain is due to a reduction in K_m from 37 mM to 17 mM aspartate is shown in Figure 2B by the different slopes of the linear Eadie plots, which demonstrate the absence of cooperativity. The various kinetic parameters, K_m, V_{max}, and n_H (Hill coefficient) are summarized in Table 2 along with the results for wild-type ATCase and the mutant containing the Lys 164 → Glu substitution. For the mutant C trimer containing the Gln 231 → Ile substitution, the increase in enzyme activity at low substrate concentration resulting from the interaction of the C trimer with the zinc domain is illustrated in Figure 2C. Because of the increased affinity of the active sites for aspartate as a result of the interaction of the C trimer with the zinc domain, it is possible at low aspartate concentration to titrate the enzyme activity as a function of the ratio of zinc domain to C trimer. As seen in Figure 2C, there is a marked increase in activity upon the addition of zinc domain to the mutant C trimer. Moreover, the maximum enhancement in activity is achieved at a molar ratio of about one zinc domain per c chain.

The striking difference in enzyme activity between the holoenzyme and the complex between C trimer and zinc

Table 2. *Summary of kinetic parameters for various C subunits, holoenzymes, zinc domain-C subunit complexes*

	K_m or $K_{0.5}$ (mM)[a]	V_{max} (mmol/mg/h)	n_H	V_m/K_m
Wild type				
C subunit	5.6	21	1.0	3.8
Holoenzyme	5.0	12	1.5	
Zinc domain-C subunit	2.4	11	1.0	4.6
Lys 164 → Glu[b]				
C subunit	≫300[c]	4.0	1.0	≪0.01
Holoenzyme	100	4.6	1.0	0.05
Zinc domain-C subunit	77	4.2	1.0	0.05
Gln 231 → Ile				
C subunit	36	2.1	1.0	0.06
Holoenzyme[d]	56	0.03	1.1	0.0005
Zinc domain-C subunit	17	2.0	1.0	0.12

[a] The cooperative kinetic saturation curves are characterized by $K_{0.5}$, and the noncooperative saturation curves by K_m.
[b] For the Lys 164 → Glu mutant, the parameters for C subunit and holoenzyme are from Newell and Schachman (1990); the parameters for the complex are from Markby et al. (1991).
[c] The curve for mutant C subunit does not attain half saturation even at 300 mM aspartate, so K_m is assumed to be much larger than 300 mM.
[d] Concentration of Gln 231 → Ile holoenzyme was 130 nM with about a fourfold excess of R subunit added to the holoenzyme.

