Function of Serine-171 in Domain Closure, Cooperativity, and Catalysis in *Escherichia coli* Aspartate Transcarbamoylase†

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ABSTRACT: Structural studies of *Escherichia coli* aspartate transcarbamoylase suggest that the R state of the enzyme is stabilized by an interaction between Ser-171 of the aspartate domain and both the backbone carbonyl of His-134 and the side chain of Gln-133 of the carbamoyl phosphate domain of a catalytic chain [Ke, H.-M., Lipscomb, W. N., Cho, Y., & Honzatko, R. B. (1988) *J. Mol. Biol. 204*, 725-747]. In the present study, site-specific mutagenesis is used to replace Ser-171 by alanine, thereby eliminating the interactions between Ser-171 and both Gln-133 and His-134. The Ser-171 → Ala holoenzyme exhibits no cooperativity, more than a 140-fold loss of activity, little change in the carbamoyl phosphate concentration at half the maximal observed specific activity, and a 7-fold increase in the aspartate concentration at half the maximal observed specific activity. Although the Ser-171 → Ala enzyme exhibits no homotropic cooperativity, it is still activated by *N*-(phosphonacetyl)-L-aspartate (PALA), but not by succinate, in the presence of saturating carbamoyl phosphate and subsaturating aspartate. At subsaturating concentrations of aspartate, the Ser-171 → Ala enzyme is still activated by ATP but is inhibited less by CTP than is the wild-type enzyme. At saturating concentrations of aspartate, the Ser-171 → Ala enzyme is activated by ATP and inhibited by CTP to an even greater extent than at subsaturating concentrations of aspartate. At saturating aspartate, the wild-type enzyme is neither activated by ATP nor inhibited by CTP. The heterotropic effectors ATP and CTP alter the maximal velocity of the Ser-171 → Ala enzyme but do not affect the aspartate concentration at half the maximal observed specific activity. For the wild-type enzyme, the maximal velocity remains unaltered in the presence of either ATP or CTP, but these nucleotides do alter the aspartate concentration at half the maximal observed specific activity. The isolated catalytic subunit of the Ser-171 → Ala enzyme is only 6-fold less active than the wild-type catalytic subunit, and the mutant catalytic subunit is 32-fold more active than the mutant holoenzyme. The K_m for aspartate is 180 mM for the Ser-171 → Ala catalytic subunit compared to 6.0 mM for the wild-type catalytic subunit. Analysis of these data suggests that the Ser-171 → Ala enzyme exists in a low-activity and low-affinity state even when saturated with substrates, implying that the interdomain bridging interactions between Ser-171 and both the backbone carbonyl of His-134 and the side chain of Gln-133 are important for the formation of the high-activity high-affinity state of the enzyme. Furthermore, these data support the proposal [Ladjimi, M. M., Middleton, S. A., Kelleher, K. S., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 268-276] that the closure of the two domains of the catalytic chain of the enzyme is involved in the creation of the high-affinity aspartate binding site.

Aspartate transcarbamoylase (EC 2.1.3.2) from *Escherichia coli* exhibits a sigmoidal dependence of velocity on the concentration of the substrates (Gerhart & Pardee, 1962; Bethell et al., 1968). This behavior is characteristic of a class of enzymes that not only catalyze a particular biochemical reaction but also are involved in the control of a biosynthetic pathway. Aspartate transcarbamoylase catalyzes the first step in pyrimidine biosynthesis, the reaction of carbamoyl phosphate with aspartate to form *N*-carbamoyl-L-aspartate and inorganic phosphate. Control is exerted over the pathway by a combination of methods. Not only is the amount of enzyme in the cell regulated by an attenuation mechanism (Roof et al., 1982; Navre & Schachman, 1983; Turnbough et al., 1983; Levin et al., 1989), but the activity of the enzyme is also homotropically regulated by its substrates, heterotropically activated by ATP (Gerhart & Pardee, 1962), one of the products of the purine biosynthetic pathway, and heterotropically inhibited by CTP and UTP (Gerhart & Pardee, 1962; Wild et al., 1989), the end products of the pyrimidine biosynthetic pathway.

Upon treatment with mercurials or heat, the holoenzyme[1] (M_r 310000) dissociates into two larger subunits (M_r 100000) and three smaller subunits (M_r 34000). Each of the larger or catalytic subunits is composed of three identical polypeptide chains, while the smaller or regulatory subunits are composed of two identical polypeptide chains. Each catalytic subunit contains three complete active sites, each of which is shared between two adjacent polypeptide chains (Monaco et al., 1978; Robey & Schachman, 1985; Krause et al., 1985; Wente & Schachman, 1987), while each regulatory chain has a binding site for the regulatory effectors (Gerhart & Schachman, 1965), all of which bind to the same site. The biochemical properties of this enzyme have been extensively reviewed (Allewell, 1989; Kantrowitz & Lipscomb, 1988; Schachman, 1988; Schachman, 1974; Kantrowitz et al., 1980a,b; Jacobson & Stark, 1973; Gerhart, 1970).

