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The 80s loop of the catalytic chain of *Escherichia coli* aspartate transcarbamoylase is critical for catalysis and homotropic cooperativity

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Abstract

The X-ray structure of the *Escherichia coli* aspartate transcarbamoylase with the bisubstrate analog phosphonacetyl-L-aspartate (PALA) bound shows that PALA interacts with Lys84 from an adjacent catalytic chain. To probe the function of Lys84, site-specific mutagenesis was used to convert Lys84 to alanine, threonine, and asparagine. The K84N and K84T enzymes exhibited 0.08 and 0.29% of the activity of the wild-type enzyme, respectively. However, the K84A enzyme retained 12% of the activity of the wild-type enzyme. For each of these enzymes, the affinity for aspartate was reduced 5- to 10-fold, and the affinity for carbamoyl phosphate was reduced 10- to 30-fold. The enzymes K84N and K84T exhibited no appreciable cooperativity, whereas the K84A enzyme exhibited a Hill coefficient of 1.8. The residual cooperativity and enhanced activity of the K84A enzyme suggest that in this enzyme another mechanism functions to restore catalytic activity. Modeling studies as well as molecular dynamics simulations suggest that in the case of only the K84A enzyme, the lysine residue at position 83 can reorient into the active site and complement for the loss of Lys84. This hypothesis was tested by the creation and analysis of the K83A enzyme and a double mutant enzyme (DM) that has both Lys83 and Lys84 replaced by alanine. The DM enzyme has no cooperativity and exhibited 0.18% of wild-type activity, while the K83A enzyme exhibited 61% of wild-type activity. These data suggest that Lys84 is not only catalytically important, but is also essential for binding both substrates and creation of the high-activity, high-affinity active site. Since low-angle X-ray scattering demonstrated that the mutant enzymes can be converted to the R-structural state, the loss of cooperativity must be related to the inability of these mutant enzymes to form the high-activity, high-affinity active site characteristic of the R-functional state of the enzyme.

Keywords: allosteric enzyme; complementation; low-angle X-ray scattering; molecular dynamics

Escherichia coli aspartate transcarbamoylase catalyzes the committed step in the pyrimidine biosynthesis pathway: the condensation of L-aspartate and carbamoyl phosphate to form *N*-carbamoyl-L-aspartate and inorganic phosphate (Gerhart & Pardee, 1962). The product of the reaction, *N*-carbamoyl-L-aspartate, then undergoes a series of enzymatic reactions to yield the pyrimidine nucleotides CTP and UTP. Aspartate transcarbamoylase not only catalyzes this important metabolic reaction, but also helps regulate the biosynthesis of pyrimidines. The enzyme is subject to feedback inhibition by its end product, CTP, as well as by UTP in the presence of CTP. ATP, the end product of the parallel purine biosynthesis pathway, activates the enzyme (Gerhart & Pardee, 1962). The aspartate transcarbamoylase from *E. coli* has been extensively studied, and a number of reviews are available that cover both the structure and function of the enzyme (Gerhart, 1970; Jacobson & Stark, 1973; Schachman, 1988; Kantrowitz & Lipscomb, 1990; Lipscomb, 1994).

The holoenzyme (M_r = 310,000) is a dodecamer composed of six catalytic and six regulatory chains. The catalytic chains (M_r = 33,000 each) are organized into two trimers, while the regulatory chains (M_r = 17,000 each) are organized into three dimers. The catalytic chains contain the active sites, whereas the regulatory chains are devoid of catalytic activity, containing the binding sites for the regulatory nucleotides. Each active site is shared between two catalytic chains and residues required for maximal activity are

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Abbreviations: $[Asp]_{0.5}$, aspartate concentration at one-half the maximal observed specific activity; $[CP]_{0.5}$, carbamoyl phosphate concentration at one-half the maximal observed specific activity; DM, double mutant enzyme in which both Lys83 and Lys84 in the catalytic chain have been converted to alanine; PALA, *N*-phosphonacetyl-L-aspartate.

contributed by the 80s loop of an adjacent catalytic chain (Monaco et al., 1978; Krause et al., 1985; Robey & Schachman, 1985; Wente & Schachman, 1987). Each of the catalytic chains is composed of two structural domains—the carbamoyl phosphate domain and the aspartate domain—which contain binding loci for each of the two substrates.

The holoenzyme displays sigmoidal kinetics characteristic of allosteric enzymes. The isolated catalytic subunit, which is not subject to regulation, exhibits simple Michaelis–Menten kinetics and a maximal velocity about 50% higher than the holoenzyme. The catalytic mechanism involves ordered binding with carbamoyl phosphate binding prior to aspartate (Porter et al., 1969; Hsuanyu & Wedler, 1987). The binding of carbamoyl phosphate induces a conformational change in the enzyme that facilitates the binding of aspartate (Collins & Stark, 1969; Griffin et al., 1972; Stebbins et al., 1989), inducing the quaternary conformational change that creates the high-activity, high-affinity R-state of the enzyme. Each active site is formed by closing the gap between the carbamoyl phosphate and aspartate domains (i.e., a domain closure) along with major reorientations of the 240s loop in the aspartate domain and the 80s loop in the carbamoyl phosphate domain. The reorientation of the 80s loop shifts Ser80 and Lys84 into contact with the substrates bound in the adjacent chain.