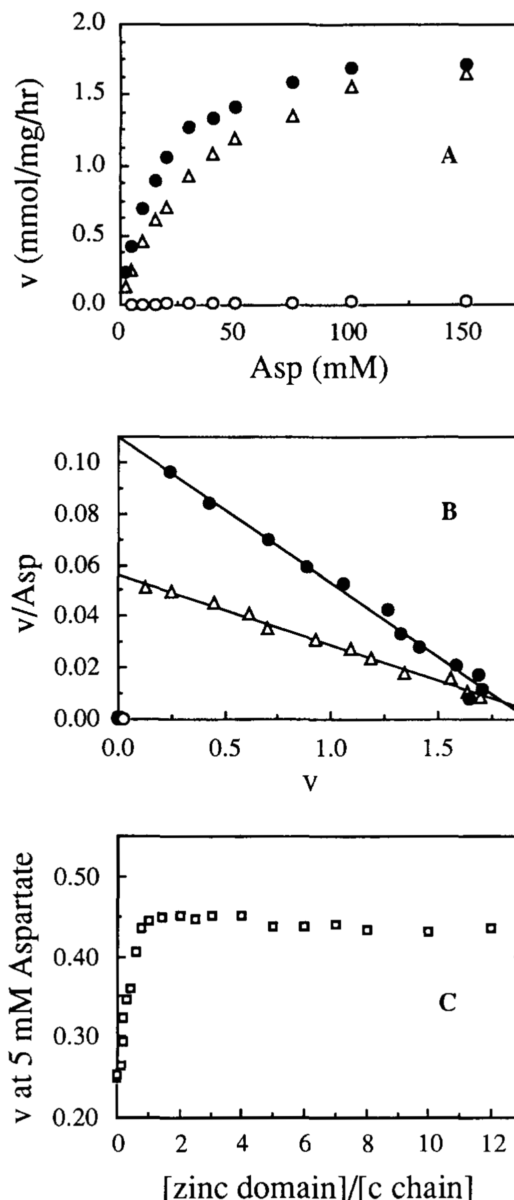


Fig. 2. Effect of zinc domain on the catalytic activity of mutant C trimer containing the Gln 231 → Ile replacement. Experiments were performed in MOPS buffer. **A:** Saturation curves are presented as initial velocity (v) as a function of aspartate concentration: △ represents mutant C trimer (2 μg/mL) alone; ● designates mixtures of mutant C trimer (2 μg/mL) and zinc domain (21.4 μg/mL); ○ represents mutant holoenzyme (40 μg/mL) plus 13.3 μg/mL excess R subunit. **B:** Eadie plots of the data. **C:** Effect of increasing amounts of zinc domain on activity of mutant C trimer containing the Gln 231 → Ile replacement. The titration experiments were performed with C trimer at 10 μg/mL. The aspartate concentration was 5 mM.

domain is illustrated further in Figure 3. When C trimer, shown in lane 1 of Figure 3A, is titrated with R subunit, there is a large decrease in electrophoretic mobility as it is converted to the holoenzyme (C_2R_3). Moreover, as seen in the gel patterns stained for enzyme activity (Bothwell, 1975), there is a substantial decrease in activity because the assembled enzyme is in the **T** state. In contrast, as shown in Figure 3B, the addition of zinc domain to the C trimer causes a smaller change in electrophoretic mobility and a large increase in activity because the complex is analogous to the **R** conformation.

Additional evidence indicating that the complex containing the C trimer and the zinc domain is representative of the **R** conformation of the holoenzyme was attained from equilibrium dialysis measurement of the binding of PALA. As seen in Table 1, the C subunit has a very low affinity for PALA with a K_d of 2,900 nM, whereas the holoenzyme, which exhibits cooperativity in the binding of PALA, has a higher average affinity ($K_{0.5}$) of 960 nM. The complex shows no cooperativity and a higher affinity for PALA represented by a K_d of 500 nM.

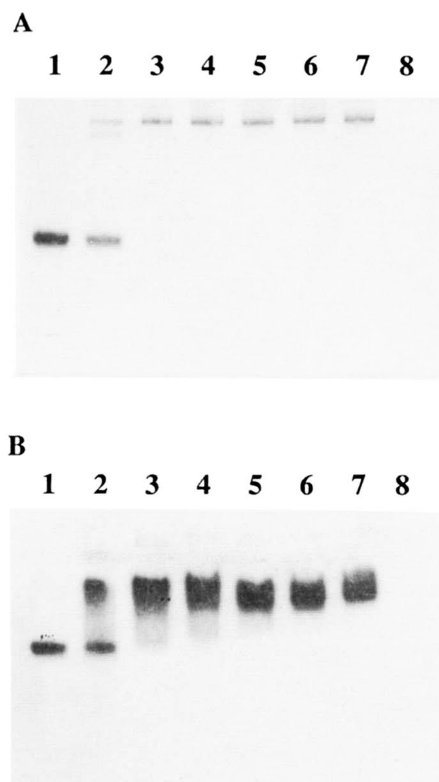


Fig. 3. Effects of zinc domain and R subunit on catalytic activities of mutant C trimer containing the Gln 231 → Ile replacement. Active species were detected by enzyme activity staining in nondenaturing polyacrylamide gels. Purified zinc domain, R subunit, and C trimer were in MOPS buffer. Experiments were performed using nondenaturing gel electrophoresis with 0.4 μg of mutant C trimer per lane and staining gels for enzyme activity under conditions corresponding to 15 mM aspartate and 1 mM carbamoyl phosphate. **A:** Lane 1, mutant C trimer alone; lanes 2–7, mutant C trimer plus R subunit at molar ratios of 0.8, 1.6, 4, 8, 20, and 36 r chains per mutant c chain; lane 8, R subunit alone. **B:** Lane 1, mutant C trimer alone; lanes 2–7, mutant C trimer plus zinc domain at molar ratios of 1, 3, 6, 10, 20, and 50 zinc domains per mutant c chain; lane 8, zinc domain alone.

