The allosteric activator ATP induces a substrate-dependent alteration of the quaternary structure of a mutant aspartate transcarbamoylase impaired in active site closure

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Abstract

Aspartate transcarbamoylase from *Escherichia coli* shows homotropic cooperativity for aspartate as well as heterotropic regulation by nucleotides. Structurally, it consists of two trimeric catalytic subunits and three dimeric regulatory subunits, each chain being comprised of two domains. Glu-50 and Ser-171 are involved in stabilizing the closed conformation of the catalytic chain. Replacement of Glu-50 or Ser-171 by Ala in the holoenzyme has been shown previously to result in marked decreases in the maximal observed specific activity, homotropic cooperativity, and affinity for aspartate (Dembowski NJ, Newton CJ, Kantrowitz ER, 1990, *Biochemistry* 29:3716–3723; Newton CJ, Kantrowitz ER, 1990, *Biochemistry* 29:1444–1451). We have constructed a double mutant enzyme combining both mutations. The resulting Glu-50/Ser-171 → Ala enzyme is 9-fold less active than the Ser-171 → Ala enzyme, 69-fold less active than the Glu-50 → Ala enzyme, and shows 1.3-fold and 1.6-fold increases in the $[S]_{0.5}^{ASP}$ as compared to the Ser-171 → Ala and Glu-50 → Ala enzymes, respectively. However, the double mutant enzyme exhibits some enhancement of homotropic cooperativity with respect to aspartate, relative to the single mutant enzymes. At subsaturating concentrations of aspartate, the Glu-50/Ser-171 → Ala enzyme is activated less by ATP than either the Glu-50 → Ala or Ser-171 → Ala enzyme, whereas CTP inhibition is intermediate between that of the two single mutants. As opposed to the wild-type enzyme, the Glu-50/Ser-171 → Ala enzyme is activated by ATP and inhibited by CTP at saturating concentrations of aspartate. Structural analysis of the Ser-171 → Ala and Glu-50/Ser-171 → Ala enzymes by solution X-ray scattering indicates that both mutants exist in the same T quaternary structure as the wild-type enzyme in the absence of ligands, and in the same R quaternary structure in the presence of saturating *N*-(phosphonoacetyl)-L-aspartate. However, saturating concentrations of carbamoyl phosphate and succinate are unable to convert a significant fraction of either mutant enzyme population to the R quaternary structure, as has been observed previously for the Glu-50 → Ala enzyme. The curves for both the Ser-171 → Ala and Glu-50/Ser-171 → Ala enzymes obtained in the presence of substoichiometric amounts of PALA are linear combinations of the two extreme T and R states. The structural consequences of nucleotide binding to these two enzymes were also investigated. Most surprisingly, the direction and amplitude of the effect of ATP upon the double mutant enzyme were shown to vary depending upon the substrate analogue used.

Keywords: domain closure; heterotropic regulation; homotropic cooperativity; protein structure–function; site-specific mutagenesis; solution X-ray scattering

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Abbreviations: CP, carbamoyl phosphate; $[S]_{0.5}^{ASP}$, the aspartate concentration at half the maximal observed specific activity; $[S]_{0.5}^{CP}$, the carbamoyl phosphate concentration at half the maximal observed specific activity; MWC, the Monod, Wyman, and Changeux model (Monod et al., 1965).

Escherichia coli aspartate transcarbamoylase (E.C. 2.1.3.2) catalyzes the condensation of L-aspartate with carbamoyl phosphate to form *N*-carbamoyl-L-aspartate and inorganic phosphate as the committed step in the biosynthesis of pyrimidines (Porter et al., 1969). This allosteric enzyme exhibits positive homotropic cooperativity for the substrate aspartate (Gerhart & Pardee, 1962), heterotropic inhibition by CTP (Gerhart & Pardee, 1962), and by UTP in the presence of CTP (Gerhart & Pardee, 1962; Wild et al.,

1989), as well as heterotropic activation by ATP (Gerhart & Pardee, 1962).

The X-ray structures of the enzyme in the absence (T structure) and in the presence (R structure) of substrate analogues have been determined (for a review see Lipscomb, 1994). A comparison of these two structures indicates that the T $\rightarrow$ R quaternary transition involves an expansion of the enzyme by 11 Å along the threefold axis, along with rotations of the catalytic subunits about this axis and the regulatory subunits about their respective twofold axes. In addition to these quaternary structural changes, there are tertiary conformational changes within both the catalytic and regulatory chains. In particular, during the T $\rightarrow$ R transition, the two domains of each catalytic chain close, whereas the two domains of each regulatory chain open. Site-specific mutagenesis experiments indicate that the closure of the two domains of the catalytic chain is critical for the formation of the high-activity, high-affinity R-state of the enzyme, for homotropic cooperativity (Kantrowitz & Lipscomb, 1988, 1990; Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988; Newton & Kantrowitz, 1990), and for attaining the proper active-site conformation needed for catalysis.

