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Function of Threonine-55 in the Carbamoyl Phosphate Binding Site 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, the carbonyl group of carbamoyl phosphate interacts with Thr-55, Arg-105, and His-134. Site-specific mutagenesis was used to create a mutant version of the enzyme in which Thr-55 was replaced by alanine in order to help define the role of this residue in the catalytic mechanism. The Thr-55 → Ala holoenzyme exhibits a 4.7-fold reduction in maximal observed specific activity, no alteration in aspartate cooperativity, and a small reduction in carbamoyl phosphate cooperativity. The mutation also causes 14-fold and 35-fold increases in the carbamoyl phosphate and aspartate concentrations required for half the maximal observed specific activity, respectively. Circular dichroism spectroscopy has shown that saturating carbamoyl phosphate does not induce a conformational change in the Thr-55 → Ala holoenzyme as it does for the wild-type holoenzyme. The kinetic properties of the Thr-55 → Ala catalytic subunit are altered to a greater extent than the mutant holoenzyme. The mutant catalytic subunit cannot be saturated by either substrate under the experimental conditions. Furthermore, as opposed to the wild-type catalytic subunit, the Thr-55 → Ala catalytic subunit shows cooperativity for aspartate and can be activated by *N*-(phosphonoacetyl)-L-aspartate in the presence of low concentrations of aspartate and high concentrations of carbamoyl phosphate. As deduced by circular dichroism spectroscopy, the conformation of the Thr-55 → Ala catalytic subunit in the absence of active-site ligands is distinctly different from the wild-type catalytic subunit. Furthermore, carbamoyl phosphate causes an alteration in the circular dichroism spectrum of the Thr-55 → Ala catalytic subunit as opposed to the mutant holoenzyme. For the Thr-55 → Ala catalytic subunit, the K_D of carbamoyl phosphate and the K_i of PALA are both increased approximately 50-fold compared to the wild-type catalytic subunit. These data suggest that in the wild-type holoenzyme Thr-55 plays an important part in the binding of carbamoyl phosphate, has a small catalytic function in polarizing the carbonyl, and is important in the conformational change that occurs upon binding of carbamoyl phosphate which in turn is important for the ordered binding of aspartate. In addition, Thr-55 also plays a role in stabilizing the native conformation of the wild-type catalytic subunit.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the condensation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate in the committed step of the pyrimidine biosynthesis pathway. The enzyme, used as a model system to study the molecular mechanisms of allosteric regulation [see reviews by Allewell (1989); Gerhart (1970), Jacobson and Stark (1973), Kantrowitz and Lipscomb (1988), Kantrowitz et al. (1980a,b), and Schachman (1974)], consists of three regulatory subunits and two catalytic subunits. The holoenzyme exhibits homotropic cooperativity for both

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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 subunit, are shared between adjacent catalytic chains within the trimer (Krause et al., 1985; Monaco et al., 1978; Robey & Schachman, 1985; Wente & Schachman, 1987). The regulatory subunit 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. The amino acid sequences of the catalytic and regulatory polypeptide chains have been determined (Hoover et al., 1983; Konigsberg & Henderson, 1983; Schachman et al., 1984; Weber, 1968), and information about the three-dimensional structure of the unliganded enzyme (Honzatko & Lipscomb, 1982; Ke et al., 1984), the CTP–enzyme complex (Honzatko & Lipscomb, 1982; Kim et al., 1987), the enzyme complexed with the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA)[1] (Krause et al., 1985, 1987; Ladner et al., 1982), and the enzyme complexed with carbamoyl phosphate plus succinate (Gouaux & Lipscomb, 1988) is available at atomic resolution.