Conformational changes in C trimers resulting both from complex formation with zinc domain and from PALA binding to the active sites

The binding of PALA to C subunit was shown by Collins and Stark (1971) to perturb the environment of tyrosine and tryptophan residues leading to a change in the ultraviolet absorption spectrum. Similarly, Markby et al. (1991) showed that the binding of the zinc domain to trinitrophenylated C trimers (C_{TNP}) resulted in a spectral change at the active sites due to a change in the environment of the spectral probes covalently attached to Lys 83 and Lys 84 (Lahue & Schachman, 1984). It was of interest, therefore, to combine these approaches to determine whether the effect of PALA on the free C subunit differed from that on the complex. Figure 4A shows the difference spectrum resulting from the binding of PALA to free C_{TNP}. The analogous difference spectrum for PALA binding to the C_{TNP}–zinc domain complex is shown in Figure 4B. Clearly, the PALA difference spectrum for the complex differs from that of the free subunit. This is illustrated by subtracting the upper difference spectrum (Fig. 4A) from the middle spectrum (Fig. 4B) to yield the difference difference spectrum in Figure 4C.

Performing the spectral studies in the opposite order as in Figure 5 yields similar results. In Figure 5A is shown the spectral change in C_{TNP} resulting from the addition of the zinc domain. If the zinc domain is added to C_{TNP}, which already contains bound PALA, the difference spectrum in Figure 5B is obtained. The striking difference between these two difference spectra is illustrated by the difference difference spectrum in Figure 5C. Moreover, as expected, the two difference difference spectra in Figures 4C and 5C are very similar despite the small spectral changes and the accompanying experimental errors.

It is clear from the spectral studies that PALA binding affects the environment at the active sites in both free C_{TNP} subunit and in the complex with the zinc domain. Similarly, the formation of the zinc domain–C_{TNP} trimer complex alters the environment at the active site of both unliganded and PALA-liganded C_{TNP} trimers.

Linkage between the strength of the C subunit–zinc domain interaction and the binding of PALA

Because the addition of the zinc domain to the C subunit leads to a large increase in the affinity of the C trimer for PALA (Fig. 1B; Table 1), it is to be expected that the binding of PALA should affect the strength of the interaction between the peptide and the protein. This linkage was tested by studies of the assembly of the complex at

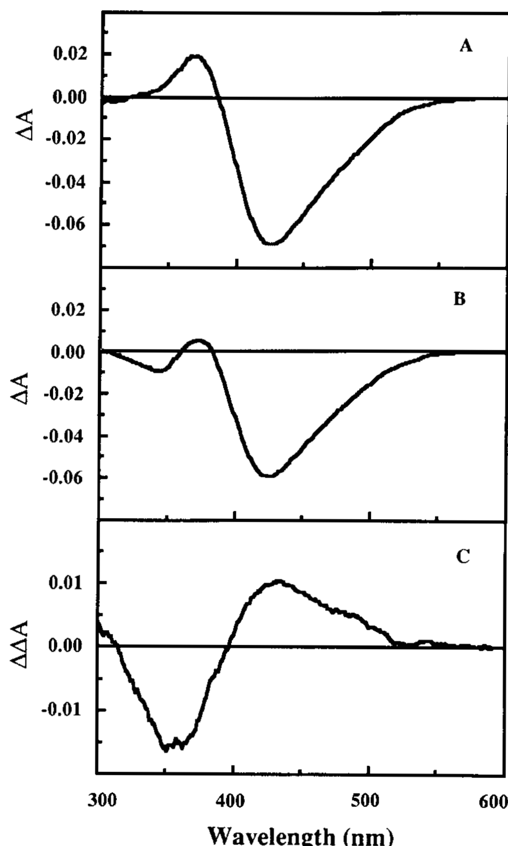


Fig. 4. Changes in absorption spectra of C_{TNP} upon PALA binding at the active sites. The experiments were performed in the absence (**A**) and presence of zinc domain (**B**). Equal volumes of C_{TNP} (2 mg/mL) or its mixture with zinc domain (2 mg/mL C_{TNP} plus 2.75 mg/mL zinc domain) in a 50 mM imidazole acetate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol and 200 μM PALA in the same buffer were placed in separate chambers of tandem cells. The baseline was recorded, contents of the sample were then mixed, and difference spectra were recorded. **A:** Difference spectrum for interaction of C_{TNP} with active site ligand PALA. **B:** Difference spectrum for interaction of zinc domain–C_{TNP} complex with PALA. **C:** Difference spectrum obtained by subtracting the PALA binding difference spectrum for C_{TNP} from the PALA binding difference spectrum for zinc domain–C_{TNP} complex.

