Function of Serine-52 and Serine-80 in the Catalytic Mechanism of *Escherichia coli* Aspartate Transcarbamoylase†

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ABSTRACT: Carbamoyl phosphate is held in the active site of *Escherichia coli* aspartate transcarbamoylase by a variety of interactions with specific side chains of the enzyme. In particular, oxygens of the phosphate of carbamoyl phosphate interact with Ser-52, Thr-53 (backbone), Arg-54, Thr-55, and Arg-105 from one catalytic chain, as well as Ser-80 and Lys-84 from an adjacent chain in the same catalytic subunit. In order to define the role of Ser-52 and Ser-80 in the catalytic mechanism, two mutant versions of the enzyme were created with Ser-52 or Ser-80 replaced by alanine. The Ser-52→Ala holoenzyme exhibits a 670-fold reduction in maximal observed specific activity, and a loss of both aspartate and carbamoyl phosphate cooperativity. This mutation also causes 23-fold and 5.6-fold increases in the carbamoyl phosphate and aspartate concentrations required for half the maximal observed specific activity, respectively. Circular dichroism spectroscopy indicates that saturating carbamoyl phosphate does not induce the same conformational change in the Ser-52→Ala holoenzyme as it does for the wild-type holoenzyme. The kinetic properties of the Ser-52→Ala catalytic subunit are altered to a lesser extent than the mutant holoenzyme. The maximal observed specific activity is reduced by 89-fold, and the carbamoyl phosphate concentration at half the maximal observed velocity increases by 53-fold while the aspartate concentration at half the maximal observed velocity increases 6-fold. For the Ser-52→Ala catalytic subunit, the K_D of carbamoyl phosphate and the K_i of PALA are both increased substantially compared to the wild-type catalytic subunit. These data suggest that in the wild-type holoenzyme Ser-52 plays an important part in the binding of carbamoyl phosphate and has an influence on the T to R transition by possibly stabilizing the enzyme–carbamoyl phosphate complex. The maximum observed specific activity of the Ser-80→Ala holoenzyme and catalytic subunit are reduced by 20% and 60% relative to the wild-type holoenzyme and catalytic subunit, respectively. For the Ser-80→Ala holoenzyme, aspartate cooperativity is increased slightly, while the carbamoyl phosphate cooperativity is eliminated. The affinity of the Ser-80→Ala holoenzyme and catalytic subunit for substrates is altered only slightly. These data suggest that Ser-80 in the wild-type enzyme may be important for communication between the catalytic chains, but does not play an important role in catalysis. The results from this study as well as previous studies of mutant enzymes at the active site of aspartate transcarbamoylase have proven that the reaction does not proceed by way of a carbamoyl-enzyme intermediate, and support a reaction mechanism that involves a tetrahedral intermediate. Furthermore, these data suggest that no side chains on the enzyme are involved as a general base in catalysis; rather the proton transfer occurs directly from the amino group of aspartate to the leaving phosphate group.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate in the committed step of the pyrimidine biosynthesis pathway. The biochemical properties of the enzyme have been reviewed extensively (Gerhart, 1970; Jacobson & Stark, 1973; Schachman, 1974, 1988; Kantrowitz et al., 1980a,b; Kantrowitz & Lipscomb, 1988, 1990; Allewell, 1989). The holoenzyme, consisting of three regulatory dimers and two catalytic trimers, exhibits homotropic cooperativity for both aspartate and carbamoyl phosphate, is activated by ATP (Gerhart & Pardee, 1962), an end product of the purine biosynthesis pathway, and is inhibited by CTP (Gerhart & Pardee, 1962) and UTP (Wild et al., 1989), the end products of the pyrimidine biosynthesis pathway. The active sites, three per catalytic trimer, are shared between adjacent catalytic chains within the trimer (Monaco et al., 1978; Robey & Schachman, 1985; Krause et al., 1985; Wente & Schachman, 1987). The regulatory dimer binds the regulatory nucleotides but does not have catalytic activity while the isolated catalytic subunit exhibits simple Michaelis–Menten kinetics and is insensitive to the allosteric effectors. Information about the three-dimensional structure of the enzyme, at medium resolution (2.5–3.0 Å), is available in the presence and absence of substrates and substrate analogues, as well as in combination with the heterotropic effectors (Krause et al., 1985, 1987; Honzatko & Lipscomb, 1982; Ladner et al., 1982; Ke et al., 1984, 1988; Kim et al., 1987; Gouaux & Lipscomb, 1988, 1990; Stevens et al., 1990; Gouaux et al., 1990).

The catalytic mechanism involves the ordered binding of the substrates, with carbamoyl phosphate binding before aspartate (Porter et al., 1969; Wedler & Gasser, 1974; Hsuanyu & Wedler, 1987). The binding of carbamoyl phosphate induces a conformational change in the enzyme (Collins & Stark, 1969; Griffin et al., 1972) that allows for the binding of aspartate (Collins & Stark, 1969). The sequential binding of aspartate results in further conformational changes that bring the aspartate and carbamoyl phosphate domains together. This closure of the domains not only assists in catalysis but also is involved in the concerted quaternary conformational

