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A Loop Involving Catalytic Chain Residues 230–245 Is Essential for the Stabilization of Both Allosteric Forms of *Escherichia coli* Aspartate Transcarbamylase†

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ABSTRACT: The allosteric transition of *Escherichia coli* aspartate transcarbamylase involves significant alterations in structure at both the quaternary and tertiary levels. On the tertiary level, the 240s loop (residues 230–245 of the catalytic chain) repositions, influencing the conformation of Arg-229, a residue near the aspartate binding site. In the T state, Arg-229 is bent out of the active site and may be stabilized in this position by an interaction with Glu-272. In the R state, the conformation of Arg-229 changes, allowing it to interact with the β-carboxylate of aspartate, and is stabilized in this position by a specific interaction with Glu-233. In order to ascertain the function of Arg-229, Glu-233, and Glu-272 in the catalytic and cooperative interactions of the enzyme, three mutant enzymes were created by site-specific mutagenesis. Arg-229 was replaced by Ala, while both Glu-233 and Glu-272 were replaced by Ser. The Arg-229 → Ala and Glu-233 → Ser enzymes exhibit 10000-fold and 80-fold decreases in maximal activity, respectively, and they both exhibit a 2-fold increase in the aspartate concentration at half the maximal observed velocity, $[S]_{0.5}$. The Arg-229 → Ala enzyme still exhibits substantial homotropic cooperativity, but all cooperativity is lost in the Glu-233 → Ser enzyme. The Glu-233 → Ser enzyme also shows a 4-fold decrease in the carbamyl phosphate $[S]_{0.5}$, while the Arg-229 → Ala enzyme shows no change in the carbamyl phosphate $[S]_{0.5}$ compared to the wild-type enzyme. The Glu-272 to Ser mutation results in a slight reduction in maximal activity, an increase in $[S]_{0.5}$ for both aspartate and carbamyl phosphate, and reduced cooperativity. Analysis of the isolated catalytic subunits from these three mutant enzymes reveals that in each case the changes in the kinetic properties of the isolated catalytic subunit are similar to the changes caused by the mutation in the holoenzyme. PALA was able to activate the Glu-233 → Ser enzyme, at low aspartate concentrations, even though the mutant holoenzyme did not exhibit any cooperativity, indicating that cooperative interactions still exist between the active sites in this enzyme. It is proposed that Glu-233 of the 240s loop helps create the high-activity–high-affinity R state by positioning the side chain of Arg-229 for aspartate binding while Glu-272 helps stabilize the low-activity–low-affinity T state by positioning the side chain of Arg-229 so that it cannot interact with aspartate. Evidence presented here as well as from previous crystallographic and mutagenesis studies suggests that 240s loop interactions are critical for stabilizing both allosteric forms of the enzyme. A model is proposed that suggests that the stabilization of alternate conformations of the 240s loop, by specific side chain interactions, provides mechanisms by which homotropic cooperativity is manifest, and provides a molecular level explanation for a concerted allosteric transition in aspartate transcarbamylase.

Escherichia coli aspartate transcarbamylase (EC 2.1.3.2) catalyzes the first step in pyrimidine biosynthesis, the reaction of carbamyl phosphate and L-aspartate to form *N*-carbamyl-L-aspartate and inorganic phosphate. The enzyme exhibits a sigmoidal-shaped saturation curve with both of its substrates indicating cooperative interactions between the six active sites of the holoenzyme[1] (M_r 310000). The conversion of the enzyme from a T state having low substrate affinity and low catalytic activity to an R state having high substrate affinity and high catalytic activity has been proposed to occur in a concerted fashion based on kinetic, physicochemical, and structural analyses of both wild-type and mutant versions of the enzyme (Foote & Schachman, 1985; Krause et al., 1987;

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[1] Abbreviations: holoenzyme, native enzyme composed of two catalytic trimers and three regulatory dimers; PALA, *N*-(phosphonoacetyl)-L-aspartate; Tris, tris(hydroxymethyl)aminomethane; T and R states, tense and relaxed conformations of the enzyme having low activity and affinity for substrates and high activity and affinity for substrates, respectively; $[S]_{0.5}$, substrate concentration at half the maximal observed specific activity; 80s loop, flexible loop of the enzyme comprising approximately amino acid residues 76–86 of the catalytic chain; 240s loop, flexible loop of the enzyme comprising approximately amino acid residues 230–245 of the catalytic chain.

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Ladjimi & Kantrowitz, 1988). Comparison of the X-ray structures in the T (Kim et al., 1987) and R forms (Krause et al., 1985, 1987) reveals that during the conversion to the R state the enzyme undergoes dramatic tertiary and quaternary conformational changes. Changes within a catalytic chain at the tertiary level include domain closure, in which the aspartate domain moves approximately 3 Å toward the carbamyl phosphate domain, resulting in the formation of the active site. This movement of the aspartate domain is accompanied by the movement of two loops, a loop involving residues 76–86 (80s loop) and a loop involving residues 230–245 (240s loop). The reorientation of residues in the 240s loop results in the breaking of a specific set of interactions in the T state and the formation of another set of interactions in the R state (Krause et al., 1987).

We have previously investigated a number of residues within the 240s loop which form different interactions depending upon which allosteric state the enzyme is in. The interaction between Tyr-240 and Asp-271 and the intersubunit interactions between Glu-239 and both Lys-164 and Tyr-165 have been shown to be important for the stabilization of the T state (Middleton & Kantrowitz, 1986, 1988; Ladjimi & Kantrowitz, 1988) while the interdomain bridging interactions between Glu-50 and both Arg-234 and Arg-167, within a catalytic chain, have been shown to be important for the stabilization of the R state (Ladjimi et al., 1988; Middleton & Kantrowitz, 1988). Since all of these interactions involve residues of the 240s loop, it appears that this loop plays a central role in the stabilization of both allosteric forms of the enzyme.

Further examination of the T-state (Kim et al., 1987) and R-state (Krause et al., 1987) structures in the region of the 240s loop reveals another residue, Glu-233, which forms different interactions between the two conformational states of the enzyme. In the R-state structure, Glu-233 forms an intrachain salt link with Arg-229, a residue which interacts with the portion of PALA corresponding to the β-carboxylate of aspartate (Krause et al., 1987). In the T-state structure, the Glu-233–Arg-229 interaction does not occur, and Arg-229 interacts with a different glutamate, Glu-272.[2] Furthermore, in the R state, Arg-229 appears to be bent into the active site and held in position by the salt link with Glu-233 while in the T state Arg-229 is bent out of the active site and held in position by the salt link with Glu-272. Thus, it appears that Arg-229 may be involved in a switching mechanism between two conformational states of the active site which may have functional significance in the catalytic and regulatory properties of the enzyme.

In order to ascertain the function of Glu-233, Arg-229, and Glu-272 in the catalytic and cooperative interactions of aspartate transcarbamylase, we decided to create three mutant enzymes by site-directed mutagenesis techniques. Arg-229 was replaced with alanine (Arg-229 → Ala enzyme[3]), Glu-233 was replaced with serine (Glu-233 → Ser enzyme), and Glu-272 was also replaced with serine (Glu-272 → Ser enzyme). Following the functional analyses of these mutant enzymes, we present a model for the central role of the 240s loop in optimizing catalysis and in the concerted allosteric transition of aspartate transcarbamylase.

[2] The T-state interaction between Glu-272 and Arg-229 is only observed in one of the two catalytic chains in the asymmetric unit of the crystal (Kim et al., 1987).

[3] The notation used to name the mutant enzymes is, for example, the Arg-229 → 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.

