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Glu-50 in the catalytic chain of *Escherichia coli* aspartate transcarbamoylase plays a crucial role in the stability of the R quaternary structure

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Abstract

Glu-50 of aspartate transcarbamoylase from *Escherichia coli* forms a set of interdomain bridging interactions between the 2 domains of the catalytic chain; these interactions are critical for stabilization of the high-activity high-affinity form of the enzyme. The mutant enzyme with an alanine substituted for Glu-50 (Glu-50 → Ala) exhibits significantly reduced activity, little cooperativity, and altered regulatory behavior (Newton CJ, Kantrowitz ER, 1990, *Biochemistry 29*:1444–1451). A study of the structural consequences of replacing Glu-50 by alanine using solution X-ray scattering is reported here. Correspondingly, in the absence of substrates, the mutant enzyme is in the same, so-called T quaternary conformation as is the wild-type enzyme. In the presence of a saturating concentration of the bisubstrate analog *N*-phosphonacetyl-L-aspartate (PALA), the mutant enzyme is in the same, so-called R quaternary conformation as the wild-type enzyme. However, the Glu-50 → Ala enzyme differs from the wild-type enzyme, in that its scattering pattern is hardly altered by a combination of carbamoyl phosphate and succinate. Addition of ATP under these conditions does result in a slight shift toward the R structure. Steady-state kinetic studies indicate that, in contrast to the wild-type enzyme, the Glu-50 → Ala enzyme is activated by PALA at saturating concentrations of carbamoyl phosphate and aspartate, and that PALA increases the affinity of the mutant enzyme for aspartate. These data suggest that the enzyme does not undergo the normal T to R transition upon binding of the physiological substrates and verifies the previous suggestion that the interdomain bridging interactions involving Glu-50 are critical for the creation of the high-activity, high-affinity R state of the enzyme.

Keywords: aspartate carbamoyltransferase; quaternary structure; single amino acid mutation; solution X-ray scattering

Aspartate transcarbamoylase (EC 2.1.3.2.) from *Escherichia coli* catalyzes the first step of pyrimidine biosynthesis, the condensation of carbamoyl phosphate and L-aspartate to form *N*-carbamoyl-L-aspartate and phosphate. The reaction mechanism is "preferred order" with carbamoyl phosphate binding before aspartate, and *N*-carbamoylaspartate leaving before phosphate (Hsuanyu & Wedler, 1987). The *E. coli* enzyme is endowed with a very complex system for the regulation of its activity: it shows homotropic cooperativity for aspartate, is activated by ATP and inhibited by CTP (Gerhart & Pardee, 1962), and is inhibited synergistically by UTP in the presence of CTP (Wild et al., 1989). This extensively studied allosteric enzyme is composed of 2 trimers of 34,000 M_r catalytic chains and 3 dimers of 17,000 M_r regulatory chains carrying the effector binding sites. Two neighboring catalytic chains contribute amino acids to the active site (for reviews, see Kantrowitz & Lipscomb, 1988, 1990; Schachman, 1988; Hervé, 1989; Lipscomb, 1994). The enzyme exists in at least 2 functional and structural states that have been well characterized by a variety of methods. The T state (T stands for taut or tense), as originally proposed by Monod et al. (1965), shows low activity and low affinity for aspartate and is predominant in the absence of substrates. The R (relaxed) state, which is obtained in the presence of saturating concentrations of substrates, shows high activity and high affinity for aspartate. CTP

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Abbreviations: PALA, *N*-phosphonacetyl-L-aspartate; Glu-50 → Ala, mutant version of aspartate transcarbamoylase in which glutamic acid 50 in the catalytic chain is replaced by alanine; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; SAXS, small angle X-ray scattering.

stabilizes the T state, whereas ATP, substrates, or substrate analogues such as PALA (Collins & Stark, 1971) stabilize the R state. The 3-dimensional structures of both forms have been determined by Lipscomb and coworkers (Kim et al., 1987; Ke et al., 1988). In the transition from T to the R state, the enzyme undergoes an expansion by 11 Å along the 3-fold axis while the catalytic trimers rotate by 10° relative to one another and the regulatory dimers rotate by 15° along their respective 2-fold axes (Ke et al., 1988).