The generally accepted mechanism for catalysis in aspartate transcarbamoylase was proposed by Gouaux et al. (1987). It involves nucleophilic attack by the amino group of aspartate on the carbonyl carbon of carbamoyl phosphate to form a tetrahedral intermediate. Collapse of the tetrahedral intermediate occurs upon transfer of a proton directly from the amino group of aspartate to the closest oxygen of the leaving phosphate group of carbamoyl phosphate, followed by the release of products.

The analysis of X-ray structures of the enzyme bound with either the bisubstrate analog PALA (Krause et al., 1987), carbamoyl phosphate plus succinate (an aspartate analog) (Gouaux & Lipscomb, 1988), or phosphonacetamide and malonate (Gouaux & Lipscomb, 1990) provides information on the specific residues of the enzyme that interact with the substrates. The residues that are believed to be critical for catalytic activity and have been investigated via site-specific mutagenesis include Ser52, Thr53, Arg54, Thr55, Arg105, His134, Gln137, Arg167, Arg229, and Gln231 from one catalytic chain, and Ser80 and Lys84 from an adjacent chain (Stevens et al., 1991). Lys84 is a residue of particular importance as it forms bridging interactions to both substrates. The importance of Lys84 in catalysis has been demonstrated through chemical modification (Greenwell et al., 1973; Kempe & Stark, 1975; Lauritzen & Lipscomb, 1982; Lahue & Schachman, 1984) and site-specific mutagenesis studies (Robey et al., 1986). Loss of catalytic activity was observed when Lys84 was pyridoxylated (Greenwell et al., 1973; Kempe & Stark, 1975), and when both Lys83 and Lys84 were modified with bromosuccinate or with trinitrobenzene sulfonate (Lauritzen & Lipscomb, 1982; Lahue & Schachman, 1984). Subsequent site-specific mutagenesis studies demonstrated that there was only a slight reduction in activity when Lys83 was substituted with Gln (K83Q), but replacement of Lys84 with Gln (K84Q) or Arg (K84R) resulted in significant loss of enzymatic activity (Robey et al., 1986). In both cases bulky groups were substituted in place of lysine. The effect of this change on carbamoyl phosphate binding and on the homotropic and heterotropic interactions of the enzyme were not reported. This investigation was undertaken to further study the role of the 80s loop and of Lys84 for catalysis in aspartate transcarbamoylase.

Results

Kinetic comparison of the wild-type and mutant holoenzymes

To further evaluate the function of the 80s loop in aspartate transcarbamoylase in general and the role of the side chain of Lys84 in particular, site-specific mutagenesis was used to create a series of mutant versions of the enzyme. The amino acids Ala, Thr, and Asn were selected to replace Lys84, because they were shorter than Lys, and in the cases of Thr and Asn, retained some hydrophilic character. As seen in Table 1, the K84N and the K84T enzymes exhibited greatly reduced activities, ranging from 0.08 to 0.29% of the wild-type activity. On the other hand, the K84A mutant enzyme had about 12% of wild-type activity, significantly higher than the K84N and K84T enzymes. Aspartate saturation curves (see Fig. 1) reveal that the mutant enzymes have lost all detectable cooperativity, except for the K84A enzyme, that retains substantial cooperativity as reflected by a Hill coefficient of 1.8. The apparent affinity for aspartate was reduced fivefold to ninefold for all the mutant enzymes, while the affinity for carbamoyl phosphate was reduced 10–30-fold (see Table 1).

Effect of PALA on the wild-type and mutant holoenzymes

At low concentrations of aspartate, the wild-type holoenzyme is activated by the bisubstrate analog PALA (Collins & Stark, 1971). This reflects the allosteric transition of the enzyme from the T to the R state. The inability of PALA to activate a mutant enzyme (at low concentrations of aspartate) can therefore be used to verify that cooperativity has been completely abolished by a particular mutation. The K84N, K84T, and DM enzymes exhibited activation that was just above background, while the K84A enzyme was activated 1.6-fold compared to fivefold for the wild-type enzyme (data not shown). However, the concentration of PALA required for maximal activation of the K84A enzyme was significantly higher (100 μM) than for the wild-type enzyme (2 μM). The higher concentration of PALA necessary to activate the K84A enzyme is most likely due to the diminished substrate affinity of the mutant enzyme. Only the amino acid substitution with the smallest side chain (Ala) retained significant homotropic cooperativity.

Table 1. *Kinetic parameters of wild-type and mutant holoenzymes at pH 8.3*[a]

Enzyme	V_{max}[a] (mmol h^{-1} mg^{-1})	$[Asp]_{0.5}$[a] (mM)	n_H	$[CP]_{0.5}$[b] (mM)
Wild-type	17 ± 0.1	11.3 ± 0.9	2.7 ± 0.2	0.25 ± 0.05
K84A	2.1 ± 0.3	96 ± 3	1.8 ± 0.2	2.7 ± 0.2
K84N	0.014 ± 0.01	71 ± 10	1	6.3 ± 0.5
K84T	0.05 ± 0.02	55 ± 5	1	2.8 ± 0.4

[a]These data were determined from aspartate saturation curves. Colorimetric assays were performed at 25 °C in 0.05 M Tris-acetate, pH 8.3. Aspartate saturation curves were determined at the saturating carbamoyl phosphate concentrations for the respective enzymes. The errors indicated are standard deviations obtained from three or more trials.