Experimental Procedures

Materials

Potassium phosphate (monobasic), EDTA (disodium salt), carbamyl phosphate, *N*-carbamyl-L-aspartate, agar, L-aspartate, ampicillin, and uracil were purchased from Sigma Chemical Co. The carbamyl phosphate was purified in 5-g batches by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C (Gerhart & Pardee, 1962). Electrophoresis-grade acrylamide, urea, agarose, Tris, and enzyme-grade ammonium sulfate were obtained from ICN Biochemicals. Casamino acids, yeast extract, and tryptone were from Difco, and 2-mercaptoethanol was from Baker. Restriction endonucleases were purchased from U.S. Biochemicals or New England Biolabs and used according to the supplier's recommendations. T4 DNA ligase, T4 polynucleotide kinase, and the Klenow fragment of DNA polymerase I were purchased from U.S. Biochemicals. NA45 paper and nitrocellulose filters were obtained 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. Plasmid pUC119 and the M13 phage M13K07 were obtained from J. Messing, Rutgers University.

Methods

Enzyme Isolation and Purification. The wild-type and mutant versions of aspartate transcarbamylase were isolated as described by Nowlan and Kantrowitz (1985), from *E. coli* strain EK1104 [F⁻*ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *rspL*, *pyrF*±] containing either plasmid pEK38 (Ladjimi & Kantrowitz, 1988), pEK63, pEK71, or pEK75 for the wild-type, the Arg-229 → Ala, the Glu-233 → Ser, or the Glu-272 → Ser enzyme, respectively.

The purity of the enzyme preparations was checked by electrophoresis on nondenaturing polyacrylamide gels (Ornstein, 1964; Davis, 1964), and the concentrations of the purified wild-type and mutant enzymes were determined by Bradford's dye binding assay (Bradford, 1976) as modified by Bio-Rad Laboratories.

Isolation and Purification of Catalytic Subunits. The catalytic subunits of the wild-type and Glu-272 → Ser enzymes were isolated as described previously (Kantrowitz & Lipscomb, 1977). The catalytic subunits for the mutant enzymes with lower activity, Arg-229 → Ala and Glu-233 → Ser, were isolated in large quantities by using EK1104 containing the appropriate plasmid, pEK66 or pEK72, in which the majority of the gene coding for the regulatory subunit (*pyrI*) had been deleted by methods described previously (Nowlan & Kantrowitz, 1985). Due to the deletion in *pyrI*, EK1104 carrying these plasmids will only overproduce the catalytic subunit. For the isolation of catalytic subunit, 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 cells were harvested, they were ruptured by sonication (Branson Cell Disruptor 200), and cellular debris was removed by centrifugation. Following 70% ammonium sulfate precipitation, the protein was dialyzed into Tris buffer (0.05 M Tris–acetate, pH 8.3) followed by ion-exchange chromatography (Q-Sepharose Fast Flow resin from Pharmacia) on a 2.5 cm × 20 cm column using a 300-mL linear gradient of 0–0.5 M KCl in Tris buffer at a flow rate of 0.75 mL/min. Fractions containing catalytic subunit were identified by polyacrylamide gel electrophoresis, pooled, concentrated, and dialyzed into phosphate buffer (40 mM KH_2PO_4, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.0). The mutant catalytic subunits were then purified by size exclusion chromatography (Ultrogel AcA 34 resin from IBF Biotech-

nics) on a 1.5 cm × 100 cm column which was eluted with phosphate buffer at a flow rate of 7.2 mL/h.

Aspartate Transcarbamylase Assay and Data Analysis. The transcarbamylase activity of the wild-type and mutant enzymes was determined at 25 °C and pH 8.3 using a colorimetric assay (Pastra-Landis et al., 1981). All colorimetric assays were performed in duplicate, and the data points shown in the figures are the average. The steady-state kinetic data were analyzed as described by Silver et al. (1983), and the structural data were analyzed on the basis of three-dimensional coordinates of the CTP-liganded (Kim et al., 1987) and PALA-liganded enzymes (Krause et al., 1987) using the program FRODO (Department of Biochemistry, Rice University) on an Evans & Sutherland PS390 interfaced to a MicroVAX Q5.

Oligonucleotide Synthesis. The various oligonucleotides used in the site-directed mutagenesis procedure and the sequencing primers used were synthesized on an Applied Biosystems 381A DNA synthesizer.

RESULTS

Site-Directed Mutagenesis. (i) Construction of the Arg-229 to Ala Mutation. The substitution of alanine for Arg-229 in the catalytic chain of aspartate transcarbamylase was achieved by site-directed mutagenesis using the method of Zoller and Smith (1982) with the modifications described previously (Carter et al., 1985; Ladjimi et al., 1988). A total of 48 plaques were picked off the transformation plate and inoculated into 2X YT medium (Miller, 1972) from which single-stranded M13 DNA was isolated. Selection was accomplished by dot blot hybridization (Carter et al., 1984), and after dideoxy sequencing (Sanger et al., 1977), of eight candidates that exhibited dark spots on the nitrocellulose filters, four were observed to have the desired Arg to Ala change. The Arg-229 to Ala mutation was then cloned into pEK54 (Xu et al., 1988) by cutting both the mutant M13 RF DNA and pEK54 with *Bst*EII and *Bgl*II and, after separation by agarose gel electrophoresis, isolating the appropriate fragments using NA45 paper. The 630 base pair fragment containing the Arg-229 to Ala mutation and the pEK54 fragment containing the vector pUC119 and the remainder of the *pyrBI* operon were mixed, and after ligation and selection by transformation into U39a, a strain deleted for *pyrB*, a plasmid containing the Arg-229 to Ala mutation (pEK63) was isolated by alkaline lysis (Maniatis et al., 1982). Single-stranded plasmid DNA from pEK63 was isolated (Vieira & Messing, 1987), and the Arg-229 to Ala mutation was confirmed by dideoxy sequencing.

(ii) Construction of the Glu-233 to Ser and Glu-272 to Ser Mutations. The substitutions of serine for Glu-233 and serine for Glu-272 in the catalytic chain of aspartate transcarbamylase were accomplished as described above for the Arg-229 to Ala mutation with the following exceptions. After the 2 transformation mixtures were plated, 10 plaques from each were chosen at random and grown for the production of single-stranded M13 DNA. After dideoxy sequencing, 4 of the 10 Glu-233 to Ser mutant candidates and 2 of the 10 Glu-272 to Ser mutant candidates showed the desired Glu to Ser change. The Glu-233 to Ser and Glu-272 to Ser mutations were then moved into plasmid pEK38 by cutting each mutant M13 RF DNA and pEK38 with *Bst*EII and *Eco*RV and isolating the appropriate fragments as described above for the Arg-229 to Ala mutation. The two 864 base pair DNA fragments carrying the mutations were mixed separately with the pEK38 fragment carrying the vector pUC119 and the remainder of the *pyrBI* operon, and after ligation and transformation into U39a, plasmids pEK71 and pEK75 containing the Glu-233 to Ser and Glu-272 to Ser mutations, respectively, were isolated by alkaline lysis and checked for intact *Bst*EII and *Eco*RV sites and correct size by restriction analysis. Single-stranded plasmid DNA from pEK71 and pEK75 was isolated (Vieira & Messing, 1987), and the mutations were confirmed by dideoxy sequencing.