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change of the holoenzyme from the T[1] to the R state (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988). NMR data suggests that the carbonyl oxygen of carbamoyl phosphate is hydrogen-bonded to a group on the enzyme or is protonated by an enzymatic group, activating the carbonyl carbon to be more susceptible to nucleophilic attack by the amino group of aspartate (Roberts et al., 1976). The analysis of X-ray structures of the enzyme with either *N*-phosphonoacetyl-L-aspartate (PALA) (Krause et al., 1987) or carbamoyl phosphate plus succinate (an aspartate analogue) bound (Gouaux & Lipscomb, 1988) provides information concerning the specific groups on the enzyme that interact with the substrates. The residues that interact with carbamoyl phosphate include Ser-52, Thr-53, Arg-54, Thr-55, Arg-105, His-134, and Gln-137 from one catalytic chain plus Ser-80 and Lys-84 from the adjacent catalytic chain. Site-specific mutagenesis has been used to investigate the role of many of these residues in binding and catalysis (Robey et al., 1986; Stebbins et al., 1989, 1990; Xu & Kantrowitz, 1989). Of particular importance is Arg-54 that is thought to stabilize the negative charge on the phosphate leaving group, as well as Arg-105 and Lys-84 which can interact with both of the substrates simultaneously in the transition state (Robey et al., 1986; Stebbins et al., 1989).

The functions of the two serine residues in the active site have not been previously investigated by site-specific mutagenesis, although Ser-52 was replaced with phenylalanine by random mutagenesis (Jenness & Schachman, 1983). The Ser-52→Phe[2] enzyme was inactive and was locked in the T state even in the presence of PALA (Schachman et al., 1984). The introduction of phenylalanine, a bulky hydrophobic amino acid, to replace serine in the hydrophilic environment of the active site may cause loss of activity by blocking substrate binding. Therefore, the nature of the interaction between the oxygen phosphate and Ser-52 cannot be conclusively rationalized based on the kinetic properties of the Ser-52→Phe enzyme. In order to determine the role of the two serine residues in the active site of aspartate transcarbamoylase, we report here the construction and characterization of two mutant versions of aspartate transcarbamoylase, Ser-52→Ala and Ser-80→Ala.

Experimental Procedures

Materials

Agar, ampicillin, L-aspartate, *N*-carbamoyl-L-aspartate, carbamoyl phosphate, and potassium dihydrogen phosphate were purchased from Sigma Chemical Co. The carbamoyl phosphate was purified before use by precipitation from 50% (v/v) ethanol, and stored desiccated at −20 °C (Gerhart & Pardee, 1962). Electrophoresis-grade acrylamide, agarose, urea, Tris, and enzyme-grade ammonium sulfate were obtained from ICN Biomedicals. Restriction endonucleases were obtained from either U.S. Biochemicals or New England Biolabs and used according to the supplier's recommendations. The Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, and T4 ligase were products of U.S. Biochemicals. NA45 paper used for the isolation of DNA fragments from agarose gels was purchased from Schleicher & Schuell.

Escherichia coli strain U39a [F⁻ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *rpsL*] was obtained from J. Wild, Texas A&M University. The plasmid pUC119 and the M13 phage M13K07 were obtained from J. Messing, Rutgers University.

Methods

Oligonucleotide Synthesis. The oligonucleotide required for the site-specific mutagenesis and also the sequencing primers were synthesized by using an Applied Biosystems 381A DNA synthesizer.

Construction of the Ser-52→Ala and Ser-80→Ala Mutations by Site-Specific Mutagenesis. The Ser-52→Ala and Ser-80→Ala substitutions in the catalytic chain of aspartate transcarbamoylase were accomplished by site-specific mutagenesis using the method of Zoller & Smith (1982) with the modifications previously described (Carter et al., 1985; Ladjimi et al., 1988). Single-stranded DNAs from 10 candidates of each were isolated and sequenced by the dideoxy method (Sanger et al., 1977). After verification of the mutation, a small fragment of the gene was removed with restriction enzymes and inserted into a plasmid, pEK54 (Xu et al., 1988), that had the corresponding section of the wild-type gene removed by restriction enzymes. Selection was accomplished after transformation in U39a, a strain which has a deletion in the *pyrBI* region. The plasmids pEK111 and pEK120 containing the Ser-52→Ala and Ser-80→Ala mutations, respectively, were isolated and purified by retransformation at low density. The mutations were verified a second time by directly sequencing the gene fragment which was cloned into the pEK54 backbone by employing single-stranded DNA copied from the plasmid using the helper phage M13K07 (Vieira & Messing, 1987).

Wild-Type and Mutant Holoenzyme Purification. Wild-type and the mutant versions of aspartate transcarbamoylase were purified as described by Nowlan and Kantrowitz (1985), from *E. coli* strain EK1104 [F⁻ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *pyrF*±, *rpsL*], containing the plasmid pEK2 for the wild-type (Smith et al., 1986), pEK111 for the Ser-52→Ala, and pEK120 for the Ser-80→Ala enzyme.

Wild-Type and Mutant Catalytic Subunit Overproduction and Purification. The purification of catalytic subunit of the wild-type, Ser-52→Ala, and Ser-80→Ala aspartate transcarbamoylases was accomplished after the in vivo overproduction of catalytic subunit using strain EK1104 harboring a plasmid which had a portion of the *pyrI* gene for the regulatory chain deleted (Nowlan & Kantrowitz, 1985). The plasmids pEK17, pEK113, and pEK127 used for the production of the wild-type, Ser-52→Ala, and Ser-80→Ala catalytic subunits, respectively, were constructed as previously described (Nowlan & Kantrowitz, 1985). The catalytic subunit was purified by the procedure of Stebbins et al. (1989).

