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# Weakening of the interface between adjacent catalytic chains promotes domain closure in *Escherichia coli* aspartate transcarbamoylase*

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**Abstract**

Aspartate transcarbamoylase from *Escherichia coli* is a dodecameric enzyme consisting of two trimeric catalytic subunits and three dimeric regulatory subunits. Asp-100, from one catalytic chain, is involved in stabilizing the C1–C2 interface by means of its interaction with Arg-65 from an adjacent catalytic chain. Replacement of Asp-100 by Ala has been shown previously to result in increases in the maximal specific activity, homotropic cooperativity, and the affinity for aspartate (Baker DP, Kantrowitz ER, 1993, *Biochemistry 32*:10150–10158). In order to determine whether these properties were due to promotion of domain closure induced by the weakening of the C1–C2 interface, we constructed a double mutant version of aspartate transcarbamoylase in which the Asp-100 → Ala mutation was introduced into the Glu-50 → Ala holoenzyme, a mutant in which domain closure is impaired. The Glu-50/Asp-100 → Ala enzyme is fourfold more active than the Glu-50 → Ala enzyme, and exhibits significant restoration of homotropic cooperativity with respect to aspartate. In addition, the Asp-100 → Ala mutation restores the ability of the Glu-50 → Ala enzyme to be activated by succinate and increases the affinity of the enzyme for the bisubstrate analogue *N*-(phosphonacetyl)-L-aspartate (PALA). At subsaturating concentrations of aspartate, the Glu-50/Asp-100 → Ala enzyme is activated more by ATP than the Glu-50 → Ala enzyme and is also inhibited more by CTP than either the wild-type or the Glu-50 → Ala enzyme. As opposed to the wild-type enzyme, the Glu-50/Asp-100 → Ala enzyme is activated by ATP and inhibited by CTP at saturating concentrations of aspartate. Structural analysis of the Glu-50/Asp-100 → Ala enzyme by solution X-ray scattering indicates that the double mutant exists 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 PALA. However, saturating concentrations of carbamoyl phosphate and succinate only convert a fraction of the Glu-50/Asp-100 → Ala enzyme population to the R quaternary structure, a behavior intermediate between that observed for the Glu-50 → Ala and wild-type enzymes. Solution X-ray scattering was also used to investigate the structural consequences of nucleotide binding to the Glu-50/Asp-100 → Ala enzyme.

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

* We dedicate this paper to the memory of our friend and colleague Frederick C. Wedler, whose work on the catalytic mechanism of aspartate transcarbamoylase has been fundamental to the understanding of this complex enzyme. His personal and intellectual contributions to the scientific community will be sorely missed.

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*Abbreviations:* PALA, *N*-(phosphonacetyl)-L-aspartate; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; $[S]_{0.5}^{CP}$, carbamoyl phosphate concentration at half the maximal observed specific activity.

*Escherichia coli* aspartate transcarbamoylase (ATCase; EC 2.1.3.2) catalyzes the committed step in the biosynthesis of pyrimidine nucleotides: the reaction between carbamoyl phosphate and L-aspartate to form *N*-carbamoyl-L-aspartate and inorganic phosphate (Jones et al., 1955; Reichard & Hanshoff, 1956). The reaction proceeds by a preferred order mechanism with carbamoyl phosphate binding before aspartate, and *N*-carbamoyl-L-aspartate leaving before inorganic phosphate (Hsuanyu & Wedler, 1987). The enzyme exhibits homotropic cooperativity for aspartate (Gerhart & Pardee, 1962), but not for carbamoyl

phosphate (England et al., 1994), is heterotropically activated by ATP, the end product of the purine biosynthetic pathway (Gerhart & Pardee, 1962), and is heterotropically inhibited by CTP (Gerhart & Pardee, 1962) and UTP (in the presence of CTP) (Wild et al., 1989), the end products of the pyrimidine biosynthetic pathway.

The dodecameric holoenzyme is composed of six catalytic chains ($M_r$ 33,000), which are associated as two trimers (catalytic subunits), and six regulatory chains ($M_r$ 17,000), which are associated as three dimers. Each of the catalytic chains is composed of two structural domains, the carbamoyl phosphate domain and the aspartate domain. The active sites are located at the interface between the carbamoyl phosphate and aspartate domains of one catalytic chain and the carbamoyl phosphate domain from an adjacent catalytic chain (Monaco et al., 1978; Robey & Schachman, 1985; Krause et al., 1987; Wente & Schachman, 1987).

In the absence of substrates, the holoenzyme exists in the low-affinity, low-activity T state, but is converted into the high-affinity, high-activity R state upon the binding of carbamoyl phosphate and aspartate. The three-dimensional crystal structure of the holoenzyme in the T state has been determined in the absence of ligands (Honzatko et al., 1982; Ke et al., 1984; Stevens et al., 1990) and in the presence of CTP (Honzatko et al., 1982; Kim et al., 1987; Stevens et al., 1990; Kosman et al., 1993), whereas the R-state structure has been determined in the presence of carbamoyl phosphate and succinate (Gouaux & Lipscomb, 1988), phosphonoacetamide and malonate (Gouaux & Lipscomb, 1990), and in the presence of the bisubstrate analogue *N*-(phosphonacetyl)-L-aspartate (Krause et al., 1987; Ke et al., 1988). Analysis of these structures indicates that the holoenzyme undergoes substantial conformational changes upon the T to R transition. Changes at the quaternary level include an elongation of the molecule along its threefold axis of 12 Å, the relative rotation of the catalytic subunits about their threefold axis by 10°, and the rotation of the regulatory dimers about their twofold axis by 15° (Krause et al., 1987; Ke et al., 1988; Gouaux & Lipscomb, 1990). Changes at the tertiary level include the closure of the carbamoyl phosphate and aspartate domains to form the active site, as well as the rearrangement of the 80's and 240's loops by the breakage of specific interactions that stabilize the T structure, and their replacement by alternative interactions that stabilize the R structure (Ke et al., 1988; Gouaux & Lipscomb, 1990).