The catalytic mechanism involves the ordered binding of the substrates, with carbamoyl phosphate binding before aspartate (Hsuanyu & Wedler, 1987; Porter et al., 1969; Wedler & Gasser, 1974). The binding of carbamoyl phosphate induces a conformational change in the enzyme (Collins & Stark, 1969; Griffin et al., 1972) that allows the sequential binding of aspartate (Collins & Stark, 1969), resulting in further conformational changes that force the aspartate and carbamoyl phosphate domains together. This domain closure not only assists in the catalysis but also initiates the concerted quaternary conformational change of the holoenzyme from the T to R state (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988). NMR data suggest that the carbonyl oxygen of carbamoyl phosphate is hydrogen bonded to a group of the enzyme, or is protonated by that 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 PALA (Krause et al., 1987) or carbamoyl phosphate plus succinate (Gouaux & Lipscomb, 1988) provides information concerning the specific groups on the enzyme which interact with carbamoyl phosphate. The residues that interact with carbamoyl phosphate include Ser-52, Thr-53, Arg-54, Thr-55, Arg-105, His-134, and Gin-137 from one catalytic chain plus Ser-80 and Lys-84 from the adjacent catalytic chain. Of these residues, Thr-55, Arg-105, and His-134 interact with the carbonyl group of carbamoyl phosphate and are conserved in all the aspartate and ornithine transcarbamoylases that have been sequenced (Bencini et al., 1983; Hoover et al., 1983; Lerner & Switzer, 1986; Shigesada et al., 1985; Van Vliet et al., 1984), which suggests that these residues may be essential for carbamoyl phosphate binding. Furthermore, these residues may also be important for the quaternary conformational change that occurs upon carbamoyl phosphate binding or may be directly involved in catalysis. Of the three residues, His-134 (Robey et al., 1986) and Arg-105 (Stebbins et al., 1989) have been replaced by using site-specific mutagenesis. In order to determine if the specific interaction between Thr-55 and the carbonyl oxygen of carbamoyl phosphate is important for substrate binding and catalysis, we report here the construction and characterization of a mutant version of aspartate transcarbamoylase with alanine in place of Thr-55 in the catalytic chain.

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*, *rspL*] 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 as well as the sequencing primers was synthesized by using an Applied Biosystems 381A DNA synthesizer.

Construction of the Thr-55 → Ala[2] *Mutation by Site-Directed Mutagenesis.* The substitution of alanine in place of Thr-55 of the catalytic chain of aspartate transcarbamoylase was accomplished by site-specific mutagenesis using the method of Zoller and Smith (1982) with the modifications previously described (Carter et al., 1985; Ladjimi et al., 1988). Single-stranded DNA from 10 candidates was 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 which had the corresponding section of the wild-type gene removed by restriction enzymes.

After digestion with *Pst*I and *Bst*EII, a fragment of 758 base pairs which contains the desired mutation was isolated from the purified M13RF after agarose gel electrophoresis with NA45 paper. The plasmid pEK54 (Xu et al., 1988) was digested with the same two enzymes, and the larger fragment was isolated in a similar fashion. This larger fragment, which contains the vector pUC119 plus the remainder of the *pyrBI* operon, was combined with the fragment from the mutant M13RF and was treated with T4 DNA ligase. Selection was accomplished after transformation in U39a, a strain which has a deletion in the *pyrBI* region. The plasmid pEK93 which contains the desired mutation was purified by retransformation at low density, and the mutation was verified a second time

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

[2] The notation used to name the mutant enzymes is, for example, the Thr-55 → 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.

by directly sequencing the gene fragment which was cloned into the pEK54 backbone 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 Thr-55 → Ala aspartate transcarbamoylases were purified as described by Nowlan and Kantrowitz (1985), from *E. coli* strain EK1104 [F⁻ *ara, thi, Δpro-lac, ΔpyrB, pyrF⁻, rpsL*], containing the plasmid pEK2 for the wild type (Smith et al., 1986) or pEK93 for the Thr-55 → Ala enzyme.

Wild-Type and Mutant Catalytic Subunit Overproduction and Purification. The purification of the catalytic subunit of the wild-type or Thr-55 → Ala aspartate transcarbamoylase 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 and pEK96, used for the production of the wild-type and Thr-55 → Ala catalytic subunit, respectively, were constructed as previously described (Nowlan & Kantrowitz, 1985). 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 cm^2/mg, respectively (Gerhart & Holoubek, 1967). The protein concentrations of the mutant holoenzyme and catalytic subunit were determined by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976).

Circular Dichroism. CD spectra were recorded by using 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 per minute, 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 and Thr-55 → Ala Holoenzymes. The substrate saturation curves of the wild-type and the Thr-55 → Ala holoenzymes are shown in Figures 1 and 2, and a summary of kinetic parameters is given in Table I. Although the specific activity of the Thr-55 → Ala holoenzyme is approximately 5-fold lower than the wild-type holoenzyme, the mutant holoenzyme still retains normal cooperativity for aspartate, as measured by the Hill coefficient, but slightly reduced cooperativity for carbamoyl phosphate. The substrate concentration at half the maximal observed specific activity, $[S]_{0.5}$, is increased substantially by this amino acid replacement. The $[S]_{0.5}^{CP}$ increases from 0.2 mM for the wild-type holoenzyme to 6.9 mM for the Thr-55 → Ala holoenzyme, and the $[S]_{0.5}^{Asp}$ increases from 11.8 mM for the wild-type holoenzyme to 163 mM for the Thr-55 → Ala holoenzyme.

