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A 70-amino acid zinc-binding polypeptide fragment from the regulatory chain of aspartate transcarbamoylase causes marked changes in the kinetic mechanism of the catalytic trimer

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Abstract

Interaction between a 70-amino acid and zinc-binding polypeptide from the regulatory chain and the catalytic (C) trimer of aspartate transcarbamoylase (ATCase) leads to dramatic changes in enzyme activity and affinity for active site ligands. The hypothesis that the complex between a C trimer and 3 polypeptide fragments (zinc domain) is an analog of **R** state ATCase has been examined by steady-state kinetics, heavy-atom isotope effects, and isotope trapping experiments. Inhibition by the bisubstrate ligand, *N*-(phosphonacetyl)-L-aspartate (PALA), or the substrate analog, succinate, at varying concentrations of substrates, aspartate, or carbamoyl phosphate indicated a compulsory ordered kinetic mechanism with carbamoyl phosphate binding prior to aspartate. In contrast, inhibition studies on C trimer were consistent with a preferred order mechanism. Similarly, ^{13}C kinetic isotope effects in carbamoyl phosphate at infinite aspartate indicated a partially random kinetic mechanism for C trimer, whereas results for the complex of C trimer and zinc domain were consistent with a compulsory ordered mechanism of substrate binding. The dependence of isotope effect on aspartate concentration observed for the Zn domain–C trimer complex was similar to that obtained earlier for intact ATCase. Isotope trapping experiments showed that the compulsory ordered mechanism for the complex was attributable to increased "stickiness" of carbamoyl phosphate to the Zn domain–C trimer complex as compared to C trimer alone. The rate of dissociation of carbamoyl phosphate from the Zn domain–C trimer complex was about 10^{-2} that from C trimer. Additional evidence for a change in binding of carbamoyl phosphate as a result of interaction of zinc domain and C trimer was obtained from the pH profile for K_{ia} for carbamoyl phosphate. Whereas participation of only 1 protonated residue is implicated in binding of carbamoyl phosphate to C trimer, 2 residues must be ionized for binding to the Zn domain–C trimer complex.

Keywords: catalytic mechanism; communication; conformational change; enzyme kinetics; peptide–protein interaction; zinc domain

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Abbreviations: ATCase, aspartate transcarbamoylase; C trimer or subunit, catalytic trimer or subunit; CbmP, carbamoyl phosphate; PALA, *N*-(phosphonacetyl)-L-aspartate; MES, (2-[*N*-morpholino]ethane sulfonic acid); MOPS, (3-[*N*-morpholino]propane sulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

The regulatory enzyme aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase, carbamoyl phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2), which catalyzes the formation of *N*-carbamoyl-L-aspartate and inorganic phosphate from L-aspartate and carbamoyl phosphate in the first committed step in pyrimidine biosynthesis in *Escherichia coli*, exists as a dodecamer composed of 6 catalytic and 6 regulatory polypeptide chains. Treatment of ATCase with mercurials leads to dissociation of the holoenzyme into catalytic trimers and regulatory dimers. Whereas isolated enzymatically active C trimers exhibit

Michaelis-Menten kinetics (Gerhart & Schachman, 1965), the holoenzyme exhibits a sigmoidal dependence of enzyme activity on the concentration of the substrates carbamoyl phosphate (Bethell et al., 1968) and aspartate (Gerhart & Pardee, 1962). Moreover, the holoenzyme is activated by ATP and inhibited by CTP (Gerhart & Pardee, 1962). These allosteric properties (Monod et al., 1965) are attributable to a ligand-promoted global conformational change in the enzyme from a low-activity, compact **T** state to the high-activity, relaxed **R** state, and the allosteric transition is generally assumed to be mediated by changes in intersubunit interactions (Howlett et al., 1977; Schachman, 1988). Although ATCase has become a model enzyme for analyzing the allosteric transition in terms of changes in interchain interactions, it has been difficult to determine the effects of individual interchain interactions on 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, in part, by studying a stable complex containing only 1 C trimer and 3 zinc-containing 70-amino acid polypeptide fragments (zinc domain) of the regulatory chain of ATCase (Markby et al., 1991).

Results from both enzyme kinetics and equilibrium binding studies indicate that the interaction between zinc domain and the C trimer converts the active sites in the complex to a form analogous to the **R** state of the holoenzyme (Markby et al., 1991; Zhou & Schachman, 1993 [including kinemage file]). A preliminary kinetic analysis showed that addition of the Zn domain to the C trimer resulted in a 50% decrease in V_{max} and K_m for aspartate, which is consistent with V_{max} for the holoenzyme being about one-half that for the C trimer (Markby et al., 1991). However, unlike the holoenzyme, the Zn domain-C trimer complex conforms to Michaelis-Menten kinetics. The present study was undertaken to investigate in more detail how the Zn domain-C trimer interaction affects the chemical and kinetic mechanisms of the C trimer. For example, what is the order of substrate addition (if any) to the Zn domain-C trimer complex? Is substrate addition ordered as with the holoenzyme or is it random? Does the Zn domain-C trimer interaction affect the on and off rates of substrate binding? Does the Zn domain-C trimer interaction alter the pH dependence of the reaction? In an attempt to answer these questions, the Zn domain-C trimer complex was subjected to a kinetic analysis using steady-state kinetics, heavy-atom isotope effects, and isotope-trapping studies. The results from these investigations, taken together with those on intact ATCase, provide strong evidence that the Zn domain-C trimer complex is similar to the holoenzyme in the **R** state.