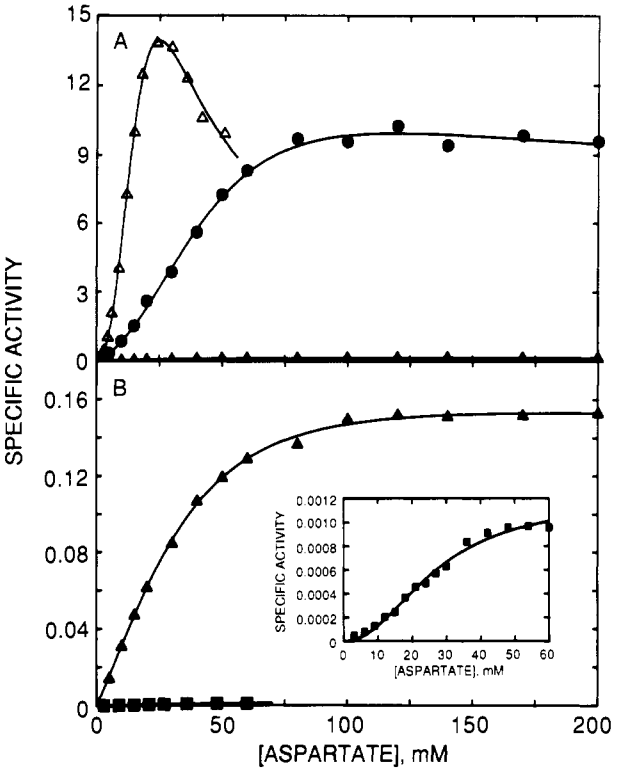

FIGURE 1: Aspartate saturation curves for the wild-type and mutant holoenzymes. (A) Data for the wild-type (▲), the Glu-272 → Ser (●), and the Glu-233 → Ser (▲) holoenzymes. (B) Region of low specific activity showing the data for the Glu-233 → Ser (▲) and the Arg-229 → Ala (■) holoenzymes. (B, inset) Detail of the low specific activity data for the Arg-229 → Ala holoenzyme (■). Assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) in the presence of saturating carbamyl phosphate (4.8 mM). Specific activity is in units of millimoles per hour per milligram.

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

	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	13.9	11.7	0.21	2.3	2.2
Arg-229 → Ala	0.0012	24	0.28	2.1	1.0
Glu-233 → Ser	0.15	25.7	0.046	1.0	1.0
Glu-272 → Ser	9.9	35.3	0.66	1.4	1.5

[a] These data were extracted from the saturation curves shown in Figures 1 and 2. [b] The V_{max} values are the maximal observed specific activities. For the wild-type, Arg-229 → Ala, and Glu-272 → Ser enzymes, the V_{max} and Hill coefficient (n_H) were calculated by a nonlinear least-squares procedure with a modified Hill equation that incorporates substrate inhibition (Pastra-Landis et al., 1978). The data for the Glu-233 → Ser enzyme were fit to a modified Michaelis–Menten equation that incorporates substrate inhibition.

Kinetic Properties of the Wild-Type and Mutant Holoenzymes. Comparison of the kinetic data presented in Figure 1 and summarized in Table I shows that the Arg-229 to Ala and Glu-233 to Ser mutations result in dramatic alterations in the enzyme while the changes caused by the Glu-272 to Ser mutation are less extreme. The Arg-229 to Ala mutation results in a more than 10000-fold decrease in maximal activity and a 2-fold increase in the $[S]_{0.5}$ for aspartate when compared with the wild-type enzyme (Figure 1 and Table I). The

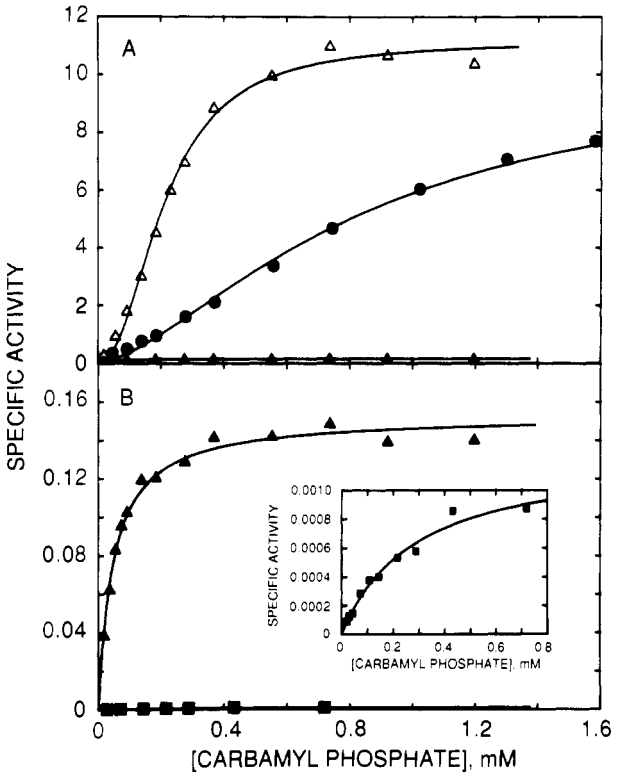

FIGURE 2: Carbamyl phosphate saturation curves for the wild-type and mutant holoenzymes. (A) Data for the wild-type (△), the Glu-272 → Ser (●), and the Glu-233 → Ser (▲) holoenzymes. (B) Region of low specific activity showing the data for the Glu-233 → Ser (▲) and the Arg-229 → Ala (■) holoenzymes. (B, inset) Detail of the low specific activity data for the Arg-229 → Ala holoenzyme (■). Assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) in the presence of saturating concentrations of aspartate (25 mM for the wild-type, 60 mM for the Arg-229 → Ala enzyme, and 100 mM for the Glu-272 → Ser and Glu-233 → Ser enzymes). Specific activity is in units of millimoles per hour per milligram.

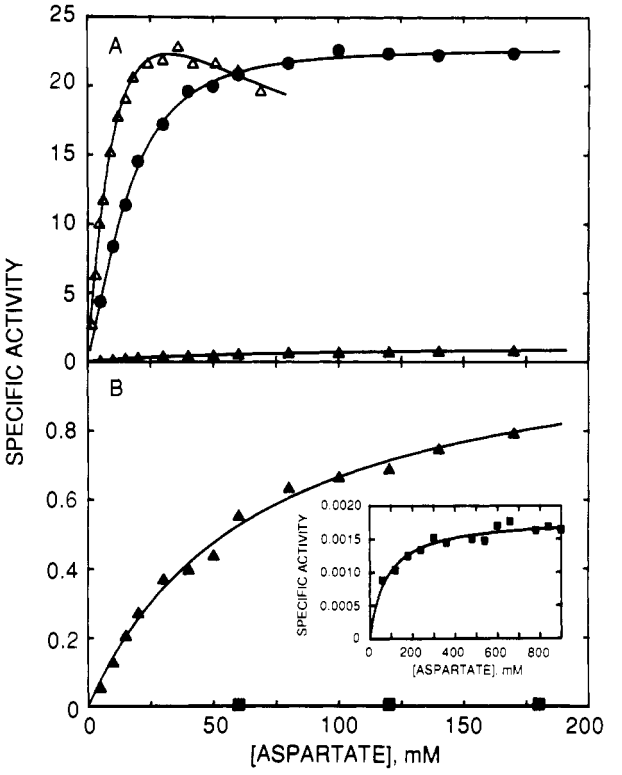

FIGURE 3: Aspartate saturation curves for the wild-type and mutant isolated catalytic subunits. (A) Data for the wild-type (△), the Glu-272 → Ser (●), and the Glu-233 → Ser (▲) catalytic subunits. (B) Region of low specific activity showing the data for the Glu-233 → Ser (▲) and the Arg-229 → Ala (■) catalytic subunits. (B, inset) Detail of the low specific activity data for the Arg-229 → Ala catalytic subunit. Specific activity is in units of millimoles per hour per milligram, and assays were performed under the conditions described in the legend to Figure 1.

Glu-233 to Ser mutation also causes a 2-fold increase in the $[S]_{0.5}$ for aspartate but results in a much less dramatic 80-fold reduction in maximal activity. Although the Arg-229 → Ala enzyme still exhibits substantial homotropic cooperativity as measured by the Hill coefficient, the Glu-233 → Ser enzyme shows no cooperative interactions (Figure 1 and Table I).