varying concentrations of zinc domain with a constant amount of the mutant C trimer containing the Lys 164 → Glu replacement. As seen in the polyacrylamide gel electrophoresis patterns in Figure 6, there is a dramatic effect of PALA on complex formation. In the absence of PALA, the conversion of the bulk of the free mutant C trimer into complex required an 18-fold molar excess of zinc domain (Fig. 6A). In contrast, when the C trimer was ligated with PALA, the conversion into complexes with zinc domain was achieved with only a twofold excess of the polypeptide.

Discussion

Much of the research on ATCase as an allosteric enzyme has focused on the global conformational changes in the enzyme resulting from the binding of substrates and analogs (Gerhart & Schachman, 1968; Howlett et al., 1977; Kim et al., 1987; Ke et al., 1988; Schachman, 1988; Eisenstein et al., 1990). Attempts to account for the ligand-promoted changes in structural and functional properties in terms of alterations in subunit interactions (Subramani et al., 1977; Eisenstein et al., 1989), however, have met with only limited success because of the complexity of the structure and the interplay of the multiple interactions. Hence the relatively simple complex containing only one C trimer and three zinc-containing polypeptide fragments of the r chain provides a valuable model for probing some aspects of the structural basis of the allosteric transition.

Kinetic measurements of enzyme activity described ear-

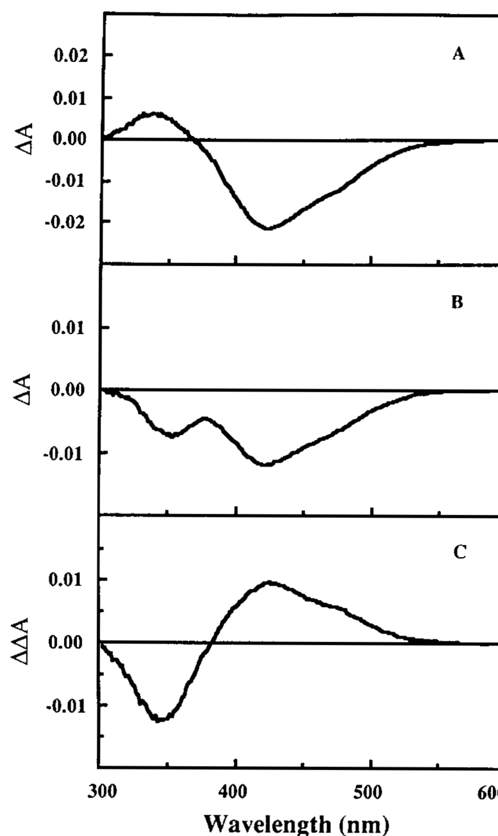


Fig. 5. Changes in absorption spectra of C_{TNP} upon formation of complex with zinc domain. The experiments were performed in the absence (**A**) and presence of active site ligand PALA (**B**). Equal volumes of C_{TNP} (2 mg/mL) or its mixture with PALA (2 mg/mL C_{TNP} plus 200 μM PALA) in a 50 mM imidazole acetate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol and Zn domain (2.75 mg/mL) in the same buffer were placed in separate chambers of tandem cells. The baseline was recorded, contents of the sample were then mixed, and difference spectra were recorded. **A:** Difference spectrum for interaction of unliganded C_{TNP} with zinc domain. **B:** Difference spectrum for interaction of PALA liganded C_{TNP} with zinc domain. **C:** Difference spectrum obtained by subtracting the assembly difference spectrum for unliganded zinc domain–C_{TNP} complex in the absence of PALA from the reconstitution difference spectrum for liganded zinc domain–C_{TNP} complex in the presence of saturating PALA.