This quaternary rearrangement is accompanied by tertiary changes in the chains. Some of the major changes involve movements of domains; in particular, the 2 domains of the catalytic chain each responsible for binding 1 substrate (the aspartate and carbamoyl phosphate domains) move closer together in the R structure (Ke et al., 1988). The domain-closed R structure is stabilized by interactions between Glu-50 of the carbamoyl phosphate domain and Arg-167 and Arg-234 of the aspartate domain (Fig. 1; Kinemage 1). The role of these interactions has been probed by the analysis of the functional properties of single amino acid substitution mutants created by site-specific mutagenesis (Ladjimi et al., 1988; Newton & Kantrowitz, 1990). One particularly important mutation is the substitution of Glu-50 by alanine (Newton & Kantrowitz, 1990). The Glu-50 → Ala enzyme exhibits strongly altered regulatory properties: the homotropic cooperativity for aspartate is almost completely abolished, while the nucleotide effectors modify the activity of the mutant enzyme not only at low aspartate concentrations, but also at high concentrations of aspartate relative to the $[S]_{0.5}^{Asp}$ (Newton & Kantrowitz, 1990). Similarly, the activity of the enzyme is stimulated by PALA not only in the presence of low concentrations of aspartate, but even at saturating concentrations of aspartate; this behavior is at variance with what is observed for the wild-type enzyme (Newton & Kantrowitz, 1990).

The analysis of the functional kinetic properties of mutants at Glu-50 suggests that this residue is crucial for the stabilization of the R state of the enzyme and can be counted among a small number of residues shown to be critical for the stabilization of either the T or R states of the enzyme. However, the kinetic characterization of mutants like Glu-50 → Ala does not provide data concerning the potential structural transition of the mutant enzyme from the T structural state to the R structural state. This missing structural information can be obtained using small angle X-ray scattering (SAXS). The SAXS pattern of aspartate transcarbamoylase has been shown to undergo major changes upon addition of substrates and substrate analogues, which reflect the T to R structural transition (Moody et al., 1979; Hervé et al., 1985). Thus, it constitutes a sensitive and specific probe for the quaternary structure of the enzyme. In order to determine what structural alterations occur in the Glu-50 → Ala enzyme, we present here a solution X-ray scattering study of the mutant aspartate transcarbamoylase in which Glu-50 is replaced by alanine.

Results and discussion

PALA induces a structural transition in the Glu-50 → Ala enzyme

The X-ray scattering spectra of the wild-type and the Glu-50 → Ala enzymes were recorded in the absence of substrates as well as in the presence of a saturating concentration of PALA. In the absence of ligands both the wild-type and the Glu-50 → Ala enzymes have virtually identical scattering curves, as they do in the presence of PALA (Fig. 2). This is direct proof that PALA, via its interactions with both domains of the catalytic chain, is able to promote the T to R structural transition even in the absence of the contribution from the salt links involving the side chain of Glu-50. However, the absence of these interactions is apparent in the approximately 1,000-fold reduced affinity of the Glu-50 → Ala enzyme for PALA compared to the wild-type enzyme