As can be seen from the carbamyl phosphate saturation curves (Figure 2), the $[S]_{0.5}$ for carbamyl phosphate for the Glu-233 → Ser enzyme actually decreases relative to the wild-type enzyme. A comparison of the $[S]_{0.5}$ for carbamyl phosphate of the Glu-233 → Ser and Arg-229 → Ala enzymes with that of the wild-type enzyme (Table I) indicates that while there is a decrease of over 4-fold for the Glu-233 → Ser enzyme, the Arg-229 → Ala enzyme has an unchanged $[S]_{0.5}$ for carbamyl phosphate. Both the Glu-233 → Ser and Arg-229 → Ala enzymes lack homotropic cooperative interactions involving carbamyl phosphate (Figure 2 and Table I).

The Glu-272 to Ser mutation causes much smaller changes in the kinetic properties of the enzyme when compared with the other mutant enzymes. This mutation results in a slight reduction in activity, an increased $[S]_{0.5}$ for both aspartate and carbamyl phosphate, and reduced cooperativity (Figures 1 and 2). When the values of $[S]_{0.5}$ for aspartate and carbamyl phosphate for the Glu-272 → Ser enzyme are compared (Table I), it can be seen that both increase by about a factor of 3 relative to the values for the wild-type enzyme. The amount of cooperativity observed for both substrates as measured by the Hill coefficient is significantly reduced in the Glu-272 → Ser enzyme, dropping from 2.6 to 1.4 and from 2.2 to 1.5 for aspartate and carbamyl phosphate, respectively.

Kinetic Properties of the Wild-Type and Mutant Catalytic Subunits. The Arg-229 to Ala, Glu-233 to Ser, and Glu-272 to Ser mutations cause changes in the kinetic properties of the isolated catalytic subunit that are similar to the changes caused by these mutations in the holoenzyme. As is the case for the Arg-229 → Ala holoenzyme, the Arg-229 → Ala catalytic subunit exhibits dramatic changes in kinetic properties when compared to the wild type. The Arg-229 to Ala mutation results in an over 12 000-fold decrease in maximal activity and a 13-fold increase in the K_m for aspartate in the isolated catalytic subunit (Table II and Figure 3). However, the K_m for carbamyl phosphate for the Arg-229 → Ala catalytic subunit is unchanged relative to the wild-type catalytic subunit.

Similar to the Arg-229 to Ala mutation, the Glu-233 to Ser mutation results in almost a 13-fold increase in the K_m for aspartate in the isolated catalytic subunit; however, the maximal activity is reduced by only 20-fold relative to the wild-type catalytic subunit (Table II and Figure 3). The K_m for carbamyl phosphate of the Glu-233 → Ser catalytic subunit, while slightly greater than the value for the wild-type catalytic subunit (Table II), is almost identical with the K_m for carbamyl phosphate of the Glu-233 → Ser holoenzyme (Table I).

The effects of the Glu-272 to Ser mutation on the kinetic properties of the isolated catalytic subunit are similar in magnitude and direction to the effects of this mutation on the kinetic properties of the holoenzyme. While the V_{max} remains unchanged, the K_m for aspartate and the K_m for carbamyl phosphate are both about 3-fold greater than the same values for the wild-type catalytic subunit (Table II). These results

Table II: Kinetic Parameters of the Wild-Type and Mutant Catalytic Subunits[a]

	V_{max}[b] (mmol·h^{-1}·mg^{-1})	K_m^{Asp} (mM)	K_m^{CP} (mM)[c]	k_{cat}[d] (s^{-1})	k_{cat}/K_m^{Asp} (s^{-1}·mM^{-1})
wild-type	22.3	5.7	0.027	214.7	37.7
Arg-229 → Ala	0.0018	77.3	0.027	0.017	0.0002
Glu-233 → Ser	1.1	66.8	0.043	10.6	0.16
Glu-272 → Ser	22.6	14.4	0.098	217.6	15.1

[a] The values of V_{max} and K_m for aspartate were calculated from the saturation data shown in Figure 3 by using a nonlinear least-squares procedure with the Michaelis–Menten equation. The data for the wild-type and Glu-233 → Ser enzymes were fit by the same procedure to a modified Michaelis–Menten equation that incorporates substrate inhibition. [b] The maximum observed specific activity. [c] The K_m values for carbamyl phosphate were determined in 0.05 M Tris–acetate buffer, pH 8.3, in the presence of saturating concentrations of aspartate, 30, 200, 200, and 100 mM for the wild-type, Arg-229 → Ala, Glu-233 → Ser, and Glu-272 → Ser catalytic subunits, respectively. [d] The turnover number for each enzyme was calculated per active site from the V_{max} values shown. Due to the presence of substrate inhibition in the aspartate saturation data (see Figure 3), the V_{max} values used to calculate the k_{cat} for the wild-type and Glu-272 → Ser catalytic subunits were the maximum observed velocities.

are similar to the results for the Glu-272 → Ser holoenzyme in which the $[S]_{0.5}$ for both aspartate and carbamyl phosphate also showed a 3-fold increase (Table I). This trend is reflected in the value of k_{cat} for the Glu-272 → Ser catalytic subunit which is nearly identical with that of the wild type while the k_{cat}/K_m^{Asp} ratio drops nearly 3-fold (Table II).

Activation of the Wild-Type and Mutant Enzymes by PALA. As can be seen in Figure 4, the Glu-233 → Ser enzyme is activated by PALA even though no cooperativity is observed for either substrate (see Figures 1 and 2). This indicates that cooperative interactions still exist between the active sites in the Glu-233 → Ser enzyme and that PALA is able to increase the affinity for aspartate at the unoccupied active sites while aspartate and carbamyl phosphate cannot. The maximal activation of the Glu-233 → Ser enzyme, however, is significantly less than that of the wild-type enzyme and occurs at a much greater concentration of PALA indicating that the affinity for PALA in the Glu-233 → Ser enzyme is reduced. In fact, even greater relative concentrations of PALA were necessary to inhibit the Glu-233 → Ser catalytic subunit than were necessary to inhibit the wild-type catalytic subunit (data not shown). The Arg-229 → Ala enzyme is also activated by PALA but to a lesser extent and at a greater concentration of PALA than the wild type, indicating that, similar to the Glu-233 → Ser enzyme, the Arg-229 → Ala enzyme has reduced affinity for PALA (Figure 4). The Glu-272 → Ser enzyme is activated by PALA with a reduced maximal effect, reflecting the reduced cooperativity of this mutant; however, the maximal activation occurs at approximately the same PALA concentration as for the wild type.

Discussion

Effects of the Arg-229 to Ala and Glu-233 to Ser Mutations on the Kinetic Properties of the Enzyme. (i) Link between Arg-229 and Glu-233. In the PALA-liganded structure of the enzyme (Krause et al., 1985, 1987), Arg-229 forms charged interactions with the β-carboxylate of the aspartate moiety of PALA and with Glu-233 (Figure 5). The interaction between Arg-229 and Glu-233 occurs only in the R state of the enzyme, and Glu-233 appears to hold Arg-229 in position for PALA binding. These structural data indicate that Arg-229 may be important for aspartate binding and that Glu-233 may be

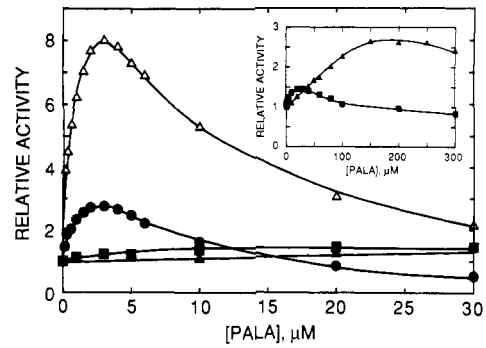

FIGURE 4: PALA saturation curves for the wild-type (△), the Glu-272 → Ser (●), the Glu-233 → Ser (▲), and the Arg-229 → Ala (■) holoenzymes. Assays were performed under the conditions described in the legend to Figure 1 at approximately $^1/_{12}$th the aspartate $[S]_{0.5}$ for the wild-type, the Glu-272 → Ser, and the Glu-233 → Ser holoenzymes (1, 3, and 2 mM aspartate, respectively) and at approximately one-fourth the aspartate $[S]_{0.5}$ for the Arg-229 → Ala holoenzyme (6 mM aspartate). (Inset) Data for the Glu-233 → Ser (▲) and Arg-229 → Ala (■) holoenzymes at higher PALA concentrations. The relative activity represents the ratio of specific activity in the absence or presence of PALA to that in its absence.

involved in stabilizing the R-state position of the side chain of Arg-229. In order to investigate the role these residues play in the catalytic and cooperative interactions of the enzyme, Arg-229 was replaced with Ala and Glu-233 was replaced with Ser using site-directed mutagenesis techniques.