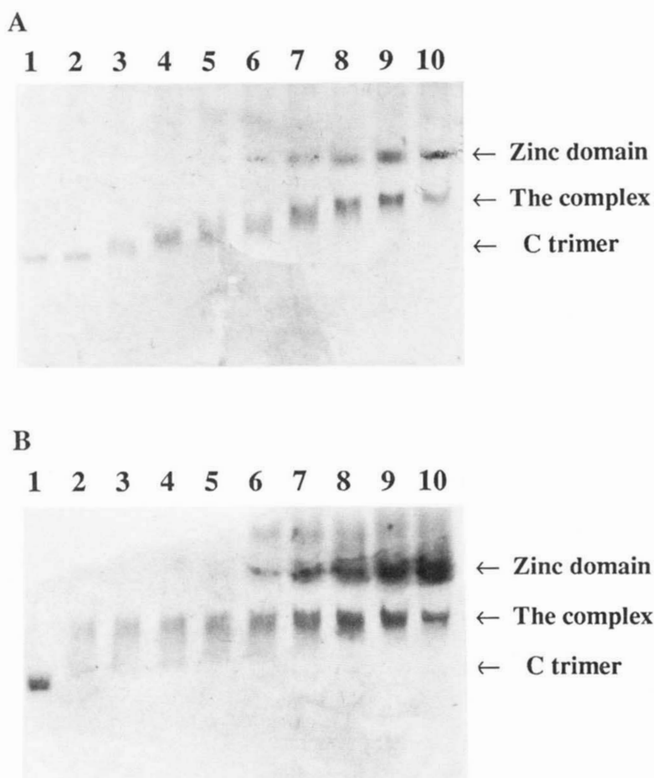


Fig. 6. Effect of PALA binding on the assembly of the zinc domain–C trimer complex. Purified zinc domain and mutant C trimer containing the Lys 164 → Glu substitution were in MOPS buffer. **A:** Experiments were performed by 7.5% PAGE nondenaturing gel electrophoresis with 0.32 μg of mutant C trimer per lane and staining gels by silver staining method. Lane 1, mutant C trimer alone; lanes 2–10, mutant C trimer plus zinc domain at molar ratios of 1, 2, 3, 5, 8, 12, 18, 25, and 40 zinc domains per mutant c chain. **B:** Samples were prepared in the presence of 2 mM PALA, and experiments were done by 7.5% nondenaturing polyacrylamide gel electrophoresis containing 2 mM PALA in the gel with 0.32 μg of mutant C trimer per lane. Lane 1, mutant C trimer alone; lanes 2–10, mutant C trimer plus zinc domain at molar ratios of 1, 2, 3, 5, 8, 12, 18, 25, and 40 zinc domains per mutant c chain.

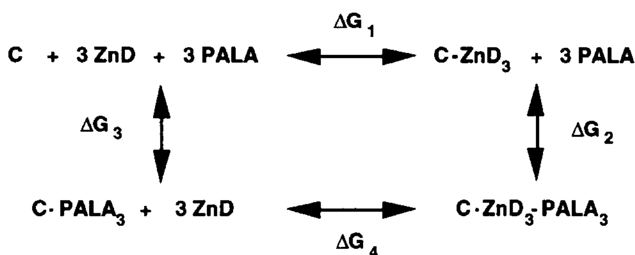
lier (Markby et al., 1991) and presented in Figure 2 and Table 2 demonstrate clearly that the interaction of the zinc domain with wild-type and mutant C trimers leads to marked changes in both V_{max} and K_m. Moreover, the enzymatic properties of the complexes are very similar to those of the respective holoenzymes when they are converted to the **R** conformation. Additional support for the hypothesis that the C trimer–zinc domain complex is an analog of the **R** state holoenzyme was obtained from equilibrium dialysis measurements of the binding of the bisubstrate ligand PALA (Fig. 1; Table 1). The complex containing C subunit from wild-type ATCase exhibits a hyperbolic saturation curve in contrast to the sigmoidal curve obtained with holoenzyme (Newell et al., 1989). This is demonstrated by the linear Scatchard plot in Figure 1A for the C trimer–zinc domain complex as contrasted to the curved plot showing the cooperativity of binding PALA to the holoenzyme. It is important to note that the slope of the line for the complex corresponds closely to that attained at a high degree of saturation of the holoenzyme, under which circumstances the bulk of the ATCase molecules are in the **R** state (Howlett et al., 1977; Foote & Schachman, 1985).

The increase in affinity for PALA binding at the active sites upon interaction of the wild-type C trimer with the zinc domain is shown by the decrease in K_d from 100 nM to 60 nM. Because this effect is relatively small, we used the mutant ATCase in which Lys 164 in the c chains is replaced by Glu. As shown by Newell and Schachman (1990), this mutant holoenzyme, unlike wild-type ATCase, exhibits Michaelis–Menten kinetics and has hydrodynamic properties characteristic of the **R** state even in the absence of active site ligands. In addition, the mutant holoenzyme was shown to have a significantly lower K_m for aspartate and K_d for PALA than the isolated C trimer (Newell & Schachman, 1990). As seen in Figure 1, the interaction of the Lys 164 → Glu C trimer with zinc domain results in a 50-fold increase in affinity for PALA, and the saturation curve for the complex is virtually identical to that for the holoenzyme.