Results

Inhibition by PALA and succinate

The inhibition of enzyme activity by the bisubstrate ligand, PALA, and the aspartate analog, succinate, was examined to establish the order of substrate addition to the Zn domain-C trimer complex. If carbamoyl phosphate binds prior to aspartate, then the pattern of inhibition by PALA will be competitive with respect to carbamoyl phosphate and noncompetitive with respect to aspartate (Collins & Stark, 1971). Velocity data obtained for the Zn domain-C trimer complex at varying concentrations of aspartate, constant carbamoyl phosphate, and fixed concentrations of PALA indicated noncompetitive inhibition (Fig. 1A). This result is consistent with a kinetic mechanism in which carbamoyl phosphate binds prior to aspartate. However, these observations cannot distinguish between a preferred ordered kinetic mechanism and one in which substrate binding is compulsory ordered.[4] A noncompetitive inhibition pattern is also seen for the isolated C subunit, even though the kinetic mechanism was found to be preferred ordered (Hsuanyu & Wedler, 1988; Parmentier et al., 1992). The random feature of a partially ordered mechanism can be detected by examining the inhibition pattern of succinate, a competitive inhibitor of aspartate (Porter et al., 1969), with respect to carbamoyl phosphate. A random component is manifested by a noncompetitive inhibition pattern for succinate with respect to carbamoyl phosphate, as was the case for the C trimer (Heyde & Morrison, 1973), whereas an uncompetitive pattern is expected for a completely ordered mechanism. Velocity data obtained for the Zn domain-C trimer complex by varying the concentration of carbamoyl phosphate at a constant amount of aspartate and fixed concentrations of succinate gave rise to a set of parallel lines (Fig. 1B). This pattern, indicating

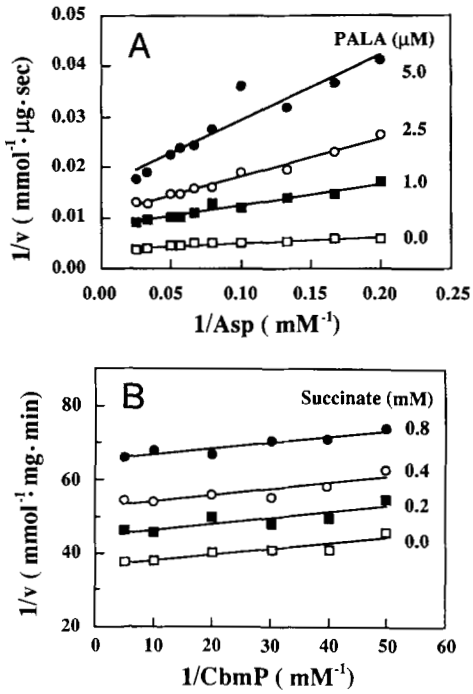

Fig. 1. Dead-end inhibition of the reaction catalyzed by the Zn domain-C trimer complex. **A:** Inhibition by PALA with respect to aspartate at a concentration of 5 mM carbamoyl phosphate. Enzyme activity was measured at 30 °C in 50 mM MOPS buffer at pH 7.5. Velocities are expressed as millimoles of carbamoyl aspartate formed per microgram of C trimer per second. **B:** Inhibition of the Zn domain-C trimer-catalyzed reaction by succinate with respect to carbamoyl phosphate. Carbamoyl phosphate concentration was varied at a fixed concentration (2 mM) of aspartate. Enzyme activities were measured in 50 mM TES buffer at pH 8.0. Velocities are expressed as millimoles of carbamoyl aspartate formed per milligram of C trimer per minute.

[4] A compulsory ordered kinetic mechanism is one in which the substrates must bind in a specific order before turnover can occur. A preferred ordered kinetic mechanism is a random mechanism where one substrate binds to the enzyme first most of the time, giving the appearance of an ordered kinetic mechanism.

that succinate acts as an uncompetitive inhibitor with respect to carbamoyl phosphate, is consistent with a compulsory ordered mechanism.

^{13}C kinetic isotope effects on the reaction catalyzed by the Zn domain-C trimer complex

A more sensitive measure of the order of the kinetic mechanism was obtained by examining the ^{13}C isotope effect of the catalytic reaction as a function of the concentration of aspartate (Parmentier et al., 1992). Results from ^{13}C isotope effect experiments on the Zn domain-C trimer complex at saturating carbamoyl phosphate and varying amounts of L-aspartate show a hyperbolic relationship (Fig. 2) between the ^{13}C isotope effect and aspartate concentration as observed previously for C trimer and intact ATCase (Parmentier et al., 1992). The data were fitted to Equation 4 (Materials and methods), which showed that the isotope effect at infinite concentration of aspartate was unity, suggesting that substrate addition is ordered. As shown by Parmentier et al. (1992), the ^{13}C isotope effect at infinite concentration of aspartate was unity for the holoenzyme (the curve accounting for their results is reproduced in Fig. 2 without the data). In contrast, the ^{13}C isotope effect for the C trimer did not approach unity at infinite concentration of aspartate (Parmentier et al., 1992; see Fig. 2). When the data for the Zn domain-C trimer complex were fitted to Equation 5 (Materials and methods) with the asymptote fixed at 1.0, the $^{13}(V/K_{CbmP})$ value extrapolated to 0 concentration of aspartate was 1.0223 ± 0.0007. This result is similar to the values (Table 1) for C trimer and the holoenzyme (Parmentier et al., 1992) and indicates that the rates of the chemical steps in the reaction catalyzed by the Zn domain-C trimer complex are not significantly different from those catalyzed by the holoenzyme or C trimer alone. The concentration of aspartate corresponding to 50% elimination of the isotope effect, K_{id}, was 1.46 ± 0.21 mM, which was slightly lower than the values determined for the holoenzyme and C trimer (Parmentier et al., 1992).