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 autoburet. All colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

Determination of Protein Concentration. Concentrations of pure wild-type holoenzyme and catalytic subunit were determined by absorbance measurements at 280 nm using extinction coefficients of 0.59 and 0.72 cm² mg, respectively (Gerhart & Holoubek, 1967). The protein concentration of

[1] Abbreviations: T and R states, tense and relaxed states of the enzyme having low and high affinity, respectively, for the substrates; PALA, *N*-phosphonoacetyl-L-aspartate; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; $[S]_{0.5}^{CP}$, carbamoyl phosphate concentration at half the maximal observed specific activity; holoenzyme, entire aspartate transcarbamoylase molecule composed of two catalytic subunits and three regulatory subunits.

[2] The notation used to name the mutant enzymes is, for example, the Ser-52→Ala enzyme. The wild-type amino acid and location within the catalytic chain are indicated to the left of the arrow while the new amino acid is indicated to the right of the arrow.

Table I: Kinetic Parameters for the Wild-Type and Mutant Holoenzymes[a]

enzyme	maximal velocity[b] (mmol·h^{-1}·mg^{-1})	$[S]_{0.5}^{Asp}$ (mM)	$[S]_{0.5}^{CP}$ (mM)	n_H^{Asp}	n_H^{CP}
wild-type	17.4 (0.6)	11.6 (0.2)	0.20 (0.04)	2.3 (0.2)	2.2 (0.2)
Ser-52→Ala	0.026 (0.001)	64.7 (2.2)	4.6 (0.1)	1.0 (0)	1.0 (0)
Ser-80→Ala	13.8 (0.3)	20.0 (1.1)	0.19 (0.01)	2.7 (0.3)	1.0 (0)

[a] These data were derived from Figure 1. The maximal velocity and Hill coefficients for aspartate and carbamoyl phosphate (n_H^{Asp} and n_H^{CP}) were obtained by a nonlinear least-squares procedure using a modified Hill equation which incorporates a term for substrate inhibition (Pastra-Landis et al., 1978). For the saturation curves without cooperativity, the data were fit by the same procedure to the Michaelis–Menten equation. [b] For the enzymes that exhibit substrate inhibition, the maximal velocity represents the maximal observed specific activity from the aspartate saturation curves. The data shown are averages of two determinations. The average deviations are shown in parentheses.

the mutant holoenzyme and catalytic subunit was determined by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976).

Circular Dichroism. Circular dichroism spectra were recorded with an Auto-Dichrograph Mark V (Jobin Yvon) interfaced to an Apple IIE computer. All spectra were scanned from 260 to 320 nm at a rate of 12 nm/min, recording points every 0.2 nm. Each scan was repeated 4 times and then averaged. Before use, enzyme samples were dialyzed against 0.04 M imidazole acetate, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.0 (Griffin et al., 1972). For the wild-type and mutant enzymes, spectra were recorded both in the absence and in the presence of saturating concentrations of carbamoyl phosphate.

Data Analysis. The analysis of the steady-state kinetic data was carried out as previously described by Silver et al. (1983). The analysis of the structural data, based on the three-dimensional coordinates of the CTP–enzyme complex (Kim et al., 1987), the PALA–enzyme complex (Krause et al., 1987), and the enzyme complexed with carbamoyl phosphate plus succinate (Gouaux & Lipscomb, 1988), was accomplished by using the program FRODO (Department of Biochemistry, Rice University) on an Evans & Sutherland PS390 interfaced to a MicroVAX Q5.

Results

Kinetic Properties of the Wild-Type, Ser-52→Ala, and Ser-80→Ala Holoenzymes. The substrate saturation curves of the wild-type, the Ser-52→Ala, and Ser-80→Ala holoenzymes are shown in Figure 1, and a summary of the kinetic parameters is given in Table I. The replacement of serine with alanine at position 52 results in a mutant holoenzyme with a 670-fold reduction in the observed maximal velocity as compared to the wild-type enzyme, and loss of cooperativity for both substrates (Table I). The aspartate concentration at half of the maximal velocity, $[S]_{0.5}^{Asp}$, is increased by 5.6-fold, while the concentration of carbamoyl phosphate at half of the maximal observed activity, $[S]_{0.5}^{CP}$, is increased by 23-fold.

The replacement of serine with alanine at position 80 does not drastically alter the properties of the holoenzyme (see Figure 1 and Table I). The maximal velocity is decreased by only 20% as compared to the wild-type enzyme; the cooperativity with respect to aspartate increases slightly, while the cooperativity with respect to carbamoyl phosphate is abolished. The K_m^{CP} for the Ser-80→Ala enzyme is the same as $[S]_{0.5}^{CP}$ of the wild-type enzyme, and the $[S]_{0.5}^{Asp}$ of the Ser-80→Ala enzyme is increased by approximately 2-fold.

Does the Ser-52→Ala Holoenzyme Still Retain any Homotropic Cooperativity? For enzymes which show no cooperativity, it is often difficult to determine if any residual cooperativity is present. For the wild-type holoenzyme, PALA or succinate is able to activate the enzyme at saturating carbamoyl phosphate and low concentrations of aspartate (approximately 0.1 $[S]_{0.5}^{Asp}$) (Gerhart & Pardee, 1963; Collins & Stark, 1971). At low aspartate concentrations, in the presence of saturating carbamoyl phosphate, the wild-type holoenzyme is in the low-activity low-affinity T state. Under these conditions, the addition of PALA or succinate will induce the allosteric transition to the high-affinity high-activity R state, resulting in increased activity (Gerhart & Schachman, 1968; Howlett et al., 1977; Blackburn & Schachman, 1977). At higher concentrations of PALA or succinate, inhibition occurs since both of these analogues can compete for the active site.