In contrast to Lys 164 → Glu ATCase, which exists in the **R** state in both the absence and presence of substrates or the bisubstrate analog PALA, the mutant form in which Gln 231 in the c chains is replaced by Ile exists predominantly in the **T** state even at high substrate concentrations (Peterson et al., 1992). For this mutant, V_{max} for the isolated C subunit is much larger than that for the holoenzyme. Moreover, as shown in Figure 2, the addition of zinc domain to Gln 231 → Ile C trimer leads to a significant increase in affinity for aspartate. Also the affinity for PALA is increased about sixfold (Table 1). It is striking, as shown in Figure 2A, that the complex, an analog of the **R** state of the holoenzyme, has a 60-fold greater activity (V_{max}) than the holoenzyme in the **T** state (Peterson et al., 1992). Thus, as demonstrated by the results of Peterson et al. (1992) and those presented here, this mutant form of ATCase when it is in the **R** conformation has a higher affinity for both aspartate (K_m) and PALA (K_d) as well as a greater enzyme activity (V_{max}) than when it is in the **T** state.

On the basis of these results of both enzyme kinetics and equilibrium binding of PALA, we conclude that the association of the zinc domain with C trimer is sufficient to convert the active sites in the complex to a form analogous to the **R** state of the holoenzyme. No interactions with an apposing trimer are required.

The functional changes at the active sites resulting from the association of the C trimer with the zinc domain are attributable to the conformational changes that are manifested by the alterations in the environment of the spectral probes on C_{TNP}. It is clear that zinc domain, though binding to the C subunit at a distance remote from the vicinity of Lys 83 and Lys 84 containing the trinitrophe-



Scheme 1. Thermodynamic linkage between zinc domain–C trimer complex assembly and PALA binding.

nyl groups, causes a significant change at that location (Fig. 5A; Markby et al., 1991). Further evidence of that altered environment is the difference in the spectral perturbations caused by PALA binding to the complex as compared to free C_{TNP} (Fig. 4A,B).

Ligand binding at the active sites and formation of the complex between C subunit and zinc domain are coupled energetically. This thermodynamic linkage between complex assembly and PALA binding, which is shown in Scheme 1, leads to predictions that the strength of the interaction between the peptide and the protein will vary depending on the relative affinity for PALA exhibited by the complex and the free C subunit.

According to Scheme 1, the free energy of formation of the unliganded complex is ΔG_1 and that for the binding of PALA to the complex is ΔG_2. Clearly, $\Delta G_1 + \Delta G_2$ must equal $\Delta G_3 + \Delta G_4$ where ΔG_3 is the free energy of binding PALA to the free C trimer and ΔG_4 is the value for the assembly of the complex from PALA-liganded C trimer and zinc domain. Because the binding of the zinc domain increases the affinity of the C trimer for PALA (Table 1), ΔG_2 is larger than ΔG_3. Accordingly ΔG_4 must be larger than ΔG_1. Thus there must be an increase in the strength of the interaction between the zinc domain and the C trimer when PALA is bound. Experimental evidence confirming this prediction is shown in Figure 6 and demonstrates the marked strengthening of the interaction between the C subunit and zinc domain when PALA is bound at the active sites about 20 Å from the interface between the protein and peptide.[1]