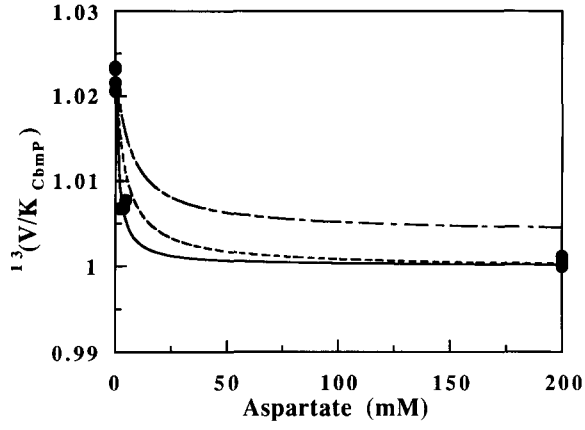

Fig. 2. ^{13}C isotope effects for the Zn domain-C trimer complex compared to those for the isolated C trimer and holoenzyme. Experiments at saturating carbamoyl phosphate and varying L-aspartate concentration were performed in a 50 mM HEPES buffer containing 2 mM dithiothreitol at pH 7.5 and 25 °C. Experimental points for the C trimer and the holoenzyme have been omitted for clarity. The curves for the C trimer (— — —) and the holoenzyme (– – –) are taken from Parmentier et al. (1992). Results for the Zn domain-C trimer complex are designated by ● and the solid curve. The data were fit to Equation 5.

Isotope trapping studies

The inhibition and isotope effect measurements clearly demonstrate that the major difference between the Zn domain-C trimer complex and the C trimer is that catalysis by the complex follows a compulsory ordered kinetic mechanism, whereas that by the C trimer is partially ordered. This finding leads to the question: How does the Zn domain interaction with the C trimer change the kinetic mechanism? A partial answer to this question was obtained from isotope trapping experiments according to the method of Rose et al. (1974), as described in Materials and methods. The percentage (P*) of labeled carbamoyl phosphate in the binary complex that appears as product after 3 s of reaction in the presence of varying concentrations of aspartate is shown in Figure 3A. In experiments with the Zn domain-C trimer complex, the maximum value of P* was about 85%, whereas with the C trimer alone, the maximum measured value was only 65%. It is striking that the concentration of aspartate for half-maximal trapping was 0.22 mM as compared to the much larger value (about 20.0 mM aspartate) in experiments with C trimer. These results were analyzed in terms of the scheme shown in Figure 4 (Cleland, 1977) to yield values for the ratio of the off-rate of carbamoyl phosphate relative to the turnover number in the binary and ternary complexes.

The values for k_1/k_3 and k_2/k_3 were calculated from the data in Figure 3A as described in the Materials and methods. For the Zn domain-C trimer complex, k_1/k_3 is 0.042 ± 0.004 and k_2/k_3 is 0.06 ± 0.05. The large error in k_2/k_3 arises because it represents the small difference between nearly equal numbers. With C trimer alone, k_1/k_3 and k_2/k_3 were 2.9 ± 0.1 and 0.38 ± 0.05, respectively. Individual values of k_1 and k_2 were calculated from these ratios and the values for k_3. For C trimer, k_3 is 600 s^{-1} and for the Zn domain-C trimer complex, k_3 is 310 s^{-1}. Thus for the C trimer alone, k_1 = 1,740 ± 60 s^{-1} and k_2 =

Table 1. *^{13}C kinetic isotope effects for the reactions catalyzed by C trimer, holoenzyme, and Zn domain-C trimer complex*

Parameters determined[a]	C trimer[b]	Holoenzyme[b]	Zn domain-C trimer
$^{13}(V/K_{CbmP})$ at 0 aspartate	1.0240 ± 0.0005	1.0217 ± 0.0005	1.0223 ± 0.0007
$^{13}(V/K_{CbmP})$ at infinite aspartate	1.0039 ± 0.0003	Unity	Unity

[a] Experiments conducted at 12 mM carbamoyl phosphate and aspartate concentrations from about 0 to 200 mM.
[b] Results from Parmentier et al. (1992).

