Function of Arginine-234 and Aspartic Acid-271 in Domain Closure, Cooperativity, and Catalysis in *Escherichia coli* Aspartate Transcarbamylase†

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Received July 1, 1988

ABSTRACT: Two mutant versions of *Escherichia coli* aspartate transcarbamylase were created by site-specific mutagenesis. Arg-234 of the 240s loop was replaced by serine in order to help deduce the function of the interactions that normally occur between Arg-234 and both Glu-50 and Gln-231 in the R state of the enzyme. The other mutation involved the replacement of Asp-271 by asparagine to further test the functional importance of the Tyr-240–Asp-271 link that has previously been proposed to stabilize the T state of the enzyme [Middleton, S. A., & Kantrowitz, E. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5866–5870]. The Arg-234 → Ser holoenzyme exhibits no cooperativity, a 24-fold reduction in maximal velocity, normal affinity for carbamyl phosphate, and substantially reduced affinity for aspartate and *N*-(phosphonoacetyl)-L-aspartate (PALA). Unlike the wild-type enzyme, the heterotropic effectors ATP and CTP are able to influence the activity of the Arg-234 → Ser enzyme at saturating aspartate concentrations. The Arg-234 → Ser catalytic subunit exhibits a 33-fold reduction in maximal activity, an aspartate K_m of 261 mM, compared to 5.7 mM for the wild-type catalytic subunit, and only a small alteration in the K_m for carbamyl phosphate. Together these results provide additional evidence that the interdomain bridging interactions between Glu-50 of the carbamyl phosphate domain and both Arg-167 and Arg-234 of the aspartate domain are necessary for the stabilization of the high-activity–high-affinity configuration of the active site of the enzyme. Furthermore, without the interdomain bridging interactions, the holoenzyme no longer exhibits homotropic cooperativity. These data also suggest that the interaction between Gln-231 and Arg-234, in the R state, is important for the formation of the high-affinity aspartate binding site. The Asp-271 → Asn holoenzyme exhibits normal maximal velocity, reduced cooperativity, and a lower substrate concentration at half the maximal activity. The properties of this mutant are identical with the Tyr-240 → Phe enzyme which indicates that it is the loss of the Asp-271–Tyr-240 interaction and not the replacement of the amino acids themselves which causes the observed effects in these two mutant enzymes. The link between Tyr-240 and Asp-271 stabilizes the position of the 240s loop in its T-state position, and these data further indicate the functional role that the 240s loop plays in the homotropic interactions of aspartate transcarbamylase.

Aspartate transcarbamylase from *Escherichia coli* (EC 2.1.3.2) has been the subject of extensive research over the last 3 decades, and its general properties have been reviewed (Gerhart, 1970; Jacobson & Stark, 1973; Schachman, 1974; Kantrowitz et al., 1980a,b). The enzyme catalyzes the first step in the pyrimidine pathway, the condensation of carbamyl phosphate with L-aspartate to form *N*-carbamyl-L-aspartate and phosphate. Homotropic cooperative interactions are observed for both substrates, and the enzyme exhibits heterotropic inhibition by CTP, the end product of the pyrimidine pathway, and heterotropic activation by ATP, the end product of the parallel purine pathway (Gerhart & Pardee, 1962; Bethel et al., 1968). These homotropic and heterotropic interactions provide aspartate transcarbamylase with a mechanism for sensing levels of purines and pyrimidines in the cell and maintaining a balance between them. The enzyme is composed of six catalytic polypeptide chains which combine to form two catalytic trimers (subunits) and six regulatory polypeptide chains which combine to form three regulatory dimers (subunits). The catalytic and regulatory subunits assemble spontaneously to form the holoenzyme[1] (M_r 310 000). The holoenzyme can be dissociated into catalytic and regulatory subunits which can be readily isolated (Gerhart & Pardee, 1962; Gerhart & Schachman, 1965; Rosenbusch & Weber, 1971). The isolated catalytic subunit retains activity but loses homotropic cooperative interactions, exhibiting hyperbolic kinetics, and is insensitive to the heterotropic effectors CTP and ATP. The isolated regulatory subunit still binds CTP and ATP but has no catalytic activity. The catalytic subunit can be further dissociated into folded or unfolded inactive monomers whose reassociation is correlated with restored activity (Burns & Schachman, 1982a,b), and ample evidence has shown that the active sites are shared between polypeptide chains within a catalytic subunit (Honzatko et al., 1982; Robey & Schachman, 1985; Krause et al., 1985, 1987; Wente & Schachman, 1987). The amino acid sequences of the catalytic and regulatory chains have been determined directly (Weber,

† This work was supported by grants from the National Institutes of Health (DK1429 and GM26237).

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[1] Abbreviations: 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; holoenzyme, native enzyme composed of two catalytic trimers and three regulatory dimers; 80s loop, flexible loop of the enzyme comprising amino acid residues 76–86; 240s loop, flexible loop of the enzyme comprising amino acid residues 231–240; pHMB, *p*-(hydroxymercuri)benzoate; Arg-234 → Ser enzyme, mutant enzyme with Ser in place of Arg-234; Asp-271 → Asn enzyme, mutant enzyme with Asn in place of Asp-271; Glu-50 → Gln enzyme, mutant enzyme with Gln in place of Glu-50; Tyr-240 → Phe enzyme, mutant enzyme with Phe in place of Tyr-240; Glu-239 → Gln enzyme, mutant enzyme with Gln in place of Glu-239.

