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Three Residues Involved in Binding and Catalysis in the Carbamyl Phosphate Binding Site of *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: Site-directed mutagenesis was used to create four mutant versions of *Escherichia coli* aspartate transcarbamylase at three positions in the catalytic chain of the enzyme. The location of all the amino acid substitutions was near the carbamyl phosphate binding site as previously determined by X-ray crystallography. Arg-54, which interacts with both the anhydride oxygen and a phosphate oxygen of carbamyl phosphate, was replaced by alanine. This mutant enzyme was approximately 17 000-fold less active than the wild type, although the binding of substrates and substrate analogues was not altered substantially. Arg-105, which interacts with both the carbonyl oxygen and a phosphate oxygen of carbamyl phosphate, was replaced by alanine. This mutant enzyme exhibited an approximate 1000-fold loss of activity, while the activity of catalytic subunit isolated from this mutant enzyme was reduced by 170-fold compared to the wild-type catalytic subunit. The K_D of carbamyl phosphate and the inhibition constants for acetyl phosphate and *N*-(phosphonoacetyl)-L-aspartate (PALA) were increased substantially by this amino acid substitution. Furthermore, this loss in substrate and substrate analogue binding can be correlated with the large increases in the aspartate and carbamyl phosphate concentrations at half of the maximum observed specific activity, $[S]_{0.5}$. Gln-137, which interacts with the amino group of carbamyl phosphate, was replaced by both asparagine and alanine. The asparagine mutant exhibited only a small reduction in activity while the alanine mutant was approximately 50-fold less active than the wild type. The catalytic subunits of both these mutant enzymes were substantially more active than the corresponding holoenzymes. Both mutant enzymes at position 137 exhibited an approximate 40-fold higher K_D for carbamyl phosphate than the wild-type catalytic subunit. The inhibition constants for acetyl phosphate were hardly altered by these mutations, while both mutations exhibited higher inhibition constants for PALA. The holoenzymes with alanine substitutions at both Arg-54 and Gln-137 exhibited loss of cooperativity, while the Arg-105 → Ala catalytic subunit exhibited cooperativity with a Hill coefficient of 2.0. The pH dependence of the reaction was unaltered by the mutations at Gln-137, although the substitutions at Arg-54 and Arg-105 both resulted in an increase in the pH optimum of the reaction. Circular dichroism (CD) spectroscopy was used to determine if the conformational change normally induced by the binding of carbamyl phosphate had been altered by any of the mutations. In every case, the magnitude of the CD difference spectrum at saturating carbamyl phosphate concentrations was reduced. The alanine substitution at Arg-105 exhibited the largest reduction, while the asparagine substitution at Gln-137 exhibited the smallest reduction. New insights into the catalytic mechanism of aspartate transcarbamylase have been obtained by combining the results of the analysis of these four mutant enzymes with previously proposed mechanisms.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) catalyzes the committed step of the pyrimidine biosynthesis pathway, the formation of *N*-carbamyl-L-aspartate from carbamyl phosphate and L-aspartate. The enzyme, used as a model system to study the molecular mechanisms of allosteric regulation [see reviews by Gerhart (1970), Jacobson and Stark (1973), Schachman (1974), Kantrowitz et al. (1980a,b), and Kantrowitz and Lipscomb (1988)], exhibits positive cooperativity for both substrates (Gerhart & Pardee, 1962; Bethell et al., 1968), and its activity is inhibited by CTP and activated by ATP, the end products of the pyrimidine and purine pathways, respectively. The enzyme is composed of three regulatory dimers (i.e., regulatory subunits) and two catalytic trimers (i.e., catalytic subunits). The regulatory subunit binds CTP and ATP but is devoid of catalytic activity while the isolated catalytic subunit exhibits no homotropic cooperativity

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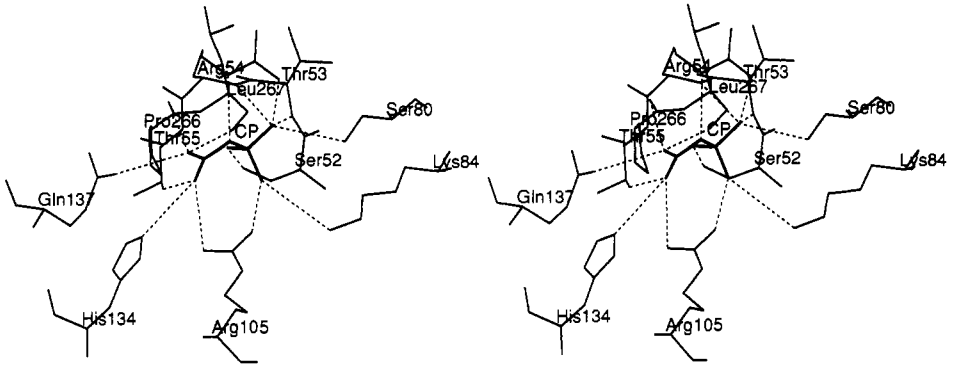

FIGURE 1: Stereoview of the CP binding site in aspartate transcarbamylase. The CP site is composed of residues from two adjacent catalytic chains. In this figure, all the side chains that interact with CP are shown. All the residues come from one catalytic chain except for Ser-80 and Lys-84 which come from an adjacent catalytic chain. The hydrogen-bonding interactions which stabilize CP in the active site are shown as dashed lines. CP interacts only with the backbone of residues Thr-53, Pro-266, and Leu-267. The data used to draw this figure are from Gouaux and Lipscomb (1988).

and is insensitive to the allosteric effectors. The active sites, three per catalytic subunit, are shared between adjacent catalytic chains within the trimer (Monaco et al., 1978; Robey & Schachman, 1985; Krause et al., 1985; Wente & Schachman, 1987). The amino acid sequences of the catalytic and regulatory polypeptide chains have been determined (Weber, 1968; Konigsberg & Henderson, 1983; Hoover et al., 1983; Schachman et al., 1984), and information about the three-dimensional structure of the unliganded enzyme (Honzatko et al., 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] (Ladner et al., 1982; Krause et al., 1985, 1987), and the enzyme complexed with CP and succinate (Gouaux & Lipscomb, 1988) is available at atomic resolution.