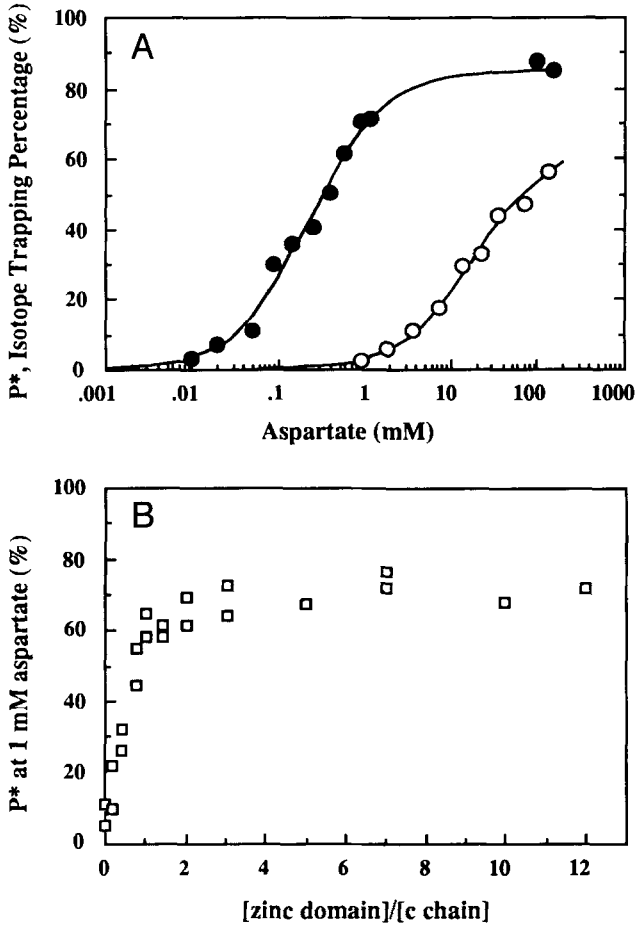

Fig. 3. Isotope trapping of carbamoyl phosphate by the Zn domain–C trimer complex and isolated C trimer. Experiments were performed at room temperature (about 23 °C) in 50 mM MOPS buffer at pH 7.5 containing 2 mM β-mercaptoethanol. **A:** Saturation curves are presented as isotope trapping percentage (P*) as a function of logarithmic aspartate concentrations. ○ Represents the C trimer alone at 0.275 mg/mL; ● designates mixture of the C trimer (0.275 mg/mL) and zinc domain (0.756 mg/mL). **B:** Effect of increasing amounts of Zn domain per c chain on P*. The titration experiment was performed with C trimer at 0.275 mg/mL and an aspartate concentration of 1 mM.

228 ± 30 s^{-1}. In contrast, $k_1 = 13.0 \pm 1.2$ s^{-1} and $k_2 = 19 \pm 16$ s^{-1} for the Zn domain–C trimer complex. For a completely ordered kinetic mechanism, k_2, the rate of dissociation of carbamoyl phosphate from the ternary complex should be 0. Although the value calculated for k_2 for the experiments with the Zn domain–C trimer complex is clearly measurable, it should be noted that the precision in the determination of the maximum amount of labeled carbamoyl phosphate trapped decreases as the amount trapped approaches the enzyme concentration. A very small amount of carbamoyl phosphate may indeed be able to dissociate from the ternary complex. However, the results from the isotope trapping experiments demonstrate clearly that the rate of dissociation of carbamoyl phosphate from the binary and ternary enzyme forms has been decreased dramatically as a result of the interaction of the zinc domain with the C trimer.

Because of the large decrease in the off-rate of carbamoyl phosphate, resulting from the interaction of the zinc domain with the C trimer, it was possible to measure the percentage trapping at 1 mM aspartate as a function of the ratio of zinc domain to C trimer. As seen in Figure 3B, there is a marked increase in trapping percentage upon the addition of zinc domain to C trimer. Moreover, the titration curve shows that the maximum enhancement in the percentage of labeled carbamoyl phosphate trapped is achieved at a ratio of about 1 zinc domain per catalytic chain.

Carbamoyl phosphate binding

The dissociation constant (K_{ia}) for the release of carbamoyl phosphate was determined from measurements of initial velocities at varying carbamoyl phosphate concentrations and a fixed aspartate concentration that was 1/50 the Michaelis constant for aspartate. Under these conditions, the reaction examined was that of carbamoyl phosphate with free enzyme to form the binary complex, and the apparent K_m equals the dissociation constant (Porter et al., 1969). For C trimer, the value of K_{ia} is 9.0 ± 1.1 μM, and $K_{ia} = 8.2 \pm 0.9$ μM for the Zn domain–C trimer complex. Because the off-rate for carbamoyl phosphate from the binary enzyme form was known from the isotope trapping experiments, the on-rate for carbamoyl phosphate binding can be calculated to be 1.9×10^8 s^{-1} M^{-1} for C trimer alone. In contrast, this value is only 1.6×10^6 s^{-1} M^{-1} for the Zn domain–C trimer complex. These results indicate that the on-rate and off-rate for carbamoyl phosphate binding were decreased by more than 100-fold as a result of the peptide–protein interaction. This decrease in rate corresponds to a 2.9-kcal/mol free energy increase in activation energy.

pH dependence of carbamoyl phosphate binding

In an effort to determine how the zinc domain causes a decrease in the off-rate of carbamoyl phosphate from the C trimer, we examined the effect of pH on the binding of carbamoyl phosphate to the Zn domain–C trimer complex for comparison with that for the C trimer alone. The dissociation constant for carbamoyl phosphate (expressed as pK_{ia}) vs. pH was a bell-shaped curve (Fig. 5), and fitting the data to Equation 2 (Materials and methods) yielded pK values (Table 2) for the ionizing residues observed on the acidic (6.68 ± 0.18) and basic (9.01 ± 0.18) limbs of the profile. In comparable experiments with C trimer alone (Turnbull et al., 1992) carbamoyl phosphate binding was shown to require the participation of a single protonated residue with