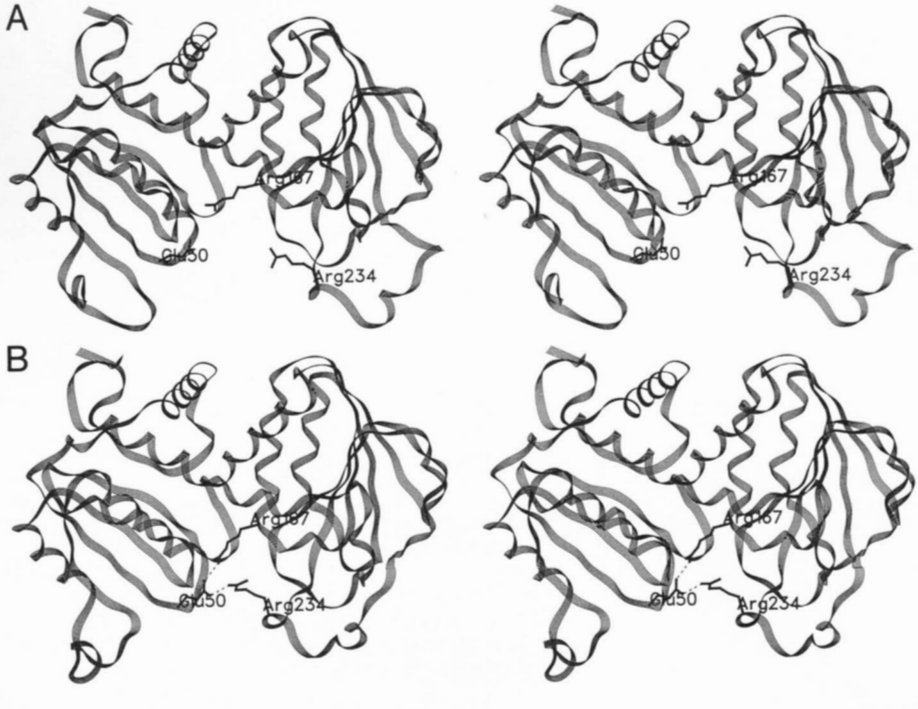

Fig. 1. Stereo view of the interface between the carbamoyl phosphate and aspartate domains of 1 catalytic chain of *E. coli* aspartate transcarbamoylase. The carbamoyl phosphate domain is on the left and the aspartate domain is on the right. **A:** The unligated T form (Kim et al., 1987). **B:** The PALA-ligated R form (Ke et al., 1988). The side chains of residues Glu-50, Arg-167, and Arg-234 are shown with heavy lines. The interactions found in the R form are indicated by dotted lines.

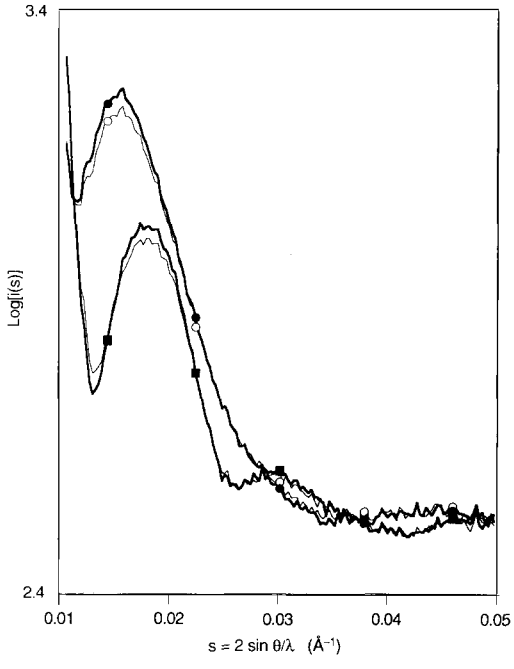

Fig. 2. Solution X-ray scattering spectra of the wild-type and the Glu-50 → Ala enzymes. Spectra are shown for the unligated wild-type enzyme (□, thin lines), unligated Glu-50 → Ala enzyme (■, heavy lines); wild-type enzyme in the presence of 2 molar excess of PALA (○, thin lines); and the Glu-50 → Ala enzyme in the presence of 2 molar excess PALA (●, heavy lines).

(Newton & Kantrowitz, 1990). The addition of a subsaturating concentration of PALA results in an intermediate curve between T and R, as observed with the wild-type enzyme.