Critical for the domain-closed R-state are two sets of inter-domain bridging interactions: one set involves interactions between Glu-50 and both Arg-167 and Arg-234, whereas the other involves interactions between Ser-171 and both the side chain of Gln-133 and the backbone of His-134. The mutants Glu-50 $\rightarrow$ Ala⁵ (Newton & Kantrowitz, 1990), Glu-50 $\rightarrow$ Gln (Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988), Arg-234 $\rightarrow$ Ser (Middleton & Kantrowitz, 1988) and Ser-171 $\rightarrow$ Ala (Dembowski et al., 1990) show significantly reduced maximal velocities, decreased affinities for aspartate, and reduced or eliminated cooperativity. Another characteristic of mutants impaired in domain closure is that, in contrast to the wild-type enzyme, they can be activated by ATP or PALA and inhibited by CTP, even in the presence of saturating concentrations of carbamoyl phosphate and aspartate (Ladjimi et al., 1988; Dembowski et al., 1990; Newton & Kantrowitz, 1990). Low-angle X-ray scattering data for the Glu-50 $\rightarrow$ Ala enzyme have indicated that PALA can convert the enzyme from the T to the R-structural state; however, the combination of carbamoyl phosphate and the aspartate analogue succinate does not cause any quaternary structural alteration (Tauc et al., 1994). In contrast, the combination of carbamoyl phosphate and succinate is sufficient to convert the wild-type enzyme completely into the R-structural state (Tsuruta et al., 1994).

Kinetic isotope effects and equilibrium isotope exchange kinetics have been used to probe the dynamic properties of the wild-type and Glu-50 $\rightarrow$ Ala enzymes (Lee et al., 1995). For the wild-type enzyme, both kinetic isotope effects and equilibrium isotope exchange kinetics suggest that the mechanism may vary from random to ordered depending on a number of factors, including the degree to which the enzyme has undergone the T $\rightarrow$ R transition. In particular, with complete closure of the two domains of the catalytic chain, compulsory ordered substrate binding occurs. The Glu-50 $\rightarrow$ Ala enzyme operates by a more random mechanism, indicating that the closure of the domains is critical for normal

catalysis as well as for the allosteric transition between the T and R states. To investigate further the role of domain closure in aspartate transcarbamoylase, we present kinetic and structural studies for a double mutant version of the enzyme, in which each of the individual mutations has been shown to be critical for domain closure and for the transition of the enzyme to the R state. If this enzyme does not make the transition to the R state, the properties of the T state of the enzyme could be evaluated independent of the complications arising from the equilibrium between the T and R states.

Results

Kinetic properties of wild-type and mutant holoenzymes with respect to aspartate and carbamoyl phosphate

The aspartate saturation curves for Glu-50 $\rightarrow$ Ala, Ser-171 $\rightarrow$ Ala and Glu-50/Ser-171 $\rightarrow$ Ala holoenzymes are shown in Figure 1, and the kinetic parameters for these enzymes and the wild-type enzyme are summarized in Table 1. It is clear from the kinetic data that the Glu-50 $\rightarrow$ Ala mutation has a similar effect on the enzyme as that caused by the Ser-171 $\rightarrow$ Ala mutation. Whereas replacement of Glu-50 by Ala results in a 19-fold reduction in the maximal activity, a seven-fold increase in the $[S]_{0.5}^{Asp}$, and a significant decrease in the cooperativity toward aspartate, replacement of Ser-171 by Ala results in a 148-fold decrease in the maximal activity, a nine-fold increase in the $[S]_{0.5}^{Asp}$, and an identical reduction in cooperativity (Table 1). Introduction of the Glu-50 $\rightarrow$ Ala mutation in the Ser-171 $\rightarrow$ Ala mutant results in a 1,300-fold decrease in the maximal activity and an 11-fold increase in the $[S]_{0.5}^{Asp}$ with respect to the wild-type enzyme; however, the double mutant regains some cooperativity compared to the single Glu-50 $\rightarrow$ Ala and Ser-171 $\rightarrow$ Ala enzymes (Table 1).

The $[S]_{0.5}^{Cp}$ values calculated from the carbamoyl phosphate saturation curves of the wild-type, Glu-50 $\rightarrow$ Ala, and the Ser-171 $\rightarrow$ Ala enzymes resemble those of the wild-type enzyme, with the exception of the $[S]_{0.5}^{Cp}$ for the Glu-50/Ser-171 $\rightarrow$ Ala enzyme, which is approximately two-fold larger, indicating a slight decrease in the affinity for carbamoyl phosphate (Table 1).

Fig. 1. Aspartate saturation curves of the Glu-50 $\rightarrow$ Ala (○), Ser-171 $\rightarrow$ Ala (●), and the Glu-50/Ser-171 $\rightarrow$ Ala (■) enzymes in the presence of saturating carbamoyl phosphate (4.8 mM). Assays were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. Specific activity is reported in units of $\text{mmol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$. Inset: expanded region for the data from the Glu-50/Ser-171 $\rightarrow$ Ala enzyme.

⁵The notation used to name the mutant enzymes is, for example, the Ser-171 $\rightarrow$ Ala enzyme. The wild-type amino acid and its location within the catalytic chain are indicated to the left of the arrow and the new amino acid is indicated to the right of the arrow. Glu-50/Ser-171 $\rightarrow$ Ala corresponds to the double mutant that has alanine at both positions 50 and 171 in the catalytic chain.