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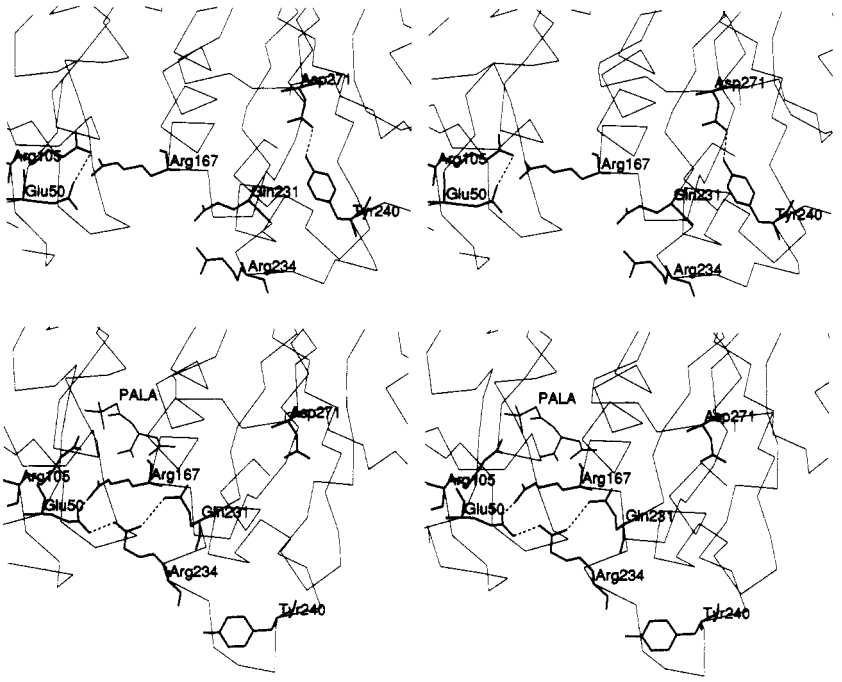

FIGURE 1: Stereoview of the region around the active site in aspartate transcarbamylase. In the T state (upper stereopair in figure), the active-site pocket is open, and Glu-50 and Arg-105 of the carbamyl phosphate domain form a charged interaction while Asp-271 and Tyr-240 of the aspartate domain are linked by a hydrogen bond which contributes to the stability of the T state (Middleton & Kantrowitz, 1986). In the R state (lower stereopair in figure), the active-site pocket (indicated by PALA) is closed, and the interaction between Asp-271 and Tyr-240 is lost. The movement and reorientation of the 240s loop allow new interactions between Glu-50 and both Arg-234 and Arg-167, which help to stabilize the domain-closed conformation (Ladjimi et al., 1988), while an interaction between Arg-234 and Gln-231 stabilizes the position of Gln-231, a residue which interacts with PALA.

1968; Konigsberg & Henderson, 1983) and by DNA sequencing of the structural genes (Hoover et al., 1983; Schachman et al., 1984). High-resolution X-ray crystallography has been used to solve the three-dimensional structures of the unliganded enzyme (Honzatko et al., 1982; Ke et al., 1984), the enzyme in the presence of CTP (Honzatko & Lipscomb, 1982; Kim et al., 1987), and the enzyme in the presence of *N*-(phosphonoacetyl)-L-aspartate (PALA) (Ladner et al., 1982; Krause et al., 1985, 1987), an inhibitor which is thought to bind in a conformation resembling the transition state of the reaction of the natural substrates (Collins & Stark, 1971).

For aspartate transcarbamylase, the allosteric transition from the T to R state occurs upon the binding of substrates or substrate analogues such as PALA and involves extensive structural changes at both the tertiary and quaternary levels. Comparison of the crystal structures of the T conformation of the enzyme (Kim et al., 1987) with the R conformation of the enzyme (Krause et al., 1987) reveals that on the quaternary level, the catalytic subunits rotate by 10° relative to one another, the regulatory subunits rotate 15° about the 2-fold axes, and the enzyme elongates by 12 Å. On the tertiary level, within a catalytic chain, the aspartate domain moves closer to the carbamyl phosphate domain by 3 Å in a domain closure motion (Krause et al., 1987), along with major rearrangements of the 80s loop (residues 76–86) and the 240s loop (residues 230–245).

The closure of the two domains of the catalytic chain, upon the binding of the substrates, is critical for the homotropic cooperativity of the enzyme (Ladjimi & Kantrowitz, 1988). Previous studies involving site-directed mutations at Glu-50 and Tyr-240 suggested that the interdomain bridging interactions of Glu-50 with both Arg-167 and Arg-234 stabilize the high-activity–high-affinity R state of the enzyme (Ladjimi et al., 1988) while the link between Tyr-240 and Asp-271 helps stabilize the low-activity–low-affinity T state of the enzyme (Middleton & Kantrowitz, 1986) (see Figure 1). In these studies, Glu-50 was replaced with Gln, eliminating the charged interaction with both Arg-167 and Arg-234 but still allowing hydrogen-bonding interactions with these residues, and Tyr-240 was replaced with Phe, eliminating the hydrogen-bonding interaction with Asp-271. In order to further investigate the importance of the 240s loop for the homotropic cooperative interactions of aspartate transcarbamylase, we replaced Arg-234 with Ser and Asp-271 with Asn. The substitution of Ser for Arg-234 eliminates any possible interactions with both Glu-50 and Gln-231 but should still allow the interaction between Glu-50 and Arg-167 while the substitution of Asn for Asp-271 eliminates the charged hydrogen bond with Tyr-240 but should still allow hydrogen bonding with this residue.