[b]The values for $[CP]_{0.5}$ were determined from the carbamoyl phosphate saturation curves at pH 8.3 in 0.05 M Tris-acetate buffer, at saturating aspartate concentrations of the respective enzymes.

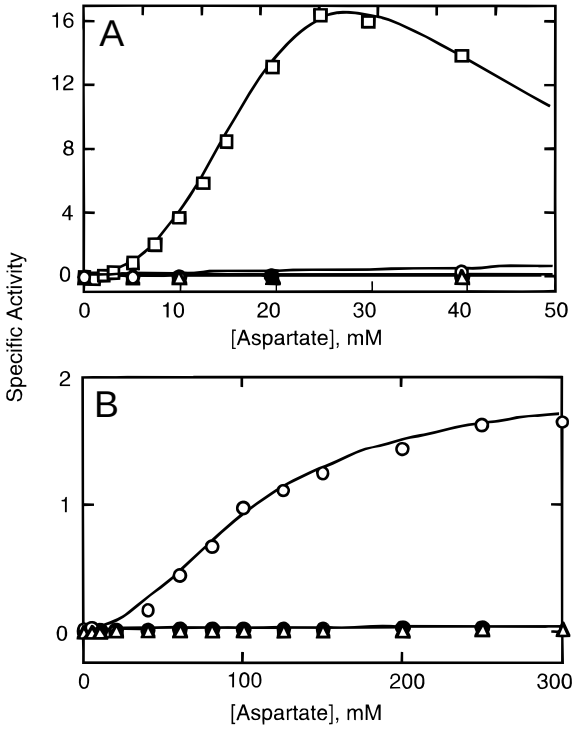

Fig. 1. Aspartate saturation curves for the wild-type and the mutant holoenzymes carried out at 25 °C in 0.05 M Tris-acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 28 mM. (**A**) Data are shown for the wild-type (□), the K84A (○), the K84N (△), and the K84T (●) enzymes and (**B**) for the K84A (○), the K84N (△), and K84T (●) enzymes.

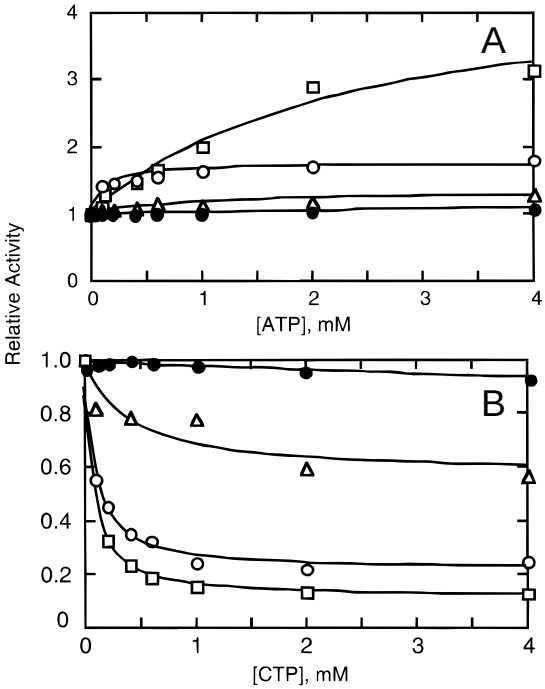

Fig. 2. Influence of (**A**) ATP and (**B**) CTP on the activity of the wild-type (□), the K84A (○), the K84N (△), and the K84T (●) enzymes. Assays were carried out at one-half $[Asp]_{0.5,}$ 25 °C in 0.05 M Tris-acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 28 mM.

Heterotropic response of the enzymes with mutations at position 84

The influence of ATP and CTP on the activity of the wild-type and mutant enzymes was investigated. The nucleotide effectors, ATP and CTP, modify the activity of the enzyme, however, they do not alter the maximal velocity at saturating substrate concentrations. Figure 2 shows the relative activity of the mutant and wild-type enzymes as a function of ATP or CTP concentration.

None of the mutant enzymes exhibited a normal response to either ATP or CTP. The K84A enzyme was the most similar to the wild-type enzyme. ATP activates the wild-type enzyme approximately fourfold (at one-half $[Asp]_{0.5}$) compared to approximately twofold for the K84A. The wild-type enzyme exhibits a residual activity of 11% when maximally inhibited by CTP, while the K84A exhibits 24% residual activity, under these conditions.

The K84T and K84N enzymes showed substantially reduced activation by ATP as well as substantially reduced inhibition by CTP compared to the wild-type and K84A enzymes. As seen in Figure 2, the K84T enzyme exhibited almost no activation nor inhibition. For either maximal activation by ATP or maximal inhibition by CTP the order of the response of the enzymes is K84T < K84N < K84A < wild-type.

To more fully evaluate the response of the mutant enzymes to the heterotropic effectors, aspartate saturation curves were carried out in the absence of nucleotides and in the presence of ATP and CTP (see Fig. 3). For the K84T and K84N enzymes, even in the presence of CTP, no cooperativity is observed. Furthermore, as seen in Figure 3C, the aspartate saturation curve for the K84T enzyme exhibits almost no alteration when performed in the presence of either ATP or CTP.