Table 1. Kinetic parameters of the wild-type and mutant holoenzymes^{a,b}

Enzyme	Maximal velocity ^c (mmol·h ⁻¹ ·mg ⁻¹)	[S] _{0.5} ^{Asp} (mM)	<i>n</i> _H ^{Asp}	[S] _{0.5} ^{CTP} (mM)
Wild-type ^d	20.7 ± 0.4	12.2 ± 0.2	2.6 ± <0.1	0.26 ± <0.01
Glu-50 → Ala	1.1 ± 0.05	89.2 ± 1.0	1.6 ± <0.1	0.24 ^e
Ser-171 → Ala	0.14 ± 0.05	104 ± 3.5	1.6 ± <0.1	0.17 ^f
Glu-50/Ser-171 → Ala	0.016 ± 0.004	139 ± 3	1.9 ± 0.1	0.45 ± 0.04

^aData are the average of two independent determinations, except the maximal velocity, [S]_{0.5}^{Asp}, and *n*_H^{Asp} values of the wild-type and Glu-50/Ser-171 → Ala enzymes, which are the average of three independent determinations.

^b*V*_{max} and the Hill coefficient (*n*_H) were calculated by a nonlinear least-squares procedure using the Hill equation, incorporating a term for substrate inhibition when necessary (Pastra-Landis et al., 1978).

^cMaximal velocity represents the maximal observed specific activity from the aspartate saturation curve.

^dData taken from Baker and Kantrowitz (1993).

^eData taken from Newton and Kantrowitz (1990).

^fData taken from Dembowski et al. (1990).

Influence of allosteric effectors on wild-type and mutant holoenzymes

In order to determine whether the heterotropic effects induced by the regulatory nucleotides were altered by the introduction of the single and double mutations, the wild-type, Glu-50 → Ala, Ser-171 → Ala, and Glu-50/Ser-171 → Ala enzymes were assayed in the presence of ATP and CTP, at both subsaturating and saturating concentrations of aspartate.

At an aspartate concentration corresponding to one-half the [S]_{0.5}^{Asp}, ATP activates the wild-type and Ser-171 → Ala enzymes to about the same extent, whereas the activation of the Glu-50 → Ala and Glu-50/Ser-171 → Ala enzymes is significantly lower than that of the wild-type enzyme (Table 2). The maximal inhibition of the wild-type and Glu-50 → Ala enzymes by CTP is almost identical (Table 2). However, the Ser-171 → Ala enzyme is inhibited significantly less than either the wild-type or the Glu-50 → Ala enzyme (Table 2). For the Glu-50/Ser-171 → Ala enzyme, the maximal inhibition by CTP is intermediate between that of the Ser-171 → Ala enzyme and the Glu-50 → Ala enzyme (Table 2). Furthermore, the concentration of CTP required for half-maximal inhibition (*K*_{CTP}) for the double mutant is higher than that required for either the wild-type, the Glu-50 → Ala, or the Ser-171 → Ala enzyme (Table 2). The concentration of ATP required for half-maximal activation (*K*_{ATP}) of the Glu-50/Ser-171 → Ala enzyme is lower than either of the single mutants, and is the same as for the wild-type enzyme (Table 2).

At a concentration of aspartate corresponding to three times the [S]_{0.5}^{Asp}, conditions under which ATP is unable to activate the wild-type enzyme because it is already fully in the R-state, the Glu-50 → Ala, Ser-171 → Ala, and Glu-50/Ser-171 → Ala enzymes show significant activation (Table 2), although the Glu-50/Ser-171 → Ala enzyme is activated the least of the three. Similarly, at a concentration of aspartate corresponding to five times the [S]_{0.5}^{Asp}, CTP fails to inhibit the wild-type enzyme significantly, whereas the Glu-50 → Ala, Ser-171 → Ala and Glu-50/Ser-171 → Ala enzymes are inhibited (Table 2); however, the Glu-50/Ser-171 → Ala enzyme was inhibited less than either of the single-mutant enzymes (Table 2).

Effects of succinate and PALA on wild-type and mutant holoenzymes

Although succinate activates the wild-type enzyme approximately sixfold (Fig. 2) at an aspartate concentration corresponding to one-fifth the [S]_{0.5}^{Asp}, it is unable to activate the Glu-50 → Ala, Ser-171 → Ala, or the Glu-50/Ser-171 → Ala enzyme (Fig. 2), even at a concentration of 200 mM. In contrast, PALA induces significant activation of all three mutant enzymes, although the concentration required for maximal activation is increased significantly (Fig. 3).

Table 2. ATP activation and CTP inhibition of the wild-type and mutant holoenzymes^a

Enzyme	Activity with ATP ^b	<i>K</i> _{ATP} (mM)	Activity with CTP ^c	<i>K</i> _{CTP} (mM)
Asp = 0.5 × [S]_{0.5}^{Asp}				
Wild-type	4.7	1.1	0.19	0.12
Glu-50 → Ala	3.4	1.4	0.18	0.08
Ser-171 → Ala	4.5	1.9	0.47	0.11
Glu-50/Ser-171 → Ala	1.9	1.1	0.35	0.18
Asp = 3.0 × [S]_{0.5}^{Asp} for ATP activation and 5.0 × [S]_{0.5}^{Asp} for CTP inhibition				
Wild-type	1	—	0.90	—
Glu-50 → Ala	2.7	4.7	0.19	0.27
Ser-171 → Ala	3.8	7.9	0.33	0.24
Glu-50/Ser-171 → Ala	1.3	3.1	0.59	0.76

^aAssays were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. Carbamoyl phosphate concentration was held constant at 4.8 mM for all enzymes. The concentration of aspartate used was either half, three, or five times the [S]_{0.5}^{Asp} of the respective enzymes (see Table 1).