EXPERIMENTAL PROCEDURES

Materials

ATP, CTP, carbamyl phosphate, *N*-carbamyl-L-aspartate, ampicillin, agar, agarose, L-aspartate, and Tris 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). Enzyme-grade ammonium sulfate was obtained from ICN Biochemicals, and casamino acids were from Difco. 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.

E. coli strain U39a [F⁻ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *rspL*] was obtained from J. Wild, Texas A&M University, while *E. coli* strain HB2154 [F′ *ara*, *thi*, Δ*pro-lac*, *mutL*::Tn10(tet^r)/F′ *proAB*, *lacI*^{q}, *lacZ*ΔM15] was obtained from G. Winter, MRC

Laboratory of Molecular Biology, Cambridge, U.K. The M13 phage M13K07 and the plasmid pUC119 were obtained from J. Messing, Rutgers University, while the plasmid pGC2 was obtained from T. Maniatis, Harvard University.

Methods

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.

Enzyme Purification and Determination of Protein Concentration. 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*$^{\pm}$] containing either plasmid pEK38 (Ladjimi & Kantrowitz, 1988), pEK49, or pEK50 for the wild-type, the Arg-234 → Ser, or the Asp-271 → Asn enzymes, respectively (see Results). The wild-type and mutant catalytic subunits were isolated as described previously (Kantrowitz & Lipscomb, 1977).

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.

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 the 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 VAX 11/750.

Reaction of the Wild-Type and Mutant Enzymes with pHMB. The measurements of the rates of reaction of the wild-type and the mutant enzymes with pHMB were performed according to the method of Gerhart and Schachman (1968).

Plasmids Used. The plasmid pEK38 contains the vector pUC119 and the entire *pyrBI* operon coding for the catalytic and regulatory chains of aspartate transcarbamylase (Ladjimi & Kantrowitz, 1988). Prior to the construction of this plasmid, another plasmid, pEK24, was created by cloning the *pyrBI* operon into the vector pGC2 (Myers et al., 1985). This was accomplished by using the same restriction endonucleases and methods as described by Ladjimi and Kantrowitz (1988) for the creation of pEK38. The vectors pUC119 and pGC2 both contain the M13 intergenic region and thus, upon the addition of a helper phage such as M13K07, produce single-stranded plasmid DNA (Vieira & Messing, 1987). The advantage of these plasmids is that a mutation created in the *pyrBI* operon in M13 can be moved into pEK24 or pEK38, single-stranded plasmid DNA can be isolated, and the presence of the mutation in the newly created plasmid can be confirmed by dideoxy sequencing. Alternatively, the mutagenesis can be performed by using single-stranded plasmid DNA instead of single-stranded M13 DNA as template for the mutagenic oligonucleotide. This eliminates the need to isolate double-stranded M13 RF DNA for subsequent cloning steps, and this method, using single-stranded pEK24 as template, was used to create the Arg-234 → Ser and the Asp-271 → Asn enzymes (see Results).

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

	V_{max}[a] (mmol·h^{-1}·mg^{-1})	$[S]_{0.5}^{Asp}$ (mM)	n_H^{Asp}
wild type	13.9	11.7	2.3
Arg-234 → Ser	0.54	99.7[b]	1.0
Asp-271 → Asn	15.3	4.5	1.8

[a] The V_{max} values are the maximal observed specific activities. For the wild-type and Asp-271 → Asn enzymes, the kinetic parameters were calculated from the data shown in Figure 2 by a nonlinear least-squares procedure with a modified Hill equation that incorporates substrate inhibition (Pastra-Landis et al., 1978). The data for the Arg-234 → Ser enzyme were fit by the same procedure to the Michaelis–Menten equation. [b] For the Arg-234 → Ser enzyme, which lacks cooperativity, the $[S]_{0.5}$ value corresponds to the apparent K_m for asparatate.

RESULTS

Creation of the Arg-234 → Ser and Asp-271 → Asn Enzymes by Site-Directed Mutagenesis. The replacement of Arg-234 with Ser and Asp-271 with Asn by site-directed mutagenesis was accomplished by using the method of Zoller and Smith (1982) except that single-stranded plasmid pEK24 DNA was used as template in the fill-out/ligation step instead of single-stranded M13 DNA (see Experimental Procedures). The filled-out pEK24 was transformed into HB2154 (Carter et al., 1985), and the cells were grown for 1 h in YT medium (Miller, 1972) at 37 °C and then plated for colonies. Selection was accomplished by colony blot hybridization (Carter et al., 1984), and 10 candidates from each mutagenesis that showed dark spots on the nitrocellulose filters were grown for the production of single-stranded plasmid DNA using the helper phage M13K07 (Viera & Messing, 1987). According to dideoxy sequencing (Sanger et al., 1977), one of the Arg-234 → Ser candidates was shown to contain an Arg to Ser-234 change, and two of the Asp-271 → Asn candidates were shown to contain an Asp to Asn-271 change. The Ser-234 mutation in pEK24 and the Asn-271 mutation in pEK24 were then moved into pEK38. This was accomplished by cutting pEK24 carrying the mutations and pEK38 with *Bst*EII and *Eco*RV and, after separation by agarose gel electrophoresis, isolating the appropriate fragments using NA45 paper. 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 pEK49 and pEK50 containing the Arg-234 → Ser and Asp-271 → Asn mutations, respectively, were isolated by alkaline lysis and checked for intact *Bst*EII and *Eco*RV sites and correct size by restriction analysis. The entire region between the *Bst*EII and *Eco*RV sites in pEK49 and pEK50 was sequenced, and analysis of the sequence data revealed no mutations other than the Arg to Ser-234 change in pEK49 and the Asp to Asn-271 change in pEK50.