The ability of PALA to induce the T to R structural transition was also confirmed by measurements of the radius of gyration, via Guinier plots, for the Glu-50 → Ala enzyme (see Table 1). Analysis of these data revealed that the radii of gyration of the Glu-50 → Ala enzyme in the absence of ligands and in the presence of PALA were identical to the corresponding values for the wild-type enzyme.

X-ray scattering spectra were also measured at subsaturating concentrations of PALA. In spite of the reduced affinity of the mutant enzyme for PALA, the concentration of active sites in these experiments (1.4 mM) is such that, in substoichiometric conditions, most of the PALA molecules are bound. The addition of 0.3 PALA molecules per active site results in a scattering pattern that is intermediate between the T and the R patterns.

Table 1. *Radii of gyration for the wild-type and Glu-50 → Ala enzymes*

	No substrates (Å)	With PALA[a] (Å)
Wild type	47.0 ± 0.4	50.0 ± 0.4
Glu-50 → Ala	47.2 ± 0.4	50.2 ± 0.4

[a] The ratio [PALA]/[active sites] was 2.0.

More precisely, the curve is identical to a linear combination of T and R scattering patterns in equal proportion (50% T and 50% R), which is the exact proportion observed with the wild-type enzyme using the same relative concentration of PALA (data not shown). Therefore, with the exception of the reduced affinity of the mutant enzyme for PALA, the structural effects of PALA on the mutant enzyme are indistinguishable from those observed with the wild-type enzyme.

Carbamoyl phosphate and succinate do not induce a structural transition in the Glu-50 → Ala enzyme

The addition of 3–4 mM succinate, a nonreactive analogue of aspartate, to the wild-type enzyme in the presence of a saturating concentration of carbamoyl phosphate triggers the full T to R transition (Hervé et al., 1985). In contrast, the scattering pattern for the Glu-50 → Ala enzyme is barely affected by concentrations of succinate as high as 140 mM (Fig. 3). This is a structural demonstration that succinate is unable to convert a large fraction of the enzyme to the R structure and provides an explanation for the failure of succinate to stimulate the activity of the mutant enzyme at concentrations up to 200 mM (Baker & Kantrowitz, unpubl. obs.). However, this small but significant change in the scattering pattern of the Glu-50 → Ala enzyme indicates that succinate does bind to the enzyme.

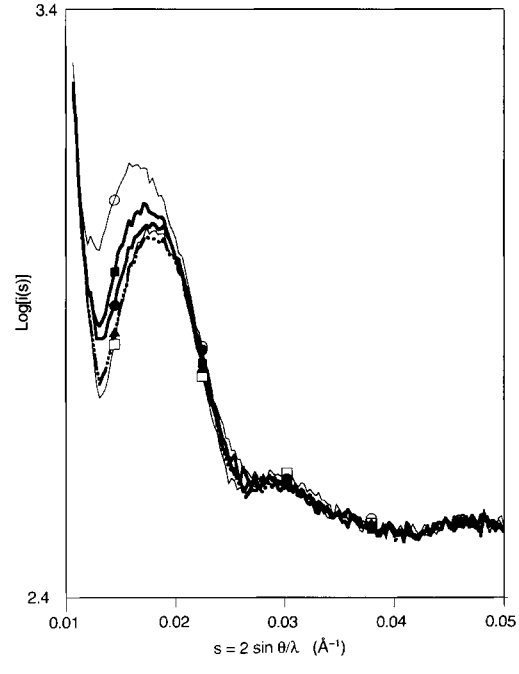

Fig. 3. Solution X-ray scattering spectra of the Glu-50 → Ala enzyme in the absence and presence of ligands. Spectra are shown for the unligated Glu-50 → Ala enzyme (□, thin lines); the Glu-50 → Ala enzyme in the presence of 5 mM carbamoyl phosphate and 140 mM succinate (▲, heavy long-dashed and dotted lines); the Glu-50 → Ala enzyme in the presence of 5 mM carbamoyl phosphate, 140 mM succinate and 5 mM ATP (●, heavy lines); the Glu-50 → Ala enzyme in the presence of 0.3 mol of PALA per mol of active site (○, thin lines); and the Glu-50 → Ala enzyme in the presence of 5 mM carbamoyl phosphate, 70 mM succinate, and 0.3 mol of PALA per mol of active site (■, heavy lines).