Kinetic comparison of the double mutant and the K84A holoenzymes

A comparison of the kinetic data in Table 1 indicates something rather unusual about the K84A substitution when compared to the other substitutions reported here, as well as those previously introduced at this position (Robey et al., 1986). Attempts to rationalize these results raised serious questions (e.g., why should the alanine substitute at this location have substantially more activity than the mutant enzymes with other amino acids at this position and also retain cooperativity?). Examination of the amino acid sequence for the catalytic chain of the enzyme reveals another lysine residue at position 83. We propose that Lys83 may be able to substitute for Lys84 in the active site. Computer modeling indicates that a relatively small alteration in the conformation of the 80s loop is sufficient to position Lys83 in the active site, allowing it to interact with the substrate, which may allow it to partially compensate for the loss of Lys84 (see Fig. 4). The modeling also indicates that only when Lys84 is converted to an alanine or glycine can this conformational change of the 80s loop occur. Due to steric hindrance, larger side chains would prevent the 80s loop from taking on the required conformation for Lys83 to reach into the active site.

To test whether Lys83 can replace the function of Lys84, two additional site-specific mutant enzymes were created. One enzyme

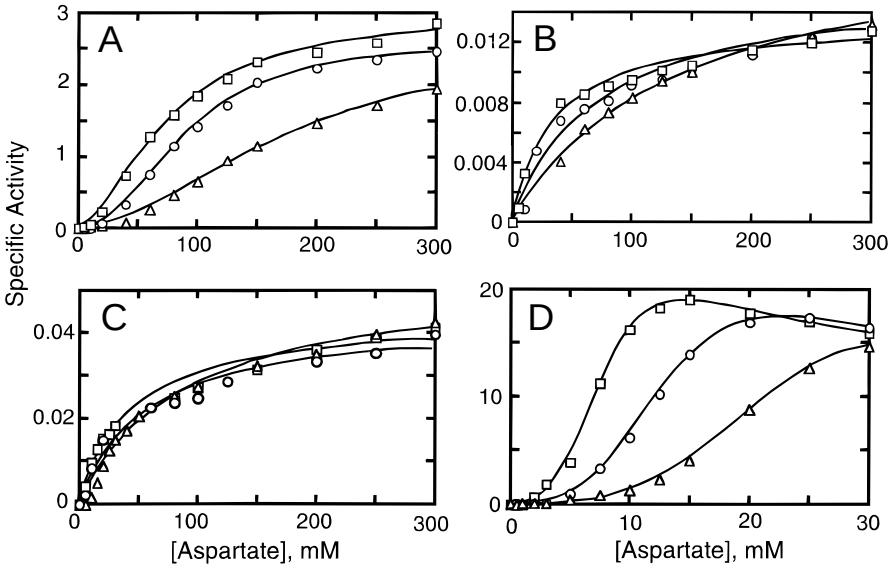

Fig. 3. Aspartate saturation curves for (**A**) the K84A, (**B**) the K84N, (**C**) the K84T, and (**D**) the wild-type enzymes in the absence (○) and presence saturating concentrations of ATP (5.0 mM) (□) and CTP(2.0 mM) (△). Enzyme activity was determined at 25 °C in 0.05 M Tris-acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 28 mM.

has Lys83 converted to alanine (K83A), and the other has Lys83 and Lys84 both converted to alanine (DM). The DM enzyme exhibited only 0.6% of wild-type activity—comparable to the K84T and K84N enzymes. Furthermore, the DM enzyme lost all cooperativity. However, the mutant enzyme that had only Lys83 replaced by alanine retained 60% of wild-type activity and cooperativity (see Table 2), indicating that the lysine at position 83 is not critical for the function of the enzyme.

Molecular dynamics simulation

The stability of the protein's conformation in the native and K84A mutant enzymes was studied through an extensive molecular dynamics simulation. The initial model included the entire molecule, composed of two catalytic and three regulatory subunits. As detailed in Materials and methods, the upper catalytic subunit has Lys84 replaced by Ala, while the lower catalytic subunit has in