Kinetic Properties of the Arg-234 → Ser and Asp-271 → Asn Mutant Holoenzymes. The Arg-234 → Ser mutation results in large alterations in the kinetic properties of the enzyme. Compared to the wild-type enzyme, the maximal actvity of the Arg-234 → Ser enzyme is reduced by 24-fold, the $[S]_{0.5}$ for aspartate increases by about 8-fold, and the cooperativity is abolished (Table I and Figure 2). Although the $[S]_{0.5}$ for aspartate increases significantly for the Arg-234 → Ser enzyme, the $[S]_{0.5}$ as well as the K_D for carbamyl phosphate is unchanged relative to the wild type (data not shown).

Table II: Kinetic Parameters of the Wild-Type and Arg-234 → Ser 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-234 → Ser	0.66	261.3	0.060	6.4	0.02

[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 enzyme were fit by the same procedure to a modified Michaelis–Menten equation that incorporates substrate inhibition. [b] The maximal 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 mM and 300 mM for the wild-type and Arg-234 → 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 2B), the V_{max} value used to calculate the k_{cat} for the wild-type catalytic subunit was the maximal observed velocity.

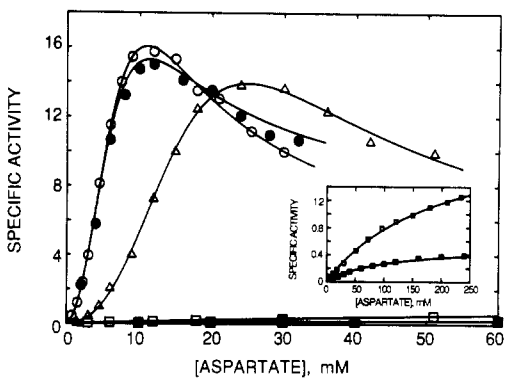

FIGURE 2: Aspartate saturation curves of the wild-type (△), the Arg-234 → Ser (■), and the Asp-271 → Asn (●) holoenzymes. For comparison purposes, the aspartate saturation data for the Glu-50 → Gln (□) (Ladjimi et al., 1988) and Tyr-240 → Phe (○) (Middleton & Kantrowitz, 1986) holoenzymes are also shown. (Inset) Region of low specific activity showing the data for the Arg-234 → Ser (■) and the Glu-50 → Gln (□) enzymes. 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.

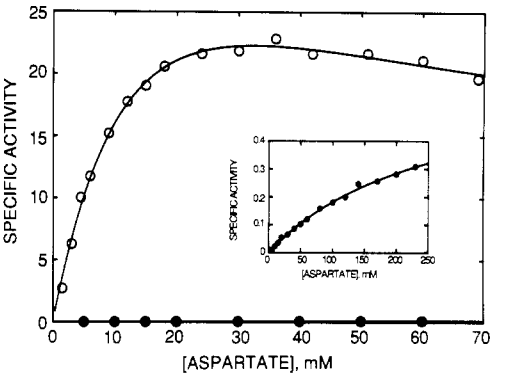

FIGURE 3: Aspartate saturation curves of the wild-type (○) and the Arg-234 → Ser (●) isolated catalytic subunits. (Inset) Region of low specific activity showing the data for the Arg-234 → Ser (●) catalytic subunit. Specific activity is in units of millimoles per hour per milligram, and assays were performed under the conditions described in the legend to Figure 2.

The Asp-271 → Asn mutation causes a significant decrease both in the $[S]_{0.5}$ for aspartate and in cooperativity but has no effect on the maximal activity of the enzyme (Figure 2). As can be seen in Table I, the $[S]_{0.5}$ for aspartate is decreased almost 3-fold in the Asp-271 → Asn enzyme compared to the wild type.

Kinetic Properties of the Arg-234 → Ser Mutant Catalytic Subunit. Unlike the wild-type holoenzyme, the isolated wild-type catalytic subunit exhibits Michaelis–Menten kinetics. The catalytic subunit not only lacks cooperativity but also has an increased affinity for substrates and a higher maximal velocity compared to the holoenzyme. This suggests that, without the constraints imposed on the active sites by the regulatory subunits, the catalytic subunit exists in a high-activity–high-affinity state. This does not imply, however, that the isolated catalytic subunit exists in the same high-activity–high-affinity state as the R state of the holoenzyme. Figure 3 shows the aspartate saturation curves for the wild-type and Arg-234 → Ser catalytic subunits, and a summary of the kinetic parameters is presented in Table II. The Arg-234 → Ser catalytic subunit exhibits changes in kinetic properties similar to the changes exhibited by the Arg-234 → Ser holoenzyme. While the maximal velocity of the Arg-234 → Ser catalytic subunit increases only slightly over the value for the mutant holoenzyme, the K_m for aspartate increases by over 2-fold relative to the apparent K_m for aspartate of the mutant holoenzyme. Compared to the wild-type catalytic subunit, the K_m for aspartate of the Arg-234 → Ser catalytic subunit increases over 45-fold, and the activity drops over 33-fold (Table II). Since the K_m for carbamyl phosphate is increased by only about 2-fold in the Arg-234 → Ser catalytic subunit (Table II) and the K_D for carbamyl phosphate of the mutant holoenzyme is nearly unchanged, the major effect of this mutation with respect to substrate is seen in the dramatically increased K_m for aspartate.