Closure of the carbamoyl phosphate and aspartate domains is particularly important for homotropic cooperativity. In the CTP-ligated T structure, the two domains are held apart (Stevens et al., 1990; Kosman et al., 1993), whereas in the (phosphonoacetamide + malonate)-ligated and PALA-ligated R structures the domains are held together by interdomain bridging interactions between the side chains of Glu-50 of the carbamoyl phosphate domain and both Arg-167 and Arg-234 of the aspartate domain (Krause et al., 1987; Ke et al., 1988; Gouaux & Lipscomb, 1990). The role of these interactions in domain closure has been confirmed by site-specific mutagenesis; replacement of Glu-50 by Gln and Ala and Arg-234 by Ser results in enzymes that exhibit low activity, low affinity for aspartate, and much reduced cooperativity (Middleton & Kantrowitz, 1988; Newton & Kantrowitz, 1990).

Although the carbamoyl phosphate and aspartate domains move toward each other during the T to R transition, there is little overall movement of the catalytic chains relative to one another because the catalytic subunits move as a rigid unit. Each chain within the catalytic subunit is tethered to its neighbor by a complex network of interactions that forms the C1–C2[4] interface. Recently, Baker and Kantrowitz (1993) showed that His-41, Asp-100, and Asp-90 from C1, which form interactions with Glu-37, Arg-65, and Arg-269 from C2, respectively, are important for the structural stabilization of the catalytic subunit. Interestingly, replacement of Asp-100 by Ala results in a mutant that exhibits a higher specific activity than the wild-type holoenzyme, as well as a twofold increase in the affinity for aspartate and an increase in homotropic cooperativity (Baker & Kantrowitz, 1993). Because the interaction between Asp-100 and Arg-65 is maintained in both the T and R structures (Ke et al., 1988; Gouaux & Lipscomb, 1990; Stevens et al., 1990), and because neither residue has any direct role in substrate binding or catalysis, it was suspected that the increase in activity, affinity for aspartate, and cooperativity observed for the Asp-100 → Ala[5] enzyme might be due to promotion of domain closure induced by the weakening of the C1–C2 interface. To test this hypothesis, we constructed a double mutant of aspartate transcarbamoylase in which the Asp-100 → Ala mutation was introduced into the Glu-50 → Ala holoenzyme, a mutant in which domain closure is impaired (Newton & Kantrowitz, 1990; Tauc et al., 1994).

Here we report the kinetic and structural characterization of the Glu-50/Asp-100 → Ala[5] double mutant of aspartate transcarbamoylase and its comparison with the wild-type, Glu-50 → Ala, and Asp-100 → Ala holoenzymes.

## Results

### *Construction of plasmid pEK246*

The plasmid pEK246, which carries the gene for the Glu-50/Asp-100 → Ala double mutant of aspartate transcarbamoylase, was constructed by fusion of appropriate DNA fragments derived from plasmids carrying the individual mutations. Plasmid pEK91 and pEK214 were digested with *Eco* RI and *Xcm* I and, in each case, the two product fragments were separated by agarose gel electrophoresis. The larger, 3.6-kb fragment of pEK91, carrying the Glu-50 → Ala mutation, and the smaller, 2.4-kb fragment of pEK214, carrying the Asp-100 → 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).

[4] The catalytic chains C1, C2, and C3 comprise the upper catalytic subunit whereas the C4, C5, and C6 chains comprise the lower catalytic subunit. Chain C4 is below C1, whereas C5 and C6 are below chains C2 and C3, respectively.

[5] The notation used to name the mutant enzymes is, for example, the Asp-100 → 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. For the double mutant, the wild-type amino acids are indicated to the left of the arrow and the new amino acids are located to the right of the arrow.

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

The kinetic parameters of the wild-type, Glu-50 → Ala, Asp-100 → Ala, and Glu-50/Asp-100 → Ala holoenzymes are summarized in Table 1. It is clear from the kinetic data that the Glu-50 → Ala mutation has the opposite effect on the enzyme to the Asp-100 → Ala mutation. Whereas replacement of Glu-50 by Ala results in a 19-fold reduction in the maximal activity, a sevenfold increase in the $[S]_{0.5}^{Asp}$, and a significant decrease in the cooperativity toward aspartate, replacement of Asp-100 by Ala results in a slight increase in the maximal activity, a twofold reduction in the $[S]_{0.5}^{Asp}$, and an increase in the cooperativity (Table 1). Similarly, introduction of the Asp-100 → Ala mutation in the Glu-50 → Ala mutant results in a fourfold increase in the maximal activity, a small decrease in the $[S]_{0.5}^{Asp}$, as well as a significant restoration of cooperativity when compared to the Glu-50 → Ala enzyme (Table 1).

The carbamoyl phosphate saturation curves of the wild-type, Glu-50 → Ala, Asp-100 → Ala, and Glu-50/Asp-100 → Ala enzymes indicate that the affinity for carbamoyl phosphate remains essentially the same, irrespective of the presence of one or both mutations (Table 1).

*Influence of the allosteric effectors on the 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, Asp-100 → Ala, and Glu-50/Asp-100 → 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 Asp-100 → Ala enzymes to about the same extent, whereas the activation of the Glu-50/Asp-100 → Ala enzyme is intermediate between that of the wild-type and Glu-50 → Ala enzyme (Table 2). By contrast, the maximal inhibition of the Glu-50/Asp-100 → Ala enzyme by CTP is essentially identical to that of the Asp-100 → Ala enzyme and not intermediate between the wild-type and Glu-50 → Ala enzymes (Table 2). Furthermore, the affinity of the double mutant for ATP seems to be dependent upon the presence of the Asp-100 → Ala mutation because the concentration of ATP required for half maximal activation ($K_{ATP}$) is not changed by the introduction of the Glu-50 → Ala mutation, whereas it is reduced 1.6- and 1.8-fold in the Asp-100 → Ala and Glu-50/Asp-100 → Ala enzymes, respectively (Table 2). Likewise, the concentration of CTP required for half-maximal inhibition ($K_{CTP}$) seems to be dependent on the presence of the Asp-100 → Ala mutation, because the Asp-100 → Ala and Glu-50/Asp-100 → Ala enzymes exhibit identical 3.5-fold reductions in the $K_{CTP}$, whereas the Glu-50 → Ala enzyme shows a 1.4-fold increase (Table 2).