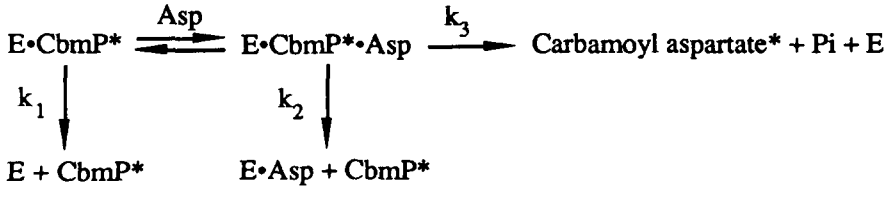

Fig. 4. Examples of isotope trapping reactions involving ATCase (E), carbamoyl phosphate (CbmP), and aspartate.

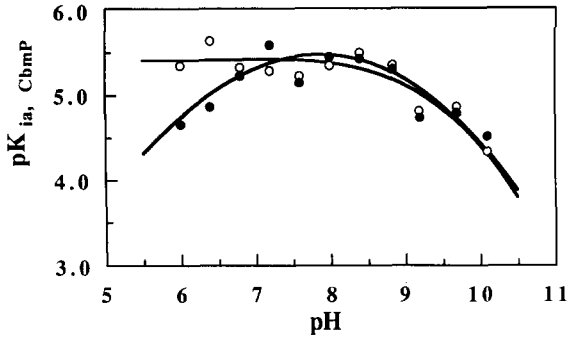

Fig. 5. pH dependence of pK_{ia} for carbamoyl phosphate in the Zn domain-C trimer complex and isolated C trimer. Initial velocity data for K_{ia} were obtained by varying carbamoyl phosphate concentration at 0.1 mM aspartate. The curves represent the best fits of the data to Equation 2 for the Zn domain-C trimer complex (●) and Equation 3 for the C trimer (○). Curves were drawn using the parameters given in Table 2. The units for K_{ia} are M.

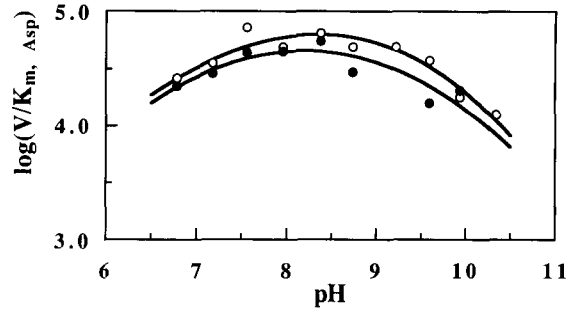

Fig. 6. pH dependence of log($V/K_{m,Asp}$) for the reaction catalyzed by the Zn domain-C trimer complex and C trimer alone. Initial velocity data were obtained by varying aspartate concentrations at 5 mM carbamoyl phosphate. The curves represent best fits of the data to Equation 2, with ● representing the Zn domain-C trimer complex and ○ the isolated C trimer. The values of the parameters used to draw the curves are given in Table 2. The units for $V/K_{m,Asp}$ are M^{-1} s^{-1}.

a basic pK value of 9.03 ± 0.08. This difference in pH dependence is consistent with the previous results showing that zinc domain binding to C trimers causes a conformational change in the active sites of the C trimer (Markby et al., 1991; Zhou & Schachman, 1993). It is of interest that in the pH range from 7.5 to 10.0, the pK_{ia} does not change significantly as a result of zinc domain binding. Moreover, the pH-independent parameters for carbamoyl phosphate binding to the Zn domain-C trimer complex and the C trimer alone are not significantly different (Table 2).

pH dependence of the reaction catalyzed by the Zn domain-C trimer complex

The effect of pH on the reaction catalyzed by the Zn domain-C trimer complex was determined over the pH range of 6.8-10.3 by varying aspartate concentrations at a fixed, saturating level (5 mM) of carbamoyl phosphate at each pH. Under these conditions, the reaction examined was that of aspartate with the enzyme-carbamoyl phosphate complex. The V/K profiles for both the Zn domain-C trimer complex and the C trimer alone show a decrease in the rate of reaction of aspartate at low and high pH values (Fig. 6). The fit of the data to Equation 2 yielded pK values of 6.82 ± 0.31 and 9.60 ± 0.22 for the Zn domain-C trimer complex, in agreement with those for C trimer alone, 6.94 ± 0.17 and 9.66 ± 0.11. These bell-shaped profiles and pK values are similar to those obtained with the slow substrate cysteine sulfinate (Foote et al., 1985) and with a relatively inactive mutant form of ATCase in which His 134 in the catalytic chains was replaced by Ala (Waldrop et al., 1992). In both of these cases, it was shown that aspartate was not a sticky substrate.[5] Therefore, according to the discussion by Turnbull et al. (1992), the pK values for the Zn domain-C trimer complex are intrinsic and aspartate is not a sticky substrate.