PALA Inhibition of the Arg-234→Ser Enzyme. In the presence of low concentrations of aspartate, the majority of the enzyme is in the T state, and low concentrations of PALA actually increase enzyme activity (Collins & Stark, 1971). Foote and Schachman (1985) have shown that the binding of a molecule of PALA at one active site in aspartate transcarbamylase is sufficient to convert all of the active sites to a high-activity–high-affinity state. Thus, the binding of PALA to one active site induces the quaternary conformational change, increasing the activity and affinity for the substrates in the unoccupied active sites. As can be seen in Table I, the Arg-234 → Ser mutation causes a significant loss of activity and a dramatic increase in the $[S]_{0.5}$ for aspartate. These characteristics of low activity and low affinity for substrate suggest that the T ⇌ R equilibrium in this mutant enzyme is shifted toward the T state. It is possible that the mutant enzyme is unable to undergo the conformational transition to the R state and, thus, the active sites would be unable to attain a high-activity–high-affinity conformation. Alternatively, the gross conformational change could still occur in the Arg-234 → Ser enzyme, but the structure of the active sites could be affected such that the T- and R-state active-site conformations possess similar affinities for substrate. In order to determine whether there are interacting active sites and a T to R transition in the Arg-234 → Ser enzyme, PALA activation experiments were performed. As can be seen in Figure 4, the Arg-234 → Ser enzyme shows no activation by PALA even at low concentrations, and, in addition, unusually high concentrations of PALA are necessary to inhibit activity.

In order to further evaluate the interactions of PALA with the Arg-234 → Ser enzyme, the PALA K_i was determined by the method of Collins and Stark (1971). The K_i for PALA

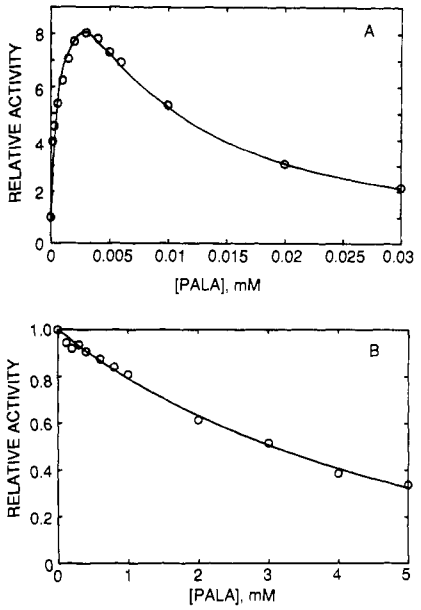

FIGURE 4: PALA saturation curves of the wild-type (A) and the Arg-234 → Ser (B) holoenzymes. Assays were performed under the conditions described in the legend to Figure 2 at approximately $^1/_{12}$th the $[S]_{0.5}$ for aspartate (1 and 9 mM aspartate for the wild-type and Arg-234 → Ser enzymes, respectively). The relative activity represents the ratio of specific activity in the absence or presence of PALA to that in its absence.

determined for the Arg-234 → Ser enzyme was found to be 65 μM, approximately 2400-fold higher than the value reported for the wild-type enzyme (Collins & Stark, 1971).

Influence of Effectors on the Arg-234 → Ser Mutant Enzyme. In a previously described mutant enzyme in which Glu-50 in the catalytic chain of aspartate transcarbamylase was replaced by Gln, activation by ATP and inhibition by CTP were observed at saturating concentrations of aspartate (Ladjimi et al., 1988). This is in contrast to the wild type which is an enzyme of the K system (Monod et al., 1965) and is not activated or inhibited by the effectors at saturating concentrations of aspartate. Since in the wild-type enzyme Arg-234 forms an interdomain charged interaction with Glu-50, it was of interest to determine whether the Arg-234 → Ser and Glu-50 → Gln enzymes have similarly altered properties with respect to effectors. Therefore, saturation kinetics with ATP and CTP were performed on the Arg-234 → Ser enzyme at the K_m and at $3K_m$ for aspartate. At the K_m for aspartate, the Arg-234 → Ser enzyme is activated to 157% by ATP and inhibited to 29% by CTP. These values are only slightly less than the activation and inhibition observed for the wild type at the $[S]_{0.5}$ for aspartate (Ladjimi et al., 1988). At $3K_m$ for aspartate, however, where the wild type is not activated or inhibited, the Arg-234 → Ser enzyme is still activated to 133% by ATP and still inhibited to 32% by CTP (Figure 5). Thus, unlike the wild type and similar to the Glu-50 → Gln enzyme, the Arg-234 → Ser enzyme is further activated by ATP and inhibited by CTP at high concentrations of aspartate.