At a concentration of aspartate corresponding to three times the $[S]_{0.5}^{Asp}$, ATP is unable to activate either the wild-type or the Asp-100 → Ala enzyme, whereas the Glu-50 → Ala and Glu-50/Asp-100 → Ala enzymes show significant activation (Fig. 1). Similarly, at a concentration of aspartate corresponding to five times the $[S]_{0.5}^{Asp}$, CTP fails to inhibit significantly either the wild-type or the Asp-100 → Ala enzyme, whereas the Glu-50 → Ala and Glu-50/Asp-100 → Ala enzymes are inhibited (Fig. 1).

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

At an aspartate concentration corresponding to one fifth the $[S]_{0.5}^{Asp}$, succinate activates the wild-type, Asp-100 → Ala, and Glu-50/Asp-100 → Ala enzymes, although the concentration required to maximally activate the double mutant is approximately 60 mM compared to 6 and 3 mM for the wild-type and Asp-100 → Ala enzymes, respectively (Fig. 2). By contrast, succinate has no effect on the Glu-50 → Ala enzyme, even at a concentration of 200 mM (Fig. 2).

Whereas succinate cannot activate the Glu-50 → Ala enzyme, PALA induces significant activation, although the concentration required for maximal activation is approximately 300 $\mu$M compared to 3, 1, and 50 $\mu$M for the wild-type, Asp-100 → Ala, and Glu-50/Asp-100 → Ala enzymes, respectively (Fig. 3).

*Effect of substrate analogues on the quaternary structure of the wild-type and Glu-50/Asp-100 → Ala holoenzymes*

Recently, Tauc et al. (1994) used solution X-ray scattering to determine the structural consequences of effector binding to the Glu-50 → Ala mutant of aspartate transcarbamoylase. Whereas

**Table 1.** *Kinetic parameters of the wild-type and mutant holoenzymes*[a,b]

| Enzyme | Maximal velocity[c] ($mmol \cdot h^{-1} \cdot mg^{-1}$) | $[S]_{0.5}^{Asp}$ (mM) | $n_H^{Asp}$ | $[S]_{0.5}^{CP}$ (mM) |
|---|---|---|---|---|
| Wild type[d] | 20.7 ± 0.4 | 12.2 ± 0.2 | 2.6 ± 0.0 | 0.26 ± 0.0 |
| Glu-50 → Ala | 1.1 ± 0.05 | 89.2 ± 1.0 | 1.6 ± 0.0 | 0.24[e] |
| Asp-100 → Ala[d] | 24.2 ± 0.5 | 6.6 ± 0.1 | 3.0 ± 0.0 | 0.26 ± 0.0 |
| Glu-50/Asp-100 → Ala | 4.5 ± 0.1 | 76.8 ± 1.6 | 2.1 ± 0.2 | 0.32 ± 0.0 |

[a] Data are the average of two independent determinations, except the maximal velocity, $[S]_{0.5}^{Asp}$, and $n_H^{Asp}$ values of the wild-type, Asp-100 → Ala and Glu-50/Asp-100 → Ala enzymes, which are the average of three independent determinations.

[b] $V_{max}$ and 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).

[c] Maximal velocity represents the maximal observed specific activity from the aspartate saturation curve.

[d] Data taken from Baker and Kantrowitz (1993).

[e] Data taken from Newton and Kantrowitz (1990).

**Table 2.** *ATP activation and CTP inhibition of the wild-type and mutant holoenzymes at subsaturating aspartate*[a]

| Enzyme | Activity with ATP[b] | $K_{ATP}$ (mM) | Activity with CTP[c] | $K_{CTP}$ (mM) |
|---|---|---|---|---|
| Wild type | 5.3 | 1.1 | 0.15 | 0.14 |
| Glu-50 → Ala | 1.7 | 1.1 | 0.22 | 0.20 |
| Asp-100 → Ala | 4.8 | 0.7 | 0.11 | 0.04 |
| Glu-50/Asp-100 → Ala | 2.2 | 0.6 | 0.10 | 0.04 |

[a] Assays were performed at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM for all enzymes. The concentration of aspartate used was half the $[S]_{0.5}^{Asp}$, which corresponds to 6.1, 44.6, 3.3, and 38.4 mM for the wild-type, Glu-50 → Ala, Asp-100 → Ala, and Glu-50/Asp-100 → Ala enzymes, respectively.

[b] Activity 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.

[c] Activity 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.

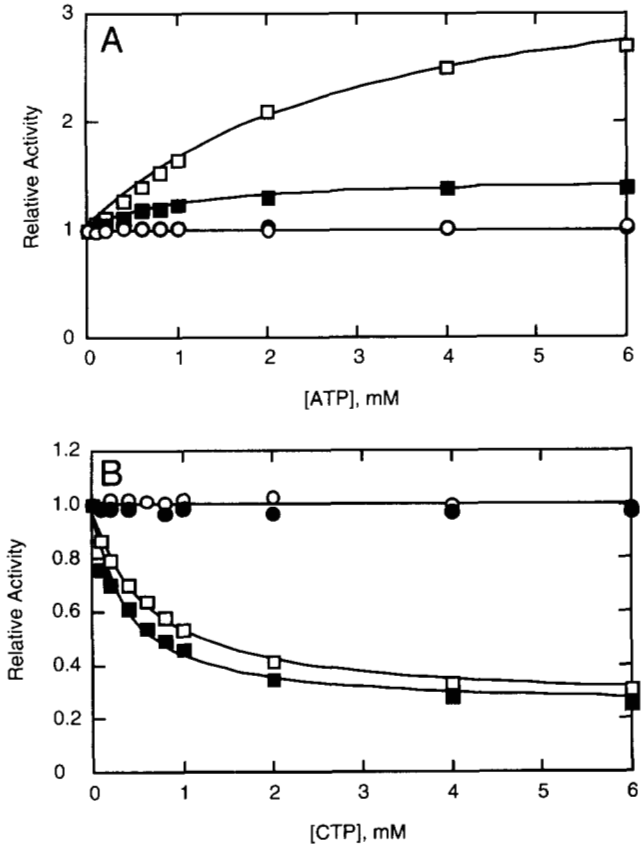


**Fig. 1.** ATP activation (**A**) and CTP inhibition (**B**) of the wild-type and mutant holoenzymes at a high aspartate concentration relative to the $[S]_{0.5}^{Asp}$. Assays were carried out at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. Aspartate concentration was held constant at three times the $[S]_{0.5}^{Asp}$ for the ATP activation experiments and five times the $[S]_{0.5}^{Asp}$ for the CTP inhibition experiments. Carbamoyl phosphate concentration was held constant at 4.8 mM for all the enzymes. Data are shown for ○, wild-type; ●, Asp-100 → Ala; □, Glu-50 → Ala; and ■, Glu-50/Asp-100 → Ala enzymes.