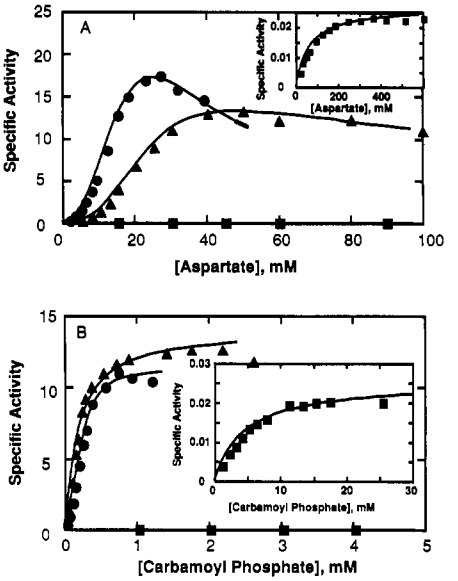

FIGURE 1: Substrate saturation curves of the wild-type (●), Ser-80→Ala (▲), and Ser-52→Ala (■) holoenzymes. The reactions were carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3, in the presence of either saturating carbamoyl phosphate or saturating aspartate. The specific activity is in millimoles of carbamoyl aspartate formed per hour per milligram of protein. (A) Aspartate saturation curves for the wild-type and the mutant enzymes. For these experiments, the concentrations of carbamoyl phosphate were 4.8 mM for the wild-type, 15 mM for Ser-52→Ala, and 12 mM for Ser-80→Ala enzymes. (Inset) Shown is an expanded region corresponding to high aspartate concentrations for the Ser-52→Ala enzyme. (B) Carbamoyl phosphate saturation curves for the wild-type and mutant enzymes. The carbamoyl phosphate saturation curves were performed at 30, 450, and 60 mM aspartate for the wild-type, the Ser-52→Ala, and the Ser-80→Ala enzymes, respectively. (Inset) Expanded region corresponding to high carbamoyl phosphate concentrations for the Ser-52→Ala enzyme.

As seen in Figure 2, in the presence of 6 mM aspartate and saturating carbamoyl phosphate, PALA can activate the Ser-52→Ala holoenzyme slightly. However, under identical conditions of aspartate saturation, the wild-type enzyme is activated approximately 8-fold.

Kinetic Properties of the Wild-Type, Ser-52→Ala, and Ser-80→Ala Catalytic Subunits. For the catalytic subunit of the Ser-52→Ala enzyme, the maximal velocity is decreased by 89-fold, the K_m^{CP} is increased by 53-fold, and the K_m^{Asp} is only increased by 6-fold (Table II). On the other hand, the Ser-80→Ala catalytic subunit exhibits a normal K_m for as-

Table II: Kinetic Parameters for the Wild-Type and Mutant Catalytic Subunits

enzyme	maximal velocity[a] (mmol·h^{-1}·mg^{-1})	K_m^{CP} (mM)	K_m^{Asp} (mM)	K_{cat}/K_m^{Asp} (s^{-1}·mM^{-1})
wild-type	25.9 (1.5)	0.02 (0.003)	6.7 (0.9)	35.8
Ser-52→Ala	0.29 (0.02)	1.07 (0.1)	40.8 (3.3)	0.066
Ser-80→Ala	10.3 (0.4)	0.06 (0.002)	5.6 (0.7)	17.0

[a] The maximum velocity represents the maximal observed specific activity from the aspartate saturation curve. Colorimetric assays were performed at 25 °C in 0.05 M Tris-acetate (pH 8.3). Aspartate saturation curves were determined at saturating levels of carbamoyl phosphate (4.8 mM for the wild-type, 15 mM for the Ser-52→Ala, and 4.8 mM for the Ser-80→Ala enzymes, respectively). Carbamoyl phosphate saturation curves were determined at saturating levels of aspartate (30 mM for the wild-type, 225 mM for the Ser-52→Ala, and 30 mM for the Ser-80→Ala enzymes, respectively). The numbers in parentheses are the standard deviations from the computer fit.

Table III: Affinity Parameters for the Wild-Type and Mutant Catalytic Subunits

enzyme	K_D^{CP} (mM)[a]	K_i^{PALA} (μM)[b]
wild-type	0.02 (0.003)	0.026 (0.003)
Ser-52→Ala	1.5 (0.2)	0.15 (0.02)
Ser-80→Ala	0.04 (0.01)	0.009 (0.001)

[a] The binding constant of carbamoyl phosphate was determined kinetically by the method of Porter et al. (1969). Because of the low activity of the Ser-52→Ala enzyme, the binding constant of carbamoyl phosphate was determined at 0.2 K_m^{Asp}. The inhibition constants of PALA for the wild-type and mutant enzymes are averages of two determinations; the deviations are shown in parentheses. [b] The K_i^{PALA} was determined kinetically by the method of Collins and Stark (1971).