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

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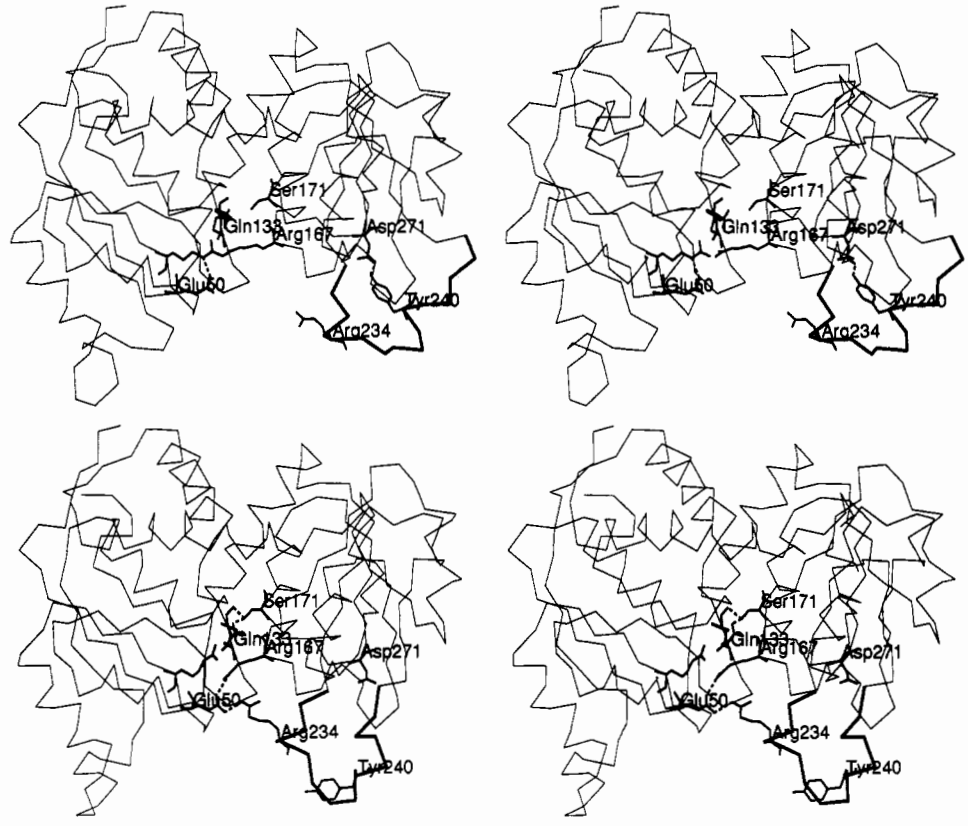

FIGURE 1: Stereoview showing the α-carbon trace of a single unliganded (top panel) and PALA-liganded (bottom panel) catalytic polypeptide chain. In each panel the carbamoyl phosphate domain is on the left and aspartate domain is on the right. Ser-171 (aspartate domain) and Gln-133 and His-134 (carbamoyl phosphate domain) do not interact in the unliganded enzyme, but become involved in a core interaction in the PALA-liganded enzyme. The PALA molecule and the label for His-134 have been omitted for clarity. The unliganded and PALA-liganded crystallographic data are from Kim et al. (1987) and Krause et al. (1987), respectively.

The three-dimensional arrangement of the regulatory and catalytic subunits has been deduced by X-ray crystallography of the holoenzyme (Honzatko et al., 1982; Kim et al., 1987). In addition, X-ray crystallography has also been used to determine the alteration in quaternary structure that takes place upon the binding of the substrates and substrate analogues (Krause et al., 1985, 1987; Ke et al., 1988; Gouaux & Lipscomb, 1988). When the substrates bind to aspartate transcarbamoylase, the molecule undergoes a quaternary conformational change from the T to the R state (Monod et al., 1965) that involves an expansion of 12 Å along the 3-fold axis along with rotations of the catalytic subunits about the 3-fold axis and rotations of the regulatory subunits about their respective 2-fold axes. These quaternary changes are accompanied by alterations in the tertiary structure of both the catalytic and regulatory subunits, the major of which involves movements of domains and reorientations of loops (Kantrowitz & Lipscomb, 1988; Ke et al., 1988). In particular, the aspartate and carbamoyl phosphate domains of the catalytic chain close together. This domain closure along with a major reorientation of the 240s loop is involved with the formation of the aspartate binding site (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988; Middleton & Kantrowitz, 1988; Middleton et al., 1989). These tertiary conformational changes involve the breaking of interactions that appear to stabilize the T state and are replaced by other interactions that appear to stabilize the R state of the enzyme.

In order to determine if the interactions observed by X-ray crystallography that appear to stabilize either the T or R states are functionally important, we have evaluated the properties of mutant enzymes that have specific interactions perturbed by site-specific mutagenesis. In this manner the interactions that are functionally important can be identified and mapped in the structure of the enzyme. For example, the X-ray structures of the T and R states (Kim et al., 1987; Krause et al., 1987; Ke et al., 1988) reveals that there is a set of interdomain bridging interactions between Glu-50 of the carbamoyl phosphate domain and both Arg-167 and Arg-234 of the aspartate domain that are formed upon the closure of the two catalytic chain domains. Experiments employing site-specific mutations have determined that these interdomain bridging interactions are important for the formation of the R structure and for cooperativity in aspartate transcarbamoylase (Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988; Middleton & Kantrowitz, 1988).