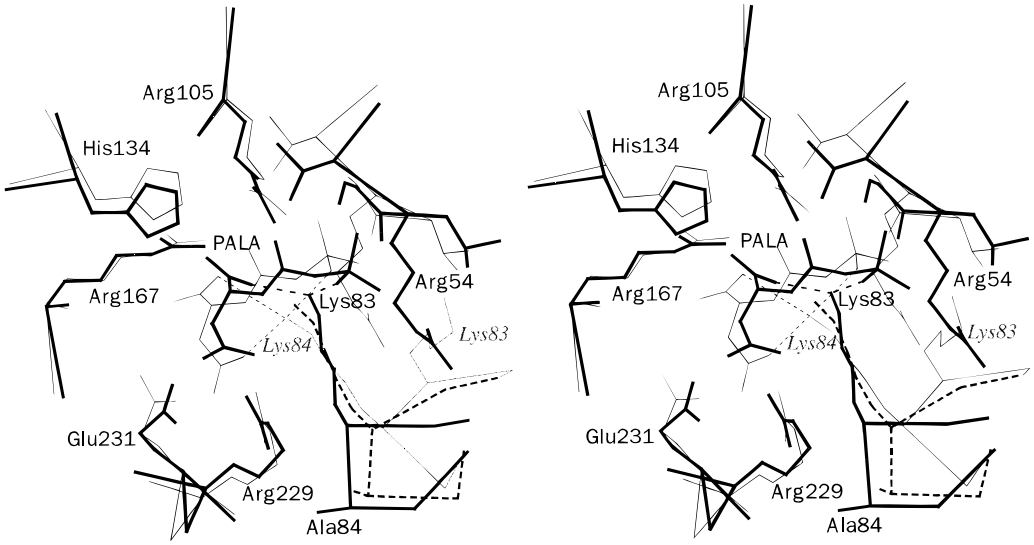

Fig. 4. Stereoview of the active site region of aspartate transcarbamoylase. Shown with thin lines is the structure of the enzyme with PALA bound. The 80s loop from the adjacent catalytic chain is shown with the side chains of Lys83 and Lys84, which are labeled in italics. The broken lines show the initial modeled position of the 80s loop in the K84A mutant enzyme showing Lys83 pointed toward PALA in approximately the same position as Lys84 in the wild-type structure. The active site area of the K84A mutant at the end of the molecular dynamics and energy minimization is shown in bold. Note that Lys83 is involved in two interactions with PALA as opposed to the three observed for Lys84 in the wild-type structure.

Table 2. *Kinetic parameters of the wild-type and mutant holoenzymes at pH 8.3*[a]

Enzyme	V_{max}[a] (mmol h^{-1} mg^{-1})	$[Asp]_{0.5}$[a] (mM)	n_H
Wild-type	17 ± 0.1	11.3 ± 0.9	2.7 ± 0.2
K84A	2.1 ± 0.3	96 ± 3	1.8 ± 0.2
K83A	10.3 ± 0.8	5.1 ± 0.9	1.5 ± 0.3
DM	0.03 ± 0.005	195 ± 30	1

[a]These data were determined from aspartate saturation curves. Colorimetric assays were performed at 25 °C in 0.05 M Tris-acetate buffer, pH 8.3, at saturating carbamoyl phosphate concentrations for the respective enzymes. The errors indicated are standard deviations obtained from three or more trials.

[b]The maximal velocity represents the maximal observed specific activity from the aspartate saturation curve.

addition an altered conformation of the 80s loop, allowing the side chain of Lys83 to point into the active site. An initial Powell minimization resulted in a significant lowering of the total energy. This procedure generated a total RMS deviation (RMSD) of only 0.24 Å from the initial structure, leaving the 80s loop in both the upper and lower subunits practically unchanged. The heating, molecular dynamics, cooling, and final minimization steps result in the RMSD for all the atoms being 0.93 Å from the initial structure. However, the 80s loop behavior is trimer dependent. The lower trimer, in which Lys83 was modeled into the active site, has minimal changes; however, electrostatic contacts of the ϵ-amino group of Lys83 with PALA are improved (see Fig. 4). The upper trimer shows significantly different behavior. The model of the 80s loop became significantly asymmetric between the three chains, and the loops showed significant unfolding and displacement. The maximal displacement was as high as 5 Å, and residues Ala84 and Lys83 were at the tip of the most displaced fragment. In this model, Lys83 moves into a position where it can initiate the approach toward the active site.

Low-angle X-ray scattering

Low-angle X-ray scattering has been used extensively in studying aspartate transcarbamoylase because this technique allows a rapid determination of the quaternary state of the enzyme in solution (Hervé et al., 1985; Fetler et al., 1995). The scattering pattern of the wild-type enzyme in the absence of PALA is characteristic of the T-structural state while the scattering pattern in the presence of PALA is characteristic of the R-structural state (see Fig. 5). The low activity and lack of cooperativity for the K84N enzyme may be due to the inability of the enzyme to shift to the R-state. To determine if this enzyme could be converted to the R-structural state, low-angle X-ray scattering patterns were recorded in the absence and presence of PALA. As seen in Figure 5, the K84N enzyme exhibits scattering patterns that are virtually identical to that of the wild-type enzyme, indicating that PALA can convert the mutant enzyme into the same structural R-state as it can for the wild-type enzyme. As expected from the observed cooperativity of the K84A enzyme, it could also be converted to the structural R-state by PALA (data not shown).

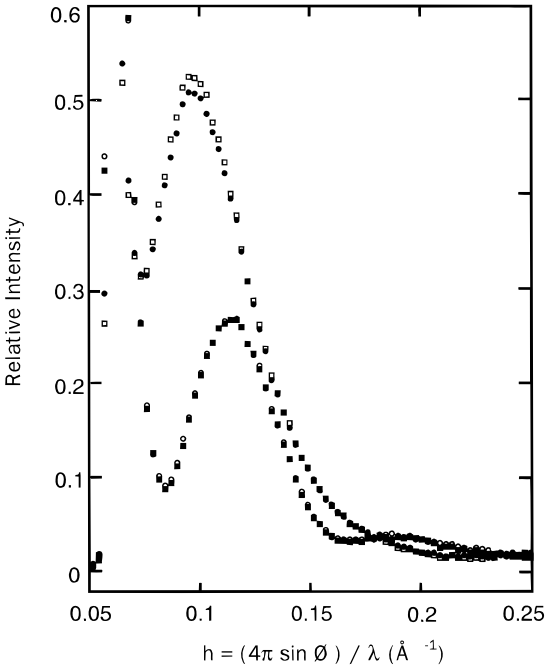

Fig. 5. Solution low-angle X-ray scattering curves for the wild-type and K84N enzymes. The wild-type (■) and K84N (○) enzymes in the absence the wild-type (□) and K84N (●) enzymes in the presence of 1 mM PALA.