^bActivity with ATP is defined as *A*^{ATP}/*A* where *A*^{ATP} is the activity extrapolated to an infinite concentration of ATP and *A* is the activity in the absence of ATP.

^cActivity with CTP is defined as *A*^{CTP}/*A* where *A*^{CTP} is the activity extrapolated to an infinite concentration of CTP and *A* is the activity in the absence of CTP.

Fig. 2. Effect of succinate on the activity of the wild-type (□), Glu-50 → Ala (○), Ser-171 → Ala (●), and the Glu-50/Ser-171 → Ala (■) enzymes. Assays were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. In each case, the carbamoyl phosphate concentration was held constant at 4.8 mM, whereas the aspartate concentration used was one-fifth their respective $[S]_{0.5}^{Asp}$ (see Table 1).

Fig. 3. Effect of PALA on the activity of the wild-type (□), Glu-50 → Ala (○), Ser-171 → Ala (●), and the Glu-50/Ser-171 → Ala (■) enzymes. Assays were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. In each case, the carbamoyl phosphate concentration was held constant at 4.8 mM, whereas the aspartate concentration used was one-fifth their respective $[S]_{0.5}^{Asp}$ (see Table 1).

Effect of substrate analogues on quaternary structure of wild-type, Ser-171 → Ala, and the Glu-50/Ser-171 → Ala enzymes

The X-ray scattering spectra of the wild-type, Ser-171 → Ala, and the Glu-50/Ser-171 → Ala enzymes were recorded in the absence as well as in the presence of a twofold molar excess of PALA. In the absence of ligands, the wild-type and both mutants enzymes

have virtually identical scattering patterns, as is also the case in the presence of PALA (Fig. 4A,B). The slight differences in scattering curves observed between the Glu-50/Ser-171 → Ala mutant and the wild-type enzyme can be accounted for by the presence of a small fraction of dissociated enzyme, revealed by SDS-PAGE.

Addition of a saturating concentration of carbamoyl phosphate (5 mM) alone gave scattering curves very close to those obtained in the absence of any substrate for the three enzymes examined

Fig. 4. Solution X-ray scattering spectra of the wild-type and Ser-171 → Ala enzymes (A), and the wild-type and Glu-50/Ser-171 → Ala enzymes (B). Spectra are shown for the unligated enzymes (lower curves, circles) and for the enzymes in the presence of a twofold molar excess of PALA (upper curves, squares). Thin lines, empty symbols: wild-type enzyme; thick lines, filled symbols: mutant enzymes.

(data not shown). As observed previously, carbamoyl phosphate alone is unable to provoke the structural transition to the R state for the wild-type enzyme (Hervé et al., 1985). However, in the presence of 3–4 mM succinate and a saturating concentration of carbamoyl phosphate, the wild-type enzyme undergoes the T $\rightarrow$ R transition (Hervé et al., 1985). By contrast, the scattering patterns of the Ser-171 $\rightarrow$ Ala and Glu-50/Ser-171 $\rightarrow$ Ala enzymes are barely affected by succinate concentrations as high as 250 mM in the presence of saturating carbamoyl phosphate (Fig. 5A,B). This clearly indicates that both mutant enzymes are unable to undergo the normal quaternary structure change in the presence of succinate, as observed previously with the Glu-50 $\rightarrow$ Ala enzyme (Tauc et al., 1994).

Effect of subsaturating concentrations of PALA on quaternary structure of Ser-171 $\rightarrow$ Ala and Glu-50/Ser-171 $\rightarrow$ Ala enzymes

X-ray scattering patterns were recorded with subsaturating concentrations of PALA, giving intermediate curves between T and R, as observed for the wild-type enzyme. In the case of the Ser-171 $\rightarrow$ Ala enzyme, the addition of 0.33 PALA molecules per active site resulted in a scattering pattern identical to a linear combination of 60% T and 40% R scattering patterns (Fig. 6A), close to the 50% T and 50% R observed for the wild-type enzyme under the same experimental conditions (Fetler et al., 1995). This slight retardation of the T to R transition observed for the Ser-171 $\rightarrow$ Ala enzyme during the PALA titration experiment is very much en-

hanced for the double mutant, the scattering pattern of which under the same conditions corresponds to only 6% of the enzyme molecules in the R form (Fig. 6B). This prompted us to record scattering patterns using five different total PALA concentrations. The various intermediate curves are well approximated by linear combinations of the two extreme T and R states corresponding to the following percentages of the R form: 16% R for 0.50 PALA/active site, 56% R for 1.0 PALA/active site (Fig. 6B), 90% R for 1.7 PALA/active site, and 100% R for 2 PALA/active site. The transition appears to be concerted. It is thus tempting to use the equations of the classical MWC model for a concerted transition (Monod et al., 1965) in order to determine the range of acceptable values for the model parameters L and c , as well as for the affinity constant K_R for PALA. The calculations, performed as explained in Materials and methods, yielded the following acceptable ranges for the three parameters: $80 \mu\text{M} < K_R < 250 \mu\text{M}$, $2,000 < L < 30,000$, and $0.003 < c < 0.05$, where the boundaries are narrowly coupled, the lower boundary for L being associated with the upper boundary on c and vice versa, and where the L value decreases with increasing K_R . One of the best fits to the data is presented in Figure 7. The calculated curve is in excellent agreement with the experimental points with only a minor deviation observed for the largest values of $[\text{PALA}]_{\text{tot}}$. Though large, the ranges of acceptable values for each parameter clearly indicate the effects of the amino acid substitutions when compared with the values derived for the wild-type enzyme, namely $K_R = 20 \text{ nM}$ for the catalytic subunit at pH 7 (Collins & Stark, 1971), $L = 140$, and $c = 0.07$ (Fetler et al., 1995).