As originally identified by Ke et al. (1988), there is another set of interdomain bridging interactions between the aspartate and carbamoyl phosphate domains that is absent in the T state and present in the R state. As seen in Figure 1, Ser-171 of the aspartate domain interacts with both the backbone carbonyl of His-134 as well as the side chain of Gln-133 in the R state. This set of interactions is not observed when the domains are open since the residues are too far apart. Here we report the construction and analysis of a mutant version of aspartate transcarbamoylase in which Ser-171 has been replaced by alanine in order to determine if this set of interdomain bridging interactions is important for the homotropic or heterotropic properties of the enzyme.

Experimental Procedures

Materials

Agar, ampicillin, L-aspartate, *N*-carbamoyl-L-aspartate, carbamoyl phosphate, and potassium dihydrogen phosphate were purchased from Sigma Chemical Co. The carbamoyl phosphate was purified before use by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C (Gerhart &

Pardee, 1962). Electrophoresis-grade acrylamide, agarose, urea, Tris, and enzyme-grade ammonium sulfate were obtained from ICN Biomedicals. Restriction endonucleases were obtained from either U.S. Biochemicals or New England Biolabs and used according to the supplier's recommendations. The Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, and T4 ligase were products of U.S. Biochemicals.

Methods

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

Construction of the Ser-171 → Ala[2] *Mutation by Site-Specific Mutagenesis.* The replacement of serine by alanine at position 171 in the catalytic chain of aspartate transcarbamoylase was accomplished by site-specific mutagenesis using the method of Zoller and Smith (1982), with modifications as previously described (Ladjimi & Kantrowitz, 1987).

In order to overproduce the mutant enzyme, a small fragment of the *pyrBI* gene was removed from the M13RF with the restriction enzymes *Eco*RV and *Bst*EII and inserted into a plasmid which had the corresponding section of the wild-type gene removed. Details of the recloning steps have been previously reported (Ladjimi et al., 1988). The plasmid pEK98 for the Ser-171 → Ala mutation was isolated and then transformed into strain EK1104 for the overproduction of enzyme (see below).

Wild-Type and Mutant Holoenzyme Purification. Wild-type and the mutant aspartate transcarbamoylases were isolated as described by Nowlan and Kantrowitz (1985), from *Escherichia coli* strain EK1104 [F⁻ *ara, thi,* Δ*pro-lac,* Δ*pyrB, pyrF*⁻, *rpsL*], containing the plasmids pEK2 (Smith et al., 1986) and pEK98 for the wild-type and the Ser-171 → Ala enzymes, respectively.

Wild-Type and Mutant Catalytic Subunit Overproduction and Purification. The isolation of catalytic subunit from the wild-type and the Ser-171 → Ala aspartate transcarbamoylases was accomplished after the in vivo overproduction of catalytic subunit by using strain EK1104 harboring a plasmid which had a portion of the gene for the regulatory subunit deleted (Nowlan & Kantrowitz, 1985). The plasmids pEK17 and pEK106 used for the production of the wild-type and the Ser-171 → Ala catalytic subunits, respectively, were constructed as previously described (Nowlan & Kantrowitz, 1985). Catalytic subunit was purified by the procedure previously described (Stebbins et al., 1989).

Aspartate Transcarbamoylase Assay. The transcarbamoylase activity was measured at 25 °C by either the colorimetric (Pastra-Landis et al., 1981) or the pH-stat method (Wu & Hammes, 1973). pH-stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburet. All colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

Determination of Protein Concentration. Concentration of pure wild-type holoenzyme and catalytic subunit were determined by absorbance measurements at 280 nm using extinction coefficients of 0.59 and 0.72 cm²/mg respectively (Gerhart & Holoubek, 1967). The protein concentration of the mutant holoenzyme and catalytic subunit were determined by the Bio-Rad version of Bradford's dye-binding assay (Bradford, 1976).

[2] The notation used to name the mutant enzyme is, for example, the Ser-171 → 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.

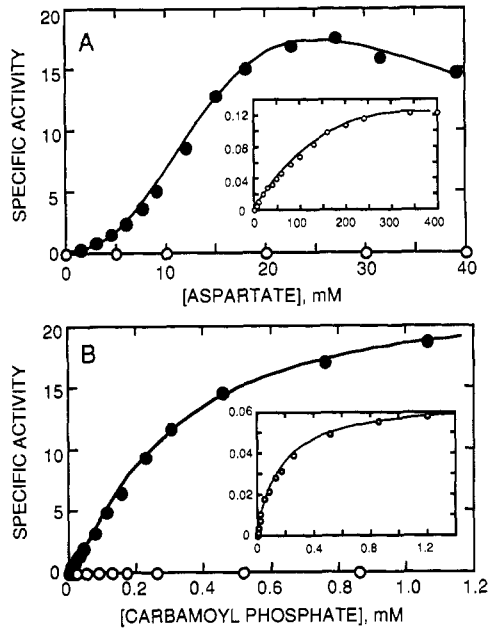

FIGURE 2: Aspartate and carbamoyl phosphate saturation curves of the wild-type (●) and the Ser-171 → Ala (○) holoenzymes. (A) Aspartate saturation curves were performed in the presence of saturating carbamoyl phosphate (4.8 mM). (B) The carbamoyl phosphate saturation curves were performed in presence of 25 and 100 mM aspartate for the wild-type and Ser-171 → Ala enzymes, respectively. (Insets) Region of low specific activity showing the data for the Ser-171 → Ala enzyme. All assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3). Specific activity is in units of $mmol \cdot h^{-1} \cdot mg^{-1}$.