The catalytic mechanism involves the binding of CP first (Porter et al., 1969; Wedler & Gasser, 1974; Hsuanyu & Wedler, 1987), which induces a conformational change in the enzyme (Collins & Stark, 1969; Griffin et al., 1972) that allows the binding of aspartate (Collins & Stark, 1969). NMR data suggest that the carbonyl oxygen of CP is hydrogen bonded to a group on the enzyme or is protonated by that group, activating the carbonyl carbon for nucleophilic attack by the amino group of aspartate (Roberts et al., 1976). The binding of aspartate induces a further conformational change which forces the aspartate and CP binding 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 the R state (Ladjimi & Kantrowitz, 1988; Kantrowitz & Lipscomb, 1988).

Information concerning the specific groups on the enzyme which interact with CP has come from the analysis of X-ray structures of the enzyme with either PALA (Krause et al., 1987) or CP plus succinate bound (Gouaux & Lipscomb, 1988). As seen in Figure 1, the residues which interact with CP are 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. Lys-84, which is thought to also interact with aspartate (Gouaux & Lipscomb, 1988), has been replaced by both Gln and Arg. Neither substitution affects the $[S]_{0.5}^{Asp}$ but both cause more than a 1000-fold loss of activity (Robey et al., 1986). The replacement of His-134 by alanine leads to a 20-fold reduction in activity and a substantial increase in $[S]_{0.5}^{Asp}$ (Robey et al., 1986), while the replacement of Ser-52 with the much larger phenylalanine side chain, by random mutagenesis, results in almost total loss of activity (Schachman et al., 1984). The small effect that the removal of His-134 has on the activity of the enzyme suggests that although His-134 is positioned properly to polarize the carbonyl of CP it is not essential for catalysis.

To determine the function of specific residues at the CP binding site of aspartate transcarbamylase, a set of mutations were constructed, each eliminating an interaction between the enzyme and CP. On the basis of the X-ray structure of the enzyme with CP plus succinate bound (Gouaux & Lipscomb, 1988), we have selected Arg-54, Arg-105, and Gln-137 for replacement by site-directed mutagenesis. Arg-54 interacts with the anhydride oxygen and the phosphate of CP while Arg-105 interacts with both the carbonyl and the phosphate. Gln-137 interacts specifically with the amide by a hydrogen bond from the carbonyl oxygen of the Gln-137 side chain. Here we report the results of the analysis of this set of mutant versions of aspartate transcarbamylase and use these results to gain new insights about the catalytic mechanism of the enzyme.

Experimental Procedures

Materials

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

[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; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; $[S]_{0.5}^{CP}$, carbamyl phosphate concentration at half the maximal observed specific activity; Tris, tris(hydroxymethyl)aminomethane; holoenzyme, entire aspartate transcarbamylase molecule composed of two catalytic subunits and three regulatory subunits; CP, carbamyl phosphate; AP, acetyl phosphate.

obtained from J. Messing, Rutgers University.

Methods

Oligonucleotide Synthesis. The oligonucleotides required for the site-directed mutagenesis as well as the sequencing primers were synthesized by using an Applied Biosystems 381A DNA synthesizer.

Determination of Protein Concentration. Concentrations of pure wild-type holoenzyme and isolated 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 enzymes 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/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 in the absence and presence of varying concentrations of CP. At each concentration of CP, difference spectra were calculated by subtracting the spectrum without CP from the spectrum with CP. To determine a CP binding constant, a plot of the maximum change in difference ellipticity over the wavelength range between 260 and 320 nm versus CP concentration was fit to a hyperbolic function by nonlinear least squares. The volume change due to the addition of CP was never greater than 6%.

Aspartate Transcarbamylase Assay. The transcarbamylase activity was measured at 25 °C by either a colorimetric (Pastra-Landis et al., 1981) or a 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.

Site-Directed Mutagenesis. The introduction of specific base changes in the *pyrB* gene to create altered versions of aspartate transcarbamylase was accomplished by site-directed mutagenesis (Zoller & Smith, 1982) with the modifications previously described (Carter et al., 1985; Ladjimi et al., 1988). Single-stranded DNA from several candidates were isolated and sequenced by the dideoxy method (Sanger et al., 1977) to identify the mutations. In some cases, candidates were screened by blot hybridization (Carter et al., 1984) prior to sequencing. In these experiments, the mutagenesis frequency varied between 15 and 20%.

After verification of the mutations, 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. In each case, a *Pst*I–*Bst*EII fragment of 758 base pairs, containing the desired mutation, was isolated from the purified M13RF after agarose gel electrophoresis, with NA45 paper. In addition, the plasmid pEK54 (Xu et al., 1988) was cut with the same two restriction enzymes, and the larger fragment was isolated in a similar fashion. This fragment, containing the vector pUC119 plus the remainder of the *pyrBI* operon, was combined with the fragment from the mutant M13 RF and treated with T4 DNA ligase. Selection was accomplished after transformation in U39a, a strain which has a deletion in the *pyrBI* region. The plasmid was purified by retransformation at low density, and the mutation was verified a second time by directly sequencing the *pyrB* gene employing single-stranded DNA isolated from the plasmid (Vieira & Messing, 1987). In this fashion, plasmids pEK69, pEK76, pEK85, and pEK89 were isolated which carried the *pyrB* gene for the Arg-54 → Ala,[2] the Arg-105 → Ala, the Gln-137 → Asn, and the Gln-137 → Ala enzymes, respectively.

Wild-Type and Mutant Holoenzyme Purification. Wild-type and mutant aspartate transcarbamylases 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 plasmid pEK2 for the wild type (Smith et al., 1986) or pEK69, pEK76, pEK85, or pEK89 for the Arg-54 → Ala, the Arg-105 → Ala, the Gln-137 → Asn, and the Gln-137 → Ala enzymes, respectively.

Wild-Type and Mutant Catalytic Subunit Overproduction. The isolation of catalytic subunit of the wild-type and mutant versions of aspartate transcarbamylase was accomplished after the in vivo overproduction of catalytic subunit using plasmids which had a portion of the gene for the regulatory subunit deleted (Nowlan & Kantrowitz, 1985) in strain EK1104. The plasmids pEK17, pEK78, pEK87, and pEK90, which were used for the production of the wild-type, the Arg-105 → Ala, Gln-137 → Asn, and Gln-137 → Ala enzymes, respectively, were isolated as previously described (Nowlan & Kantrowitz, 1985).