In order to investigate further the influence of carbamoyl phosphate and succinate on the Glu → Ala enzyme, scattering patterns were recorded in the presence of succinate, carbamoyl phosphate and a subsaturating concentration of PALA. A subsaturating concentration of PALA produces a mixture of T and R states (see above). Under these conditions, a small shift in the relative populations of the T and R states would be detectable if produced by the binding of carbamoyl phosphate and succinate. When carbamoyl phosphate and succinate are added to the Glu-50 → Ala enzyme in the presence of a subsaturating concentration of PALA, the resulting scattering pattern is intermediate between the T pattern and the pattern observed with the same concentration of PALA only, corresponding to a mixture of 80% T and 20% R (Fig. 3). For the wild-type enzyme, the corresponding scattering pattern corresponds to a mixture of 10% T and 90% R, a distribution practically symmetrically opposite to that observed with the mutant enzyme (data not shown). This seemingly paradoxical result can be understood by considering the higher affinity of the Glu-50 → Ala enzyme for carbamoyl phosphate than for PALA. Thus, carbamoyl phosphate can partially displace PALA from the active sites resulting in part of the enzyme population reverting back to the T structure.

Influence of nucleotide effectors on the quaternary structure of the Glu-50 → Ala enzyme

The behavior of the mutant enzyme in the presence of the nucleotide effectors was investigated by adding ATP or CTP to a solution of the Glu-50 → Ala enzyme already containing a subsaturating concentration of PALA. Sufficient PALA was added so as to bring the pattern approximately halfway between the T and the R patterns, where effects of the nucleotides would be expected to be maximal. The addition of ATP leaves the pattern unchanged, whereas CTP causes a shift toward the T curve (Fig. 4), which is similar to observations made with the wild-type enzyme (Hervé et al., 1985). CTP has a larger effect on the curve for the Glu-50 → Ala enzyme than for the wild-type, which provides evidence for the destabilization of the R structure of the Glu-50 → Ala enzyme. At variance with the wild-type behavior is the weak but significant shift of the scattering curve toward the R pattern caused by the addition of ATP in the presence of high concentrations of carbamoyl phosphate and succinate (Fig. 3). The scattering pattern is fairly well approximated by a linear combination of 10–15% R with 90–85% T curves. Assuming a similar behavior with aspartate, which cannot be directly studied because of its reaction with carbamoyl phosphate, this result is in agreement with the observation of a stimulation of the activity by ATP at an aspartate concentration as high as 4 times the $[S]_{0.5}^{Asp}$ (Newton & Kantrowitz, 1990).

PALA enhances the activity and substrate affinity of the Glu-50 → Ala enzyme

Additional steady-state kinetic experiments were also carried out on the Glu-50 → Ala enzyme especially in the absence and presence of PALA. The shift in the scattering pattern observed upon the addition of PALA indicated that PALA, but not the substrates, shifts the enzyme into the R state. Therefore, PALA should be able to activate the Glu-50 → Ala enzyme at *saturating concentrations* of aspartate and carbamoyl phosphate, conditions at which the wild-type enzyme is inhibited by PALA. As