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

enzyme	maximal velocity[b] ($mmol \cdot h^{-1} \cdot mg^{-1}$)	$[S]_{0.5}^{Asp}$ (mM)	n_H^{Asp}	$[S]_{0.5}^{CP}$ (mM)	n_H^{CP}
wild type	17.2	11.7	2.2	0.29	1.3
Ser-171 → Ala	0.12	85	1	0.17	1.0

[a] The data and experimental conditions used to determine the parameters in this table are presented in Figure 2. The maximal velocities were calculated by a nonlinear least-squares procedure using a modified Hill equation which incorporates a term for substrate inhibition (Pastra-Landis et al., 1978). [b] Maximal observed specific activity.

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

RESULTS

Kinetic Properties of the Wild-Type and Ser-171 → Ala Holoenzymes. The aspartate saturation curves for the wild-type and Ser-171 → Ala enzymes are compared in Figure 2. The replacement of Ser-171 of the catalytic chain of aspartate transcarbamoylase with alanine causes more than a 140-fold decrease in the maximal observed specific activity, and cooperativity is eliminated as measured by the Hill coefficient (see Table I). In addition, the mutation also causes the aspartate concentration at half the maximal observed specific activity ($[S]_{0.5}^{Asp}$) to increase from 11.7 mM for the wild-type

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

enzyme	maximal velocity[b] (mmol·h^{-1}·mg^{-1})	K_m^{Asp} (mM)	K_m^{CP} (mM)
wild type	23	6.0	0.027
Ser-171 → Ala	3.9	180	0.38

[a] Assays were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. In all cases the carbamoyl phosphate concentration was held constant at 4.8 mM. The maximal velocities and K_m values were calculated by a nonlinear least-squares procedure using a modified Michaelis-Menten equation which incorporates a term for substrate inhibition (Pastra-Landis et al., 1978) when necessary. [b] Maximal observed specificity activity.

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

catalytic subunit	K_D[a] for carbamoyl phosphate (mM)	K_i[b] for succinate (mM)	K_i[b] for PALA (μM)
wild type	0.02	0.22	0.024
Ser-171 → Ala	0.02	147	4

[a] The K_D of carbamoyl phosphate was determined kinetically by the method of Porter et al. (1969). [b] The K_i for PALA and succinate was determined kinetically by the method of Collins and Stark (1971).

to 85 mM for the Ser-171 → Ala enzyme. When carbamoyl phosphate is used as the varied substrate, and aspartate is held constant, the carbamoyl phosphate concentration at half the maximal observed specific activity ($[S]_{0.5}^{CP}$) is not altered significantly for the Ser-171 → Ala enzyme; however, cooperativity is eliminated (see Figure 2B and Table I).

Kinetic Properties of the Wild-Type and Ser-171 → Ala Catalytic Subunits. The Ser-171 → Ala catalytic subunit exhibits a 6-fold decrease in activity compared to the wild-type catalytic subunit. However, relative to the Ser-171 → Ala holoenzyme, the catalytic subunit exhibits a 32-fold increase in activity. This is compared to less than a 50% increase in specific activity between the wild-type holoenzyme and its catalytic subunit. As shown in Table II, the $[S]_{0.5}^{Asp}$ is substantially increased to 180 mM for the Ser-171 → Ala catalytic subunit compared to the 6.0 mM for the wild-type catalytic subunit. In addition, the $[S]_{0.5}^{CP}$ increases to 0.38 mM compared to the 0.03 mM for the wild-type catalytic subunit.

Binding of Substrates and Substrate Analogues to the Wild-Type and Ser-171 → Ala Enzymes. In order to determine if the Ser-171 → Ala mutation influences the binding of the substrates directly, the dissociation constant for carbamoyl phosphate (K_D^{CP}) was determined along with the K_i for both succinate and PALA. The K_D^{CP} for the Ser-171 → Ala catalytic subunit is virtually identical with the K_D^{CP} of the wild-type catalytic subunit. This indicates that the Ser-171 → Ala mutation has little effect on the affinity of the enzyme for carbamoyl phosphate.

To investigate the alterations at the aspartate binding site due to the Ser-171 → Ala substitution, the K_i for succinate and PALA were determined for the Ser-171 → Ala catalytic subunit. As seen in Table III, the Ser-171 → Ala substitution results in large increases in these constants. The K_i for succinate and the K_i for PALA increase approximately 670-fold and 170-fold, respectively, relative to the wild-type catalytic subunit.