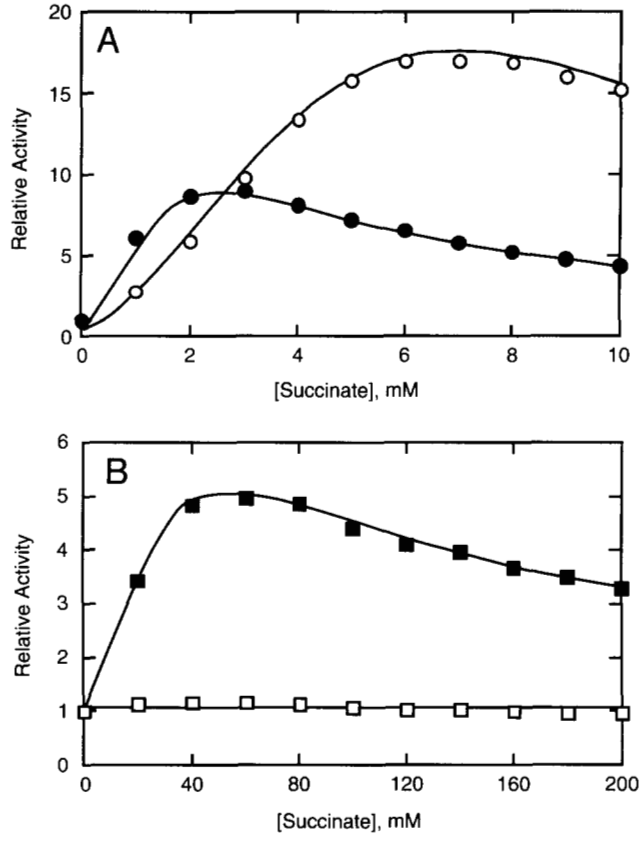


**Fig. 2.** Succinate activation of the wild-type and mutant holoenzymes was carried out at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. Aspartate concentration was held constant at one fifth the $[S]_{0.5}^{Asp}$, and carbamoyl phosphate concentration was held constant at 4.8 mM for all the enzymes. **A:** ○, Wild type; ●, Asp-100 → Ala. **B:** □, Glu-50 → Ala; ■, Glu-50/Asp-100 → Ala.

the wild-type and Glu-50 → Ala enzymes exist in the same T quaternary structure in the absence of ligands and in the same R quaternary structure in the presence of a saturating concentration of PALA, carbamoyl phosphate and succinate, which induce the full T to R transition in the wild-type enzyme (Tsuruta et al., 1994), have practically no effect on the Glu-50 → Ala enzyme (Tauc et al., 1994). Because the Glu-50/Asp-100 → Ala enzyme is obviously activated by succinate (Fig. 2), it was of significant interest to determine the structural effects of ligand binding on the double mutant.

Figure 4 shows that the scattering curves of the wild-type and Glu-50/Asp-100 → Ala enzymes are practically identical in both the absence of ligands and in the presence of a twofold molar excess of PALA. In the absence of ligands, both enzymes are in the T structural state, whereas in the presence of PALA, both enzymes are in the R structural state. Furthermore, the radius of gyration is the same for both enzymes in the absence of ligands and in the presence of saturating PALA (Table 3). Addition of 0.3 mol of PALA per mol of active sites yields similar scattering patterns, each of which can be accounted for by a linear combination of the corresponding scattering pattern in the absence of ligands and of the scattering pattern in the presence of excess PALA (54% T:46% R for the wild-type enzyme and 52% T:48% R for the Glu-50/Asp-100 → Ala enzyme) (Fig. 4).

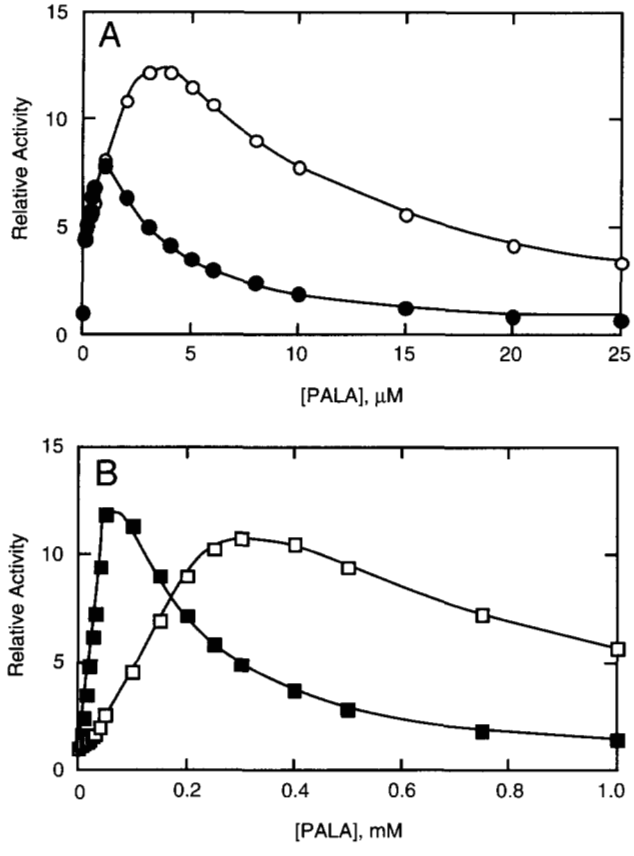


**Fig. 3.** PALA activation of the wild-type and mutant holoenzymes was carried out at 25 °C in 50 mM Tris-acetate buffer, pH 8.3. Aspartate concentration was held constant at one fifth the $[S]_{0.5}^{Asp}$, and carbamoyl phosphate concentration was held constant at 4.8 mM for all the enzymes. **A:** ○, Wild type; ●, Asp-100 → Ala. **B:** □, Glu-50 → Ala; ■, Glu-50/Asp-100 → Ala.