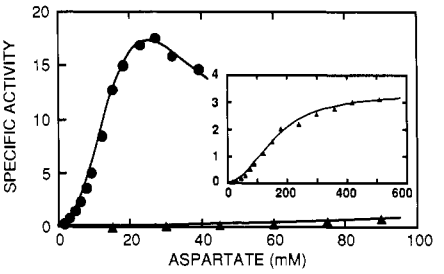

FIGURE 1: Aspartate saturation curves for the Thr-55 → Ala (▲) and wild-type (●) holoenzymes. Specific activity is reported in millimoles of *N*-carbamoylaspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer, pH 8.3. The aspartate saturation curves were performed at 4.8 and 15 mM carbamoyl phosphate for the wild-type and the Thr-55 → Ala enzymes, respectively. (Inset) Expanded region corresponding to high aspartate concentrations for the Thr-55 → Ala enzyme.

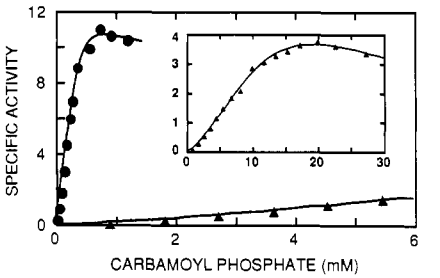

FIGURE 2: Carbamoyl phosphate saturation curves for the Thr-55 → Ala (▲) and wild-type (●) holoenzymes. Specific activity is reported in millimoles of *N*-carbamoylaspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer, pH 8.3. The carbamoyl phosphate saturation curves were performed at 30 and 300 mM aspartate for the wild-type and the Thr-55 → Ala enzymes, respectively. (Inset) Expanded region corresponding to high carbamoyl phosphate concentrations for the Thr-55 → Ala enzyme.

Table I: Kinetic Parameters for the Wild-Type and Thr-55 → Ala Holoenzymes[a]

enzyme	max velocity[b] ($mmol \cdot h^{-1} \cdot mg^{-1}$)	$[S]_{0.5}^{Asp}$ (mM)	n_H^{Asp}	$[S]_{0.5}^{CP}$ (mM)	n_H^{CP}
wild type	17.2	11.8	2.2	0.20	2.0
Thr-55 → Ala	3.6	163	2.2	6.9	1.4

[a] These data are extracted from Figures 1 and 2. The maximal velocity and Hill coefficients (n_H) were calculated by a nonlinear least-squares procedure using a modified Hill equation which incorporates a term for substrate inhibition (Pastra-Landis et al., 1978). [b] Maximal observed specific activity.

Kinetic Properties of the Wild-Type and Thr-55 → Ala Catalytic Subunits. The kinetic properties of the Thr-55 → Ala catalytic subunit are altered significantly more than the mutant holoenzyme. Both $[S]_{0.5}^{Asp}$ and $[S]_{0.5}^{CP}$ increased dramatically for the Thr-55 → Ala catalytic subunit compared to the Thr-55 → Ala holoenzyme. Furthermore, because of the limited solubility of the carbamoyl phosphate, and the rate of the nonenzymatic reaction at high aspartate and carbamoyl phosphate concentrations, it was impossible to obtain aspartate and carbamoyl phosphate saturation curves under conditions where the nonvaried substrate was saturating. At a constant

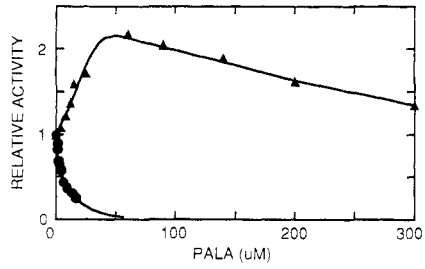

FIGURE 3: PALA activation of wild-type (●) and Thr-55 → Ala (▲) catalytic subunits. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer, pH 8.3. Activity was measured at a constant concentration of aspartate and carbamoyl phosphate as the concentration of PALA was varied. The assay was performed at 6 mM aspartate and 4.8 mM carbamoyl phosphate for the wild-type catalytic subunit, and at 60 mM aspartate and 15 mM carbamoyl phosphate for the Thr-55 → Ala catalytic subunit.