Influence of the Allosteric Effectors on the Activity of the Ser-171 → Ala and Wild-Type Enzymes. Saturating concentrations of the allosteric effectors ATP and CTP alter the aspartate saturation curves of the Ser-171 → Ala enzyme quite differently than the wild type. As seen in Figure 3A, ATP

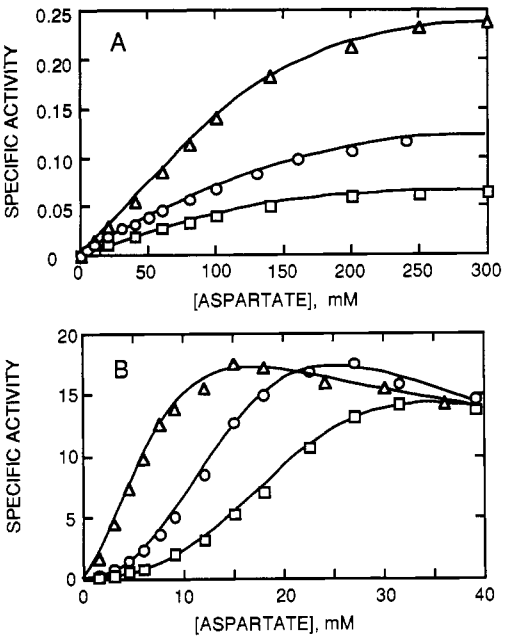

FIGURE 3: Aspartate saturation curves of the Ser-171 → Ala (A) and the wild-type (B) enzymes in the absence and presence of either ATP or CTP. Assays were carried out at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM. Data are shown for the enzyme in the absence of effectors (○), in the presence of 6 mM ATP (△), and in the presence of 2 mM CTP (□). The theoretical curves shown were determined by a nonlinear least-squares fit to the Hill equation incorporating a term for substrate inhibition (Pastra-Landis et al., 1978) (A) or the Michealis-Menten equation (B).

Table IV: Kinetic Parameters for the Wild-Type and Mutant Enzymes in the Absence and Presence of ATP and CTP[a]

enzyme	ATP[b]	control	CTP[c]
	Maximal Velocity[d] (mmol·h^{-1}·mg^{-1})		
wild type	17.3	17.2	14.2
Ser-171 → Ala	0.24	0.12	0.07
	$[S]_{0.5}^{Asp}$ Values (mM)		
wild type	5.2	11.7	17.4
Ser-171 → Ala	84	85	78
	Hill Coefficients[e]		
wild type	1.3	2.2	2.5
Ser-171 → Ala	1	1	1

[a] These data are extracted from the aspartate saturation curves. [b] Assays were performed in the presence of 4 and 6 mM ATP for the wild-type and Ser-171 → Ala enzymes, respectively. [c] Assays were performed in the presence of 1 and 2 mM CTP for the wild-type and Ser-171 → Ala enzymes, respectively. [d] Maximal observed specific activity. [e] Hill coefficients for the wild-type enzyme were calculated by a nonlinear least-squares procedure employing a modified Hill equation which incorporates substrate inhibition (Pastra-Landis et al., 1978).

and CTP are able to alter the maximal velocity of the Ser-171 → Ala enzyme without significantly affecting the $[S]_{0.5}^{Asp}$ (Table IV). This behavior is different from that observed for the wild-type enzyme, in which case ATP and CTP do not alter the maximal velocity but do cause alterations in the $[S]_{0.5}^{Asp}$ (Figure 3B and Table IV). The Ser-171 → Ala substitution has therefore converted the enzyme from a K to a V system (Monod et al., 1965). UTP in the presence of CTP (Wild et al., 1989) inhibits the Ser-171 → Ala enzyme to the same extent as the wild type (data not shown).

The alteration in the behavior of the Ser-171 → Ala enzyme with respect to the nucleotide effectors is more clearly shown in Figure 4. Here the activation by ATP and inhibition by CTP were determined at saturating concentrations of aspartate, conditions under which the wild-type enzyme is neither

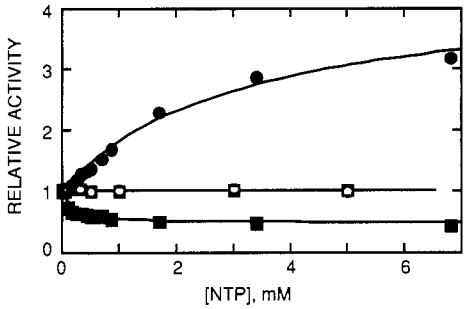

FIGURE 4: Influence of the effectors ATP and CTP on the activity of the wild-type and the Ser-171 → Ala enzymes at high concentrations of aspartate (above $2K_m$). The assays were performed as described in the legend to Figure 2, in the presence of saturating levels of carbamoyl phosphate (4.8 mM) and approximately 30 and 240 mM of aspartate for the wild-type and mutant enzymes, respectively. ATP effect on the wild-type (○) and the Ser-171 → Ala enzymes (●). CTP effect on the wild-type (□) and the Ser-171 → Ala enzymes (■).

activated nor inhibited. For the wild-type enzyme, the activation by ATP is greatest at low aspartate concentrations and decreases to zero as the concentration of aspartate is increased. In the case of Ser-171 → Ala enzyme, activation by ATP and inhibition by CTP occur at even saturating concentrations of carbamoyl phosphate and aspartate. These data indicate that, at saturating concentrations of aspartate,[3] the mutant enzyme is still not in the high-affinity high-activity form, suggesting that the combination of carbamoyl phosphate and aspartate are not sufficient to convert the enzyme to a functional state with high activity and affinity for aspartate.