Fig. 5. Solution X-ray scattering spectra of the Ser-171 $\rightarrow$ Ala (A) and Glu-50/Ser-171 $\rightarrow$ Ala (B) enzymes. Spectra are shown for the unligated enzymes (thin line, $\circ$), for the enzymes in the presence of 5 mM carbamoyl phosphate and 250 mM succinate (thick line, $\square$), and for the enzymes in the presence of 5 mM carbamoyl phosphate, 250 mM succinate, and 10 mM ATP (thick line, $\blacksquare$).

Fig. 6. Effect of a subsaturating concentration of PALA on the scattering patterns of the Ser-171 → Ala (A) and Glu-50/Ser-171 → Ala (B) enzymes. Thin line, best linear combination of T and R patterns; thick line, experimental curve. For A, the curve was recorded in the presence of 0.33 PALA molecules per active site, = 40% R; for B (lower curve), the curve was recorded in the presence of 0.33 PALA molecules per active site, = 6% R; for B (upper curve), the curve was recorded in the presence of 1.0 PALA molecule per active site, = 56% R.

Influence of nucleotide effectors on quaternary structure change of Ser-171 → Ala and Glu-50/Ser-171 → Ala enzymes

The behavior of the mutant enzymes in the presence of nucleotide effectors was investigated by adding ATP, CTP, or CTP plus UTP to a solution of either mutant already containing substrate analogues. The effect of ATP was investigated in the presence of a saturating concentration of carbamoyl phosphate and 250 mM succinate. Under these conditions, the scattering patterns of both mutant enzymes showed a marked R shift (0% to 18% R for the Ser-171 → Ala enzyme (Fig. 5A), and 0% to about 24% R for the Glu-50/Ser-171 → Ala enzyme (Fig. 5B)). The influence of CTP alone or with UTP was not tested under these conditions because carbamoyl phosphate and succinate did not alter the scattering pattern from the T-state curve.

In a second series of experiments, PALA was used in sufficient quantity to bring the scattering pattern approximately halfway between the T and R patterns. For the Ser-171 → Ala mutant, at a PALA concentration of 0.33 molecule per active site (40% R), addition of CTP or CTP plus UTP caused an identical T shift (to 32% R), similar to observations already made with the wild-type enzyme (Fetler et al., 1995). In contrast to the wild-type enzyme where ATP has no detectable effect on the T to R transition, the scattering pattern of the Ser-171 → Ala enzyme was shifted toward R by about 10% upon the addition of ATP (Fig. 8A). The results for the Glu-50/Ser-171 → Ala enzyme were in marked contrast to those indicated above (Fig. 8B). As already shown, one PALA molecule per active site was necessary to obtain a scattering pat-

tern halfway between T and R (56% R). Subsequent addition of CTP or CTP plus UTP shifts the scattering patterns nearly all the way back to the T pattern, i.e., 6% R and 2% R, respectively. Most unexpectedly, addition of ATP to the same solution of the Glu-50/Ser-171 → Ala enzyme with one PALA molecule per active site, causes a T shift, the scattering pattern being fairly well approximated by a linear combination corresponding to 32% R and 68% T curves.

Discussion

In the R quaternary structure, the two domains of the catalytic chain are close to one another in a conformation that confers to the active site a high affinity for aspartate. This closed conformation is stabilized by two sets of interdomain bridging interactions between Glu-50 of the carbamoyl phosphate domain and both Arg-167 and Arg-234 of the aspartate domain, and between Ser-171 of the aspartate domain, which interacts with both the backbone carbonyl of His-134 as well as the side chain of Gln-133. In order to investigate the role of these residues in domain closure, site-specific mutagenesis has been used to disrupt these interactions and the functional consequences of the mutations have been determined (Robey et al., 1986; Middleton & Kantrowitz, 1988; Dembowski et al., 1990; Newton & Kantrowitz, 1990; Stebbins et al., 1990). Moreover, the structural consequences of the Glu-50 → Ala mutation have been investigated using solution X-ray scattering (Tauc et al., 1994). To elaborate further on our previous studies, we constructed a double mutant in which both sets of interdomain interactions were disrupted simultaneously.

Fig. 7. Titration curve with PALA of the structural transition for the Glu-50/Ser-171 → Ala enzyme. Circles, fraction of molecules in the R structure determined by fitting linear combinations of T and R patterns to the experimental scattering curves as in Figure 8. Continuous line, calculated curve obtained with Equations 1–4 from the MWC model: $K_R = 160 \mu\text{M}$, $L = 5,000$, $c = 0.014$ (see text for details).