carbamoyl phosphate concentration of 15 mM, approximately as high as is possible to use experimentally, the aspartate saturation curve does not begin to saturate at 500 mM aspartate, and furthermore the curve looks sigmoidal in shape. At 15 mM carbamoyl phosphate and 500 mM aspartate, the activity of the Thr-55 → Ala catalytic subunit is 12-fold lower than the maximal activity of Thr-55 → Ala holoenzyme. Because of this very unusual kinetic behavior, the standard kinetic parameters could not be determined.

Does the Thr-55 → Ala Catalytic Subunit Exhibit Homotropic Cooperativity. For the wild-type holoenzyme, PALA or succinate is able to activate the enzyme at saturating carbamoyl phosphate and low concentrations of aspartate (approximately $^1/_6[S]_{0.5}^{Asp}$ (Collins & Stark, 1971; Gerhart & Pardee, 1963). 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 (Blackburn & Schachman, 1977; Gerhart & Schachman, 1968; Howlett et al., 1977). At higher concentrations of PALA or succinate, inhibition occurs since both of these analogues can compete for the active site.

As seen in Figure 3, PALA can activate the Thr-55 → Ala catalytic subunit at low concentrations of aspartate. However, under identical conditions, the wild-type catalytic subunit is not activated by PALA. Succinate (in the presence of saturating carbamoyl phosphate) does not activate either the Thr-55 → Ala or the wild-type catalytic subunits (data not shown).

Carbamoyl Phosphate Does Not Induce a Conformational Change in the Thr-55 → Ala Holoenzyme. As mentioned above, the binding of carbamoyl phosphate to the wild-type holoenzyme causes a conformational change that allows the subsequent binding of aspartate. This conformational change can be monitored by UV (Collins & Stark, 1969) and circular dichroism (Griffin et al., 1972) difference spectroscopy. As shown in Figure 4A, saturating carbamoyl phosphate causes the ellipticity at both the 273- and 297-nm regions to increase for the wild-type enzyme. This alteration in the CD spectrum of wild-type holoenzyme suggests that carbamoyl phosphate induces a conformational change that affects the orientation of the side chains of some aromatic amino acids. A K_D of 0.02 mM for carbamoyl phosphate was determined by using the alteration in the CD spectrum as a function of carbamoyl phosphate concentration (Stebbins et al., 1989).

When carbamoyl phosphate is added to the Thr-55 → Ala holoenzyme, the ellipticity does not change in the region between 260 and 320 nm, indicating either that carbamoyl

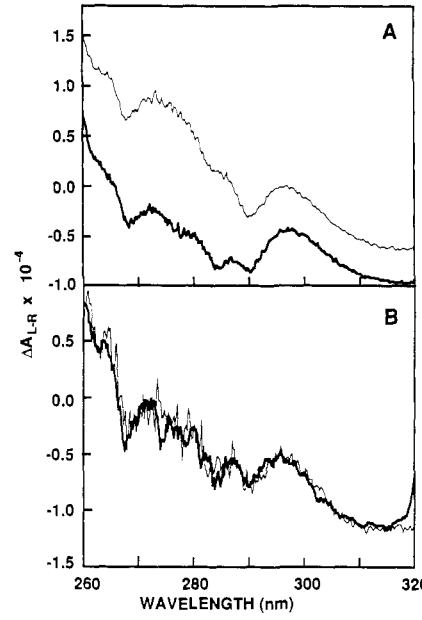

FIGURE 4: Circular dichroism spectra of wild-type (A) and Thr-55 → Ala (B) holoenzymes (2.0 mg/mL) in the absence and presence of carbamoyl phosphate. The spectra of the enzymes were recorded at 25 °C in 0.04 mM imidazole–acetate, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.0. The darker lines represent 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 Thr-55 → Ala holoenzymes were 0.13 and 0.78 mM, respectively.