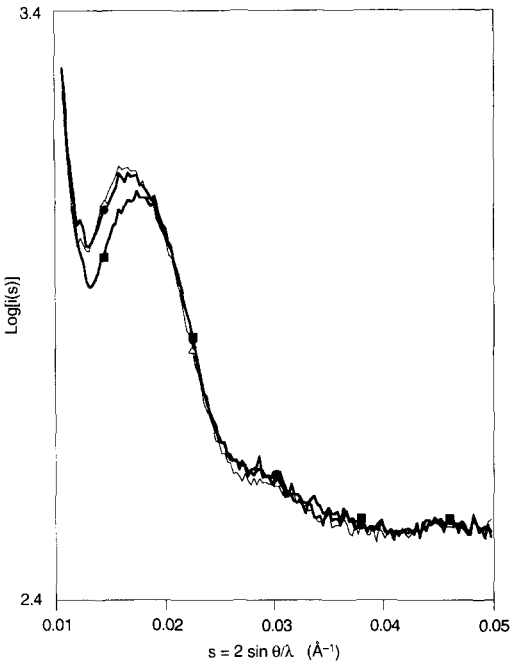

Fig. 4. Effect of the nucleotide effectors on solution X-ray scattering spectra of the Glu-50 → Ala enzyme. Spectra are shown for the Glu-50 → Ala enzyme in the presence 0.3 mol of PALA per mol of active sites (△, thin lines); the Glu-50 → Ala enzyme in the presence of 0.3 mol of PALA per mol of active sites plus 10 mM CTP (■, heavy lines); and the Glu-50 → Ala enzyme in the presence of 0.3 mol of PALA per mol of active sites plus 10 mM ATP (●, heavy lines).

seen in Figure 5, at an aspartate concentration of 3 × $[S]_{0.5}^{Asp}$, PALA at concentrations up to 1 mM activated the Glu-50 → Ala enzyme. In fact, the Glu-50 → Ala enzyme is activated 3-fold, whereas under the same saturating concentrations of substrates the wild-type enzyme is completely inhibited (see Fig. 5). The increase in activity upon the addition of less than saturating concentrations of PALA to the Glu-50 → Ala enzyme must be due to the ability of PALA to convert the mutant enzyme into the

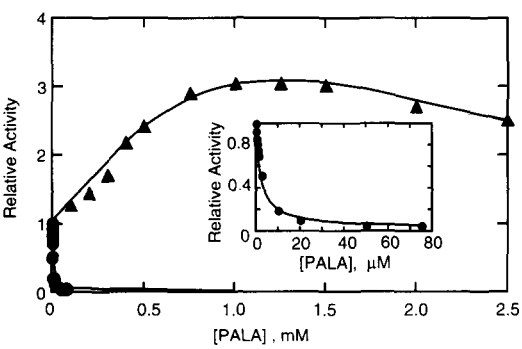

Fig. 5. Effect of PALA on the activity of the wild-type (●) and Glu-50 → Ala (▲) enzymes at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM. The aspartate concentration was held constant at 36 mM for the wild-type enzyme, and 240 mM for the Glu-50 → Ala enzyme. Inset, data for the PALA inhibition of the wild-type enzyme shown in expanded format for the low concentrations of PALA.

more active R state, even when carbamoyl phosphate and aspartate are already saturating. However, at PALA concentrations higher than 1 mM the expected inhibition is observed, as PALA blocks a majority of the active sites.

As indicated by the results from the X-ray scattering and suggested from the kinetic experiment above, subsaturating concentrations of PALA at saturating concentrations of the physiological substrates shift the T to R equilibrium toward R. Therefore, subsaturating concentrations of PALA should be effective not only in enhancing the activity of the Glu-50 → Ala enzyme but also in enhancing the affinity of the mutant enzyme for aspartate. In order to test this hypothesis we determined the aspartate saturation curves of the wild-type and the Glu-50→Ala enzymes in the presence of PALA. As seen in Figure 6A, PALA has only a small effect on the aspartate saturation curve of the wild-type enzyme but has a dramatic effect on the aspartate saturation curve of the Glu-50 → Ala enzyme (see Fig. 6B). The maximal velocity for the Glu-50 → Ala enzyme more than doubles and the $[S]_{0.5}^{Asp}$ decreases from 90 mM to 37 mM in the presence of 0.5 mM PALA. The small amount of cooperativity observed here for the Glu-50 → Ala enzyme in the absence of PALA was not detected previously (Newton & Kantrowitz, 1990).