(ii) Arg-229–Glu-233 Link Is Important for Optimal Catalytic Activity. The Arg-229 to Ala and Glu-233 to Ser mutations both result in dramatic reductions in the maximal activity of the enzyme (Figure 2 and Table I). The Arg-229 to Ala mutation causes a reduction in activity of over 10 000-fold while the Glu-233 to Ser mutation causes a reduction in activity of over 80-fold. These results indicate that both Arg-229 and Glu-233 are important for optimal catalytic activity. Since the $[S]_{0.5}$ for aspartate is nearly unchanged in both the Arg-229 → Ala and Glu-233 → Ser enzymes, the marked reduction in catalysis in each mutant enzyme is most likely due to improper orientation of aspartate. The structural evidence indicates that Arg-229 interacts directly with the β-carboxylate of aspartate (Volz et al., 1986; Krause et al., 1985, 1987; Gouaux & Lipscomb, 1988), suggesting that the improper orientation of aspartate and large reduction in catalysis in the Arg-229 → Ala enzyme are due to the loss of this interaction. Since Glu-233 does not interact directly with aspartate, the reduction in activity in the Glu-233 → Ser enzyme is probably due to the loss of the Glu-233–Arg-229 charged interaction. From the analysis of these two mutant enzymes, it seems likely that in the R state Glu-233 positions the side chain of Arg-229 such that aspartate is in the correct orientation for optimal catalysis.

The loss of activity due to the replacement of Arg-229 by Ala is significantly greater than that due to the replacement of His-134 by Ala (Robey et al., 1986), a residue implicated in the catalytic mechanism. Evidence suggests that His-134 is involved in the polarization of the carbonyl group of carbamyl phosphate (Roberts et al., 1976) and has been postulated to act as a general base to deprotonate the amino group of aspartate (Gouaux & Lipscomb, 1988). The greater reduction in activity of the Arg-229 → Ala relative to the His-134 → Ala enzyme suggests that for aspartate transcarbamylase, catalysis relies more on the correct binding and especially orientation of the substrates than on the actual bond formation [for a review, see Jencks (1987)].

(iii) Arg-229–Glu-233 Link Is Not Critical for Substrate Binding. The Arg-229 to Ala and Glu-233 to Ser mutations

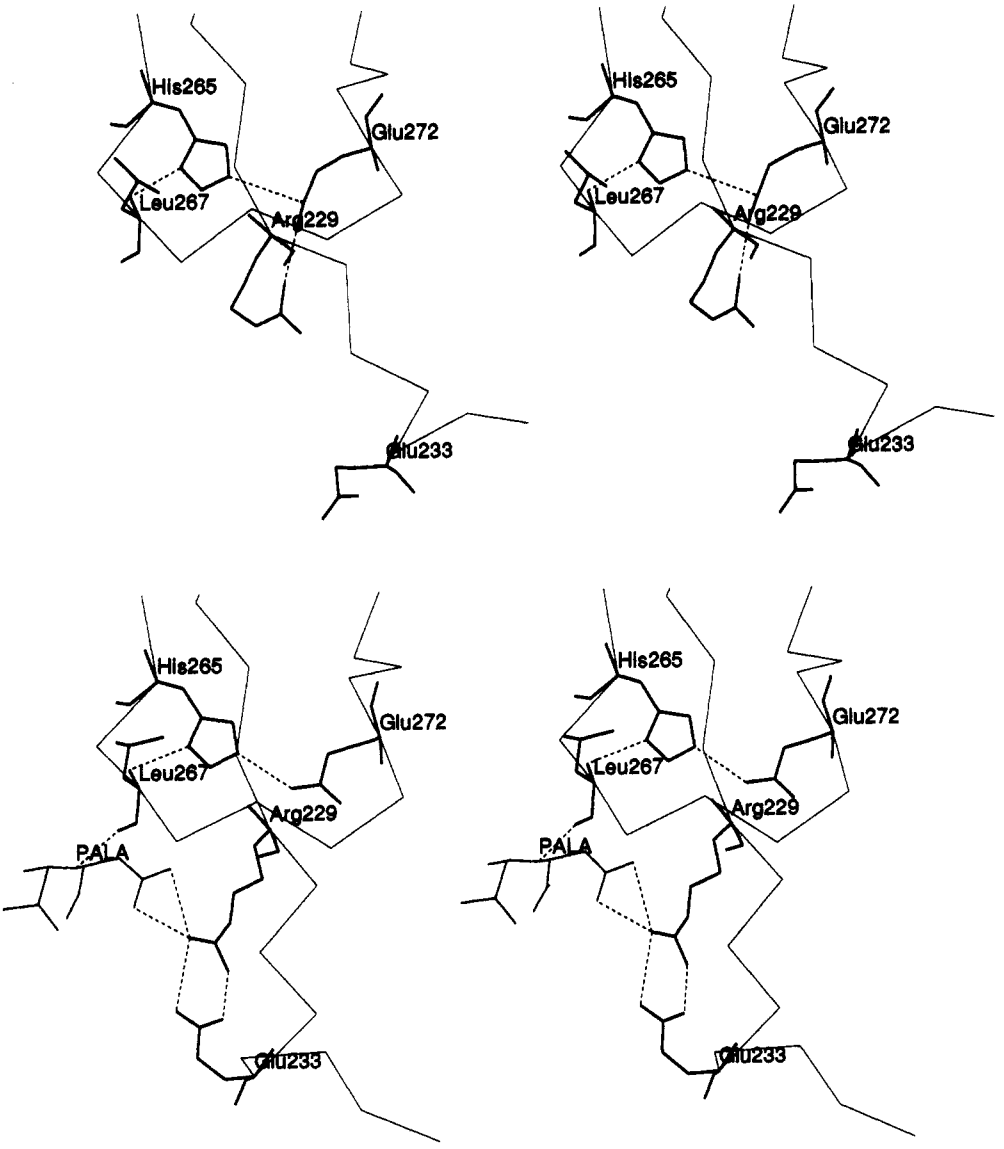

FIGURE 5: Stereoview of the region of the active site around Glu-233, Arg-229, Glu-272, and His-265 in the T (top) and R (bottom) states. In the T state, Arg-229 is bent out of the active site and may be stabilized in this position by an interaction with Glu-272. The carbonyl of Leu-267, which forms a hydrogen bond to the amide nitrogen of carbamyl phosphate, in the R state, is held in position by an interaction with His-265, and, in turn, His-265 interacts with Glu-272. In the R state, Arg-229 interacts with PALA, and by analogy with the β-carboxylate of aspartate. The position of Arg-229 in this state is stabilized by the salt link with Glu-233 of the 240s loop. His-265 interacts with both Glu-272 and the backbone nitrogen of Leu-267 as it does in the T state.

both result in an increase in the $[S]_{0.5}$ for aspartate of only 2-fold in the mutant holoenzymes relative to the wild-type holoenzyme (Table I). If the $[S]_{0.5}$ values are representative of the relative affinities of the enzymes for aspartate, then the Arg-229 to Ala and Glu-233 to Ser mutations do not have a dramatic effect on aspartate binding. The results for these two mutant enzymes suggest that the interaction between Glu-233 and Arg-229 in the R state is not critical for optimal aspartate binding. On the other hand, both the Arg-229 to Ala and the Glu-233 to Ser mutations cause a significant increase in the aspartate K_m of the isolated catalytic subunit (Table II). In the holoenzyme, the motions of the 240s loops of one catalytic subunit are sterically restricted by the 240s loops of the other catalytic subunit (see Figure 6). These restrictions would not be expected to occur in the isolated catalytic subunit where the 240s loops would be on the surface and interactions which might stabilize the position of the 240s loops (e.g., Arg-229–Glu-233) would be of greater importance. These results suggest that the Arg-229–Glu-233 interaction may be more important for the correct conformation of the 240s loop in the isolated catalytic subunit than in the holoenzyme.