Discussion

The residue Lys84 in the catalytic chain of aspartate transcarbamoylase is atypical because it is not only important for catalysis (Robey et al., 1986), but also for substrate binding. Furthermore, Lys84 is part of the flexible 80s loop, which along with the 240s loop undergoes a major reorientation and repositioning during the quaternary transition of the enzyme (Ladjimi et al., 1988; Middleton & Kantrowitz, 1988; Middleton et al., 1989). The R-state position of these loops, which requires the observed quaternary conformational change, allows the creation of the high-activity, high-affinity active sites. Although previous studies using chemical modification (Lauritzen & Lipscomb, 1982; Lahue & Schachman, 1984) or site-specific mutagenesis (Robey et al., 1986) of Lys84 concluded that this residue is important for catalytic activity, they did not determine the exact role of Lys84 in the catalytic process. This study was undertaken to probe the function of Lys84 in greater detail.

The active site of aspartate transcarbamoylase is well defined, and many of the residues that have side chains in direct contact with either substrate have their roles clearly defined from site-specific mutagenesis studies. Arg105 (Stebbins et al., 1989) and His134 (Robey et al., 1986) are involved in the polarization of the carbonyl group of carbamoyl phosphate. Ser52 aids in the binding and proper orientation of carbamoyl phosphate (Xu & Kantrowitz, 1991). Thr55 binds to carbamoyl phosphate and is important for the conformational change that accompanies carbamoyl phosphate binding (Xu & Kantrowitz, 1989). Arg54 is one of the most critical residues in the active site, interacting with the anhydride oxygen of carbamoyl phosphate, and serving to stabilize the developing negative charge on the leaving phosphate group (Stebbins et al., 1992).

Arg167 (Stebbins et al., 1990), Arg229 (Middleton et al., 1989), and Gln231 (Stebbins et al., 1990) are important residues for the binding of aspartate.

The roles of the active site residues, as determined from site-specific mutagenesis studies, agree with their predicted roles from structural studies that are based on observed interactions with substrate analogs. However, the experimental kinetic data of the Lys84 mutant enzymes are inconsistent with previous predications based on structural studies. The X-ray structure of the wild-type enzyme with PALA bound suggests that Lys84 has interactions with both substrates, yet the K84Q and K84R enzymes have relatively normal values for aspartate affinity as measured from the $[Asp]_{0.5}$. Although it was reported that the addition of carbamoyl phosphate to the K84Q enzyme causes a change in the ultraviolet difference spectrum that is similar to the wild-type enzyme, the $[CP]_{0.5}$ was not reported for either of the mutant enzymes. The PALA-promoted difference spectra as well as sedimentation velocity studies showed that the mutant enzymes bind PALA in a similar fashion to the wild-type enzyme (Robey et al., 1986), suggesting that these enzymes retain their ability to undergo the quaternary conformational change necessary for cooperativity. However, the tetrahedral transition-state model proposed by Gouaux et al. (1987) suggests that Lys84 stabilizes the transition state by binding both substrates, implying a role of this residue in both the binding of carbamoyl phosphate and cooperativity.

Role of Lys84 in catalysis

Substitution of Lys84 by arginine (Robey et al., 1986), glutamine (Robey et al., 1986), threonine, and asparagine all result in mutant enzymes with less than 0.3% residual activity compared to the wild-type enzyme. In the case of the K84T and K84N enzymes reported in this study, these replacements at position 84 also dramatically reduce the affinity of the enzyme for both aspartate and carbamoyl phosphate, and eliminate homotropic cooperativity. When considered altogether, these substitutions suggest an important role of Lys84 in the proper function of aspartate transcarbamoylase. We propose that this residue is required for normal levels of catalytic activity, substrate binding, and cooperativity. However, the replacement of Lys84 by alanine results in a mutant enzyme with about 12% of wild-type activity that exhibits both homotropic and heterotropic interactions. Because position 84 in the catalytic chain of aspartate transcarbamoylase is part of the flexible 80s loop, different residues in this position might be expected to alter the kinetic parameters of the enzyme differently. The unusually high activity of the K84A enzyme and retention of homotropic cooperativity, compared to the other mutants, suggest something unusual about this substitution. One possible explanation might be that Lys84 is less critical for the catalytic function of the enzyme than had been thought. However, kinetic results obtained from the other substitutions in this position, including a conservative change to arginine, make this an unreasonable explanation. A more likely argument is that for the K84A enzyme there is partial complementation of the function of Lys84 by its preceding residue, Lys83. Because both of these residues reside on the 80s loop, we believe that there may be sufficient flexibility in this loop to allow Lys83 to partially compensate for the loss of Lys84; and thereby explain why the K84A enzyme is more active than the other mutant enzymes, and retains cooperativity, yet is not able to bind either substrate well. The kinetic data collected on the K83A and DM enzymes support this conclusion. If Lys83 is replaced by alanine or glutamine (Robey et al., 1986), little alteration in catalytic activity is observed. However, if both Lys83 and Lys84 are converted to alanine, the resulting double mutant enzyme has properties similar to the enzymes with single mutations at position 84 with the exception of alanine.