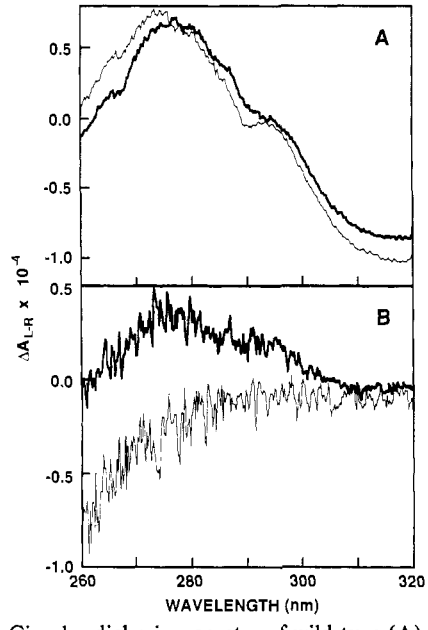

FIGURE 5: Circular dichroism spectra of wild-type (A) and Thr-55 → Ala (B) catalytic subunits (1.0 mg/mL) in the absence and presence of carbamoyl phosphate. The conditions are identical with those reported in Figure 4 for the holoenzyme. The darker lines represent the spectra in the absence of carbamoyl phosphate while the lighter lines represent the spectra in the presence of carbamoyl phosphate. The carbamoyl phosphate concentrations for the wild-type and the Thr-55 → Ala catalytic subunits were 0.2 and 0.5 mM, respectively.

phosphate does not bind to the Thr-55 → Ala enzyme or that it binds to the mutant enzyme but does not induce the same conformational change as the wild-type holoenzyme (see Figure 4B).

Carbamoyl Phosphate Induces a Different Conformational Change in the Wild-type than the Thr-55 → Ala Catalytic Subunit. As seen in Figure 5, the CD spectra of the wild-type and the Thr-55 → Ala catalytic subunits are significantly

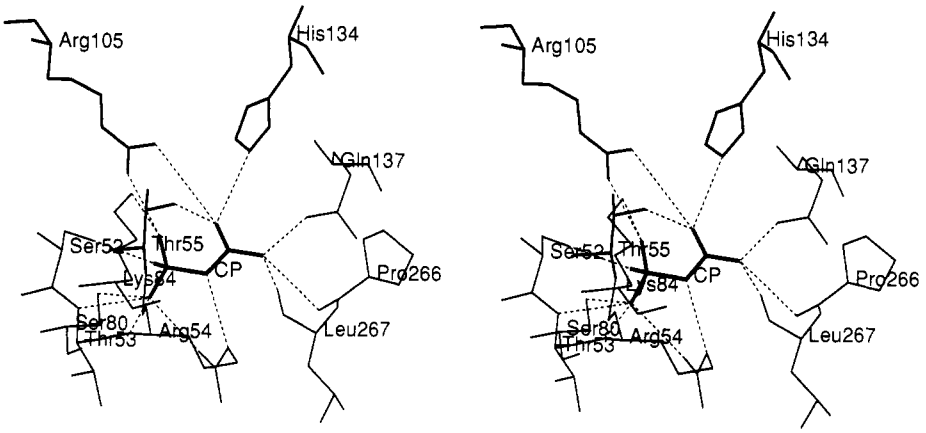

FIGURE 6: Stereoview of the carbamoyl phosphate binding site in aspartate transcarbamylase. The carbamoyl phosphate site is composed of residues from two adjacent catalytic chains. In this figure, all the side chains that interact with carbamoyl phosphate are shown. All the residues come from one catalytic 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 dashed 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).

different in the region between 260 and 320 nm. In particular, the negative peak at 318 nm observed in the wild-type spectrum is no longer present, and the intensity of the peak at 275 nm is decreased significantly for the Thr-55 → Ala catalytic subunit. These spectra indicate that the conformational state of the Thr-55 → Ala catalytic subunit is significantly different from the wild-type catalytic subunit.

The addition of carbamoyl phosphate to the Thr-55 → Ala catalytic subunit does induce a change in the CD spectra (see Figure 5B). The peak centered around 275 nm decreases in intensity as carbamoyl phosphate is added. The alteration in the CD spectra of the Thr-55 → Ala catalytic subunit is significantly different from the wild-type catalytic subunit. For the wild-type catalytic subunit, the addition of carbamoyl phosphate causes an increase below 275 nm and a decrease above 275 nm, while for the Thr-55 → Ala catalytic subunit the spectrum in the presence of carbamoyl phosphate is always lower than the spectrum without carbamoyl phosphate.