Since the carbamyl phosphate $[S]_{0.5}$ for the Arg-229 → Ala enzyme is unchanged, the decrease in the carbamyl phosphate $[S]_{0.5}$ observed for the Glu-233 → Ser enzyme is not due to the loss of the Arg-229–Glu-233 interaction in the R state. This indicates that the link between Arg-229 and Glu-233 in the R state is not critical for carbamyl phosphate binding. The 5-fold decrease in the carbamyl phosphate $[S]_{0.5}$ in the Glu-233 → Ser holoenzyme places the concentration of carbamyl phosphate required to half-saturate the mutant holoenzyme in the same range as that required to half-saturate the mutant catalytic subunit. This implies that carbamyl phosphate binding in the Glu-233 → Ser holoenzyme is the maximal binding of the R state. Thus, the Glu-233 to Ser mutation results in a holoenzyme with low activity but high affinity for carbamyl phosphate relative to the wild-type holoenzyme.

(*iv*) *Glu-233–Arg-229 Link Is Important for Optimal PALA Affinity*. Considerably greater concentrations of PALA are necessary to activate both the Arg-229 → Ala and Glu-233

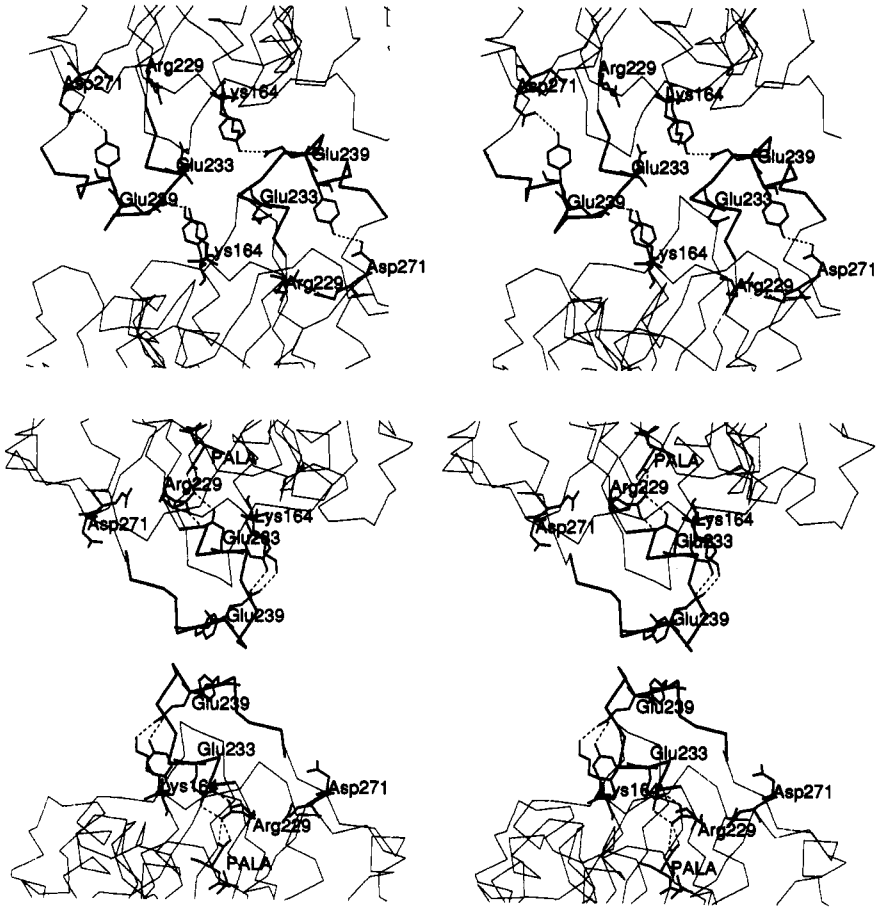

FIGURE 6: Stereoview of the C1–C4 interface of aspartate transcarbamylase in the T (Kim et al., 1987) and R states (Krause et al., 1987). The C1 and C4 catalytic chains are in different catalytic subunits of the holoenzyme. In the T state (top), the 240s loops (dark) of C1 and C4 are side by side, allowing the intersubunit interaction between Glu-239 and Tyr-165. The position of the 240s loop is constrained in this position by this intersubunit interaction as well as an intrachain interaction between Tyr-240 and Asp-271. In this state, the catalytically important Arg-229 is bent out of the active site and held in this position by a link to Glu-272 (in C1 only). In the R state (bottom), the 240s loops (dark) of C1 and C4 are on top of one another. The intersubunit link between Glu-239 and Tyr-165 is lost and is replaced by an intrachain link between Glu-239 and both Lys-164 and Tyr-165 that helps to stabilize the position of the 240s loop in this state. The link between Tyr-240 and Asp-271 is also lost, while a new link forms between Glu-233 and Arg-229. This link both helps to stabilize the R-state position of the 240s loop and positions Arg-229 to interact with the portion of PALA corresponding to the β-carboxylate of aspartate. For clarity, the side chains of Tyr-165, Tyr-240, and Glu-272 are shown but are not labeled.

→ Ser enzymes than are required to activate the wild-type enzyme (Figure 4). Moreover, millimolar quantities of PALA are required to fully inhibit the Glu-233 → Ser catalytic subunit compared to the micromolar quantities necessary to inhibit the wild-type catalytic subunit. These results indicate that the Arg-229 → Ala and Glu-233 → Ser enzymes have reduced affinities for PALA. Since the reduction in catalysis in the mutant enzymes is most likely due to the improper orientation of aspartate in the transition state and PALA is a reasonable transition-state analogue (Collins & Stark, 1971), the lower affinity for PALA in the Arg-229 → Ala and Glu-233 → Ser enzymes reflects the lower affinity of these mutant enzymes for the transition state.

(*v*) *Glu-233 Is Important for the Cooperative Interactions of the Enzyme.* If the absence of cooperativity in the Glu-233 → Ser enzyme were the result of the loss of the Glu-233–Arg-229 interaction in the R state, then we would expect the Arg-229 → Ala enzyme to also exhibit no cooperativity, since the Glu-233–Arg-229 interaction cannot form in the Arg-229 → Ala enzyme. However, the Arg-229 → Ala enzyme exhibits significant aspartate cooperativity (see Table I), indicating that it is the replacement of Glu-233 by Ser and not the loss of the Glu-233–Arg-229 interaction that is responsible for the lack of cooperativity in the Glu-233 → Ser enzyme. The absence of cooperativity in the Glu-233 → Ser enzyme does not mean that this mutant is locked in one conformational state, since PALA can still activate the Glu-233 → Ser enzyme. The activation by PALA of the Glu-233 → Ser enzyme demonstrates that there is still interaction between the active sites in this mutant and that PALA binding at one active site can still force the unoccupied active sites into a high-activity–high-affinity conformation while aspartate binding to one active site (in the presence of carbamyl phosphate) cannot.