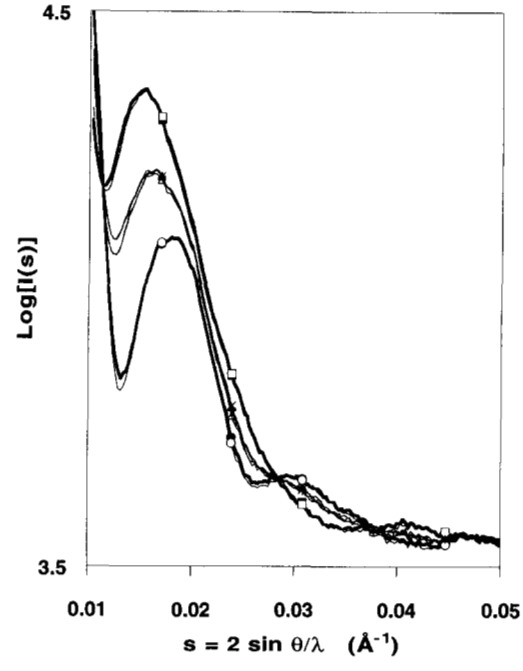


**Fig. 4.** Solution X-ray scattering spectra of the wild-type and Glu-50/Asp-100 → Ala enzymes. Spectra are shown for the ○, thin line: unligated wild-type enzyme; ●, heavy line: unligated Glu-50/Asp-100 → Ala enzyme; □, thin line: wild-type enzyme in the presence of a twofold molar excess of PALA; ■, heavy line: Glu-50/Asp-100 → Ala enzyme in the presence of a twofold molar excess of PALA; △, thin line: wild-type enzyme in the presence of 0.3 mol of PALA per mol of active sites; +, thin line: linear combination of 54% wild-type T and 46% wild-type R scattering patterns; ▲, thin line: Glu-50/Asp-100 → Ala enzyme in the presence of 0.3 mol of PALA per mol of active sites; ×, thin line: linear combination of 52% Glu-50/Asp-100 → Ala T and 48% Glu-50/Asp-100 → Ala R scattering patterns.

The addition of 5 mM carbamoyl phosphate alone gives very similar curves for both enzymes (data not shown). Further addition of succinate to the Glu-50/Asp-100 → Ala enzyme results in a shift toward the R pattern, but it falls short from achieving the complete transition. Data were recorded for several succinate concentrations between 60 and 400 mM. The scattering pattern is about half-way beween the T and R patterns at 100 mM succinate (curve in thin line in Fig. 5), and this shifts only slightly further toward R between 100 and 200 mM succinate (from 50% R to less than 60% R). At 400 mM succinate, however, the curve shifts markedly back toward the T pattern, probably because of the well-documented substrate inhibition at high substrate concentration (Reichard & Hanshoff, 1956).

The combined addition of 5 mM carbamoyl phosphate, 60 mM succinate, and 0.3 mol of PALA per mol of active sites results in a scattering pattern corresponding to about 70% R, as compared to about 40% R in the presence of 5 mM carbamoyl phosphate and 60 mM succinate, and 48% R in the presence of 0.3 mol of PALA per mol of active sites alone (Fig. 6).

**Table 3.** *Radii of gyration for the wild-type and Glu-50/Asp-100 → Ala enzymes*[a]

| Enzyme | No ligands (Å) | Saturating PALA (Å) |
|---|---|---|
| Wild type | 45.8 ± 0.3 | 49.5 ± 0.3 |
| Glu-50/Asp-100 → Ala | 45.6 ± 0.3 | 49.3 ± 0.3 |

[a] The small angle X-ray scattering pattern of dilute solutions of the wild-type and Glu-50/Asp-100 → Ala enzymes (3–7 mg·mL$^{-1}$) were recorded in the absence of ligands and in the presence of a twofold molar excess of PALA. The value of the radius of gyration was derived from a Guinier analysis (Guinier & Fournet, 1955).

### *Influence of nucleotide effectors on the quaternary structure of the wild-type and Glu-50/Asp-100 → Ala holoenzymes*

The effects of ATP, CTP, and CTP + UTP on the Glu-50/Asp-100 → Ala enzyme were investigated by adding them to a solution of the enzyme containing either 0.3 mol of PALA per mol of active sites or 5 mM carbamoyl phosphate and 100 mM succinate. These conditions were chosen so as to bring the scattering pattern roughly half-way between the T and R patterns, where effects of the nucleotides would be expected to be maximal. In the presence of 5 mM carbamoyl phosphate and 100 mM succinate, ATP causes a strong R shift (from 50% R to about

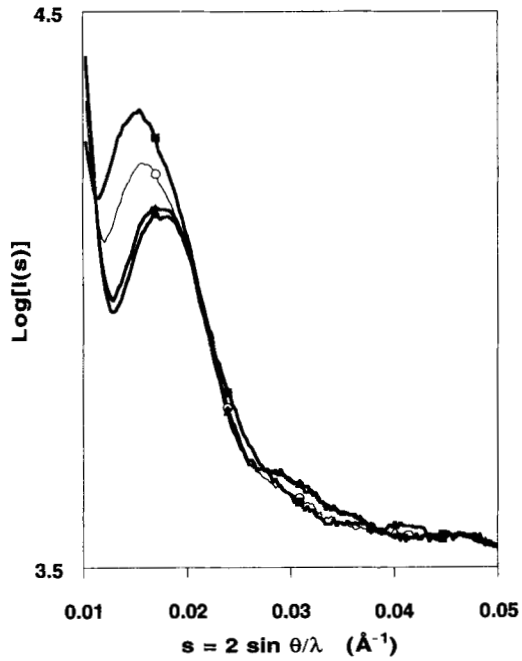


**Fig. 5.** Effect of nucleotides on the solution X-ray scattering spectra of the Glu-50/Asp-100 → Ala enzyme. All solutions contained 5 mM carbamoyl phosphate and 100 mM succinate. ○, thin line: no nucleotide; ■, heavy line: + 5 mM ATP; ▲, heavy line: + 5 mM CTP; ◆, heavy line: + 2.5 mM CTP + 2.5 mM UTP.