Mutation of Glu-50 increases homotropic cooperativity of the Ser-171 → Ala enzyme

A comparison of the binding properties of the Glu-50/Ser-171 → Ala enzyme with those of the single mutants shows that the presence of both mutations reduced the affinity for aspartate. Indeed, the $[S]_{0.5}^{\text{ASP}}$ for the double mutant is increased with respect to that of each of the enzymes with single mutations. This is to be expected, because the closure of the active-site cleft, which has been observed to be crucial for the establishment of the high-affinity aspartate binding site, is hindered by the individual Glu-50 → Ala and Ser-171 → Ala mutations. The twofold increase of the $[S]_{0.5}^{\text{CP}}$ is more surprising because neither single mutation altered the affinity of the enzyme for carbamoyl phosphate. In fact, carbamoyl phosphate binds to the carbamoyl phosphate domain without involvement of the aspartate domain. However, carbamoyl phosphate binding does alter the local conformation of the active site because the binding of substrates has been shown to follow a preferred order (Hsuanyu & Wedler, 1987). The two mutations may make this modification more difficult and the extra energy cost may be apparent through a slightly reduced affinity for carbamoyl phosphate.

The two single mutants, Glu-50 → Ala and Ser-171 → Ala, displayed reduced cooperativity (Table 1). Both mutants have been shown to be activated by substoichiometric amounts of PALA, and stimulated by the addition of ATP, even in the presence of saturating concentrations of aspartate (Dembowski et al., 1990; New-

ton & Kantrowitz, 1990). This activation has been interpreted by assuming that, in the presence of PALA, the mutant enzymes were able to adopt a higher-affinity state, which they could not reach with the physiological substrates or with the substrate analogue succinate.

Quaternary structures accessible to the mutants

Does this higher activity state correspond to the R structure or is there a different, less effective quaternary structure that is now accessible to the double mutant? Scattering patterns obtained in the presence of saturating amounts of PALA with both the single and the double mutant enzymes are identical to that of the wild-type enzyme. Together with the patterns recorded in the absence of substrates, they show that the two extreme quaternary structures of the T and R states are accessible to both the Ser-171 → Ala and Glu-50/Ser-171 → Ala enzymes, as they are to the Glu-50 → Ala enzyme (Tauc et al., 1994).

Effect of succinate in the presence of saturating carbamoyl phosphate

Concentrations of succinate as high as 250 mM are unable to alter significantly the scattering patterns of the Ser-171 → Ala and Glu-50/Ser-171 → Ala enzymes, as was seen previously with the Glu-50 → Ala mutant (Tauc et al., 1994). All three mutants remain predominantly in the T quaternary structure, accounting for the observed lack of stimulation of activity by succinate (Fig. 2). These results point toward a destabilization of the R quaternary structure due to the individual mutations or their combination. The differences between the levels of the residual cooperativity for aspartate express differences in the amplitude of the perturbation, whereas the similar absence of effect with succinate suggest that the ability of this analogue to trigger the T → R transition is more readily lost than with the physiological substrate. This has been confirmed recently by time-resolved solution X-ray scattering experiments performed with the Glu-50 → Ala enzyme, which show a partial T → R transition after mixing the enzyme with carbamoyl phosphate and aspartate (H. Tsuruta, D.P. Baker, P. Vachette, B. Stec, E.R. Kantrowitz, in prep.).

Titration of the quaternary structure transition with PALA

The effect of varying substoichiometric concentrations of PALA points to differences between the two mutants. Addition of 0.33 molecules of PALA per active site converts about half of the population of the Ser-171 → Ala enzyme into the R quaternary structure, as observed with the wild-type enzyme, and also is analogous to previous results obtained with the Glu-50 → Ala enzyme (Tauc et al., 1994), although the affinity of the latter enzyme for PALA appears to be reduced by several orders of magnitude (Newton & Kantrowitz, 1990). Under the same conditions, only 6% of the double-mutant enzyme is converted into the R quaternary structure. The analysis of the titration experiment performed with the double-mutant enzyme using the equations of the concerted model by Monod, Wyman, and Changeux shows a four-order of magnitude increase of K_R (lower affinity), a stronger partition coefficient of PALA between molecules in the T and R conformations, and a 40-fold increase on average in the equilibrium constant between T and R quaternary structures. The lower affinity reflects the destabilization of the closed conformation of the active site cleft due to the elimination of stabilizing bonds. The lower c value might be

Fig. 8. Effect of nucleotides on the scattering pattern of the Ser-171 → Ala (A) and Glu-50/Ser-171 → Ala (B) enzymes. For the Ser-171 → Ala enzyme, the curves were recorded in the presence of 0.33 PALA molecules per active site, whereas for the Glu-50/Ser-171 → Ala enzyme, the curves were recorded in the presence of 1.0 PALA molecule per active site. Thin lines, curves recorded in the absence of nucleotide; thick dots, curves recorded with the addition of 10 mM CTP; thick dashed lines, curves recorded with the addition of 5 mM CTP plus 5 mM UTP; thick continuous lines, curves recorded with the addition of 10 mM ATP.

due to the increased premium for substrate binding to the closed active site of a molecule in the R conformation, whereas the large L value is a direct expression of the destabilization of the R conformation. The last two results are in agreement with the partial restoration of cooperativity for aspartate observed in the activity measurements.