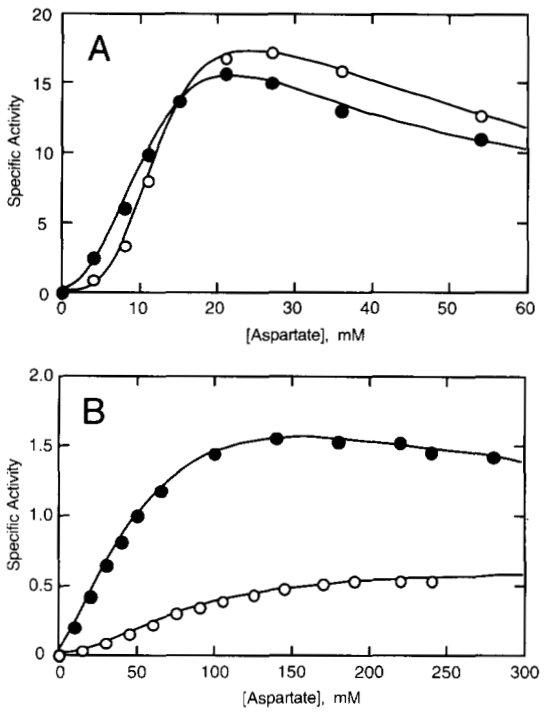

Fig. 6. Aspartate saturation curves for the wild-type (**A**) and the Glu-50 → Ala (**B**) enzymes in the absence (○) and presence (●) of PALA. Assays were carried out at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. The carbamoyl phosphate concentrations was held constant at 4.8 mM. The PALA concentration used for the wild-type and Glu-50 → Ala enzymes were 0.5 μM and 500 μM, respectively. The curves were drawn by a nonlinear least-squares fit to the Hill equation incorporating a term for substrate inhibition (Pastra-Landis et al., 1978).

Only a small fraction of the Glu-50 → Ala enzyme undergoes the T to R allosteric transition with the physiological substrates

The kinetic characterization of the Glu-50 → Ala enzyme (Newton & Kantrowitz, 1990, and the additional experiments reported here) suggests that this enzyme does not undergo the allosteric transition in the same way as the wild-type enzyme upon binding of the physiological substrates. Indeed, the combination of carbamoyl phosphate and succinate barely induces the transition to the R structural state for the Glu-50 → Ala enzyme, whereas these same compounds entirely convert the wild-type enzyme into the R structural state. However, this quaternary structure is still accessible to the Glu-50 → Ala enzyme, as shown by the scattering pattern of the Glu-50 → Ala enzyme observed in the presence of the bisubstrate analog PALA. The fact that some cooperativity is observed for the Glu-50 → Ala enzyme with the natural substrates, carbamoyl phosphate and aspartate (Fig. 6), indicates that the enzyme still can shift from a state of low activity and low affinity for substrates to one that has a higher activity and higher affinity for substrates. We cannot rule out the possibility that aspartate is more effective than succinate in shifting the quaternary structural equilibrium of the mutant enzyme toward the R state. Actually, the only way to answer this question directly is to perform time-resolved SAXS experiments—mixing the enzyme and the physiological substrates rapidly, and recording successive scattering patterns while the reaction proceeds. Information is thus obtained about transient quaternary structures, as illustrated by experiments already performed with the wild-type enzyme (Tsuruta et al., 1990, 1994).