Wild-Type and Mutant Catalytic Subunit Purification. For the purification of catalytic subunit from strain EK1104 containing a plasmid having only the intact *pyrB* gene, 1 L of cells was grown for 22 h at 37 °C in M9 medium (Miller, 1972) supplemented with 5 g/L casamino acids, 12 μg/mL uracil, and 100 μg/mL ampicillin. After the bacterial growth, the cells were harvested and disrupted by sonication followed by centrifugation to remove cellular debris. Ammonium sulfate was added to 80% saturation to both precipitate and concentrate the crude cell proteins. After recovery of the precipitate by centrifugation, it was dialyzed into 0.05 M Tris–acetate buffer, pH 8.3, prior to ion-exchange chromatography (Q-Sepharose Fast Flow, Pharmacia) employing a 2.5 × 20 cm column. Elution was accomplished using a 300-mL linear gradient of 0–0.5 M NaCl in 0.05 M Tris–acetate, pH 8.3. Column fractions were monitored at 280 nm and by polyacrylamide gel electrophoresis. The fractions containing catalytic subunit were pooled and concentrated followed by dialysis into phosphate buffer (40 mM KH_2PO_4, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.0), in preparation for further purification by size-exclusion chromatography. The enzyme in a volume of approximately 1.5 mL was then applied to an Ultrogel AcA34 column (LBF Biotechnics) (1.5 × 100 cm) and was eluted with phosphate buffer at a flow rate of 7.2 mL/h. The fractions containing the catalytic subunit were pooled and concentrated before use. On the basis of nondenaturing polyacrylamide gel electrophoresis, the catalytic subunit was judged greater than 98% pure.

pH Dependence. The activity of the wild-type and the mutant catalytic subunits was measured at constant substrate concentrations as the pH was varied. To keep the ionic strength constant, a three-part buffer system of 2-(*N*-morpholino)ethanesulfonic acid, *N*-ethylmorpholine, and diethanolamine was employed (Leger & Hervé, 1988). The pH of a duplicate set of assay tubes, with all constituents except the enzyme present, was measured to verify the pH.

[2] The notation used to name the mutane enzymes is, for example, the Arg-54 → 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.

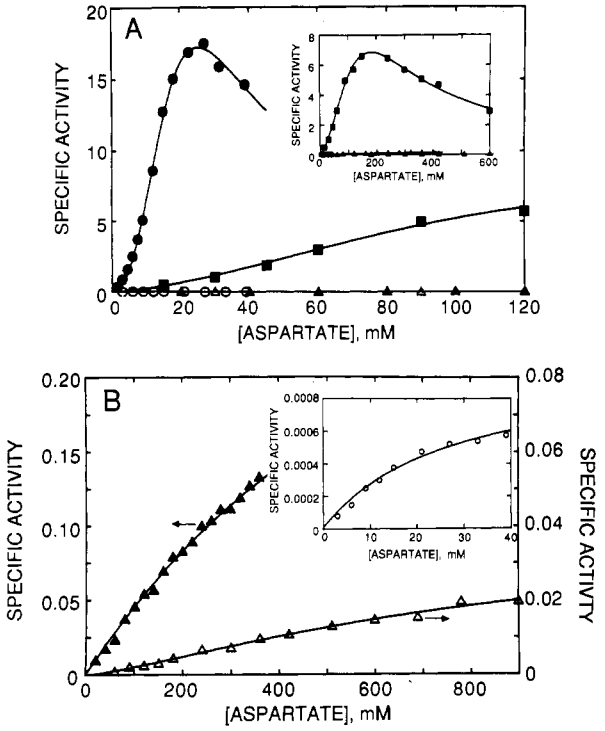

FIGURE 2: Aspartate saturation curves of mutant and wild-type holoenzymes. Specific activity is reported in millimoles of *N*-carbamylaspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate (pH 8.3) at constant levels of carbamyl phosphate (4.8 mM for the wild-type and the Arg-54 → Ala enzymes, 20 mM for the Arg-105 → Ala enzyme, and 15 mM for the Gln-137 → Ala and the Gln-137 → Asn enzymes). (A) Saturation curves for the wild-type (●), the Arg-54 → Ala (○), the Arg-105 → Ala (△), the Gln-137 → Ala (▲), and the Gln-137 → Asn (■) enzymes. (Inset) Expanded region corresponding to high aspartate concentrations; wild type not shown. (B) Saturation curves of the Arg-105 → Ala (△) and the Gln-137 → Ala (▲) enzymes. As indicated by the arrows, the left-hand ordinate corresponds to the Gln-137 → Ala enzyme, and the right-hand ordinate corresponds to the Arg-105 → Ala enzyme. (Inset) Saturation curve of the Arg-54 → Ala (○) enzyme.

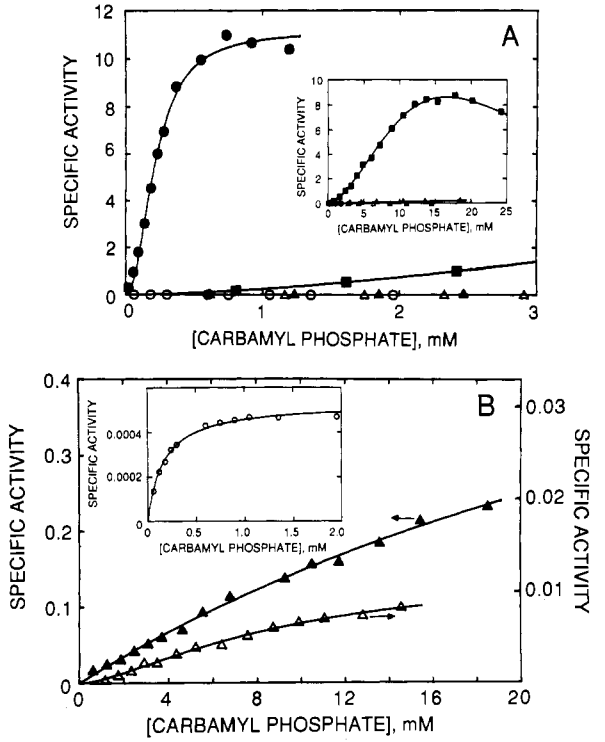

FIGURE 3: Carbamyl phosphate saturation curves of mutant and wild-type holoenzymes. Specific activity is reported in millimoles of *N*-carbamylaspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate (pH 8.3) at constant levels of aspartate (30 mM for the wild-type and the Arg-54 → Ala enzymes; 600 mM for the Arg-105 → Ala and the Gln-137 → Asn enzymes, and 200 mM for the Gln-137 → Ala enzyme). (A) Saturation curves for the wild-type (●), the Arg-54 → Ala (○), the Arg-105 → Ala (△), the Gln-137 → Ala (▲), and the Gln-137 → Asn (■) enzymes. (Inset) Expanded region corresponding to high carbamyl phosphate concentrations; wild type not shown. (B) Saturation curves of the Arg-105 → Ala (△) and the Gln-137 → Ala (▲) enzymes. As indicated by the arrows, the left-hand ordinate corresponds to the Gln-137 → Ala enzyme, and the right-hand ordinate corresponds to the Arg-105 → Ala enzyme. (Inset) Saturation curve of the Arg-54 → Ala (○) enzyme.