Molecular complementation by Lys83

The modeling and molecular dynamics experiments reported here support the proposal that only in the case of the K84A enzyme can the 80s loop adopt a conformation that allows Lys83 to complement for the loss of Lys84 in the active site. The structural model with Lys83 substituting for Lys84 successfully underwent the molecular dynamics simulation with only minor alterations (see Fig. 4). This result underscores the dynamic stability of the system and shows the feasibility of Lys83 partially substituting for the function of Lys84. The changes detected in the molecular dynamics simulation of the catalytic trimer that had just Lys84 replaced by alanine, that showed no reorientation of the 80s loop, suggests that this loop conformation has diminished stability. The direction of the observed conformational changes of the 80s loop reinforces the proposal that Lys83 can substitute for Lys84.

Role of Lys84 for homotropic cooperativity

The series of mutant enzymes at position 84 exhibit differences in homotropic cooperativity. In the case of the K84A enzyme, cooperativity is not abolished, but it is attenuated. However, for the other mutants at position 84 cooperativity is essentially eliminated even in the presence of saturating concentrations of CTP (see Fig. 3). The difference in behavior of the K84A enzyme with respect to cooperativity can be accounted for by the ability of Lys83 to compensate for the lost of Lys84.

For the K84N and K84T enzymes, the loss of cooperativity was verified by the inability of PALA to significantly activate these enzymes at subsaturating concentrations of aspartate. Loss of cooperativity could be due to the inability of these enzymes to undergo the allosteric transition from the T- to the R-state. If these enzymes cannot attain the high-activity, high-affinity R-state, then cooperativity will not be observed. Alternatively, the transition from the T to the R structural state may take place, but without Lys84 the high-activity, high-affinity functional state may not be formed. Low-angle X-ray scattering data indicate that for the K84N and K84A enzymes, PALA promotes a quaternary conformational change of similar magnitude to that observed for the wild-type enzyme (Fig. 5). Although the experiment was not carried out on the K84T enzyme, it is expected to respond in a similar fashion. These X-ray scattering results along with the previously reported sedimentation experiments on the K84R and K84Q enzymes (Robey et al., 1986) all support the proposal that these mutant enzymes can undergo a structural transition to the structural R-state, but the active site is of low activity and low affinity. Without a significant increase in activity between the T- and R-states, cooperativity is not observed.

Role of Lys84 and the 80s loop of the catalytic chain for the heterotropic interactions

For the noncooperative K84T and K84N enzymes, the nucleotide effectors have a much reduced influence on enzymatic activity (see Figs. 2, 3). In these two cases, the heterotropic and homotropic

interactions of aspartate transcarbamoylase are no longer coupled. The decoupling of homotropic and heterotropic interactions has been observed previously in other mutants (Kerbiriou & Hervé, 1972; Newton & Kantrowitz, 1990; Stebbins et al., 1990), as well as in enzymes modified by chemical modification (Kantrowitz & Lipscomb, 1977; Landfear et al., 1978a, 1978b). The X-ray scattering data indicate that, at least in the case of the K84N enzyme, the addition of PALA induces the structural change to the R-state; therefore, the retention of heterotropic interactions suggests that the binding of the effectors may globally change the T to R equilibrium. However, because the T- and R-states of the mutant enzymes have relatively equal catalytic activities, only small alterations in activity are observed in the presence of the nucleotide effectors. These data suggest that only when the active sites are completely functional will the full influence of the nucleotides be observed.

The role of Lys84 in the function of aspartate transcarbamoylase

As has been amply demonstrated here, the K84A enzyme is not useful in establishing the function of Lys84 in aspartate transcarbamoylase because of the complementation of function by Lys83. Without Lys84 (except when it is replaced by alanine) the enzyme exhibits an approximate 1,000-fold reduction in activity and loss of cooperativity along with reduced affinity for both substrates. The data presented here support the conclusion that Lys84 is primarily responsible for the observed increase in substrate affinity and activity between the T- and R-states of the enzyme. When Lys84 is replaced, homotropic cooperativity is essentially eliminated because the T- and R-states now have similar substrate affinity as well as catalytic ability. Although the role of Lys84 in catalysis has not been fully established by this work, we speculate that it may be involved as a general base, abstracting one of the two protons that must be removed from the amino group of aspartate during the reaction.

Materials and methods

Materials

Agar, ampicillin, dilithium carbamoyl phosphate, *N*-carbamoyl-L-aspartate, L-aspartate, potassium dihydrogen phosphate, and Tris were purchased from Sigma Chemical Co. (St. Louis, Missouri). The carbamoyl phosphate was purified by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C. Electrophoresis-grade acrylamide, agarose, urea, and enzyme-grade ammonium sulfate were purchased from ICN Biomedicals (Costa Mesa, California) and the oligonucleotides from Operon Technologies (Alameda, California).

Methods

Construction of the mutant enzymes by site-specific mutagenesis

The mutant enzymes were constructed using the Kunkel method (Kunkel, 1985; Kunkel et al., 1987). Uracil containing single-stranded DNA was obtained by infection of *E. coli* strain CJ236 containing the phagemid pEK152 (Baker & Kantrowitz, 1993) carrying the *pyrBI* genes, with the helper phage M13K07 (Vieira & Messing, 1987). Single-stranded candidates were isolated and screened by Sanger dideoxy sequencing (Sanger et al., 1977).