Binding of Carbamoyl Phosphate and PALA to the Thr-55 → Ala Catalytic Subunit. According to the method of Porter et al. (1969), the K_D values of carbamoyl phosphate for the wild-type and the Thr-55 → Ala catalytic subunits were determined to be 0.022 and 1.13 mM, respectively (data not shown). Therefore, the introduction of alanine at position 55 of the catalytic subunit increases the K_D of carbamoyl phosphate by more than 50-fold. The binding for PALA is affected to nearly the same extent as for carbamoyl phosphate. The K_i of PALA increases from 0.024 μM for the wild-type catalytic subunit to 1.18 μM for the Thr-55 → Ala catalytic subunit (data not shown).

Discussion

In the active site of aspartate transcarbamylase, carbamoyl phosphate is precisely oriented to react with aspartate by a large number of specific interactions (see Figure 6). The amino group interacts with both the side chain of Gln-137 and also the backbone carbonyls of Pro-266 and Leu-267; the carbonyl interacts with His-134, Thr-55, and Arg-105; the anhydride oxygen interacts with Arg-54; and the phosphate oxygens interact with Ser-52, Thr-53, Arg-54, and Arg-105 as well as Ser-80 and Lys-84 from an adjacent catalytic chain (Gouaux & Lipscomb, 1988). The orientation of the carbonyl group of carbamoyl phosphate as well as its reactivity is critical for the catalytic reaction (Stebbins et al., 1989). Previously, two of the three side chains that interact with the carbonyl of carbamoyl phosphate have been studied by site-specific mutagenesis. The replacement of His-134 (Robey et al., 1986) and Arg-105 (Stebbins et al., 1989) by alanine resulted in 20-fold and 1000-fold losses in activity, respectively. Although the replacement of Arg-105 has a larger effect on activity than the replacement of His-134, Arg-105 is also involved in interactions with the α-carboxylate of aspartate.[3] In addition to reductions in activity, both the Arg-105 → Ala and His-134 → Ala enzymes exhibit dramatically reduced affinity for aspartate (Robey et al., 1986; Stebbins et al., 1989). Furthermore, the Arg-105 → Ala enzyme binds carbamoyl phosphate more weakly than the wild-type enzyme, and the conformational change that is observed upon carbamoyl phosphate binding is reduced significantly by this amino acid replacement (Stebbins et al., 1989). Data on carbamoyl phosphate affinity and alterations in the conformational changes due to the His-134 → Ala replacement have not been reported. In this work, we have replaced Thr-55 with alanine in order to evaluate the role of Thr-55 in the catalytic mechanism of aspartate transcarbamoylase.

Thr-55 Is Not Essential for Catalysis. The 5-fold decrease in activity observed when Thr-55 is replaced by alanine suggests that this residue is involved in catalysis but is not essential. Since each of the residues that interact with the carbonyl of carbamoyl phosphate, Thr-55, Arg-105, and His-134, all cause losses in activity, they may work in a concerted fashion to both orient and polarize the carbonyl of carbamoyl phosphate.

Alterations in the Carbamoyl Phosphate Binding Site Affect the Aspartate Binding Site. Although the relatively conservative replacement of Thr-55 by alanine results in an enzyme with only a small decrease in catalytic activity, there are relatively large alterations in the $[S]_{0.5}$ not only for carbamoyl phosphate, which interacts directly with Thr-55, but also for aspartate, which binds to an entirely different domain of the enzyme. These results parallel results of other substitutions in the carbamoyl phosphate binding site of aspartate transcarbamoylase. When His-134 (Robey et al., 1986) and Arg-105 and Gln-137 (Stebbins et al., 1989) were replaced by site-specific mutagenesis, large increases in the $[S]_{0.5}^{Asp}$ were

[3] The interaction with the α-carboxylate of aspartate is based upon analogy with the structures of the enzyme with either PALA (Krause et al., 1985, 1987) or carbamoyl phosphate and succinate bound (Gouaux & Lipscomb, 1988).

observed. Stebbins et al. (1989) suggest that certain mutations at the carbamoyl phosphate binding site significantly affect the conformational change that normally occurs upon carbamoyl phosphate binding to the wild-type enzyme. If this conformational change is critical for creation of the high-affinity aspartate binding site, and it does not take place correctly, then binding of aspartate is hindered, and an increase in $[S]_{0.5}^{Asp}$ would be observed. For the Thr-55 → Ala holoenzyme, the normal conformational change induced upon carbamoyl phosphate binding cannot be detected. Therefore, the data reported here for the Thr-55 → Ala holoenzyme support the conclusion that the conformational change induced upon the binding of carbamoyl phosphate is critical for the formation of the high-affinity aspartate binding site, and is partially responsible for the ordered binding of the substrates.