Discussion

Earlier studies on the Zn domain-C trimer complex revealed that V_{max} and K_m for aspartate were about one-half of the values obtained for the C trimer (Markby et al., 1991). Moreover, equilibrium dialysis experiments showed that binding of the bisubstrate analog PALA to the Zn domain-C trimer complex was significantly tighter than to the C trimer alone (Zhou & Schach-

[5] Stickiness of a substrate in an enzyme reaction is the ratio of rates of the substrate reacting to form the product relative to dissociating away from the enzyme. By definition, sticky substrates react to give products more rapidly than they dissociate from the enzyme-substrate complex.

Table 2. *Values of pK and pH-independent kinetic parameters for reaction catalyzed by C trimer and the Zn domain-C trimer complex*

Parameters determined	Species	pH-independent value	pK_1	pK_2
[$V/K_{m,Asp}$][a]	C trimer	88 ± 10	6.94 ± 0.17	9.66 ± 0.11
	Zn domain-C trimer complex	86 ± 11	6.82 ± 0.31	9.60 ± 0.22
[$K_{ia,CbmP}$][b]	C trimer	7.23 ± 1.40		9.03 ± 0.08
	Zn domain-C trimer complex	6.98 ± 1.60	6.68 ± 0.18	9.01 ± 0.18

[a] Experiments were conducted at saturating carbamoyl phosphate and varying concentrations. $V/K_{m,Asp}$ is expressed as mM^{-1} s^{-1}.

[b] Experiments were conducted at nonsaturating aspartate and varying carbamoyl phosphate concentrations. $K_{ia,CbmP}$ is expressed as μM.

man, 1993). The detailed kinetic studies in this report were initiated to determine how the Zn domain–C trimer interaction affects the kinetic mechanism of the C trimer. The kinetic approach used here complements the activity and equilibrium binding studies (Markby et al., 1991; Zhou & Schachman, 1993) to strengthen further the argument that the Zn domain–C trimer complex resembles that of intact ATCase in the **R** state. In addition, because the Zn domain–C trimer complex exhibits Michaelis–Menten kinetics, it is an ideal model system for studying aspects of the catalytic mechanism of the **R** state of ATCase.

Dead-end inhibition studies with the bisubstrate analog PALA and the aspartate analog succinate indicate a compulsory ordered mechanism for the Zn domain–C trimer complex. The pattern of inhibition of the complex by PALA was noncompetitive with respect to aspartate, which is consistent with an ordered mechanism. Moreover, succinate exhibited uncompetitive inhibition with respect to carbamoyl phosphate, indicating that succinate does not bind until carbamoyl phosphate is already bound. In contrast to these results for the Zn domain–C trimer complex, the inhibition pattern for C trimer alone at varying succinate concentrations was noncompetitive, which led Heyde and Morrison (1973) to conclude that the kinetic mechanism for the C trimer is random. Subsequent studies using the techniques of isotope exchange (Hsuanyu & Wedler, 1988) and heavy-atom isotope effects (Parmentier et al., 1992) have confirmed that the kinetic mechanism for the C trimer is in fact preferred ordered. From one point of view the kinetic mechanism appears to be random because catalysis can occur independent of the order of substrate binding. However, due to synergistic binding of the substrates, the kinetic mechanism appears to be ordered with carbamoyl phosphate binding first about 70% of the time (Hsuanyu & Wedler, 1988; Parmentier et al., 1992).

Although dead-end inhibitors were useful for examining the kinetic mechanism of the Zn domain–C trimer complex, more detailed information regarding the kinetic mechanism was derived from a study of heavy-atom isotope effects (Parmentier et al., 1992). As seen in Figure 2, the ^{13}C isotope effect for the Zn domain–C trimer complex varies as a function of aspartate concentration and overlaps with that of intact ATCase at higher aspartate concentration. For both the Zn domain–C trimer complex and the holoenzyme, the isotope effects approach unity as the aspartate concentration is increased, indicating an ordered mechanism with carbamoyl phosphate binding first (Cook & Cleland, 1981). Although the ^{13}C isotope effect for the C trimer alone also decreases with increasing concentration of aspartate, it does not approach unity at infinite aspartate as expected for a mechanism that is not completely ordered (Parmentier et al., 1992). The decrease in the observed value of $^{13}(V/K_{CbmP})$ for the Zn domain–C trimer complex as the concentration of aspartate is increased shows that there is a large forward commitment[6] (C_f) for carbamoyl phosphate and that aspartate binding completely prevents the dissociation of carbamoyl phosphate from the ternary complex. The C_f for the Zn domain–C trimer complex at the observed $^{13}(V/K_{CbmP})$ value extrapolated to 0 aspartate concentration (Table 1) can be calculated from:

$$^{13}(V/K_{CbmP}) = (k + C_f)/(1 + C_f),$$

where k is the intrinsic isotope effect.[7] If 1.0434 is used for the intrinsic isotope effect (Waldrop et al., 1992), C_f for the peptide–protein complex is 0.95; the C_f's for the holoenzyme and C trimer at 0 concentration of aspartate are 1 and 0.81, respectively (Parmentier et al., 1992). The similarity of the C_f's for the C trimer, holoenzyme, and Zn domain–C trimer complex indicates that the extent to which the chemical step of the reaction is rate limiting after formation of the ternary complex is not significantly different among the 3 different species. Thus, the isotope effect and inhibition experiments indicate clearly that the interaction of the peptide and the C trimer affects the active sites at a distance 20 Å away so as to change the kinetic mechanism from a preferred order to compulsory ordered. However, these studies by themselves can only determine the order of substrate binding and do not provide any detailed insight as to how the peptide–protein interaction accomplishes the observed changes in the kinetic mechanism.