After verification of each mutation, a DNA fragment containing the mutations was removed and inserted into the plasmid pEK54 (Xu et al., 1988), which had the corresponding section of the wild-type gene removed. Each mutation was verified a second time by dideoxy sequencing.

Purification of the enzymes

The wild-type and mutant enzymes were isolated as described by Nowlan and Kantrowitz (1985) from *E. coli* strain EK1104 [F^- *ara, thi,* Δ*pro-lac,* Δ*pyrB, pyrF*$^{\pm}$, *rpsL*] containing the following plasmids: pEK2 (Smith et al., 1986) for the wild-type holoenzyme, pEK200 for the K84A, pEK215 for the K84N, pEK221 for the K84T, pEK408 for the K83A, and pEK409 for the double mutant in which Lys83 and Lys84 were converted to Ala.

Determination of protein concentration

The concentration of pure wild-type enzyme was determined by absorbance measurements at 280 nm with a molar extinction coefficient of 0.59 cm^2/mg for the holoenzyme. The concentrations for the mutant holoenzymes were determined by the Bio-Rad (Hercules, California) version of Bradford's dye-binding assay (Bradford, 1976) using the wild-type holoenzyme as the standard.

Aspartate transcarbamoylase assay

The transcarbamoylase activity was measured at 25 °C by either the colorimetric (Pastra-Landis et al., 1981) or the pH-stat method (Wu & Hammes, 1973). pH stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburette. The saturation curves for aspartate were performed at pH 8.3 in 0.05 M Tris-acetate. Assays with the bisubstrate analog PALA were also performed at pH 8.3 at one-fifth the $[Asp]_{0.5}$ and saturating carbamoyl phosphate. Assays with nucleotide effectors were performed at pH 8.3 at one-half the $[Asp]_{0.5}$. All the colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

Molecular dynamics

To determine the relative stability of the conformation of the protein in the native as well as the K84A mutant enzyme, an extensive molecular dynamics simulation was carried out. The calculations were performed with X-PLOR 3.1, which uses the CHARMM force field for molecular dynamics. The model of the entire dodecamer in the R-state was prepared by application of the appropriate symmetry operators to the R-state structure of the wild-type enzyme (Protein Data Bank file 8atc). The model comprised one PALA molecule as well as 20 water molecules per catalytic chain. To investigate the possible change of conformation upon mutating Lys84 to alanine and at the same time provide a good control, the upper catalytic trimer was modified by removing the side chain of Lys84 and replacing it with alanine, while in the lower trimer the backbone conformation of the 80s loop was modified such that Lys83 was directed into the active site.

The calculations were performed in the united atom model framework with explicit polarizable hydrogens and with standard charges assigned to the protein atoms. The PALA molecule was assumed to be fully deprotonated. To avoid artifacts caused by excessive electrostatic forces that result when using an internal dielectric constant (of 2), the contribution of the electrostatic term to the global forcefield was limited to 60% of its calculated value.

The protocol consisted of 300 steps of Powell minimization followed by heating from 10–300 K in incremental steps of

25 K. Every step lasted 250 fs with an integration size of 0.5 fs. As suggested by our previous experience, which indicated that cooperative motions can be seen on the multipicosecond range, the production run was carried out for 50 ps. The cooling was performed with the same protocol as heating but with the reverse temperature range (i.e., from 300–10 K). Three-hundred steps of Powell minimization ended the protocol.

The complexity and the size of the model required limiting the number of atoms directly involved in the molecular dynamics. Therefore, only atoms within a 15 Å sphere around the PALA molecule plus atoms belonging to residues 73–87 of each catalytic chain were included in the simulation. The atoms in a 3 Å zone around the atoms selected for the simulation were constrained with a 10 kcal/mol harmonic force constant. The remainder of the atoms was fixed during the simulation. This setup resulted in 7,959 atoms freely moving and 4,998 atoms being harmonically constrained out of a total 26,783 atoms in the model. The calculation was performed on the Silicon Graphics Indigo II workstation with an R8000 processor. The calculation lasted approximately 3 days.

Low-angle X-ray scattering

The low-angle X-ray scattering experiments were performed on the Beam Line 4-2 at the Stanford Synchrotron Radiation Laboratory (3.0 GeV, 50–100 mA). A significantly upgraded version of the small-angle scattering instrument was used. The specimen-to-detector distance was 95 cm, and the wavelength of the X-ray beam was 1.38 Å. A linear gas chamber detector filled with a Xe/CO_2 mixture was used in the experiment. In addition to the actual beam stop, the center part of the detector was also covered with a thin piece of lead to reduce the total counting rate on the detector below 50,000 counts per second. The scattering curves are expressed in the momentum transfer h ($h = 4\pi(\sin\theta)/\lambda$, where 2θ and λ are the scattering angle and the wavelength of the X-ray beam, respectively), which was calibrated using the (100) reflection from a cholesterol myristate powder sample held at the specimen position. Sample solutions were maintained at 25 °C. All scattering curves were normalized to incident beam intensity integrated over exposure time and the corresponding solvent scattering was subtracted.

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