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 enzyme without ligands (Ke et al., 1984), the CTP–enzyme complex (Kim et al., 1987), the PALA–enzyme complex (Krause et al., 1987), and the enzyme with CP plus succinate bound (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

Construction of the Mutant Aspartate Transcarbamylases by Site-Directed Mutagenesis. The Arg-54 → Ala, the Arg-105 → Ala, the Gln-137 → Asn, and the Gln-137 → Ala mutant versions of aspartate transcarbamylase were constructed by site-directed mutagenesis employing the method of Zoller and Smith (1982) using strains incapable of mismatch repair (Carter et al., 1985; see also Experimental Procedures).

Kinetic Properties of the Wild-Type and Mutant Holoenzymes. The substrate saturation curves for the wild-type and mutant holoenzymes are compared in Figures 2 and 3, and a summary of the kinetic data is given in Table I. The most conspicuous feature of these data is the reduced maximal velocity of these mutant enzymes. The maximal velocity for the Arg-54 → Ala enzyme is reduced by approximately 17 000-fold, while the reductions in maximal velocity for the Arg-105 → Ala, Gln-137 → Ala, and the Gln-137 → Asn

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

enzyme	V_{max}[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	11.8	0.2	2.2	2.0
Arg-54 → Ala	0.001	26	0.2	1[c]	1
Arg-105 → Ala	0.015	310	7.0	2	1.5
Gln-137 → Ala	0.4	900[d]	42[d]	1	1
Gln-137 → Asn	6.9	67	6.6	1.5	1.6

[a] These data are derived from Figures 2 and 3. For the wild-type and mutant enzymes that exhibit cooperativity, the V_{max} and Hill coefficient (n_H) were calculated by a nonlinear least-squares procedure using a modified Hill equation which incorporates substrate inhibition (Pastra-Landis et al., 1978). For the mutant enzymes without cooperativity, the data were fit by the same procedure to the Michaelis–Menten equation with or without a term for substrate inhibition as necessary. [b] For the enzymes which exhibit substrate inhibition, the V_{max} represents the maximal observed specific activity from the aspartate saturation curve. [c] Because of the very low specific activity of this enzyme, it was impossible to determine if the enzyme retains a small amount of cooperativity. [d] This enzyme requires extremely high concentrations of substrates to reach the maximal activity. Aspartate saturation curves cannot be obtained at saturating CP concentrations because of the low solubility of CP. Therefore, the values of $[S]_{0.5}^{Asp}$ and $[S]_{0.5}^{CP}$ are approximate.

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

enzyme	V_{max} (mmol·h^{-1}·mg^{-1})	K_m^{Asp} (mM)	K_m^{CP} (mM)	k_{cat}/K_m^{Asp} (s^{-1}·mM^{-1})
wild type	25.9[a]	6.7	0.02	37.2
Arg-54 → Ala	0.00036	8.3	0.09	0.004
Arg-105 → Ala	0.15	450[b]	23	0.003
Gln-137 → Ala	5.3	320	3	0.16
Gln-137 → Asn	28	47	4	5.7

[a] The V_{max} represents the maximal observed specific activity from the aspartate saturation curve. [b] The Arg-105 → Ala catalytic subunit exhibits cooperativity with aspartate but not with CP; therefore, the value reported is an $[S]_{0.5}$.

enzymes are approximately 1100-fold, 40-fold, and 2.5-fold, respectively.

The aspartate concentration at half the maximal velocity, $[S]_{0.5}^{Asp}$, is drastically increased for the Arg-105 → Ala and the Gln-137 → Ala enzymes. The $[S]_{0.5}^{Asp}$ for the wild-type enzyme of 11.8 mM increases to 310 mM for the Arg-105 → Ala enzyme and to greater than 900 mM[3] for the Gln-137 → Ala enzyme. The $[S]_{0.5}^{Asp}$ does not increase significantly for the Arg-54 → Ala enzyme but increases to 67 mM for the Gln-137 → Asn enzyme. The changes in the CP concentration at half the maximal velocity, $[S]_{0.5}^{CP}$, parallel the changes observed for the $[S]_{0.5}^{Asp}$. Specifically, the $[S]_{0.5}^{CP}$ for Gln-137 → Ala is approximately 42 mM[3] compared to 0.2 mM for the wild-type enzyme. The $[S]_{0.5}^{CP}$ is unaffected by the replacement of Arg-54 by Ala; however, both the Arg-105 → Ala and Gln-137 → Asn enzymes exhibit approximate 30-fold increases. The Arg-54 → Ala and the Gln-137 → Ala enzymes lose cooperativity for both substrates, while the Arg-105 → Ala and the Gln-137 → Asn enzymes still retain cooperativity for both substrates although their respective Hill coefficients are reduced (see Table I).

Kinetic Properties of the Wild-Type and Mutant Catalytic Subunits. As seen in Table II, the Arg-54 → Ala, Arg-105 → Ala, and Gln-137 → Ala catalytic subunits all exhibit reduced catalytic activity and increased $[S]_{0.5}^{Asp}$ and $[S]_{0.5}^{CP}$ compared to the wild-type enzyme. The Gln-137 → Asn catalytic subunit exhibits a slightly increased maximal velocity and substantially increased K_m^{Asp} and K_m^{CP} compared to the wild-type catalytic subunit (see Table II). Relative to the wild-type enzyme, the Arg-54 → Ala, Arg-105 → Ala, and Gln-137 → Ala enzymes are 70000-, 170-, and 5-fold less active, respectively. However, relative to their holoenzymes, the Arg-105 → Ala and Gln-137 → Ala catalytic subunits exhibit an approximate 10-fold increase in activity, whereas the Arg-54 → Ala catalytic subunit displays a 3-fold decrease in activity. The $[S]_{0.5}^{Asp}$ and $[S]_{0.5}^{CP}$ of the Arg-54 → Ala and Arg-105 → Ala enzymes are not significantly changed between the holoenzyme and the catalytic subunit, unlike the Gln-137 → Ala catalytic subunit, which has a 10-fold decrease in $[S]_{0.5}^{CP}$ compared to its holoenzyme.