Reaction of pHMB with the Arg-234 → Ser Enzyme. For the wild-type enzyme, the rate of the pHMB reaction in the presence of carbamyl phosphate plus succinate or PALA alone is substantially faster than when these ligands are absent. Furthermore, the rate of the pHMB reaction has been directly correlated with the quaternary structural change (Gerhart & Schachman, 1968). As seen in Table III, the rate of the pHMB reaction for the Arg-234 → Ser enzyme, without ligands, was 4.3-fold higher than for the wild type. This increased

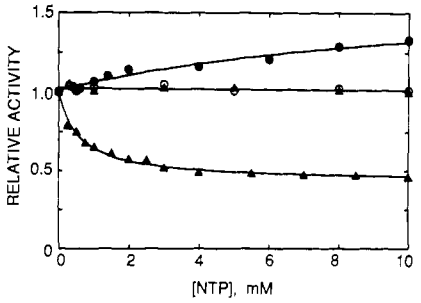

FIGURE 5: ATP and CTP saturation curves of the wild-type and the Arg-234 → Ser enzymes at high aspartate concentration (approximately $3K_m$). Assays were performed under the conditions described in the legend to Figure 2 in the presence of 30 mM aspartate and 300 mM aspartate for wild-type and Arg-234 → Ser enzymes, respectively. Influence of ATP on the wild-type (○) and Arg-234 → Ser (●) enzymes. Influence of CTP on the wild-type (△) and Arg-234 → Ser (▲) enzymes.

Table III: Reaction of the Wild-Type and Arg-234 → Ser Holoenzymes with pHMB[a]

	k ($M^{-1}\cdot s^{-1}$) for the indicated ligand			
	none	carbamyl phosphate	carbamyl phosphate + succinate	PALA
wild type	58	175	292	250
Arg-234 → Ser	253	591	602	580

[a] The reactions were carried out under the conditions described by Ladjimi and Kantrowitz (1987). Carbamyl phosphate, succinate, and PALA when present were at concentrations of 2 mM, 20 mM, and 50 μM, respectively. Higher concentrations of succinate and PALA did not further increase the reaction rates (data not shown).

reaction rate suggests that the local environment around the cysteine residues has been altered by the serine replacement at position 234.

In the presence of carbamyl phosphate, both the wild-type and Arg-234 → Ser enzymes exhibit a 2–3-fold increase in reaction rate; however, further addition of saturating succinate increases the rate of reaction for the wild-type enzyme but does not affect the rate for the Arg-234 → Ser enzyme. For the wild-type enzyme, PALA causes the same increase in rate as does the combination of carbamyl phosphate and succinate; however, for the Arg-234 → Ser mutant enzyme, PALA causes the same increase as carbamyl phosphate alone. This suggests that the conformation of the Arg-234 → Ser enzyme near the cysteine residues is identical whether PALA or carbamyl phosphate is used to induce the conformational change.

DISCUSSION

In order to further determine the interactions which stabilize the T and R states of aspartate transcarbamylase, two mutations have been made by site-directed mutagenesis. The analysis of the structural data (Krause et al., 1987; Kim et al., 1987) suggests that when aspartate binds to the wild-type enzyme (in the presence of carbamyl phosphate) the aspartate domain moves as a rigid body toward the carbamyl phosphate domain with the simultaneous reorientation of the 240s loop resulting in the closure of the active site. In the T state, the 240s loop is stabilized by a variety of interactions, including a link between Tyr-240 and Asp-271, which are lost upon the formation of the R state. On the basis of the substitution of Phe for Tyr-240 (Middleton & Kantrowitz, 1986), it has been suggested that this link is important for the stabilization of the T state of the enzyme. Here, we replace Asp-271 with Asn to further test this hypothesis. In the R state, the 240s loop is found in a completely different orientation than that

observed in the T state (Krause et al., 1987; Kim et al., 1987). The R-state position of the 240s loop, stabilized by the interdomain bridging interactions between Arg-234 and Glu-50, allows additional interactions which orient active-site residues. For example, Arg-229 and Gln-231, both of which bind to the β-carboxylate of aspartate (Krause et al., 1985, 1987; Gouaux & Lipscomb, 1988) (see Figure 1), form interactions with Glu-233 and Arg-234, respectively. In order to evaluate the role of the interdomain bridging interactions in orienting the 240s loop, we have replaced Arg-234 with serine.

Arg-234 Is Required for the Formation of the High-Activity–High-Affinity Active Site. When serine is inserted into the catalytic chain of aspartate transcarbamylase in place of Arg-234, the maximal velocity of the holoenzyme is reduced by 24-fold even though this residue does not interact directly with PALA (Krause et al., 1987) or carbamyl phosphate and succinate (Gouaux & Lipscomb, 1988). However, Arg-234 does interact indirectly with aspartate through its link with Gln-231, a residue which does interact with aspartate (Gouaux & Lipscomb, 1988) (see Figure 1).

The Arg-234 → Ser enzyme (V_{max} = 0.54 mmol·h^{-1}·mg^{-1}) has a lower maximal activity compared to the Glu-50 → Gln enzyme (V_{max} = 2.6 mmol·h^{-1}·mg^{-1}) (Ladjimi et al., 1988). The replacement of Glu-50 by Gln prevents the formation of the interdomain salt links, but this substitution still allows hydrogen-bonding interactions to occur between these residues. The higher maximal activity of the Glu-50 → Gln enzyme compared to the Arg-234 → Ser enzyme may be due to either the hydrogen-bonding interactions possible in the Glu-50 → Gln enzyme, which may assist in the stabilization of the domain-closed high-affinity–high-activity state of this mutant, or the interaction between Arg-234 and Gln-231 which is required for optimal activity.