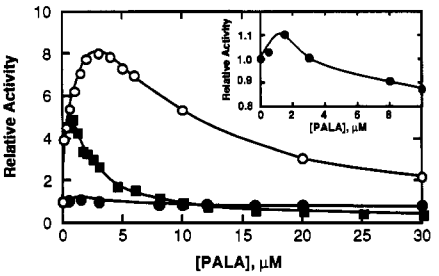

FIGURE 2: Influence of PALA on the activity of the wild-type (○), the Ser-52→Ala (●), and the Ser-80→Ala (■) holoenzymes at low concentrations of aspartate. Colorimetric assays were performed at 25 °C in 0.05 M Tris-acetate buffer, pH 8.3. Activity was measured at a constant concentration of aspartate, approximately 0.1 $[S]_{0.5}^{Asp}$, and saturating carbamoyl phosphate. The activity was measured at 1 mM aspartate and 4.8 mM carbamoyl phosphate for the wild-type holoenzyme, 6 mM aspartate and 15 mM carbamoyl phosphate for the Ser-52→Ala holoenzyme, and 2 mM aspartate and 4.8 mM carbamoyl phosphate for the Ser-80→Ala holoenzyme.

partate, only a 3-fold increase in the K_m for carbamoyl phosphate, and only a 2.5-fold decrease in the observed maximal velocity (see Table II).

Binding of Carbamoyl Phosphate and PALA to the Wild-Type and Mutant Catalytic Subunits. Since both the Ser-52 and Ser-80 residues are involved in the binding of carbamoyl phosphate, the K_D of carbamoyl phosphate, K_D^{CP}, and the K_i of PALA were determined. As seen in Table III, a K_D^{CP} of 1.5 μM was determined for the Ser-52→Ala enzyme compared to 0.02 μM for the wild-type enzyme. The K_m^{CP} is only increased by 2-fold for the Ser-80→Ala enzyme compared to the wild-type enzyme. The K_i of PALA for the Ser-52→Ala enzyme is increased by 5.8-fold while the K_i of PALA for the Ser-80→Ala enzyme is nearly the same as that of the wild-type enzyme.

Conformational Change Induced by the Binding of Carbamoyl Phosphate Is Altered for the Ser-52→Ala Holoenzyme. The binding of carbamoyl phosphate to the wild-type holoenzyme causes a conformational change that can be monitored by UV (Collins & Stark, 1969) and circular dichroism (Griffin et al., 1972) difference spectroscopy. Therefore, the circular dichroism spectrum can be used to

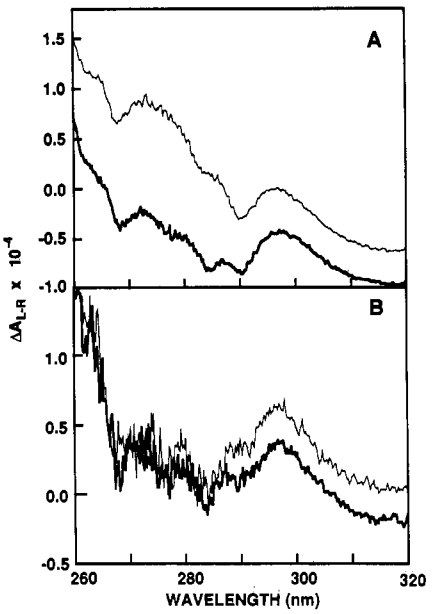

FIGURE 3: Circular dichroism spectra of the wild-type (A) and Ser-52→Ala (B) holoenzymes (2.0 mg/mL) in the absence and presence of carbamoyl phosphate. The spectra of the enzymes were recorded at 25 °C in 0.04 mM imidazole acetate, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.0. The darker lines represent the spectra in the absence of carbamoyl phosphate while the lighter lines represent the spectra in the presence of carbamoyl phosphate. The carbamoyl phosphate concentrations for the wild-type and the Ser-52→Ala holoenzymes were 0.13 and 0.37 mM, respectively.

determine if the binding of carbamoyl phosphate to the mutant enzymes has been altered. In addition, the circular dichroism spectrum can be used to determine a binding constant for carbamoyl phosphate (Stebbins et al., 1989).

In the absence of carbamoyl phosphate, the circular dichroism spectrum of the Ser-52→Ala holoenzyme is identical with that of the wild-type enzyme (Figure 3). For the wild-type holoenzyme, addition of carbamoyl phosphate increases the ellipticity in the region between 270 and 280 nm as well as the ellipticity in the region between 290 and 295 nm. For the Ser-52→Ala enzyme, the ellipticity in the range between 270 and 280 nm is not altered even in the presence of saturating carbamoyl phosphate. However, the ellipticity in the region between 290 and 295 nm is increased slightly. The alteration in the spectrum observed for the Ser-52→Ala enzyme makes the determination of K_D^{CP} difficult. However, the maximal difference in ellipticity is only 38% of that observed for the wild type-enzyme.

pH Profiles of the Wild-Type and Ser-80→Ala Holoenzymes Are Different at Low Aspartate Concentrations. At subsaturating and saturating concentrations of aspartate, the Ser-52→Ala holoenzyme exhibits an almost identical activity versus pH profile with a pH optimum of approximately 7.5 (data not shown). However, for the wild-type holoenzyme,