The Arg-105 → Ala catalytic subunit is dramatically altered from wild-type behavior; the saturation curve for aspartate exhibits cooperativity with a Hill coefficient of approximately 2.0 (see Figure 4A) and an $[S]_{0.5}^{Asp}$ of approximately 450 mM. To confirm that there is cooperativity between the active sites of this mutant catalytic subunit, a succinate activation experiment was performed. For the wild-type holoenzyme at low concentrations of aspartate and saturating CP, low concentrations of succinate will activate the enzyme (Gerhart & Pardee, 1963) because the binding of succinate will convert the enzyme into the R state which has enhanced activity. Since succinate is a competitive inhibitor of the enzyme, as its concentration increases the activity falls. As seen in Figure 4B, wild-type catalytic subunit is not activated by succinate at low aspartate concentrations while the Arg-105 → Ala catalytic subunit is.

[3] The Gln-137 → Ala enzyme requires extremely high concentrations of substrates to reach maximal activity. Aspartate saturation curves cannot be obtained at saturating CP concentrations because of the low solubility of CP. In addition, the large variation of aspartate concentrations used alters the ionic strength significantly; therefore, the values of $[S]_{0.5}^{Asp}$ and $[S]_{0.5}^{CP}$ are approximate.

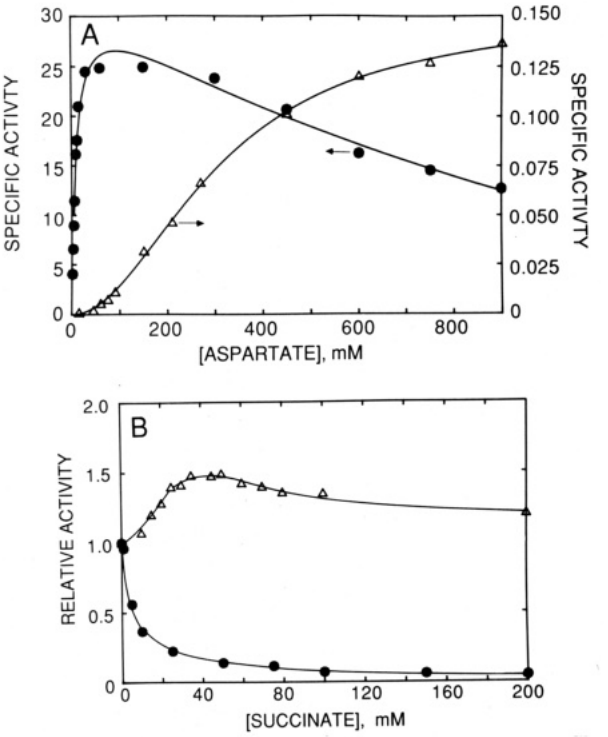

FIGURE 4: (A) Aspartate saturation curves of the wild-type (●) and the Arg-105 → Ala (△) catalytic subunits. Specific activity is reported in millimoles of *N*-carbamylaspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) at saturating levels of carbamyl phosphate (4.8 mM for the wild-type and 20 mM for the Arg-105 → Ala catalytic subunits). As indicated by the arrows, the left-hand ordinate corresponds to wild-type enzyme, and the right-hand ordinate corresponds to the Arg-105 → Ala enzyme. (B) Succinate activation curve of the wild-type (●) and the Arg-105 → Ala (△) catalytic subunits. Activity was measured at a low aspartate concentration (approximately 0.1 $[S]_{0.5}$) as the concentration of succinate was varied. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) with a saturating level of carbamyl phosphate.

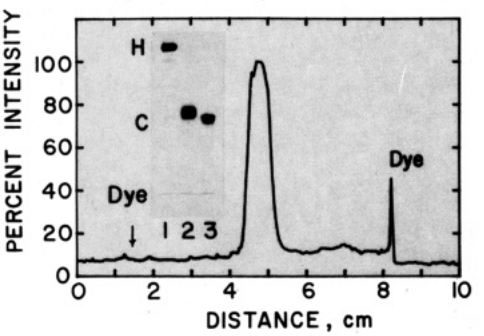

FIGURE 5: Nondenaturating gel and densitometer trace of the Arg-105 → Ala catalytic subunit. Lane 1, wild-type holoenzyme (H) of aspartate transcarbamylase; lane 2, wild-type catalytic subunit (C); lane 3, the Arg-105 → Ala catalytic subunit. The densitometer trace of lane 3 indicates the mutant catalytic subunit is greater than 98% pure, with no detectable amounts of holoenzyme even in this overloaded gel. The arrow at approximately 1.4 cm indicates the position that holoenzyme would be detected if present.

Table III: Binding of Carbamyl Phosphate to the Wild-Type and Mutant Holoenzymes

enzyme	K_D[a] for carbamyl phosphate (mM)	% difference ellipticity change[b]
wild type	0.02	100
Arg-54 → Ala	0.23	32
Arg-105 → Ala	0.16	25
Gln-137 → Ala	0.11	45
Gln-137 → Asn	0.02	85

[a] The K_D was determined by the change in the difference ellipticity as a function of CP concentration (see Experimental Procedures). [b] The difference ellipticity change is the maximum change in difference ellipticity over the wavelength range between 260 and 320 nm at saturating CP concentration and constant enzyme concentration.

Is the cooperativity of the Arg-105 → Ala catalytic subunit real? If the preparation of the Arg-105 → Ala catalytic subunit was contaminated with either wild-type holoenzyme or Arg-105 → Ala holoenzyme, a cooperative aspartate saturation curve may be observed. As seen in Figure 5, the Arg-105 → Ala catalytic subunit shows no significant amounts of a species that migrates at the position of holoenzyme. Therefore, if there is holoenzyme contamination, it must amount to less than a few percent. Furthermore, since the mutant catalytic subunit was produced from a strain lacking the *pyrI* gene, which codes for the regulatory chain, it is unlikely that either wild-type or mutant holoenzyme contamination is possible. At any level of contamination, wild-type holoenzyme could not explain the 450 mM $[S]_{0.5}^{Asp}$ observed for the Arg-105 → Ala catalytic subunit nor the requirement that nearly 40 mM succinate is required to maximally activate this enzyme at low aspartate concentrations (see Figure 4B). Contamination by the Arg-105 → Ala holoenzyme could explain the high $[S]_{0.5}^{Asp}$, but then CP cooperativity should also be observed. Furthermore, this contamination could not explain the 10-fold increase in activity of the Arg-105 → Ala catalytic subunit over the Arg-105 → Ala holoenzyme. We therefore believe that the observed cooperativity of the catalytic subunit of the Arg-105 → Ala enzyme is not an artifact.