Thr-55 → Ala Catalytic Subunit Behaves Abnormally. The kinetic properties of the Thr-55 → Ala catalytic subunit are very different from the Thr-55 → Ala holoenzyme. Under the experimental conditions used here, the mutant catalytic subunit cannot be saturated with either substrate while under the same experimental conditions the mutant holoenzyme can be. This suggests that Thr-55 in the wild-type catalytic subunit must be important in establishing the conformation of the enzyme and that Thr-55 serves a somewhat different function in the holoenzyme versus the catalytic subunit. The role of Thr-55 in establishing the native conformation of the wild-type catalytic subunit is clearly evident from the circular dichroism spectra (Figure 5). In the absence of active-site ligands, the circular dichroism spectrum of the Thr-55 → Ala catalytic subunit does not exhibit the negative peak at 317 nm that is observed for the wild-type catalytic subunit. The addition of saturating carbamoyl phosphate causes a decrease in the spectrum for the Thr-55 → Ala catalytic subunit at all wavelengths between 260 and 320 nm, but a decrease for the wild-type catalytic subunit only above 275 nm.

The increase in the K_D of carbamoyl phosphate and the K_i of PALA by approximately 50-fold each suggests that the hydroxyl group of Thr-55 plays an important role in the binding of carbamoyl phosphate. The fact that the affinity of the mutant catalytic subunit for aspartate is also dramatically reduced further suggests that the conformational change induced upon carbamoyl phosphate binding does not occur correctly.

Interacting Sites in the Catalytic Subunit. The sigmoidal shape of the aspartate saturation curve of the Thr-55 → Ala catalytic subunit as well as the fact that this catalytic subunit can be activated by PALA (Figure 3) implies that there are interacting sites in this catalytic subunit. Although the wild-type catalytic subunit exhibits no cooperativity and is not activated by PALA, Newell et al. (1989) have shown that the binding of PALA to the wild-type catalytic subunit is slightly cooperative. Furthermore, the mutant catalytic subunit with alanine in place of Arg-105 also exhibits aspartate cooperativity with a Hill coefficient of 2.0 and is activated by succinate (at low concentrations of aspartate and saturating concentrations of carbamoyl phosphate) (Stebbins et al., 1989). These data taken together confirm that there is communication between the active sites of the catalytic subunit of aspartate transcarbamoylase, i.e., the binding of substrates at one site can influence the activity at the other active sites. The most likely reason that this is observed in the mutant but not the wild-type catalytic subunits is simply that the wild-type catalytic subunit, in the presence of saturating carbamoyl phosphate, is normally in a high-affinity high-activity state. Even in the presence of carbamoyl phosphate, the Thr-55 → Ala and Arg-105 → Ala catalytic subunits are in a low-affinity low-activity state due to the amino acid substitution at the carbamoyl phosphate binding site. However, the binding of aspartate (in the presence of carbamoyl phosphate) to one active site of the Thr-55 → Ala catalytic subunit can convert the entire catalytic subunit into a high-affinity high-activity state, resulting in the observed cooperativity and ability of the enzyme to be activated by PALA or succinate.

Function of Thr-55 in Catalysis. On the basis of the results reported here, along with those of mutations at other residues in the active site of aspartate transcarbamoylase (Middleton et al., 1989; Robey et al., 1986; Stebbins et al., 1989), we suggest that Thr-55 contributes to the catalytic reaction by helping to orient carbamoyl phosphate correctly for attack by the amino group of aspartate. In addition, Thr-55 also draws electron density from the carbonyl carbon to make it more susceptible to nucleophilic attack by aspartate. Thr-55 may also be partially responsible for the ordered binding of the substrates. Previous studies have shown that the binding of the substrates to aspartate transcarbamoylase is ordered with carbamoyl phosphate binding before aspartate and carbamoyl aspartate leaving before phosphate (Hsuanyu & Wedler, 1987; Porter et al., 1969; Wedler & Gasser, 1974). The conformational change that occurs upon the binding of carbamoyl phosphate to the wild-type enzyme is a requirement for the binding of aspartate, and this conformational change is altered dramatically by the Thr-55 → Ala substitution. Additional studies are in progress to further delineate the role of Thr-55 in the catalytic mechanism of aspartate transcarbamoylase.

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