Both the Glu-50 → Gln and Arg-234 → Ser enzymes, in which the interdomain bridging interactions are either weakened or completely eliminated, have extremely high aspartate K_m values. The high aspartate K_m resulting from the loss of the link between Glu-50 and Arg-234 by substitution at either position 50 or position 234 indicates that the interdomain bridging interactions are required for the creation of the high-affinity aspartate binding site. The interdomain bridging interactions may create the high-affinity aspartate binding site by the stabilization of the 240s loop in the R-state conformation. This conformation allows for the orientation of specific residues at the active site through interactions with residues of the 240s loop. For example, in the R state, Arg-234 stabilizes the position of Gln-231, and Glu-233 stabilizes the position of Arg-229. Since both Arg-229 and Gln-231 bind the β-carboxylate of aspartate, interactions which stabilize the positions of these residues would be important for the formation of the high-affinity aspartate binding site and may be important for orienting aspartate correctly for catalysis. The altered properties of the Arg-234 → Ser catalytic subunit suggest that these conclusions are also valid for the isolated catalytic subunit.

Interdomain Bridging Interactions Are Not Required for Normal Carbamyl Phosphate Affinity. The X-ray structure of the PALA-liganded enzyme (Krause et al., 1987) suggests that the interdomain bridging interactions are not involved in the binding of carbamyl phosphate. In order to test this, the carbamyl phosphate K_D was determined by both the method of Porter et al. (1969) and spectrophotometrically (data not shown) for the Arg-234 → Ser enzyme. Both methods give values for the carbamyl phosphate K_D identical with that of the wild-type enzyme, indicating that carbamyl phosphate binds normally to the Arg-234 → Ser enzyme. These results indicate that the binding of carbamyl phosphate is not dependent on the interdomain bridging interactions.

Arg-234 Is Important for Homotropic Cooperativity. The Arg-234 → Ser enzyme no longer exhibits sigmoidal saturation curves for aspartate and carbamyl phosphate. Analysis of the Glu-50 → Gln enzyme indicated that the interdomain bridging interactions in the wild-type enzyme between Arg-234 and Glu-50 can occur only after the domain closure which induces the quaternary conformational change (Ladjimi & Kantrowitz, 1988). As was the case for the Glu-50 → Gln enzyme (Ladjimi et al., 1988), the low activity and low affinity for aspartate of the Arg-234 → Ser enzyme as well as its lack of cooperativity indicate that the interdomain bridging interactions which stabilize the domain-closed conformation are important for cooperativity in aspartate transcarbamylase. On the other hand, the rearrangement of the 240s loop during the allosteric transition to the R state produces a new interaction between Arg-234 and Gln-231. Since Gln-231 is implicated in aspartate binding, the Arg-234–Gln-231 interaction may be involved in cooperativity by helping to create the high-activity–high-affinity active site.

Additional evidence for the importance of Arg-234 for homotropic cooperativity comes from the PALA activation experiments. Previous studies have shown that the Glu-50 → Gln enzyme is activated by PALA (Ladjimi et al., 1988) while the Arg-234 → Ser enzyme is not activated and is only inhibited at much greater concentrations of PALA than are required to inhibit the wild-type enzyme (Figure 4). These results suggest that unlike the Glu-50 → Gln enzyme, the Arg-234 → Ser enzyme is not able to achieve the high-activity–high-affinity conformation of the active site presumably either because domain closure is further destabilized by the total loss of the interdomain bridging interactions or because Arg-234 is critical for positioning Gln-231 correctly for aspartate binding and catalysis.

Interdomain Bridging Interactions Are Important for Heterotropic Effects. As opposed to the wild-type enzyme, the Arg-234 → Ser enzyme is still activated by ATP and inhibited by CTP at saturating concentrations of aspartate (see Figure 5). For this mutant enzyme, as was the case for the Glu-50 → Gln enzyme (Ladjimi et al., 1988), the allosteric effectors influence the maximal activity. This behavior is in contrast to the wild-type enzyme where the effectors cause shifts in the concentration of substrate which is half-saturating but do not affect maximal activity.

For the wild-type enzyme, the domain-closed high-activity–high-affinity state of the enzyme is stabilized by the charged interdomain bridging interactions. At saturating substrate concentrations, the presence of the nucleotide effectors would be inconsequential since ATP stabilizes the domain-closed conformation which is already maximally stabilized by the saturating levels of aspartate and carbamyl phosphate. Furthermore, CTP has little if any effect under these conditions and would only inhibit at low aspartate concentrations (in the presence of carbamyl phosphate) where some enzyme exists in the low-activity–low-affinity form.

In the case of the Arg-234 → Ser enzyme, increasing the concentration of aspartate (in the presence of saturating carbamyl phosphate) does not lead to the same stabilization of the domain-closed conformation as it does for the wild-type enzyme due to the loss of the interdomain charged interaction with Glu-50. Therefore, even at saturating aspartate concentrations, ATP can still assist in the stabilization of the domain-closed high-activity–high-affinity state which results

in increased catalytic activity. Under these same saturating substrate conditions, CTP can also act to further stabilize the domain-open low-activity–low-affinity form which results in reduced catalytic activity.

At high aspartate concentrations, ATP does not activate the Arg-234 → Ser enzyme as much as the Glu-50 → Gln enzyme, indicating that ATP cannot stabilize the closure of the domains as effectively as it can with the Glu-50 → Gln enzyme (Ladjimi et al., 1988; Figure 5). On the other hand, the CTP effect on these two enzymes at high aspartate concentrations is almost identical, reflecting the fact that the interdomain bridging interactions are not involved in the stabilization of the low-activity–low-affinity form of the enzyme.