Effect of PALA on the Activity of the Wild-Type and Ser-171 → Ala Enzymes. Previous studies have shown that low concentrations of succinate or PALA activate the wild-type holoenzyme at low concentrations of aspartate (Gerhart & Pardee, 1963; Collins & Stark, 1971). These substrate analogues are able to promote the conformational transition from the low-affinity T state to the high-affinity R state (Gerhart & Schachman, 1968; Blackburn & Schachman, 1977; Howlett & Schachman, 1977; Howlett et al., 1977). Since aspartate is unable to promote the allosteric transition in the Ser-171 → Ala holoenzyme, and large concentrations of this substrate are necessary to achieve half-saturation, the replacement of Ser-171 by Ala might lock the enzyme in low-affinity state, making it unable to reach a conformation with higher affinity for aspartate. Alternatively, this specific amino acid substitution might alter the configuration of the high-affinity active site so that aspartate no longer binds tighter to the R state than the T state. Therefore, is was of interest to determine the effect of PALA and succinate[3] on the activity of the Ser-171 → Ala enzyme. As shown in Figure 5, at low aspartate concentrations the activity of the wild-type enzyme is stimulated more than 4-fold in the presence of low concentrations of PALA or succinate.[3] For the Ser-171 → Ala enzyme, by use of aspartate at the same concentration relative to the $[S]_{0.5}^{Asp}$, the mutant enzyme is still activated by PALA but not by succinate.[3] However, the concentration of PALA required for the activation of the mutant enzyme is substantially higher than that required for the wild-type enzyme.

Discussion

A comparison of the X-ray crystal structures of aspartate transcarbamoylase in the absence of ligands (Ke et al., 1984) or in the presence of CTP (Kim et al., 1987) and in the presence of the bisubstrate analogue PALA (Krause et al., 1987) reveals that upon the transition from the T to R states

[3] In the presence of saturating carbamoyl phosphate.

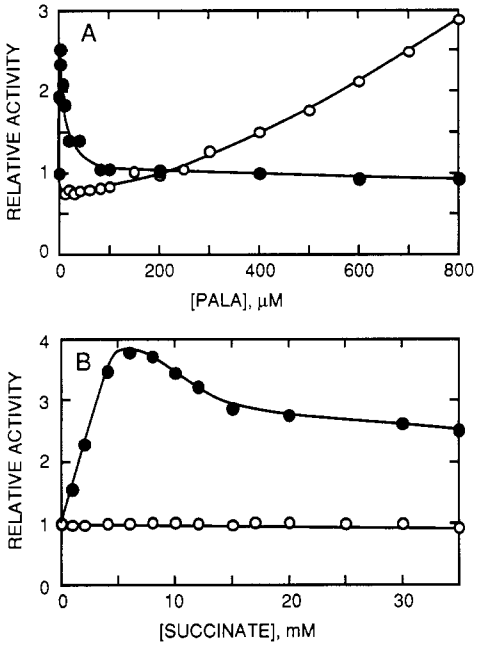

FIGURE 5: Activation of the wild-type (●) and the Ser-171 → Ala (○) enzymes by PALA (A) and succinate (B) at low concentrations of aspartate. The aspartate transcarbamoylase activity was measured as described in the legend to Figure 2 at saturating levels of carbamoyl phosphate (4.8 mM), and at an aspartate concentration of approximately $(1/12)[S]_{0.5}^{Asp}$, that is, 1 and 7 mM for the wild-type and the Ser-171 → Ala enzymes, respectively. The relative activity represents the ratio of activity in the presence of PALA (or succinate) to that in its absence.

the enzyme undergoes both tertiary and quaternary conformational changes. On the tertiary level, the two domains of the catalytic chain move together along with major reorientations of the 80s and 240s loops, resulting in the repositioning of a number of side chains that are critical for catalysis (Ladjimi et al., 1988; Middleton & Kantrowitz, 1988; Middleton et al., 1989). The closure of the two catalytic chain domains is stabilized by interactions between Glu-50 of the carbamoyl phosphate domain with both Arg-167 and Arg-234 of the aspartate domain.

The homotropic cooperativity of the enzyme can be rationalized in terms of a concerted allosteric transition between a low-activity low-affinity functional state and a high-activity high-affinity functional state. The closure of the two domains of the catalytic chain is critical for the concerted allosteric transition since domain closure in one catalytic chain cannot take place without a quaternary conformational change that forces the remaining catalytic chains into their domain-closed high-affinity high-activity state (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988; Middleton et al., 1989).

The importance of domain closure and particularly the interdomain bridging interactions between Glu-50 and both Arg-167 and Arg-234 that stabilize the R state has previously been tested by site-specific mutagenesis. Glu-50 has been replaced by glutamine (Ladjimi et al., 1988), alanine, and aspartic acid (Newton & Kantrowitz, 1990), and Arg-234 has been replaced by serine (Middleton & Kantrowitz, 1988). As originally observed by Ke et al. (1988), there is an additional interaction that exists between the aspartate and carbamoyl phosphate domains in the R state that is absent in the T state, between Ser-171 of the aspartate domain and the backbone carbonyl of His-134 and the side chain of Gln-133 (see Figure 1). The replacement of Gln-133 by alanine, which only breaks one of these interactions, causes a small decrease in velocity, an increase in $[S]_{0.5}^{Asp}$, and an increase in cooperativity (Robey

et al., 1986). In order to evaluate the importance of the interdomain bridging interactions involving Ser-171 for the function of aspartate transcarbamoylase, site-specific mutagenesis was used to replace Ser-171 with an alanine residue, thereby eliminating the interaction with both Gln-133 and His-134.