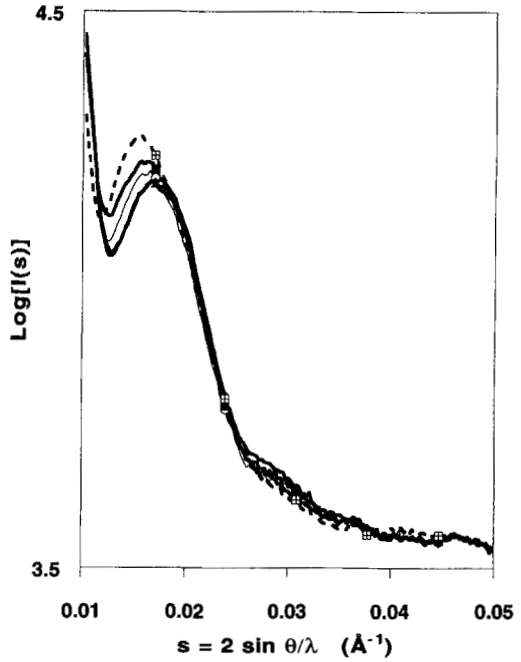


**Fig. 6.** Effect of nucleotides on the solution X-ray scattering spectra of the Glu-50/Asp-100 → Ala enzyme. All solutions contained 0.3 mol of PALA per mol of active sites. ○, thin line: no nucleotide; ■, heavy line: + 5 mM ATP; ▲, heavy line: + 5 mM CTP; ◆, heavy line: + 2.5 mM CTP + 2.5 mM UTP; ⊞, heavy dashed line: + 5 mM carbamoyl phosphate + 60 mM succinate.

85% R), whereas CTP or CTP + UTP leads to a T shift (from 50% R to about 25% R and 20% R, respectively) (Fig. 5). In the presence of 0.3 mol of PALA per mol of active sites, ATP results in a small but significant R shift (from 46% R to 54% R), whereas addition of CTP or CTP + UTP results in a small T shift (from 46% R to about 40% R) (Fig. 6).

## Discussion

### *Mutation of Asp-100 at the C1-C2 interface restores homotropic cooperativity to the Glu-50 → Ala mutant holoenzyme*

Analysis of the T and R structures of the wild-type holoenzyme indicates that it undergoes substantial conformational changes during the T to R transition. At the tertiary level, these include the closure of the carbamoyl phosphate and aspartate domains to form the active site, as well as the rearrangement of the 80's and 240's loops (Ke et al., 1988; Gouaux & Lipscomb, 1990). These tertiary structural changes are important for the concerted allosteric transition from the low-affinity, low-activity T state to the high-affinity, high-activity R state. Only after carbamoyl phosphate and aspartate bind and the domains close are the quaternary structural changes induced that allow the remaining catalytic chains to adopt their high-affinity, high-activity, domain-closed conformation (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988; Middleton et al., 1989). In such a manner, domain closure in one catalytic chain is coupled to domain closure in the remaining chains, and homotropic cooperativity is manifest.

In the X-ray structure of the T state wild-type holoenzyme (ligated to CTP), the carboxylate group of Glu-50 of the carbamoyl phosphate domain forms an intradomain interaction with the guanidinium of Arg-105 (see coordinate file 5AT1, Brookhaven Protein Data Bank). However, in the (phosphonoacetamide + malonate)-ligated and PALA-ligated R-state structures, Glu-50 "switches" to form two interdomain bridging interactions with the side chains of Arg-167 and Arg-234 from the aspartate domain (Ke et al., 1988; Gouaux & Lipscomb, 1990). These interdomain bridging interactions have been shown to be important for the establishment of the high-affinity, high-activity conformation of the active site (Newton & Kantrowitz, 1990), as well as for the stabilization of the R quaternary structure (Tauc et al., 1994), most likely through their role in the closure of the two domains of the catalytic chain. In contrast to the Glu-50 → Ala substitution, replacement of Asp-100 by Ala results in an enzyme that exhibits increased homotropic cooperativity, activity, and affinity for aspartate (Baker & Kantrowitz, 1993). Because the replacement of Asp-100 by Ala facilitates domain closure at lower aspartate concentrations (Table 1), it was suspected that the addition of the Asp-100 → Ala mutation to the Glu-50 → Ala enzyme might increase homotropic cooperativity. Indeed, kinetic analysis of the double mutant indicates that the Asp-100 → Ala mutation results in a significant restoration of homotropic cooperativity (Table 1). However, because the Glu-50/Asp-100 → Ala enzyme lacks Glu-50, it cannot form the interdomain bridging interactions with Arg-167 and Arg-234, which, in the wild-type enzyme, stabilize domain closure. Because these interdomain bridging interactions are important for the formation of the high-affinity aspartate binding site, then

it is, perhaps, not surprising that the double mutant retains the poor affinity for aspartate characteristic of the Glu-50 → Ala enzyme (Table 1) (Newton & Kantrowitz, 1990).

Despite the lack of Glu-50 and the poor affinity for aspartate, it is clear from the observed cooperativity with the natural substrate that the Glu-50/Asp-100 → Ala enzyme can shift from a state of low activity and low affinity for substrates to one with higher activity and higher affinity for substrates. Moreover, the specific activity of the double mutant is fourfold higher than the Glu-50 → Ala enzyme (Table 1). Therefore, the Asp-100 → Ala mutation has made it easier for at least some active site residues involved in catalysis to adopt a more favorable position than in the Glu-50 → Ala enzyme, although the barely improved affinity for aspartate shows that other residues involved in binding remain too far away to interact correctly with aspartate.

### *Glu-50/Asp-100 → Ala enzyme does not exist in the wild-type R functional state in the presence of saturating concentrations of carbamoyl phosphate and aspartate*

It has been shown previously that, in the presence of saturating concentrations of carbamoyl phosphate and aspartate, the Glu-50 → Ala holoenzyme cannot attain the same R functional state as the wild-type enzyme because ATP (Newton & Kantrowitz, 1990) and PALA (Tauc et al., 1994) can activate the enzyme. By contrast, ATP cannot activate the wild-type holoenzyme nor the Asp-100 → Ala enzyme in the presence of saturating substrates (Fig. 1) because both are already in the R state.