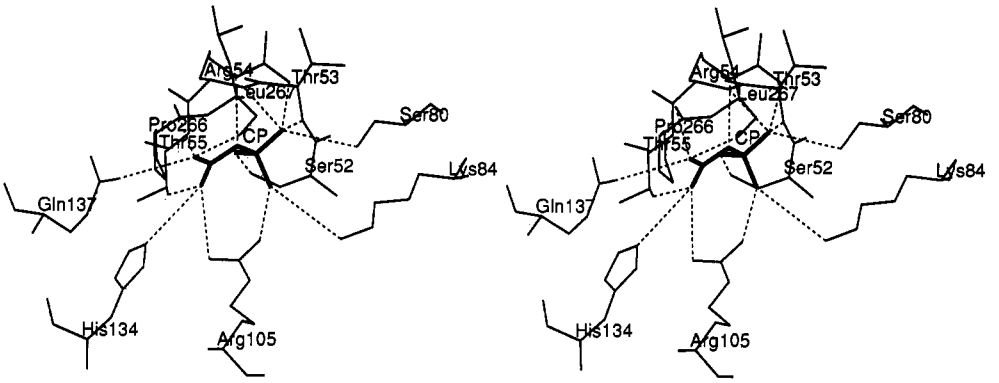

FIGURE 4: Stereoview of the carbamoyl phosphate binding site in aspartate transcarbamoylase. The carbamoyl phosphate site is composed of residues from two adjacent catalytic chains. In this figure, all the side chains that interact with carbamoyl phosphate are shown. All the residues come from one catalytic chain except for Ser-80 and Lys-84 which come from the other catalytic chain. The hydrogen-bonding interactions which stabilize carbamoyl phosphate in the active site are also shown as dotted lines. Carbamoyl phosphate interacts only with the backbone of residues Thr-53, Pro-266, and Leu-267. Thr-55, Arg-105, and His-134 (dark) all interact with the carbonyl oxygen of carbamoyl phosphate. The data used to draw this figure are from Gouaux and Lipscomb (1988).

the activity versus pH profile in the presence of subsaturating aspartate is substantially different from that in the presence of saturating aspartate. For example, in the presence of 1 mM aspartate, the pH optimum for the wild-type enzyme is 7.2, while in the presence of 30 mM aspartate, the pH optimum for the wild-type enzyme shifts to 8.7. The results for the wild-type holoenzyme are consistent with the published data (Leger & Hervé, 1988).

Discussion

As seen in Figure 4, carbamoyl phosphate is held solidly in the active site of aspartate transcarbamoylase by a series of interactions involving both side chain and backbone interactions from two different polypeptide chains. The oxygens of the phosphate of carbamoyl phosphate interact with Ser-52, Thr-53 (backbone), Arg-54, Thr-55, Arg-105, Ser-80, and Lys-84 from the adjacent chain (Gouaux & Lipscomb, 1988). When Arg-54 is replaced by alanine by employing site-specific mutagenesis, the resulting enzyme exhibits little alteration in carbamoyl phosphate binding but is more than 10000-fold less active than the wild-type enzyme (Stebbins et al., 1989). Since Arg-54 also interacts with the anhydride oxygen of carbamoyl phosphate, it has been suggested that Arg-54 functions to help neutralize the charge on the phosphate leaving group, thereby assisting in catalysis (Stebbins et al., 1989). On the other hand, when either Thr-55 or Arg-105 is replaced with alanine, the resulting enzymes exhibit both reduced activity and affinity for carbamoyl phosphate, suggesting that they are important for carbamoyl phosphate binding. These residues are also thought to be involved in polarizing the carbonyl of carbamoyl phosphate, and one or both of these interactions may be responsible for the observed loss in catalytic activity (Stebbins et al., 1989; Xu & Kantrowitz, 1989). In the structure of the enzyme in the presence of PALA (Krause et al., 1987) or carbamoyl phosphate plus succinate (Gouaux & Lipscomb, 1988), Arg-105 and Lys-84 interact with both substrates. Therefore, another function of Arg-105 and Lys-84 may be to bring the two substrates together in the transition state. In order to determine if Ser-52 and Ser-80 play a role in the binding of carbamoyl phosphate and if either of these residues is important for catalysis, we have replaced each with an alanine residue by site-specific mutagenesis.

Ser-52 Is Important for Carbamoyl Phosphate Binding in the Catalytic Subunit. The catalytic subunit of aspartate transcarbamoylase lacks both the homotropic and heterotropic interactions characteristic of the holoenzyme (Gerhart & Schachman, 1965) and has high activity and high affinity for the substrates. These properties suggest that the catalytic subunit is in a R-like state. When Ser-52 is replaced by alanine, the mutant catalytic subunit exhibits a 75-fold reduction in its affinity for carbamoyl phosphate (see Table III). These data suggest that Ser-52 is important for carbamoyl phosphate binding. The weakened binding of carbamoyl phosphate and presumably the destabilization of the transition state as well may be responsible for the loss in catalytic activity of the Ser-52→Ala catalytic subunit compared to the wild-type catalytic subunit, especially in light of the fact that this residue is not expected to be directly involved in catalysis.

Concomitant with the loss of affinity of the Ser-52→Ala enzyme for carbamoyl phosphate there is also an increase in the concentration of aspartate of half-saturation for the holoenzyme and the catalytic subunit, suggesting weakened aspartate binding as well. These data support the ordered binding mechanism (Wedler & Gasser, 1974; Hsuanyu & Wedler, 1987) by showing that an alteration in the carbamoyl phosphate binding site can affect aspartate binding.