The consequences of the interaction of the zinc domain with the two mutant C trimers described here constitute striking examples of intergenic complementation. Both the affinity for the bisubstrate ligand PALA and the catalytic efficiency, measured by V_{max}/K_m, are affected, as shown in Tables 1 and 2. Although the replacement of Lys 164 by Glu leads to marked reduction in the affinity of the mutant C trimer for PALA (Newell & Schachman, 1990), it is clear that Lys 164 is not directly involved in the binding and that the decreased affinity is attributable to an indirect effect such as altered folding of the chain. Indeed, the assembly of the mutant C trimer into a holoenzyme leads to a 50-fold enhancement in affinity for PALA. More dramatic is the observation (Fig. 2) that the 70-amino acid zinc-containing polypeptide fragment from the r chain can also lead to a correction of the incorrectly folded chains in the Lys 164 → Glu C trimer. This intergenic complementation is illustrated further with the Gln 231 → Ile ATCase. This mutant enzyme is shown to have a very low activity (Peterson et al., 1992) because it is in the **T** state and subject to the consequent quaternary constraint, which leads to a very low V_{max}. Release of this constraint by dissociating the holoenzyme to yield free C subunit produces a much more active enzyme (Fig. 2; Peterson et al., 1992). When the zinc domain is added to this mutant C trimer, the affinity for substrates is increased substantially (Fig. 2; Table 2) as is the affinity for the bisubstrate ligand, PALA (Table 1).

These studies demonstrate that the active sites of ATCase can exist in at least three distinct, relatively stable conformations represented by the isolated C trimer, the **T** state of holoenzyme, and the **R** conformation of the intact enzyme. Mutational alterations in either the c or r chains affect the energetics of the interconversion both by intrachain folding and by interchain interactions. Clearly, the free energy differences among the three conformations are not great, thereby permitting the catalytic activity to be regulated readily by various metabolites. In this respect, the C subunit of ATCase can be considered a "soft" protein whose properties are modulated by interchain interaction with zinc domain to form a relatively simple complex or with R dimers to yield a more sophisticated regulatory enzyme that can exist in either the **T** or **R** conformation.

Materials and methods

Protein preparations

Wild-type and mutant forms of ATCase were overproduced in *E. coli* strain EK1104 grown on minimal medium with the supplementations described by Nowlan and Kantrowitz (1985). Holoenzymes were purified from cell extracts using published procedures (Wall et al., 1979). Neohydrin was used to dissociate the holoenzymes into C and R subunits, and free subunits were isolated by ion exchange chromatography (Yang et al., 1978). Purity of samples was assessed by polyacrylamide gel electrophoresis using nondenaturing conditions.

[1] It should be recognized that the complex between the C trimer and the zinc domain might involve slightly different interface contacts than are present in the c1r1, c2r2, c3r3, etc. interfaces in the intact enzyme when it is in the **R** state. At present there is no information about the tertiary structure of the zinc-containing polypeptide used in this work. How different its structure may be from the domain in an isolated R dimer is not known. However, its folding is clearly dependent upon its chelation of zinc ions and it binds with high affinity ($K_d \sim 10^{-8}$ M) to the C trimer. Hence it serves as an excellent model for the domain in the intact r chain, which binds to the C subunit and contributes to the assembly and stability of the holoenzyme.

Zinc domain was overexpressed from plasmid pT7-7-ZN2 in *E. coli* strain BL21(DE3) pLysS induced by isopropyl β-D-thiogalactoside, and purified in MES buffer (20 mM 2-[*N*-morpholino]-ethanesulfonic acid, adjusted with KOH to pH 6.5, containing 1 mM 2-mercaptoethanol and 0.1 mM zinc acetate) as described previously (Markby et al., 1991). Purity of sample was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Purified protein was lyophilized and stored in powder form in the freezer.

Experimental procedures

Enzyme activities were assayed at 30 °C (Davies et al., 1970) in MOPS buffer (50 mM 4-morpholinepropanesulfonic acid, adjusted to pH 7.0 with KOH and containing 2 mM 2-mercaptoethanol). Assays were performed at varying aspartate concentrations with 4 mM carbamoyl phosphate. V_{max} and K_m were determined by nonlinear least-squares analysis. PALA binding affinities were determined by equilibrium dialysis at 23 °C (Newell et al., 1989) in standard phosphate buffer, and the Gln 231 → Ile mutant was assayed in MOPS buffer. The concentrations of free and bound PALA were calculated from the radioactivity measurements, and the results were expressed as ν, the fractional occupation of the total number of ligand binding sites, as a function of free PALA concentration.

PAGE gels (7.5%) were used for nondenaturing gel electrophoresis and were stained either for total protein using the silver staining method (Morrissey, 1981) or for ATCase activity (Bothwell, 1975).

Trinitrophenylated C subunit (C_{TNP}) was prepared as described elsewhere (Lahue & Schachman, 1984) and was used in spectral studies with a Cary model 118 double-beam spectrophotometer. Spectral changes caused by zinc domain binding and PALA binding were measured as difference spectra.

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