The Glu-50/Asp-100 → Ala enzyme is activated by ATP (Fig. 1), which suggests that carbamoyl phosphate and aspartate alone cannot bring the active site into its high-activity conformation as they do with the wild-type enzyme. However, because the level of activation of the Glu-50/Asp-100 → Ala enzyme is significantly lower than that of the Glu-50 → Ala enzyme (Fig. 1), it is likely that the physiological substrates drive the double mutant to a functional state that is intermediate between that of the Glu-50 → Ala and wild-type enzymes. In addition, the inability of the Glu-50 → Ala and Glu-50/Asp-100 → Ala enzymes to attain the fully domain-closed states is supported by the ability of CTP to inhibit these enzymes in the presence of saturating concentrations of carbamoyl phosphate and aspartate (Fig. 1).

### *PALA converts the Glu-50/Asp-100 → Ala holoenzyme to the R quaternary structure*

Although kinetic analysis of the Glu-50/Asp-100 → Ala enzyme indicates that it is not in the R state in the presence of carbamoyl phosphate and aspartate (Table 1), solution X-ray scattering of the double mutant indicates that the R quaternary structure is still accessible because the scattering pattern is identical to that of the wild-type enzyme in the presence of saturating PALA (Fig. 4). Furthermore, the ability of PALA to induce the full T to R transition in the double mutant is confirmed by measurements of the radius of gyration, which are the same for the two enzymes in the absence of ligands, as well as in the presence of saturating PALA (Table 3). The structural similarity between the wild-type and the Glu-50/Asp-100 → Ala enzymes is underlined by their behavior in the presence of a subsaturating concentration of PALA. The addition of 0.3 mol of PALA per mol of active sites can be accounted for by practically identical linear combinations of the two extreme scattering patterns (54% T: 46% R for the wild-type enzyme and 52% T:48% R for the double mutant enzyme) (Fig. 4). This strongly suggests, therefore, that under these conditions, the total population of either enzyme is composed of two discrete subpopulations of T and R structures.

Although solution X-ray scattering indicates that the structures of the wild-type, Glu-50 → Ala, and Glu-50/Asp-100 → Ala enzymes are identical in both the absence of ligands and in the presence of saturating PALA, it is evident from the PALA activation experiments that replacement of Asp-100 by Ala increases the affinity for the bisubstrate analogue (Fig. 3). Indeed, the concentration of PALA required for maximal activation of the Glu-50/Asp-100 → Ala enzyme is sixfold lower than for the Glu-50 → Ala enzyme (Fig. 3). This increase in the affinity for PALA is further apparent in the fact that the scattering pattern of the Glu-50/Asp-100 → Ala enzyme in the presence of saturating carbamoyl phosphate and subsaturating succinate is shifted toward the R pattern by the addition of subsaturating PALA (Fig. 6), whereas for the Glu-50 → Ala enzyme, which has a higher affinity for carbamoyl phosphate than for PALA (Newton & Kantrowitz, 1990), the scattering pattern is shifted in the opposite direction (Tauc et al., 1994).

### *Succinate activates the Glu-50/Asp-100 → Ala enzyme by shifting it from the T quaternary structure toward the R quaternary structure*

Succinate, a nonreactive analogue of aspartate, activates the wild-type holoenzyme by promoting the T to R transition (Collins & Stark, 1971). Although aspartate binding is inhibited at the active site to which succinate is bound, activation results from the conversion of the remaining active sites from the low-affinity, low-activity T state to the high-affinity, high-activity R state. Structural analysis of the wild-type enzyme by solution X-ray scattering indicates that succinate, in the presence of saturating carbamoyl phosphate, induces the full T to R transition (Tsuruta et al., 1994), and X-ray crystallography of the (carbamoyl phosphate + succinate)-ligated enzyme indicates that it is very similar to the PALA-ligated R-state structure (Gouaux & Lipscomb, 1988; Ke et al., 1988).

Kinetic experiments indicate that succinate can activate both the wild-type and Asp-100 → Ala enzymes, although the Asp-100 → Ala enzyme is maximally activated at a lower concentration (Fig. 2). Because succinate is an aspartate analogue, this apparent increase in the affinity for succinate presumably reflects the increase in affinity that the Asp-100 → Ala enzyme shows for aspartate (Baker & Kantrowitz, 1993). By contrast, the Glu-50 → Ala enzyme cannot be activated (Fig. 2), because it remains essentially in the T quaternary structure even in the presence of concentrations of succinate as high as 140 mM (Tauc et al., 1994).

The introduction of the Asp-100 → Ala mutation to the Glu-50 → Ala enzyme at least partially restores the ability of the enzyme to undergo the T to R transition because the scattering pattern of the Glu-50/Asp-100 → Ala enzyme, in the presence of carbamoyl phosphate and succinate, is shifted significantly toward the wild-type R curve. This shift in the scattering pattern also provides an explanation as to why succinate activates the double mutant (Fig. 2). However, because the scattering pattern of the Glu-50/Asp-100 → Ala enzyme is not shifted com-

pletely to the wild-type R curve, the double mutant does not exist fully in the R quaternary structure. Moreover, assuming a similar behavior with aspartate, this is in agreement with the observation that ATP can activate the enzyme in the presence of saturating concentrations of carbamoyl phosphate and aspartate (Fig. 1).

### *Asp-100 → Ala mutation enhances the ability of the regulatory nucleotides to alter the T to R transition*

Lower concentrations of ATP and CTP are required to shift the equilibrium between the T and R states for the Glu-50/Asp-100 → Ala enzyme than for the wild-type enzyme (Table 2). Indeed, the affinity constants for ATP and CTP of the Asp-100 → Ala and Glu-50/Asp-100 → Ala enzymes are essentially identical, indicating that it is the Asp-100 → Ala mutation that is the cause of this effect.