The isotope trapping experiments of Rose et al. (1974) demonstrated that the interaction of the zinc domain with the C trimer significantly decreased the off-rate constants for carbamoyl phosphate from both the binary and ternary complexes as compared to the rate of dissociation from C trimer alone. From the ratio of the off-rate constants to the turnover number it was possible to determine the stickiness of carbamoyl phosphate in the binary and ternary complexes. Addition of the zinc domain to the C trimer increased (relative to the C trimer alone) the stickiness of carbamoyl phosphate 70- and 6-fold in the binary and ternary complexes, respectively. This increase in the stickiness of carbamoyl phosphate in the ternary complex is consistent with the change in kinetic mechanism from preferred ordered to compulsory ordered and indicates that the completely ordered kinetic mechanism for the Zn domain–C trimer complex is a consequence of carbamoyl phosphate being a very sticky substrate in the ternary complex. These findings are consistent with the view deduced from spectral changes in a chromophore at the active site (Zhou & Schachman, 1993) that the interaction of the peptide with the C trimer leads to a conformational change at the active sites.

Although the rate of dissociation of carbamoyl phosphate from the binary complex is decreased almost 100-fold by the peptide–protein interaction, surprisingly, the dissociation constant (K_{ia}) for carbamoyl phosphate at pH 7.5 remains the same. These results could be explained by a mechanism in which the peptide–protein interaction increases the activation energy for carbamoyl phosphate binding by about 2.9 kcal/mol, whereas the relative energy levels between free enzyme and the binary complex remain unchanged.

The association of carbamoyl phosphate to isolated C trimers occurs at nearly the diffusion-controlled rate (1.9×10^8 s^{-1} M^{-1}). It is of interest that the addition of zinc domain results in a decrease in the association rate to a rate less than 10^{-2} that for the free subunit. This finding is consistent with the view that the active sites in the complex have a conformation significantly altered from those in the isolated C trimer.

Additional support for a change in the binding of carbamoyl phosphate was obtained from the results of the pH profile for the binding of carbamoyl phosphate. The pK_{ia} versus pH profile for the Zn domain–C trimer complex shows that carbam-

[6] A commitment is the ratio of the rate constant for the isotope-sensitive step to the net rate constant for release of carbamoyl phosphate from the enzyme (Cleland, 1982).

[7] The intrinsic isotope effect is the observed isotope effect when the isotope-sensitive step is completely rate limiting.

oyl phosphate binding requires 1 ionized group and 1 protonated group on the complex, whereas binding of carbamoyl phosphate to the C trimer alone requires protonation of only 1 group. This difference may arise from a conformational change in the complex that shifts the pK value of a group which is involved in binding carbamoyl phosphate to the C trimer into the experimentally accessible range of 6.0–10.0. Alternatively, the result may reflect the participation of different groups unique to the Zn domain–C trimer complex. In contrast to the pH dependence for the binding of carbamoyl phosphate, the pK values of residues in the Zn domain–C trimer complex determined from the pH dependence for the V/K for aspartate were similar to those in the profile for the C trimer alone (Fig. 6).

In summary, the major effect of the zinc domain on the kinetics of the C trimer was to change the kinetic mechanism from preferred ordered to compulsory ordered. This change is accompanied by a precipitous decrease in the off-rate constant for carbamoyl phosphate from the binary as well as the ternary complex. Because previous studies (Markby et al., 1991; Zhou & Schachman, 1993) have demonstrated that the Zn domain–C trimer complex exhibits properties very similar to the **R** state of the holoenzyme, then by inference the compulsory ordered mechanism for the holoenzyme is due to a very low off-rate for carbamoyl phosphate from the holoenzyme.

Materials and methods

Materials

L-Aspartic acid was supplied by Calbiochem, and succinic acid was purchased from Mallinckrodt. Dilithium carbamoyl phosphate and HEPES were obtained from Sigma. [^{14}C]CbmP (12.5 mCi/mmol) was supplied by New England Nuclear, and [^{14}C]aspartate (216 mCi/mmol) was obtained from Amersham. PALA was provided by Dr. Robert Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health. All other chemicals were obtained commercially and were of the highest purity available.

ATCase was overproduced in *E. coli* strain EK1104 grown on minimal medium with the supplementations described by Nowlan and Kantrowitz (1985). Holoenzyme was purified from cell extracts using published procedures (Gerhart & Holoubek, 1967). Neohydrin was used to dissociate the holoenzymes into C and R subunits, which were isolated by DEAE ion-exchange chromatography (Yang et al., 1978). C trimer was stored at 4 °C as a precipitate in 3.6 M ammonium sulfate. The zinc domain was overexpressed from plasmid pT7-7-ZN2 in *E. coli* strain BL21(DE3)pLysS induced by isopropyl β-D-thiogalactoside and purified as described previously (Markby et al., 1991; Zhou & Schachman, 1993). Purified protein was lyophilized and stored in powder form at −20 °C.