Ser-52 Is Important for both Catalysis and Allosteric Transition in the Holoenzyme. The maximal velocity for the Ser-52→Ala holoenzyme is reduced by 670-fold, while the maximal velocity for the Ser-52→Ala catalytic subunit is reduced by only 89-fold. The relatively large reduction in specific activity due to the replacement of Ser-52 by alanine is significant since this replacement only eliminates a hydroxyl group. The interaction observed in the X-ray structure (Gouaux & Lipscomb, 1988) between the hydroxyl group of Ser-52 and a phosphate oxygen of carbamoyl phosphate does not suggest a direct catalytic role for this residue. Since the orientation and stabilization of substrates, intermediates, and products are considered to be critical for the enhancement of reaction rate in enzyme catalysis (Jencks, 1987), it is more probable that Ser-52 in the wild-type enzyme is involved in both the binding and orientation of carbamoyl phosphate.

Since the Ser-52→Ala catalytic subunit does not exhibit cooperativity, the 89-fold loss in activity must represent a diminution in transition-state stabilization. Presumably, this loss in transition-state stabilization would be similar in the R state of the holoenzyme. The more significant alteration in activity of the serine to alanine substitution at position 52 in the holoenzyme compared to the catalytic subunit suggests that the mutation influences the holoenzyme in a more complex fashion. It has been determined by equilibrium isotope exchange kinetics that the rate-limiting step for the wild-type

holoenzyme is the allosteric transition accompanying aspartate binding (Wedler & Gasser, 1974; Hsuanyu & Wedler, 1987). Therefore, Ser-52 may be involved in the rate-limiting step, although not directly involved in the catalysis, if the Ser-52→Ala replacement alters the T to R transition in some fashion. For mutant or modified enzymes in which the T to R transition is altered, loss of activity is generally reduced approximately 10-fold (Kantrowitz & Lipscomb, 1988; Enns & Chan, 1979; Middleton & Kantrowitz, 1988; Newton & Kantrowitz, 1990; Dembowski et al., 1990). The total loss of activity for the Ser-52→Ala holoenzyme could be accounted for by a 10-fold loss of activity due to an alteration in the T to R transition plus and an additional 80-fold reduction due to loss of transition-state stabilization, similar to the value observed for the Ser-52→Ala catalytic subunit. Additional support for an alteration in the T to R transition for the Ser-52→Ala holoenzyme is the observation that the mutant enzyme no longer exhibits cooperativity.

Upon carbamoyl phosphate binding, the Ser-52→Ala holoenzyme undergoes a conformational change which is detected by difference circular dichroism spectroscopy. However, this conformational change only affects the ellipticity above 290 nm but does not affect the ellipticity below 290 nm, as opposed to the wild-type enzyme where there is an increase in both regions of the spectra (see Figure 3). These data imply that the Ser-52→Ala holoenzyme is unable to undergo the same conformational change upon carbamoyl phosphate binding as does the wild-type enzyme. The inability of the Ser-52→Ala enzyme to undergo this conformational change may prevent the formation of the high-activity high-affinity form of the enzyme that is necessary for cooperativity.

Additional support for an alteration in the T to R transition comes from preliminary measures of alterations in the chromatographic partition coefficient of the mutant enzyme in the presence and absence of PALA. The chromatographic partition coefficients of the Ser-52→Ala and wild-type enzymes are similar; however, PALA causes a smaller alteration for the Ser-52→Ala enzyme than for the wild-type holoenzyme (N. Allewell, personal communication).

The activity versus pH profile of the wild-type holoenzyme at subsaturating concentrations of aspartate, where the enzyme would presumably be in the T state, is different from the pH profile at saturating concentrations of aspartate, where the enzyme would presumably be in the R state (Gerhart & Pardee, 1964). The correlation of a specific activity versus pH profile with a specific quaternary structure of aspartate transcarbamoylase has been suggested (Thiry & Hervé, 1978; Baillon et al., 1985; Ladjimi et al., 1985). The fact that the pH profile of the Ser-52→Ala enzyme is unaltered at subsaturating and saturating concentrations of aspartate does suggest that alterations in the allosteric transition may have occurred. However, for a mutant enzyme, such a shift in the pH profile cannot be used as proof of a shift from the T to the R state (Ladjimi & Kantrowitz, 1987).

Comparison of the Results Obtained from Ser-52→Ala and Ser-52→Phe Enzymes. The substitution of Ser-52 with phenylalanine by random mutagenesis results in an inactive enzyme (Jenness & Schachman, 1983) that is locked in the T state even in the presence of PALA (Schachman et al., 1984). The loss of activity of the Ser-52→Phe enzyme may simply be due to the replacement of a bulky hydrophobic amino acid in the hydrophilic environment of the active site. Therefore, the nature of the interaction between the oxygen phosphate and Ser-52 cannot be rationalized based on the kinetic properties of the Ser-52→Phe enzyme. However, the total loss of activity of the Ser-52→Phe enzyme is consistent with the results presented here for the Ser-52→Ala enzyme. The replacement of Ser-52 by alanine causes a 670-fold loss of activity, and therefore the replacement of this residue by phenylalanine could easily produce an enzyme that has no detectable activity probably because carbamoyl phosphate can no longer bind to the active site.

The conformational state of the Ser-52→Ala enzyme with ligands is different from the Ser-52→Phe enzyme since PALA is able to activate the Ser-52→Ala holoenzyme slightly, while the presence of PALA is not able to decrease the sedimentation coefficient of the Ser-52→Phe holoenzyme compared to in the absence of PALA, probably because PALA is unable to bind to this mutant enzyme (Schachman et al., 1984).