Binding of Carbamyl Phosphate to the Wild-Type and Mutant Holoenzymes. Upon the binding of CP to aspartate transcarbamylase, the enzyme undergoes a conformational change that allows the subsequent binding of aspartate. This conformational change can be monitored by a variety of techniques including UV (Collins & Stark, 1969) and circular dichroism difference spectroscopy (Griffin et al., 1972). To determine if the binding of CP was altered by the amino acid substitutions that were made near the CP binding site, circular dichroism spectroscopy was employed (see Experimental Procedures). Table III gives the K_D^{CP} constants determined by circular dichroism spectroscopy plus the maximum difference in ellipticity extrapolated to infinite [CP]. The K_D^{CP} is not altered by the replacement of Gln-137 by Asn but is altered by all the other mutations. Compared to the wild type, which has a K_D^{CP} of 0.02 mM, the Arg-54 → Ala, the Arg-105 → Ala, and the Gln-137 → Ala enzymes have K_D^{CP}'s of 0.23, 0.16, and 0.11 mM, respectively. These three mutant enzymes also display a significant reduction in the magnitude of the total spectral change as compared to the wild-type enzyme (see Table III). For the Arg-54 → Ala, the Arg-105 → Ala, and the Gln-137 → Ala enzymes, these results are consistent with a reduction in affinity for CP and an alteration in the conformational change induced upon CP binding.

Binding of Carbamyl Phosphate, Acetyl Phosphate, and PALA to the Wild-Type and Mutant Catalytic Subunits. To determine the alterations in the CP binding site due to these single amino acid substitutions, the K_i values for acetyl phosphate and PALA were determined along with the K_D^{CP} values. As seen in Table IV, the Arg-105 to Ala substitution resulted in the largest alterations in these constants. The K_i^{PALA}, the K_D^{CP}, and the K_i^{AP} increase approximately 90 000-fold, 100-fold, and 10-fold, respectively, relative to the wild-type catalytic subunit. Both the Ala and Asn substitutions at Gln-137 have large effects on the K_D^{CP}, lesser effects on the K_i^{PALA}, and practically no effect on the K_i^{AP}. The Ala substitution at Arg-54 causes a 13-fold increase in the K_D^{CP} and had little effect on either K_i^{AP} or K_i^{PALA}.

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

catalytic subunit	K_D[a] for carbamyl phosphate (mM)	K_i[b] for acetyl phosphate (mM)	K_i[b] for PALA (μM)
wild type	0.022	0.58	0.024
Arg-54 → Ala	0.3	1.8	0.048
Arg-105 → Ala	2.4	5.4	2140
Gln-137 → Ala	1.1	1.2	0.16
Gln-137 → Asn	1.3	0.9	0.62

[a] The K_D of CP was determined kinetically by the method of Porter et al. (1969). Because of the low activity of some of the mutant enzymes, the K_D for all the catalytic subunits was determined at $0.2K_m^{Asp}$. [b] The K_i for PALA and acetyl phosphate was determined kinetically by the method of Collins and Stark (1971).

pH Dependences of the Arg-54 → Ala and Arg-105 → Ala Catalytic Subunits Are Altered. The profiles of activity versus pH for the wild-type and the mutant catalytic subunits were determined (data not shown). The maximum activity of the wild-type as well as the Gln-137 → Ala and Gln-137 → Asn catalytic subunits is at approximately pH 8.1. However, the pH curves for the Arg-54 → Ala and Arg-105 → Ala catalytic subunits have been shifted with the maxima occurring at approximately pH 8.6. These results are consistent with a shift in the pK_a of a catalytically important residue or residues to a higher pH.

Discussion

Conformational Change Induced by the Binding of CP Is Important for the Catalytic Reaction. A variety of physicochemical methods (Gerhart & Schachman, 1968; Collins & Stark, 1969; Griffin et al., 1972) indicate that aspartate transcarbamylase undergoes a conformational change upon the binding of CP. The importance of this conformational change has become evident from this work. When Arg-54, Arg-105, or Gln-137 is replaced, the binding of CP is weakened, and the conformational change normally induced by CP is altered, as judged by circular dichroism difference spectroscopy (see Table III). For the Arg-105 → Ala and the Gln-137 → Ala enzymes, the altered conformational change upon CP binding correlates with weakened binding of CP as measured by the K_D and is also reflected in a drastic increase in the $[S]_{0.5}^{CP}$ for the holoenzymes and catalytic subunits. Surprisingly, however, with the increase in the $[S]_{0.5}^{CP}$ for these mutant enzymes there are also large increases in the corresponding $[S]_{0.5}^{Asp}$. For example, the $[S]_{0.5}^{Asp}$ increases from 11.8 mM for the wild-type holoenzyme to 310 mM for the Arg-105 → Ala holoenzyme and to greater than 900 mM for the Gln-137 → Ala holoenzyme, and the $[S]_{0.5}^{Asp}$ increases from 5.7 mM for the wild-type catalytic subunit to 450 mM for the Arg-105 → Ala catalytic subunit and to 320 mM for the Gln-137 → Ala catalytic subunit. The alterations in $[S]_{0.5}^{Asp}$ for the Gln-137 mutants occur even though Gln-137 does not interact directly with succinate, or, by analogy, aspartate

(Gouaux & Lipscomb, 1988). The greatly reduced aspartate affinity in these mutant enzymes must be due to alterations in the conformational change that normally occurs upon the binding of CP which is thought to create the high-affinity aspartate binding site. The circular dichroism experiment provides direct evidence that the conformational change induced by CP is altered. However, this experiment cannot distinguish whether the observed spectral change corresponds to the same process that occurs in the wild-type enzyme to a lesser extent or some completely new conformational change that CP induces in these mutant enzymes. Nevertheless, the large decrease in the magnitude of the spectral change supports the contention that the same conformational change that occurs upon the binding of CP in the wild-type enzyme does not occur in these mutant enzymes. Furthermore, the kinetic and binding data of the mutant enzymes suggest that the CP-induced conformational change in the wild-type enzyme is critical for the formation of the high-affinity aspartate binding site and therefore for the catalytic reaction itself.