Interdomain Bridging Interactions Are Important both for the Formation of the Aspartate Binding Site and for Efficient Catalysis. The replacement of Ser-171 by Ala results in a substantial loss of activity for both the holoenzyme and catalytic subunit, although the mutant holoenzyme has a much lower maximal velocity relative to the wild-type holoenzyme than does the mutant catalytic subunit relative to the wild-type catalytic subunit (see Tables I and II). Thus the replacement of Ser-171 by alanine significantly diminishes the catalytic function of the enzyme. In addition, this amino acid replacement also causes an increase in the $[S]_{0.5}^{Asp}$ but only a small alteration in the $[S]_{0.5}^{CP}$, suggesting that the binding of aspartate but not carbamoyl phosphate is weakened. More direct evidence for this comes from the measurements of the K_D for carbamoyl phosphate and the K_i's for succinate and PALA. The mutation results in a 670-fold increase in the succinate K_i and a 167-fold increase in the PALA K_i. The larger increase in the K_i for succinate compared to PALA is expected since PALA interacts with both the aspartate and carbamoyl phosphate portions of the active site while succinate only interacts with the aspartate site. The unaltered K_D for carbamoyl phosphate suggests that the portion of PALA binding to the carbamoyl phosphate domain may still bind normally while the binding of the portion of the molecule to the aspartate domain is weakened. Similar behavior for mutations at the other interdomain bridging interaction, between Glu-50 and both Arg-167 and Arg-234, supports the conclusion that the interdomain bridging interactions involving both Glu-50 and Ser-171 are important for the formation of the aspartate binding site which in turn is a requirement for normal catalysis.

Importance of Domain Closure for Homotropic Cooperativity and Heterotropic Interactions. The X-ray crystal structure of aspartate transcarbamoylase in the presence of PALA shows that Ser-171 is involved in interactions with both Gln-133 and His-134 that stabilize the domain-closed structure of the R state. When Ser-171 is replaced by alanine, structurally the interdomain bridging interactions involving Ser-171 are eliminated and functionally the maximal activity is reduced dramatically, aspartate affinity is decreased, and homotropic cooperativity is not observed.

Cooperative interactions between the active sites are still present in the Ser-171 → Ala enzyme since at concentrations of aspartate low relative to the $[S]_{0.5}^{Asp}$ the mutant enzyme is activated by PALA (Figure 5). However, this amino acid substitution results in an enzyme that cannot respond cooperatively to the binding of aspartate. The loss of aspartate cooperativity could result from the R state of the enzyme no longer having increased affinity and activity for aspartate relative to the T state, or the enzyme could be restricted to one state, and therefore the binding of aspartate does not cause a structural and functional transition. The activation of the enzyme by PALA suggests that the Ser-171 → Ala enzyme can undergo a transition from a low-activity to a high-activity state. However, the activation of the enzyme by only PALA may be due to the fact that PALA can span across the two domains of the catalytic chain and perhaps induce a domain closure and the allosteric transition. Preliminary results from analytical gel chromatography indicate that the Ser-171 → Ala enzyme undergoes an expansion upon the addition of PALA (Burz and Allewell, unpublished observations), supporting the conclusion that the mutant enzyme can undergo a structural transition. However, experiments with the nucleotide effectors support the notion that in the presence of saturating substrates the enzyme exists in a state with low activity and affinity; see below.

The properties of the Ser-171 → Ala enzyme, reduced activity, loss of cooperativity, increased $[S]_{0.5}^{Asp}$, and ability to be activated by PALA at low concentrations of aspartate, are similar to the behavior of the wild-type enzyme when the alternate substrates L-alanosine (Baillon et al., 1985) or cysteinesulfinate (Foote et al., 1985) are used in place of aspartate. In the case of L-alanosine and cysteinesulfinate, the high-affinity high-activity R state is never formed because the substrates cannot induce the transition from the T to the R state. In the case of the Ser-171 → Ala enzyme, the loss of the interdomain bridging interactions destabilizes the R state sufficiently so that it is not formed when the natural substrates bind.

Heterotropic interactions are not eliminated by the replacement of Ser-171 by alanine, even though homotropic cooperativity is abolished. However, the replacement of Ser-171 by alanine alters how the nucleotides influence the activity of the enzyme. In the case of the wild-type enzyme, the nucleotide effectors alter the $[S]_{0.5}^{Asp}$ but do not alter the maximal velocity, while in the case of the Ser-171 → Ala enzyme the $[S]_{0.5}^{Asp}$ is unaltered and the maximal velocity is changed. The activation of the Ser-171 → Ala enzyme by ATP at saturating carbamoyl phosphate and aspartate shifts the enzyme into a more active state. The activation of the Ser-171 → Ala enzyme by ATP can be explained if the mutant enzyme is in a low-activity state, even in the presence of saturating carbamoyl phosphate and aspartate, and ATP causes a conversion of the mutant enzyme into a state with higher activity. CTP would function in the opposite manner by stabilizing a state with less activity.