However, this assumption about the effect of aspartate is by no means needed to account for the residual cooperativity. The residual cooperativity may be the result of a modification in the equilibrium between the 2 quaternary structures that is induced by carbamoyl phosphate and aspartate but is small enough to go practically unnoticed by SAXS as is the case with carbamoyl phosphate and succinate (Fig. 3). Indeed, the limit of detection of 1 quaternary structure in a mixture of T and R is typically about 5% in these experiments. Furthermore, the activity of the R state is several-fold higher than that of the T state, as shown by the more than 3-fold activation of the reaction by PALA at saturating carbamoyl phosphate and low concentrations of aspartate (Collins & Stark, 1971) for the wild-type enzyme, and the roughly 3-fold increase of the maximal velocity along with the marked decrease of the $[S]_{0.5}^{Asp}$ in the case of the Glu-50 → Ala enzyme (see Fig. 6B). Thus, a small amount of R, not detected unambiguously by SAXS, would significantly contribute to the activity of the solution, giving rise to the weak level of cooperativity observed. Addition of a subsaturating amount of PALA to the enzyme with carbamoyl phosphate and succinate bound causes a shift toward the R pattern (Fig. 3): a larger fraction of Glu-50 → Ala ATCase molecules are now in the R quaternary structure. The active sites on these molecules that are unoccupied by PALA are in a high-affinity conformation, which leads to an increased activity, as observed in Figure 6B. In a similar fashion, the addition of ATP causes a slight shift toward the R structure, which might account for the stimulation of the activity observed under these conditions. PALA, ATP, and CTP can induce functional changes in the Glu-50 → Ala enzyme even at saturating substrate concentrations (Newton & Kantrowitz, 1990). Our structural and functional observations all support the notion that the Glu-50 → Ala enzyme is only partially converted to the high-activity high-affinity state by the natural substrates.

Summary

Previous functional studies by site-specific mutagenesis on the allosteric mechanism of aspartate transcarbamoylase have identified the interdomain bridging interactions between Glu-50 of the carbamoyl phosphate domain with both Arg-167 and Arg-234 of the aspartate domain as critical for the concerted allosteric transition in aspartate transcarbamoylase (Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988; Newton & Kantrowitz, 1990). The studies reported here provide direct structural data confirming the importance of the interdomain bridging interactions between the 2 domains of the catalytic chain for the correct formation of the R structural state of the enzyme.

Materials and methods

Chemicals

ATP, CTP, agar, ampicillin, L-aspartate, *N*-carbamoyl-L-aspartate, carbamoyl phosphate, Tris, 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, and enzyme-grade ammonium sulfate were obtained from ICN Biomedicals.

Mutant holoenzyme purification

The Glu-50 → Ala mutant of aspartate transcarbamoylase was isolated as described by Nowlan and Kantrowitz (1985) from *E. coli* strain EK1104 (F^--*ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *pyrF*$^-$, *rpsL*), containing the plasmid pEK91 (Newton & Kantrowitz, 1990).

Solution X-ray scattering

Samples were prepared from a stock solution in 50 mM Tris-borate buffer, pH 8.3, 0.1 mM EDTA and 0.1 mM dithiothreitol as in Hervé et al. (1985), with a final protein concentration of 70 mg/mL corresponding to 1.4 mM active site. Radii of gyration were derived from a Guinier analysis of data recorded at enzyme concentrations between 5 and 11 mg/mL in the angular range $0.0016\ Å^{-1} < s < 0.0075\ Å^{-1}$ ($s = 2 \sin\theta/\lambda$ is the scattering parameter, where $\lambda = 1.608$ Å is the wavelength of the X-rays). All X-ray scattering curves were recorded on the small angle scattering instrument D24 using synchrotron radiation at LURE-DCI, Orsay, France. The instrument (Depautex et al., 1987), the data acquisition system (Bordas et al., 1980), and the experimental procedures (Hervé et al., 1985) have been described previously.

Determination of protein concentration

Concentrations of pure wild-type holoenzyme were determined by absorbance measurement at 280 nm using the extinction coefficient of 0.59 cm^2/mg (Gerhart & Holoubek, 1967). The protein concentration of the mutant holoenzyme was determined by the Bio-Rad version of Bradford's dye-binding assay (Bradford, 1976).

Aspartate transcarbamoylase assay

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

Data analysis

The analysis of the steady-state kinetic data was carried out as described previously by Silver et al. (1983). Analysis of the structural data, based on the 3-dimensional coordinates, was accomplished using the program QUANTA (Molecular Simulations, Burlington, Massachusetts) on a Silicon Graphics Indigo 2 computer.

Acknowledgments

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