In the presence of saturating carbamoyl phosphate and a subsaturating concentration of succinate, the scattering pattern of the Glu-50/Asp-100 → Ala enzyme is shifted toward the R pattern in the presence of ATP, and toward the T pattern in the presence of CTP or CTP + UTP (Fig. 5), and the magnitude of these shifts is greater than that for the wild-type enzyme under similar conditions. In the presence of a subsaturating concentration of PALA, ATP causes a small, but significant, shift toward the R pattern (Fig. 6), at variance with the absence of any detectable effect with the wild-type enzyme, whereas the effect of CTP or CTP + UTP are not markedly different from the small T shift observed with the wild-type enzyme (Hervé et al., 1985). This direct effect of ATP on the quaternary structure of the double mutant cannot be explained in detail in the absence of a high-resolution structure of this enzyme, but it does not contradict the observation made with the wild-type enzyme. Indeed, because the Asp-100 → Ala mutation weakens the interactions between neighboring catalytic chains within each trimer, it will make each catalytic chain more responsive to any alteration at the regulatory-catalytic interfaces. If, as proposed by Xi et al. (1991) or Stevens and Lipscomb (1992), the binding of nucleotides causes modifications at the subunit interfaces, this may account for the increased sensitivity of the quaternary structure of the double mutant to these nucleotides.

In summary, the introduction of the Asp-100 → Ala mutation to the Glu-50 → Ala enzyme of *E. coli* aspartate transcarbamoylase results in restoration of homotropic cooperativity, increased activity, and an increase in the affinity for PALA. Because domain closure is involved in bringing a number of active site residues into contact with the substrates (Ke et al., 1988), then it is likely that these residues are located closer to the substrates in the Glu-50/Asp-100 → Ala enzyme than in the Glu-50 → Ala enzyme. However, because the interdomain bridging interactions involving Glu-50 cannot be formed in the double mutant, the two domains are presumably not close enough to restore activity to the wild-type level.

An alternative explanation can be put forward, based on the observation that the double mutant can also be activated by succinate, activation that is correlated with a structural transition from the T quaternary structure toward the R quaternary structure. This transition remains partial, even under saturating conditions, with less than 60% of the molecules in the R quaternary structure, whereas this transition is complete with the wild-type enzyme (Tsuruta et al., 1994) and negligible with the Glu-50 → Ala enzyme (Tauc et al., 1994). Assuming a similar behavior with aspartate, this could account for the intermediate level of activity of the Glu-50/Asp-100 → Ala enzyme. Most likely, the properties of the double mutant result from the combination of an incomplete quaternary structure transition and of a different local conformation at the active site. Indeed, although about half the population of the Glu-50/Asp-100 → Ala enzyme can be converted to the R quaternary structure, the activity is only about 20% of the wild-type enzyme. Therefore, even for those molecules of the Glu-50/Asp-100 → Ala enzyme that adopt the R quaternary structure, the active site is not in the same high-activity state as the wild-type enzyme, presumably because specific residues involved in catalysis are not oriented in their optimal positions.

Taken together, these results indicate that the Glu-50/Asp-100 → Ala enzyme behaves functionally and structurally in an intermediate manner between that of the wild-type and the Glu-50 → Ala enzyme. Because Asp-100 from C1 interacts with Arg-65 from C2, Asp-100 from C2 interacts with Arg-65 from C3, and Asp-100 from C3 interacts with Arg-65 from C1, then the replacement of Asp-100 by Ala weakens two interactions that link each of the carbamoyl phosphate domains to the adjacent carbamoyl phosphate domains within the catalytic subunit. It is probable, therefore, that the weakening of these interactions in the Asp-100 → Ala and Glu-50/Asp-100 → Ala enzymes enables the carbamoyl phosphate domains to move more independently of one another, which results in the promotion of domain closure, and consequently in the promotion of the T to R quaternary structure transition. Although it has been shown that the salt bridge between Asp-100 and Arg-65 is important for the structural stability of the catalytic trimer (Baker & Kantrowitz, 1993), it is evident from these experiments that this interaction reduces the catalytic efficiency of the wild-type enzyme. It is likely, therefore, that it has been maintained during evolution for structural rather than for functional advantages, especially because Asp-100 is conserved in a number of aspartate and ornithine transcarbamoylases (see Baker & Kantrowitz, 1993, and references cited therein). The Asp-100 → Ala enzyme has recently been crystallized in order to determine how the mutation affects the C1-C2 interface and the structure of the holoenzyme.

## 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 U.S. Biochemicals or New England Biolabs and used according to the manufacturers' recommendations.

## Methods

### Wild-type and mutant enzyme overproduction and purification

Wild-type and mutant aspartate transcarbamoylases were isolated from *E. coli* strain EK1104 [$F^-$ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *pyrF*$^{\pm}$, *rpsL*], containing the plasmids pEK54 (Xu et al., 1988), pEK91 (Newton & Kantrowitz, 1990), pEK214 (Baker & Kantrowitz, 1993), and pEK246, which code for the wild-type, Glu-50 → Ala, Asp-100 → Ala, and Glu-50/Asp-100 → 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

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 carried out with a radiometer TTT80 titrator and an ABU80 autoburette.

### Data analysis

Analysis of steady-state kinetic data was carried out 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 + malonate)-ligated complex (Gouaux & Lipscomb, 1990), and the PALA-ligated complex (Ke et al., 1988), was accomplished using the program QUANTA (Molecular Simulations Inc., Burlington, Massachusetts).

### 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 $cm^2\ mg^{-1}$ (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/Asp-100 → Ala enzymes were prepared from stock solutions in 50 mM Tris-acetate buffer, pH 8.3, 0.1 mM EDTA, and 0.1 mM dithiothreitol as described previously (Hervé et al., 1985). X-ray scattering curves were recorded on the small angle scattering instrument D24 using synchrotron radiation at LURE-DCI, Orsay. 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 except for the following differences: the wavelength of the X-rays was 1.4878 Å (K-edge of Ni), and the detector was filled with a $Xe/CO_2$ mixture (90%/10%) at 1.5 bar instead of $Ar/CO_2$, which leads to about a fourfold increase in detection efficiency, whereas the flux on the sample was increased by more than 50%. As a result, the overall counting rate was multiplied by about sevenfold, yielding significantly improved scattering patterns despite a twofold decrease in the protein concentration (50 mg/mL, corresponding to 1 mM in active sites).

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