Effect of the Glu-272 to Ser Change on the Kinetic Properties of the Enzyme. (*i*) *Interaction of Glu-272 with both Arg-229 and His-265.* Besides the interaction in the T state between Glu-272 and Arg-229, which appears to hold the side chain of Arg-229 out of the active site and may be important for the stability of the low-activity–low-affinity aspartate binding site, there is also an interaction between Glu-272 and His-265 in both the T and R states (Figure 5). His-265 in turn also interacts with the backbone nitrogen of Leu-267, and the region around Leu-267 is in close proximity to the carbamyl phosphate binding site. In fact, the backbone carbonyl of Leu-267 is hydrogen bonded to the amide NH of PALA (Krause et al., 1987; Figure 5), and in a structure with carbamyl phosphate and succinate bound at the active site (Gouaux & Lipscomb, 1988), the backbone carbonyls of both Pro-266 and Leu-267 hydrogen bond to the carbamyl nitrogen of carbamyl phosphate. These structural data strongly suggest

that the backbone carbonyls of Pro-266 and Leu-267 are involved in carbamyl phosphate binding. Furthermore, the interactions of Glu-272 with His-265 and His-265 with the backbone nitrogen of Leu-267 would be expected to make the backbone carbonyl of Leu-267 more electron-rich, facilitating a hydrogen bond between this carbonyl and the carbamyl nitrogen of carbamyl phosphate.

(ii) Glu-272 May Be Important for Optimal Carbamyl Phosphate Affinity through Its Interaction with His-265. The maximal activity of the Glu-272 → Ser enzyme is reduced by less than 25% relative to the wild type, indicating that Glu-272 is not critical for catalysis. Both the carbamyl phosphate $[S]_{0.5}$ and the aspartate $[S]_{0.5}$ for the Glu-272 → Ser holoenzyme and both the carbamyl phosphate K_m and the aspartate K_m for the Glu-272 → Ser catalytic subunit increase by 3-fold. The decreased affinity of the Glu-272 → Ser enzyme for aspartate could be the result of an alteration in the conformational change that normally occurs upon carbamyl phosphate binding and is a requirement for the formation of the aspartate binding site.

A reduction in carbamyl phosphate binding in the Glu-272 → Ser enzyme can be rationalized with the structural data described above. The main chain atoms of the six amino acids between Glu-272 and His-265 form a fairly rigid loop which contains two prolines at positions 266 and 268. In the Glu-272 → Ser enzyme, the loss of the interaction between Glu-272 and His-265 could destabilize this loop, leading to reduced carbamyl phosphate binding due to a change in the positions of the backbone carbonyls of Pro-266 and Leu-267.

240s Loop Is Critical for the Stability of both the Low-Activity–Low-Affinity and High-Activity–High-Affinity States of the Enzyme. In a previously proposed model for the concerted allosteric transition in aspartate transcarbamylase, it was suggested that the closure of the aspartate and carbamyl phosphate domains upon the binding of the substrates triggers the conversion of the enzyme from the T to the R state (Ladjimi & Kantrowitz, 1988). The closure of the two domains of the catalytic chain involves more than a simple hinge motion involving these two domains, however. In particular, the 240s loop of the aspartate domain undergoes a significant conformational change (Krause et al., 1985, 1987; Kim et al., 1987) that results in the breaking of one set of conformational constraints, that presumably stabilize the T state, and the formation of another set of interactions, that presumably stabilize the R state. Evidence presented here as well as from previous studies suggests that these 240s loop interactions are critical for cooperativity as well as the concerted allosteric transition in aspartate transcarbamylase.

Figure 7 shows schematically the interactions involving residues of the 240s loop that have been shown, based on the X-ray structures of the CTP-liganded (Kim et al., 1987) and PALA-liganded enzymes (Krause et al., 1985, 1987) as well as mutagenesis studies (Middleton & Kantrowitz, 1986, 1988; Ladjimi et al., 1988; Ladjimi & Kantrowitz, 1988), to be involved in the stabilization of the T or R states of the enzyme. In the T state, Tyr-240 forms a charged hydrogen bond with Asp-271. The Tyr-240 → Phe and the Asp-271 → Asn enzymes have confirmed that the Tyr-240–Asp-271 interaction stabilizes the T state (Middleton & Kantrowitz, 1986, 1988). The loss of this interaction, however, is not sufficient to convert the enzyme from the T to the R state, although the Tyr-240 → Phe enzyme does undergo a local conformational change toward the R state in the area of residue 240 (Gouaux et al., 1988). On the other hand, the analysis of the Glu-239 → Gln enzyme suggests that the loss of the intersubunit interactions in the T state between Glu-239 of one catalytic chain (C1 or C4)[4] and both Lys-164 and Tyr-165 of the opposite catalytic chain (C4 or C1) (see Figure 6) is sufficient to convert the enzyme into a R-like state (Ladjimi & Kantrowitz, 1988; P. Vachette, personal communication). The analysis of the mutations at Glu-272 and Arg-229 reported here suggests that the interaction between these residues in the T state holds Arg-229 out of the active-site pocket and in this manner stabilizes the enzyme in the low-activity–low-affinity state. In the R state, the domain-closed conformation of the catalytic chain is stabilized by the interdomain bridging interactions between Glu-50 of the carbamyl phosphate domain and both Arg-167 and Arg-234 of the aspartate domain (Ladjimi et al., 1988). Analysis of the Glu-50 → Gln and Arg-234 → Ser enzymes suggests that the interdomain bridging interaction between Glu-50 and Arg-234 is critical for the stabilization of the high-activity–high-affinity state (Ladjimi et al., 1988; Middleton & Kantrowitz, 1988). The conversion of the enzyme from the T to the R state results in the loss of the interchain link between Tyr-240 and Asp-271, as well as the symmetrically related intersubunit links between Glu-239 (C1 or C4) and both Lys-164 and Tyr-165 (C4 or C1). However, the reorientation of the 240s loop in the R state does allow Glu-239 (C1) to form a new set of stabilizing interactions with both Lys-164 and Tyr-165 of the same catalytic chain (C1) (see Figure 6). The R-state position of the 240s loop allows Glu-233 to form a salt link with Arg-229. As reported here, the link between Glu-233 and Arg-229 stabilizes the position of Arg-229 for its interaction with the β-carboxylate of aspartate, helping to position aspartate correctly for the catalytic reaction.

In order for aspartate transcarbamylase to manifest kinetic cooperativity, there must be two different states of the enzyme that have different catalytic activities and/or affinities for substrates. Furthermore, for the transition to be concerted, the binding of the first substrate must convert all of the remaining active sites from the low-activity–low-affinity state to the high-activity–high-affinity state (Monod et al., 1965). In aspartate transcarbamylase, the 240s loop of the catalytic chain is primarily responsible for determining the relative activity and affinity of the aspartate binding site as well as for providing a mechanism to convert all of the active sites from one state to the other in a concerted fashion.

One set of interactions involving the 240s loop that is essential for the activity and affinity of the aspartate binding site is the interdomain bridging interactions between Glu-50 and both Arg-167 and Arg-234 which help stabilize the domain-closed high-activity–high-affinity state. The 7.5-Å movement of Arg-234 necessary to form an interaction with Glu-50 is made possible by the large conformational change of the 240s loop during the T to R transition. The Glu-50 → Gln and Arg-234 → Ser enzymes, in which the interdomain bridging interactions are weakened or destroyed, can no longer attain the high-activity–high-affinity state, and these mutant enzymes exhibit low maximal activity, a loss of cooperativity, and substantially decreased affinity for aspartate (Ladjimi et al., 1988; Middleton & Kantrowitz, 1988). Therefore, the domain-closed conformation of the catalytic chain, stabilized by the interdomain bridging interactions which form partly as a result of the movement of the 240s loop, is a requirement for high activity and affinity for aspartate. Another part of the mechanism by which the activity and affinity of the as-

[4] C followed by a number (e.g., C1, C4) refers to a specific catalytic chain of the enzyme as described in Figure 6 of Honzatko et al. (1982).