Effect of nucleotide effectors on activity and structure of the mutants

Nucleotides alter the activity of the double-mutant enzyme both at subsaturating and at saturating aspartate concentrations, as observed previously with the Glu-50 → Ala and Ser-171 → Ala enzymes (Dembowski et al., 1990; Newton & Kantrowitz, 1990) (Table 2). The stimulation by ATP is weakest for the double mutant under each condition, whereas CTP inhibition, weakest at saturating aspartate for the double mutant, is weaker for the Ser-171 → Ala enzyme at low aspartate.

Addition of ATP in the presence of a saturating concentration of carbamoyl phosphate and succinate causes an R shift for the Ser-171 → Ala and Glu-50/Ser-171 → Ala enzymes similar to that observed with the Glu-50 → Ala enzyme; only the amplitude of the shift differs, varying between 10 and 25%. Assuming a similar behavior in the presence of aspartate, this accounts, at least qualitatively, for the observed stimulation by ATP even at saturating aspartate concentrations.

The structural consequences of nucleotide binding in the presence of a substoichiometric concentration of PALA are more varied depending upon the mutant studied. Addition of CTP or CTP

plus UTP to the Ser-171 → Ala enzyme causes a small T shift, identical to that observed with the wild-type enzyme and slightly smaller than the effect on the Glu-50 → Ala enzyme under the same conditions (Tauc et al., 1994). Under the same conditions, a quasi-complete reversal to the T structure is observed with the double mutant (Fig. 8B). The combination of the two mutations has rendered the enzyme extremely responsive to nucleotide inhibitors. It is tempting to attribute this effect to a marked destabilization of the R quaternary structure, which also accounts for the low affinities for aspartate and PALA as well as for the very low activity.

Although ATP in the presence of a substoichiometric concentration of PALA has no effect on the Glu-50 → Ala enzyme, as observed with the wild-type enzyme (Newton & Kantrowitz, 1990), it causes a small R shift in the case of the Ser-171 → Ala enzyme and, most surprisingly, a marked T shift with the double mutant, from 55% down to 32% R structure. This is apparently difficult to reconcile with the observed activation by ATP at both low and high aspartate concentrations, as well as with the R shift observed in the presence of succinate. The fact that larger amplitude effects are observed with PALA than with succinate, contrary to previous observations made with the wild-type enzyme and several mutants (Vachette et al., 1994), is only an apparent paradox: because saturating amounts of carbamoyl phosphate and succinate are unable to cause a shift in the T to R transition in any significant way, there is simply no opportunity for a CTP effect; in contrast, the effect of ATP on an enzyme population predominantly in the T structure may be smaller than that on a population distributed equally be-

tween T and R structures, conditions chosen precisely to maximize the effect.

The change of direction of the ATP effect is more difficult to explain, but supports a role for the substrate in the effect of ATP. The network of interactions between the active-site residues and PALA on the one hand, and carbamoyl phosphate and succinate on the other, have been compared by Gouaux and Lipscomb (1988). Although the two complexes have many interactions in common, there are several new bonds between carbamoyl phosphate and succinate and the enzyme, such as that between Arg-54 and the mixed anhydride oxygen of carbamoyl phosphate, whereas succinate does not make as many H-bonds and salt links with the active-site residues than does the dicarboxylic acid moiety of PALA. One of those links, in the context of the double-mutant enzyme, whose affinity for PALA appears to be reduced by about four orders of magnitude, might explain the difference in response to ATP observed using either PALA or carbamoyl phosphate and succinate.

Materials and methods

Materials

Q-Sepharose Fast Flow resin was purchased from Pharmacia. ATP, CTP, L-aspartate, *N*-carbamoyl-L-aspartate, ampicillin, potassium dihydrogen phosphate, and uracil were obtained from Sigma. Carbamoyl phosphate dilithium salt, obtained from Sigma, was purified before use by precipitation from 50% (v/v) ethanol and was stored desiccated at -20°C (Gerhart & Pardee, 1962). Agarose, electrophoresis-grade acrylamide, enzyme-grade ammonium sulfate, and Tris were obtained from ICN Biomedicals. Casamino acids, yeast extract, and tryptone were obtained from Difco. Antipyrine was obtained from Kodak. Diacetyl monoxime was obtained from Fisher Scientific. Restriction endonucleases and T4 DNA ligase were obtained from either Amersham or New England Biolabs and used according to the manufacturers' recommendations.

Construction of plasmid pEK255

The plasmid pEK255, which carries the gene for the Glu-50/Ser-171 $\rightarrow$ Ala double mutant of aspartate transcarbamoylase, was constructed by fusion of appropriate DNA fragments derived from plasmids carrying the individual mutations. Plasmid pEK91 and pEK98, were digested with *Eco*R I and *Bst*E II and, in each case, the two product fragments were separated by agarose gel electrophoresis. The larger, 3.9-kb, fragment of pEK91, carrying the Glu-50 $\rightarrow$ Ala mutation, and the smaller, 2.1-kb, fragment of pEK98, carrying the Ser-171 $\rightarrow$ Ala mutation, were isolated and the compatible ends treated with T4 DNA ligase. The construction was confirmed by restriction analysis and the presence of both mutations was verified by dideoxy sequencing with single-stranded DNA isolated after coinfection with the helper phage VCSM13 (Stratagene).