Arg-54 Is Essential for Catalysis. When Arg-54 of the catalytic chain of the enzyme is replaced by Ala, the resulting holoenzyme and catalytic subunit exhibit approximately 17 000-fold and 70 000-fold reductions in maximal activity, respectively. Along with the substantial loss of activity, the Arg-54 → Ala enzyme also exhibits an approximate 13-fold reduction in affinity for CP. The dramatic loss of activity caused by the replacement of Arg-54 by Ala suggests that Arg-54 is essential for catalysis. The inhibition constants for PALA and acetyl phosphate are essentially unchanged by the Arg-54 to Ala substitution, further suggesting that the loss in catalytic activity is the most important result of this amino acid substitution.

As seen in Figure 1, the guanidinium group of Arg-54 interacts with both the anhydride oxygen and a phosphate oxygen of CP. The functional studies reported here imply that Arg-54 is important catalytically for the enzymatic condensation of CP and aspartate but is less important for the binding of CP. The exact role that Arg-54 plays in catalysis is less clear, although the interaction between the side chain of Arg-54 and the anhydride oxygen of CP may help to promote the release of inorganic phosphate from the tetrahedral intermediate by charge neutralization of the leaving phosphate group (Kantrowitz & Lipscomb, 1988).

Arg-105 Is Essential for Catalysis. When Arg-105 of the catalytic chain of aspartate transcarbamylase is replaced by Ala, the resulting holoenzyme exhibits a 1000-fold reduction in maximal activity while the catalytic subunit exhibits only a 170-fold reduction in maximal activity. As opposed to the Arg-54 → Ala catalytic subunit, the Arg-105 → Ala catalytic subunit exhibits substantially reduced affinity for CP, acetyl phosphate, and PALA (see Table IV). The inhibition constant of PALA for the catalytic subunit of the Arg-105 → Ala enzyme is almost 100 000-fold larger than for the wild-type catalytic subunit. This tremendously weakened binding of PALA may simply be the result of a simultaneous loss in affinity at both the CP and aspartate binding sites due to the inability of CP to induce the correct conformational change upon binding and thereby preventing the formation of the high-affinity aspartate binding site.

As seen in Figure 1, the guanidinium group of Arg-105 interacts with oxygens of the carbonyl and the phosphate of CP and is close to the ring of another reported catalytically important residue, His-134 (Robey et al., 1986). The interactions of Arg-105 with PALA are almost identical with those shown in Figure 1 with CP (Krause et al., 1987). Clearly, Arg-105 is implicated in the binding of the negatively charged phosphate of CP and may also be important for catalysis via binding the transition state or could be involved more directly by polarizing the carbonyl of CP, thereby making the carbonyl carbon a better site for nucleophilic attack by aspartate.

Positive Charges in the Active Site Are Important for the Normal pH Optimum of the Catalytic Subunits. The removal of the positive charge of either Arg-54 or Arg-105 by replacement with alanine results in a slight increase in the pH optimum of the reaction, while the replacement of the uncharged Gln-137 by either alanine or asparagine has almost no effect. It has been proposed that the pH dependence of the wild-type catalytic subunit is chiefly due to His-134 (Leger & Hervé, 1988) which is close to the guanidinium groups of both Arg-105 and Arg-54 (Krause et al., 1987; Gouaux & Lipscomb, 1988). Interactions between either of these arginine residues and the histidine ring would lower the apparent pK_a of His-134 and therefore the pH optimum of the enzyme. Although these interactions may explain the alteration in the pH optimum of the enzyme, there may be other residues that are responsible. Furthermore, for the both the Arg-54 → Ala and the Arg-105 → Ala enzymes, the normal conformational change that occurs upon CP binding is altered, and therefore an altered active-site conformation may be responsible for the observed shift in the pH optimum. In this regard, it is important to determine the pH profile for the His-134 → Ala enzyme (Robey et al., 1986).

Cooperativity of the Arg-105 → Ala Catalytic Subunit. Somewhat unexpectedly, the catalytic subunit of the Arg-105 → Ala enzyme exhibits homotropic cooperativity for aspartate but not for CP. Although this is the first report of a cooperative catalytic subunit of aspartate transcarbamylase, trimeric ornithine transcarbamylase exhibits sigmoidal kinetics in the presence of the competitive inhibitor Zn^{2+} (Kuo et al., 1982), and hybrid versions of aspartate transcarbamylase with as few as two functional active sites still exhibit cooperativity (Gibbons et al., 1976). The observed cooperativity of the Arg-105 → Ala catalytic subunit can be rationalized if one assumes the wild-type catalytic subunit always has a high-affinity aspartate binding site corresponding to the R state of the wild-type holoenzyme. Since the wild-type catalytic subunit never exists in the T state, it can never manifest cooperativity. The mutation at Arg-105 results in weakened binding of aspartate, suggesting that the high-affinity aspartate binding site is not formed, and perhaps this catalytic subunit is more like the T state of the wild-type holoenzyme. If the mutant catalytic subunit is in an abnormal low-affinity form with respect to aspartate, the binding of succinate in the presence of CP may induce a conformational change which is sufficient to alter the affinity and activity at the other active sites within the trimer, and therefore succinate would activate the enzyme at low aspartate concentration (Figure 4B), and the enzyme would exhibit cooperativity (Figure 4A). Further studies are required to establish in detail the nature of the observed cooperativity of the Arg-105 → Ala catalytic subunit.

Rate-Determining Steps for the Holoenzyme and Catalytic Subunit Are Different. The catalytic subunits of the Arg-105 → Ala and the Gln-137 → Ala enzymes are approximately 10-fold more active than the corresponding holoenzymes. This increase in activity upon the dissociation of the regulatory subunits is substantially larger than the 2-fold increase observed for the wild-type enzyme. This suggests that the rate-limiting step is different for the holoenzyme and the catalytic subunit and agrees with the results from equilibrium isotope exchange kinetics which have determined that the

rate-limiting step for the wild-type holoenzyme is the allosteric transition, a step that does not occur in the catalytic subunit (Hsuanyu & Wedler, 1987). The results reported here are consistent with the Arg-105 to Ala and the Gln-137 to Ala substitutions having two different effects: an effect on the allosteric transition of the enzyme and also an effect on catalysis alone, the allosteric transition being the rate-limiting step only for the allosteric holoenzyme.

Gln-137 Is Important for the Binding of Carbamyl Phosphate. When Gln-137 of the catalytic chain of aspartate transcarbamylase is replaced by Ala, the holoenzyme and catalytic subunit exhibit approximately 40-fold and 5-fold decreases in maximal activity, respectively, while the replacement of Gln-137 by Asn has only a small effect on the maximal activity for either the holoenzyme or the catalytic subunit. In contrast, both the Gln-137 → Ala and the Gln-137 → Asn enzymes exhibit significantly reduced affinities for both CP and aspartate.