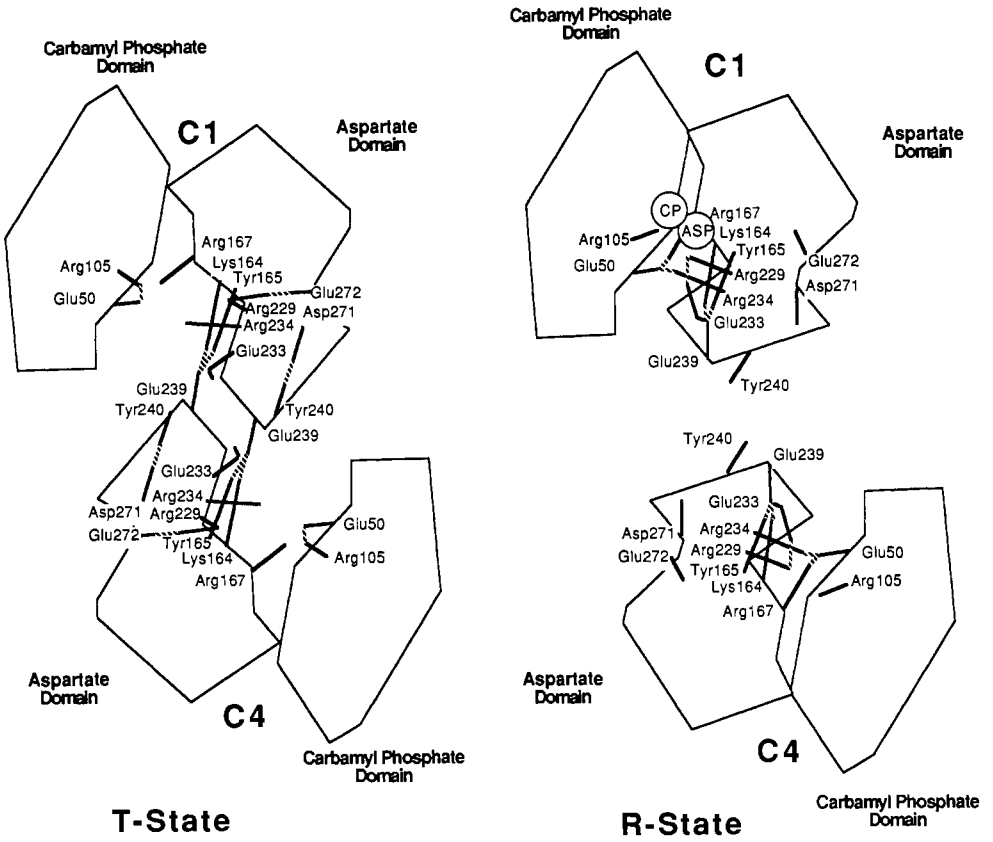

FIGURE 7: Schematic representation of the interactions, involving side chains of the 240s loop, that stabilize the T state (left) and R state (right) of aspartate transcarbamylase. For clarity, only one catalytic chain from each of the upper (C1) and lower (C4) catalytic subunits is shown. Because of the molecular 3-fold axis, the various interactions shown here are repeated in the C2–C5 and C3–C6 pairs. In the T state, the 240s loop is stabilized by interactions between Tyr-240 and Asp-271 and the intersubunit link between Glu-239 (C1 or C4) with both Lys-164 and Tyr-165 (C4 or C1). In this state, Arg-229 is bent out of the active site and may be stabilized in this position by an interaction with Glu-272. In the R state, the 240s loop is stabilized by the interdomain bridging interactions between Glu-50 with both Arg-167 and Arg-234, along with an intrachain interaction between Glu-239 and both Lys-164 and Tyr-165. In this state, the Arg-229 to Glu-233 link not only helps stabilize the 240s loop but also positions Arg-229 to interact with the β-carboxylate of aspartate. Upon aspartate binding (in the presence of carbamyl phosphate), the aspartate domain moves toward the carbamyl phosphate domain, resulting in the closure of the active site. The 240s loops of C1 and C4 undergo a large alteration in position and change from being side by side in the T state to almost one on top of the other in the R state. On the quaternary level, the catalytic subunits move apart, resulting in an elongation of the molecule. There is also a rotation about the 3-fold axis of the enzyme which is omitted in this diagram for clarity. The binding of the substrates at one active site induces the domain closure in that catalytic chain. However, steric constraints require a quaternary conformational change which allows the 240s loops of the upper and lower catalytic chains to move to their final positions. The quaternary conformational change causes the loss of the conformational constraints that stabilize the T state and are replaced by a new set of conformational constraints that stabilize the high-activity–high-affinity R state.

partate site are determined involves specific interactions between residues of the 240s loop and residues involved in the binding and orientation of aspartate. For example, during the allosteric transition to the R state, residues Glu-233 and Arg-234 of the 240s loop reposition to form interactions with Arg-229 and Gln-231, respectively, two residues which interact with the β-carboxylate of aspartate. These interactions help to stabilize the positions of Arg-229 and Gln-231 to orient aspartate in the transition state.

The rearrangement of the 240s loop provides a mechanism for a concerted allosteric transition in aspartate transcarbamylase and is the key to the entire quaternary conformational change. In the T state, the 240s loop is maintained far apart from the carbamyl phosphate domain by both the Tyr-240–Asp-271 interaction and the Glu-239–Lys-164, Glu-239–Tyr-165 links between the catalytic subunits (see Figure 6). In the Glu-239 → Gln enzyme, the loss of the interactions between the catalytic subunits results in an enzyme that is R-like both kinetically (Ladjimi et al., 1988) and structurally (P. Vachette, personal communication). Thus, not until these intersubunit interactions are broken can the catalytic subunits separate and undergo the conformational transition to the R state. In order for the allosteric transition to occur, upon aspartate binding, not only do the interactions which maintain the position of the 240s loop in the T state have to be broken but also steric constraints have to be overcome. Steric constraints arise in the central cavity between the 240s loops of the catalytic subunits which are adjacent in the T state but move further apart and are more nearly on top of each other in the R state (see Figure 6). This steric hindrance is relieved during the structural rearrangement of the 240s loop, when the symmetrically related links between Glu-239 and both Lys-164 and Tyr-165 (C1–C4) are lost and the catalytic subunits separate. However, this change cannot occur only in one C1–C4 pair, since the different catalytic chains of the upper and lower subunits are in fact held together partly by the favorable interactions between their respective 240s loops. Any change in the intersubunit interactions of one pair, e.g., C1–C4, is transmitted to the C2–C5 and C3–C6 pairs as well. Thus, when aspartate binds to one active site (in the presence of carbamyl phosphate) and the 240s loops rearrange, the entire molecule is converted to the R conformation by a 12-Å elongation along the 3-fold axis, resulting in the separation of the catalytic subunits with a concomitant reorientation of the catalytic subunits about this axis by ±5° (a relative reorientation of 10°) and a reorientation of the

regulatory dimers about the molecular 2-fold axes by 15°.

In summary, on the basis of both X-ray crystallographic and site-directed mutagenesis studies, the 240s loop of the catalytic chain of aspartate transcarbamylase is critical for maintaining both allosteric forms of the enzyme. The stabilization of alternate conformations of this loop by specific side chain interactions provides the mechanisms by which homotropic cooperativity is manifest and provides a molecular level description for a concerted allosteric transition in this oligomeric enzyme.

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Registry No. CP, 590-55-6; PALA, 51321-79-0; Arg, 74-79-3; Glu, 56-86-0; Ala, 56-41-7; Ser, 56-45-1; Asp, 56-84-8; aspartate transcarbamylase, 9012-49-1.

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