Determination of enzyme activity

Catalytic activity was measured at 30 °C using the radioactive stopped-time assay of Davies et al. (1970). For the pH-dependence studies, activities were measured in a 3-component buffer system of 0.1 M MES, 0.051 M *N*-ethylmorpholine, and 0.051 M diethanolamine that also contained 2 mM β-mercaptoethanol. Over the pH range of 6.0–10.5, for which the initial velocity was measured, the ionic strength of the buffer mixture remained constant at a value of 0.1 (Ellis & Morrison, 1982). Under the assay conditions used in this study, the velocity was a linear function of the enzyme concentration and the method of Selwyn (1965) was used to determine that there was no time-dependent inactivation of the Zn domain–C trimer complex. The complex was stable under the experimental conditions used for the entire pH range as indicated by titration experiments with zinc domain and C trimer at neutral and extreme pH values.

Isotope trapping studies

An aliquot (0.2 mL) of a solution containing 2.6 μM C trimer, 78 μM zinc domain (10-fold molar excess), and 80 μM ^{14}C-carbamoyl phosphate was added to 2 mL of a rapidly stirring solution of 100 mM carbamoyl phosphate and various amounts of aspartate as described elsewhere (Turnbull et al., 1992). The buffer for both solutions contained 50 mM MOPS, 2 mM β-mercaptoethanol at pH 7.5. The reaction was stopped after 3 s by the addition of 0.5 mL of 2 N acetic acid, and the mixture was incubated at 80 °C for 2 h to remove unreacted ^{14}C-carbamoyl phosphate and the amount of ^{14}C-carbamoylaspartate was determined by liquid scintillation counting. A control experiment was conducted at each aspartate concentration by adding 0.2 mL of 2.6 μM C trimer and 78 μM zinc domain to a 2-mL reaction mixture containing aspartate, unlabeled carbamoyl phosphate, and ^{14}C-carbamoyl phosphate, which was then allowed to react for 3 s. The amount of ^{14}C-carbamoylaspartate detected in the control reaction was subtracted from the experimental value.

^{13}C isotope effects

^{13}C isotope effects on the Zn domain–C trimer complex-catalyzed reaction were determined as described by Parmentier et al. (1992).

Isotope effect nomenclature

The nomenclature used here follows that of Northrop (1977) in which the leading superscript denotes the isotope responsible for the effect on a given kinetic parameter and the following subscript designates the substrate on which the isotope effect is measured. Thus, $^{13}(V/K_{\mathrm{CbmP}})$ represents the ratio of V/K for ^{12}C-containing carbamoyl phosphate relative to that for the ^{13}C-containing carbamoyl phosphate.

Data analysis

Initial velocities (v) obtained at each pH in experiments in which the concentration of one substrate (A) was varied at a constant level of the other substrate. The data were fitted to Equation 1,

$$v = VA/(K + A), \quad (1)$$

with the nonlinear regression computer programs of Cleland (1979) to yield values for the maximum velocity (V), the Michaelis constant (K) for the substrate, and the apparent first-order rate constant (V/K). The variations with pH of the values for V/K and $1/K_{\mathrm{ia}}$ (K_{ia} is the dissociation constant of the

enzyme-carbamoyl phosphate complex) were fitted to the appropriate equations:

$$\log y = \log[C/(1 + [H^+]/K_1 + K_2/[H^+])] \quad (2)$$

$$\log y = \log[C/(1 + K_2/[H^+])]. \quad (3)$$

In these equations, y represents the value of V/K and $1/K_{ia}$ at a particular pH and C is the pH-independent value of the parameter. K_1 and K_2 are acid dissociation constants of ionizable groups on the acid and alkaline sides of the pH profiles, respectively. ^{13}C kinetic isotope effect data were fitted to Equation 4 for a hyperbola (HYPRP; Cleland, 1979):

$$y = A(1 + x/K_{in})/(1 + x/K_{id}), \quad (4)$$

where y is the observed ^{13}C isotope effect, x is the final aspartate concentration, A is the isotope effect at an aspartate concentration extrapolated to 0, K_{id} is the aspartate concentration corresponding to half-elimination of the isotope effect, and $A(K_{id}/K_{in})$ equals the isotope effect at infinite aspartate. In the case

$$y = (A + x/K_{id})/(1 + x/K_{id}), \quad (5)$$

where the asymptote of the hyperbola is unity, the data can be fitted to Equation 5 (HYPRPL; Cleland, 1979), where x, y, K_{id}, and A are as described above. Isotope trapping data were fitted to Equations 6 and 7 derived using Figure 4 (Cleland, 1977) to yield values k_1/k_3 and k_2/k_3, which represent the ratio of the off-rate of carbamoyl phosphate to the turnover number in the binary and ternary complexes, respectively:

$$K'_{Asp}/K_{m,Asp} \le k_1/k_3 \le (K'_{Asp}/K_{m,Asp})(1/P^*_{max})\,[1/(1 + K_{ia}/[CbmP])] \quad (6)$$

$$k_2/k_3 = [(1/P^*_{max})/(1 + K_{ia}/[CbmP])] - 1, \quad (7)$$

where P^*_{max} is the maximum trapping percentage, which is equivalent to the ratio of the maximum concentration of labeled product formed to the concentration of active sites. [CbmP] is the free carbamoyl phosphate concentration, K_{ia} is the dissociation constant of the enzyme-carbamoyl phosphate complex, $K_{m,Asp}$ is the Michaelis constant for aspartate in the chemical reaction, and K'_{Asp} is the apparent Michaelis constant for trapping.

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