Domain Closure and the Formation of the High-Activity–High-Affinity Form of the Enzyme Are Required for Strong PALA Binding. In the PALA activation experiment, PALA does not activate the Arg-234 → Ser enzyme, and furthermore, substantially more PALA is required to inhibit the Arg-234 → Ser enzyme than the wild type. The approximately 2400-fold higher K_i of PALA for the Arg-234 → Ser enzyme confirms the extremely weak binding of PALA to the Arg-234 → Ser enzyme. In the Glu-50 → Gln enzyme, PALA binding is much stronger (Ladjimi et al., 1988) which suggests that in this enzyme the interaction between Arg-234 and Gln-231 positions Gln-231 for a correct interaction with PALA and presumably aspartate. Thus, the loss of the Arg-234–Gln-231 interaction in the Arg-234 → Ser enzyme results in poor PALA binding and no PALA activation due to the incorrect formation of the high-affinity aspartate binding site.

On the basis of the pHMB reactivity experiment, succinate (in the presence of saturating carbamyl phosphate) does not induce additional conformational changes in the Arg-234 → Ser enzyme over and above that obtained with carbamyl phosphate alone. Furthermore, for the Arg-234 → Ser enzyme, PALA alone induces the same conformational change as carbamyl phosphate alone. This is not the case for the wild-type enzyme where PALA induces the same conformational change as succinate (in the presence of saturating carbamyl phosphate). These data suggest that the phosphonate moiety of PALA can mimic carbamyl phosphate when it binds to the unliganded Arg-234 → Ser enzyme, causing similar conformational changes as does carbamyl phosphate but not the same as PALA induces in the wild-type enzyme. For the Arg-234 → Ser enzyme, once carbamyl phosphate is bound, however, the high-activity–high-affinity aspartate binding site is not formed correctly.

240s Loop Is Important for Homotropic Cooperativity by Stabilization of both the T and R States of the Enzyme. Both Arg-234 and Asp-271 are involved in links which stabilize the 240s loop of the enzyme. Arg-234 helps to stabilize the position of the 240s loop in the R state through an interaction with Glu-50 while Asp-271 stabilizes the position of the 240s loop in the T state through an interaction with Tyr-240 (see Figure 1).

By replacement of Tyr-240 by Phe, Middleton and Kantrowitz (1986) have shown that the Tyr-240–Asp-271 link is involved in the stabilization of the T state of the enzyme. By replacement of Glu-239 by Gln, Ladjimi and Kantrowitz (1988) have shown that Glu-239 is absolutely critical for the stabilization of the T state. In fact, the Glu-239 → Gln mutant enzyme can no longer adopt the T conformation and exists only in a state with no cooperativity and high activity and affinity for substrates. In order to further test the functionality of the link between Tyr-240 and Asp-271, we replaced Asp-271 with asparagine which would eliminate the charged hydrogen bond but still allow an uncharged hydrogen bond between these two residues. The results reported here demonstrate that when Asp-271 is replaced by Asn, a mutant enzyme is obtained with almost exactly the same kinetic properties as the Tyr-240 → Phe enzyme (see Figure 2). Neither mutation has an effect on the maximal velocity of the enzyme while changes in the $[S]_{0.5}$ for aspartate for the Asp-271 → Asn and Tyr-240 → Phe enzymes (4.5 and 4.6, respectively) and in the Hill coefficients (1.8 and 1.8, respectively) are identical (Table I; Middleton & Kantrowitz, 1986). These results indicate that it is the loss of the Asp-271–Tyr-240 interaction and not the replacement of the amino acids themselves which causes the observed effects in the Asp-271 → Asn and Tyr-240 → Phe enzymes. Furthermore, the results for the Asp-271 → Asn enzyme indicate that an uncharged hydrogen bond between these two residues is not sufficient to stabilize the T state of the enzyme. This result agrees with the suggestion of Fersht et al. (1985) that charged hydrogen bonds are substantially stronger than uncharged hydrogen bonds.

Interactions involving Arg-234 of the 240s loop are important for the stabilization of the R state of aspartate transcarbamylase. Without the Glu-50–Arg-234 interaction, the enzyme has no cooperativity. Furthermore, the analysis of the mutant enzymes Glu-50 → Gln (Ladjimi et al., 1988) and Arg-234 → Ser, reported here, suggests that the interdomain bridging interactions as well as the Arg-234–Gln-231 interaction are a necessary prerequisite for the formation of the high-affinity aspartate binding site. The analysis of the mutant enzymes Glu-50 → Gln, Glu-239 → Gln, and Tyr-240 → Phe along with the two mutant enzymes reported here, Arg-234 → Ser and Asp-271 → Asn, provides additional evidence for the critical role that the 240s loop plays in homotropic cooperativity by alternatively stabilizing the T and R conformations of aspartate transcarbamylase.

ACKNOWLEDGMENTS

We thank W. N. Lipscomb for providing the X-ray coordinates.

Registry No. EC 2.1.3.2, 9012-49-1; PALA, 51321-79-0; CTP, 65-47-4; ATP, 56-65-5; L-Asp, 56-84-8; L-Arg, 74-79-3; L-Asn, 70-47-3; L-Ser, 56-45-1; carbamyl phosphate, 590-55-6.

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