Wild-type and mutant enzyme overproduction and purification

The wild-type and mutant aspartate transcarbamoylases were isolated from *E. coli* strain EK1104 [F^{-} *ara*, *thi*, Δ *pro-lac*, Δ *pyrB*, *pyrF*⁺, *rpsL*] containing the plasmids pEK54 (Xu et al., 1988), pEK91 (Newton & Kantrowitz, 1990), pEK98 (Dembowski et al., 1990), and pEK255, which code for the wild-type, Glu-50 $\rightarrow$ Ala,

Ser-171 $\rightarrow$ Ala, and Glu-50/Ser-171 $\rightarrow$ Ala enzymes, respectively. The holoenzymes were purified to electrophoretic homogeneity essentially as described by Nowlan and Kantrowitz (1985), except that an additional ion-exchange chromatography step using Q-Sepharose Fast Flow resin (Stebbins et al., 1989) and a final isoelectric precipitation step (Nowlan & Kantrowitz, 1985) were employed.

Aspartate transcarbamoylase assay

The transcarbamoylase activity was measured at 25°C by either a colorimetric (Prescott & Jones, 1969; Pastra-Landis et al., 1981) or a pH-stat method (Wu & Hammes, 1973). Colorimetric assays were performed in 50 mM Tris-acetate buffer, pH 8.3, in duplicate, and the data points in the figures are the average values. pH-stat assays were performed with a radiometer TTT80 titrator and an ABU80 autoburette.

Data analysis

The analysis of steady-state kinetic data was conducted as described previously (Silver et al., 1983). Data points were fit by a nonlinear least-squares procedure to the Hill equation incorporating a term for substrate inhibition when necessary (Pastra-Landis et al., 1978). Analysis of the structural data of the wild-type holoenzyme, based on the three-dimensional coordinates of the CTP-ligated complex (Stevens et al., 1990), the phosphonoacetamide-plus-malonate-ligated complex (Gouaux & Lipscomb, 1990), and the PALA-ligated complex (Ke et al., 1988), was accomplished using the program QUANTA (BioSym/MSI, San Diego, California).

Determination of protein concentration

Concentrations of pure wild-type holoenzyme were determined by absorbance measurements at 280 nm using the extinction coefficient of $0.59\text{ cm}^2\text{ mg}^{-1}$ (Gerhart & Holoubek, 1967). The protein concentrations of the mutant holoenzymes were determined using the Bio-Rad version of Bradford's dye-binding assay (Bradford, 1976) with the wild-type holoenzyme as the standard.

Solution X-ray scattering

Samples of the wild-type and Glu-50/Ser-171 $\rightarrow$ Ala enzymes were prepared from stock solutions in 50 mM Tris-acetate buffer, pH 8.3 and 0.1 mM dithiothreitol as reported previously (Fetler et al., 1995). X-ray scattering curves were recorded on the small-angle scattering instrument D24 using synchrotron radiation at LURE-DCI, Orsay. The instrument (Depaetex et al., 1987), the data acquisition system (Bordas et al., 1980), and the experimental procedures (Fetler et al., 1995) have been described.

Analysis of PALA titration data for the double mutant

The PALA titration data obtained with the Glu-50/Ser-171 $\rightarrow$ Ala enzyme were analyzed in terms of the MWC model in a manner similar to that used for the wild-type enzyme by Fetler et al. (1995), with the following differences. In the case of the wild-type enzyme, virtually all PALA molecules are bound at substoichiometric concentrations of PALA, because the active site concentration used (1 mM) is very large compared with the dissociation

constant of PALA (about 20 nM for the catalytic subunit at pH 7 [Collins & Stark, 1971]). The affinity of PALA for the active site of the double mutant enzyme is unknown, but appears to be reduced markedly. Consequently, we could not make the previous assumption, and a complete calculation was made without neglecting $[PALA]_{free}$ for various K_R values, using the classical equations of the model (Monod et al., 1965):

$$\bar{Y} = \frac{\alpha(1 + \alpha)^{n-1} + Lc\alpha(1 + c\alpha)^{n-1}}{(1 + \alpha)^n + L(1 + c\alpha)^n}, \quad (1)$$

$$\bar{R} = \frac{(1 + \alpha)^n}{(1 + \alpha)^n + L(1 + c\alpha)^n}, \quad (2)$$

with

$$\alpha = [PALA]_{free}/K_R \quad (3)$$

and

$$[PALA]_{Tot} = \alpha K_R + n\bar{Y}. \quad (4)$$

n is the number of active sites ($n = 6$).

For given values of K_R , L , and c , the value of α corresponding to each of the experimental $[PALA]_{Tot}$ values was determined numerically using Equations 1, 3, and 4. Then, the corresponding values were calculated using Equation 2. Calculations were performed over five orders of magnitude of K_R values and four orders of magnitude of L and c values equally sampled on a log scale, with K_R between 10 nM and 1 mM, L between 1 and 10^4 , and c ranging from 10^{-4} to 1, with a log step of 0.1. A χ^2 value measuring the goodness of fit to the experimental values was calculated for each (L , c) couple. Inspection of the distribution of χ^2 values in the (K_R , L , c) space guided a finer exploration of a subspace centered around the region of lower values. The solutions yielding a χ^2 value lower or equal to twice the minimum value were deemed acceptable. The boundaries of the acceptable ranges are coupled narrowly, the lower boundary for L being associated with the upper boundary on c and vice-versa, whereas the L value decreases with increasing K_R .

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