Ser-80 May Be Important for Communication between Active Sites in the Wild-Type Holoenzyme. Analysis of the X-ray structure of aspartate transcarbamoylase indicates that the active site of the enzyme is shared between adjacent catalytic chains (Krause et al., 1987; Gouaux & Lipscomb, 1988). Not only the side chains of Ser-52, Arg-54, Thr-55, Arg-105, His-134, and Gln-137 from one catalytic chain but also the side chains of Ser-80 and Lys-84 from an adjacent catalytic chain interact with carbamoyl phosphate. When the wild-type holoenzyme undergoes the concerted allosteric transition, the 80s loop including Ser-80 and Lys-84 undergoes substantial movement (Kim et al., 1987; Krause et al., 1987). It has been demonstrated by Lahue and Schachman (1984) that the allosteric behavior is partially dependent on communication between the catalytic chains within the catalytic trimer in the wild-type holoenzyme. The results obtained from the Ser-80→Ala enzyme agree with the above conclusion. Although the cooperativity for aspartate is still retained, the cooperativity toward carbamoyl phosphate is totally abolished for this mutant enzyme. The total elimination of cooperativity for carbamoyl phosphate may be a result of the lack of communication between active sites in the catalytic trimer upon substrate binding at concentrations of carbamoyl phosphate less than saturating. However, the mutant enzyme exhibits aspartate cooperativity. Since PALA is able to activate the Ser-80→Ala enzyme at saturating carbamoyl phosphate, the binding of aspartate to the Ser-80→Ala enzyme, in the presence of saturating carbamoyl phosphate, can trigger the T to R transition.

Lys-84 from the adjacent catalytic chain also interacts with the phosphate oxygen of carbamoyl phosphate (Krause et al., 1987; Gouaux & Lipscomb, 1988). The replacement of Lys-84 by either glutamine or arginine results in loss of cooperativity (Robey et al., 1986). This together with the result obtained from the Ser-80→Ala enzyme emphasizes the importance of communication between the active sites for the homotropic properties of the wild-type enzyme.

Catalytic Mechanism. Gouaux et al. (1987) have proposed that the reaction catalyzed by aspartate transcarbamoylase proceeds via a tetrahedral intermediate and that the breakdown of the intermediate is facilitated by proton transfer between the amino group of aspartate and a terminal oxygen of carbamoyl phosphate. Kinetic isotope experiments support the formation of such a tetrahedral intermediate (Stark, 1971). However, in order to form the tetrahedral intermediate, the deprotonated amino group of aspartate must lose an additional proton, and there has been considerable controversy as to the means by which this proton is lost. His-134 has been proposed as a likely candidate, since the unperturbed pK_a of the imidazole is in the right pH region for optimal activity (Krause et al., 1985; Voltz et al., 1986). However, replacement of

His-134 by alanine only caused a 22-fold loss of catalytic activity (Robey et al., 1986). Furthermore, Kleanthous et al. (1988) have shown that the pK_a of His-134 is less than 6.

In an attempt to understand the catalytic mechanism of aspartate transcarbamoylase, site-specific mutagenesis has been used to investigate the residues at the active site that interact directly with the substrates. With the completion of the analysis of the Ser-52→Ala and Ser-80→Ala enzymes reported here, every side chain in direct contact with either substrate has now been modified at least once (Robey et al., 1986; Stebbins et al., 1989, 1990; Xu & Kantrowitz, 1989; Middleton et al., 1989). The site-specific mutagenesis experiments have identified Arg-54 as the most catalytically critical side chain (Stebbins et al., 1989). Replacement of Arg-54 by alanine results in 17 000-fold and 72 000-fold reductions in catalytic activity for the holoenzyme and catalytic subunit, respectively. The side chain of Arg-54, which interacts with the anhydride oxygen of carbamoyl phosphate, must be critical for the reaction by stabilizing the negative charge on the phosphate leaving group. The triad His-134 (Robey et al., 1986), Thr-55 (Xu & Kantrowitz, 1989), and Arg-105 (Stebbins et al., 1989) are all involved in polarizing the carbonyl of carbamoyl phosphate, and residues like Arg-167 (Stebbins et al., 1990), Lys-84 (Robey et al., 1986), and Arg-105 (Stebbins et al., 1989) are directly involved in stabilizing the transition state by simultaneously binding the two substrates. These interactions may very well be the molecular basis of the compression hypothesis originally proposed by Collins and Stark (1969).

The active site of aspartate transcarbamoylase with 13 groups interacting directly with the substrates in the transition state provides a great deal of transition-state stabilization. It is the sum total of this transition-state stabilization energy provided by these residues that is the most important contribution to the enhanced rate of the enzyme-catalyzed reaction. With this study, every group that is in proximity of the active site has been modified. None of these studies have identified a side chain that could be involved in a carbomyl-enzyme intermediate or one that acts as a general base in catalysis. Therefore, the results from this study as well as previous studies of mutant enzymes at the active site of aspartate transcarbamoylase have proven that the reaction does not proceed by way of a carbamoyl-enzyme intermediate, and support a reaction mechanism that involves a tetrahedral intermediate as originally proposed by Gouaux et al. (1987). Furthermore, these data suggest that no side chains on the enzyme are involved as a general base in catalysis; rather, the proton transfer occurs directly from the amino group of aspartate to the leaving phosphate group. With the availability of this set of site-specific mutants in the active site of aspartate transcarbamoylase, additional crystallographic and biochemical experiments are planned to further establish the details of the catalytic mechanism.

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Registry No. L-Ser, 56-45-1; L-Asp, 56-84-8; PALA, 51321-79-0; carbamoyl phosphate, 590-55-6; aspartate transcarbamoylase, 9012-49-1.

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