The X-ray structure of wild-type holoenzyme complexed with CP and succinate (Gouaux & Lipscomb, 1988) indicates that the carbonyl group of the side chain of Gln-137 can hydrogen bond to the amino group of CP, an interaction which is completely abolished in the Gln-137 → Ala enzyme. The fact that the Gln-137 → Ala catalytic subunit exhibits reduced affinity for CP but the maximal activity is not significantly reduced implies that Gln-137, in the wild-type enzyme, is involved in CP binding and is relatively unimportant for catalysis. Since the affinity of the Gln-137 → Asn holoenzyme is not reduced nearly as much as for the Gln-137 → Ala enzyme, it may be possible for the carbonyl group of Asn at position 137 to still interact with the amino group of CP, especially if the side chain of Asn is fully extended.

The fact that the inhibition constant for acetyl phosphate is not significantly altered by the substitutions at Gln-137, and the binding constant of CP is dramatically altered by both substitutions, suggests that Gln-137 in the wild-type enzyme interacts with the amino group of CP and it is important for CP binding. Therefore, it is not surprising that both mutant enzymes at position 137 can no longer discriminate between carbamyl phosphate and acetyl phosphate.

As mentioned above, the side chain of Gln-137 in the wild-type enzyme is important for the conformational change that occurs upon the binding of CP. The X-ray crystallographic results indicate that Gln-137 does not interact with PALA; however, the binding of PALA is altered by the mutations at position 137, further supporting the notion that the conformational change that occurs upon CP binding is necessary to create the high-affinity aspartate binding site. It is tempting to speculate that Gln-137 and the backbone carbonyls of residues 266 and 267 that interact with the amino group of CP (see Figure 1) assist in catalysis by deformation of the nitrogen–carbon bond from sp^2 toward sp^3 hybridization. This alteration in the hybridization of the carbonyl carbon would enhance catalysis and would be consistent with the NMR data. New NMR experiments are necessary with these mutant enzymes to further refine the function of Gln-137 in the catalytic mechanism.

Refinement of the Catalytic Mechanism of Aspartate Transcarbamylase. Several catalytic mechanisms have been proposed for the reaction catalyzed by aspartate transcarbamylase (Voltz et al., 1986; Collins & Stark, 1969, 1971; Jacobson & Stark, 1973; Gouaux et al., 1987; Gouaux & Lipscomb, 1988). By using site-directed mutagenesis, as a means to probe specific amino acid residues in the active site of the enzyme, we have been able to confirm previous speculation about the function of these residues in catalysis, and we have used these functional studies to further refine the catalytic mechanism of the enzyme.

Several previous studies have provided information critical to the formulation of a possible mechanism for aspartate transcarbamylase. Equilibrium isotope exchange kinetics (Wedler & Gasser, 1974; Hsuanyu & Wedler, 1987) have confirmed steady-state kinetic studies (Porter et al., 1969) establishing that the reaction is ordered with CP binding before aspartate and carbamyl aspartate being released before P_i. A variety of physicochemical methods all indicate that the enzyme undergoes a conformational change upon the binding of CP (Gerhart & Schachman, 1968; Collins & Stark, 1969; Griffin et al., 1972), and NMR studies suggest that the carbonyl of CP in the presence of succinate, is protonated (Roberts et al., 1976). The analysis of the crystal structure of the enzyme complexed with CP and succinate indicates that the side chains of residues Ser-52, Thr-53, Arg-54, Thr-55, Arg-105, His-134, and Gln-137 from one catalytic chain as well as Ser-80 and Lys-84 from an adjacent catalytic chain interact with CP. Furthermore, most of these interactions are also observed in the enzyme complex with PALA. The similarity between the interactions observed with CP plus succinate and PALA is quite reasonable since PALA may, in fact, be a better analogue of CP and succinate than CP and aspartate (Gouaux & Lipscomb, 1988). Therefore, both structures may be more representative of the structure of the transition state than the unreacted starting materials. The residues examined in this study, Arg-54, Arg-105, and Gln-137, when altered, all affect catalysis.

Using the results reported here, we can further refine the catalytic mechanism of aspartate transcarbamylase. The ordered binding of CP is critical for the reaction. Of the various amino acid side chains investigated here, Arg-105 and Gln-137 are the more important for the binding of CP, with Gln-137 also contributing to the enzymatic specificity for CP. Arg-54, Arg-105, and Gln-137 all interact with CP, and this binding energy drives the observed conformational change which positions Thr-55, His-134, and Arg-105 correctly to polarize the carbonyl of CP and make it a better site for nucleophilic attack. This conformational change also increases the affinity of the aspartate binding site, allowing aspartate to bind. Aspartate not only interacts with side chains of the aspartate domain, which correctly orient the molecule for reaction, but may also interact with Arg-105 of the CP domain. The ability of Arg-105 to simultaneously interact with both CP and aspartate may assist catalysis by drawing aspartate toward CP. The multiple interactions of Arg-105 explain the drastic loss of activity, reduced affinity for CP, and more importantly reduced affinity for aspartate when this residue is replaced. The decrease in aspartate affinity due to changes at the CP binding site could also be explained on the basis of the conformational change normally induced by CP. Since this conformational change is necessary for aspartate binding, changes in the CP site that would alter this conformational change or reduce its magnitude would be expected to also affect aspartate affinity.

Arg-54, Arg-105, His-134, and Gln-137 may all assist in catalysis. Both Arg-105 and His-134 may polarize the carbonyl of CP, and His-134 may also act as a general base to abstract a proton from the incoming aspartate (Gouaux et al., 1987). Gln-137 may also be important for catalysis if the interaction between Gln-137 and CP induces strain in the nitrogen–carbon bond which would shift the hybridization of the carbonyl carbon of CP from sp^2 toward sp^3. As the

transition state of the reaction is reached with the formation of the tetrahedral intermediate, it most likely catalyzes its own decomposition (Gouaux et al., 1987) aided by Arg-54 which promotes the release of phosphate from the tetrahedral intermediate by charge stabilization (Kantrowitz & Lipscomb, 1988). With the availability of these mutant enzymes, additional insights into the catalytic mechanism of aspartate transcarbamylase should be possible by using additional physicochemical methods.

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Registry No. Arg, 74-79-3; Gln, 56-85-9; Ala, 56-41-7; Asn, 70-47-3; Asp, 56-84-8; CP, 590-55-6; PALA, 51321-79-0; AP, 590-54-5; aspartate transcarbamylase, 9012-49-1.

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