Importance of the Regulatory Subunits. The conformational constraints imposed upon the catalytic subunits by the regulatory subunits in the Ser-171 → Ala holoenzyme result in a 32-fold decrease in specific activity. This is remarkable, considering that, for the wild-type enzyme, the decrease in activity amounts to less than 2-fold. These data imply that the interdomain bridging interactions involving Ser-171 are much more imporant for the catalytic reaction in the holoenzyme than in the catalytic subunit and may reflect the fact that the rate-determining step in the reaction for the holoenzyme is different than in the catalytic subunit (Hsuanyu & Wedler, 1987). Furthermore, the higher affinity of the Ser-171 → Ala holoenzyme for aspartate relative to the Ser-171 → Ala catalytic subunit suggests that the regulatory subunits directly influence the configuration of residues at the active site.

Extension of a Model for Homotropic Cooperativity in Aspartate Transcarbamoylase. Figure 6 shows schematically the interactions that have been shown, on the basis of the X-ray structures of the enzyme with either CTP (Kim et al., 1987) or PALA bound (Krause et al., 1985, 1987) as well as previous mutagenesis studies (Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988; Middleton & Kantrowitz, 1988; Middleton et al., 1989; Middleton & Kantrowitz, 1986), to be involved in the stabilization of the T or R states of the enzyme. The set of interdomain bridging interactions between Ser-171 and both the side chain of Gln-133 and the backbone carbonyl of His-134 described here can now be added to the model previously proposed for homotropic cooperativity in aspartate

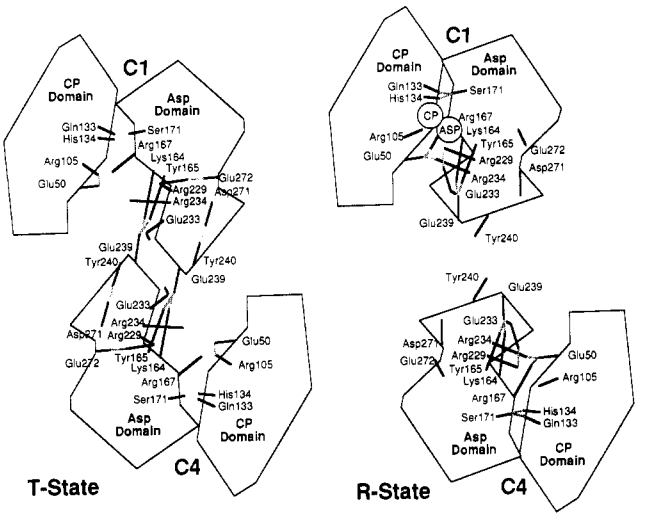

FIGURE 6: Schematic representation of the interactions that have been identified as important for the allosteric transition in aspartate transcarbamoylase by site-specific mutagenesis. For clarity, only one catalytic chain from each of the upper (C1) and lower (C4) catalytic subunits is shown. [C or R followed by a number, e.g., C1 or R1, refers to a particular polypeptide chain in aspartate transcarbamoylase as specified in Figure 6 of Honzatko et al. (1982).] 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 (left) 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 (right), the 240s loop is stabilized by the interdomain bridging interactions between Glu-50 with both Arg-167 and Arg-234, Ser-171 with both the backbone carbonyl of His-134 and the side chain of Gln-133, along with an interchain 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 carbamoyl phosphate), the aspartate domain (Asp domain) moves toward the carbamoyl phosphate domain (CP 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 from 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 conformation 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.

transcarbamoylase (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988; Middleton et al., 1989). Ser-171 is not involved in any interactions between the aspartate and carbamoyl phosphate domains of the catalytic chain that stabilize the T state (Figure 6, left). However, in the R state Ser-171 forms a link to both Gln-133 and His-134, an interaction that plays more than just a structural role in the stabilization of the R state (Figure 6, right). The interdomain bridging interactions involving the link between Ser-171 and both Gln-133 and His-134 described here along with the link between Glu-50 and both Arg-234 and Arg-167 are required for the stabilization of the R state and are directly involved in the creation of the high-activity high-affinity active site. The mutations Ser-171 → Ala, Glu-50 → Ala, Glu-50 → Gln, and Arg-234 → Ser all result in enzymes with low activity and low affinity for aspartate. The creation of the high-activity high-affinity active site is also directly related to the movement of the 240s loop. For example, Arg-234 cannot interact with Glu-50 unless the 240s loop takes on its R-state conformation. The movement of the 240s loop also moves residues like Glu-233 into position to stabilize catalytically important groups in the active site. Finally, closure of the domains of the catalytic chain and the movement of the 240s loop is directly related to homotropic cooperativity because steric constraints prevent the movement of one 240s loop without quaternary conformational changes in the enzyme. The results presented here along with previous X-ray and mutagenesis studies are beginning to provide a molecular level description of cooperativity in aspartate transcarbamoylase.

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