Importance of the loop at residues 230–245 in the allosteric interactions of *Escherichia coli* aspartate carbamoyltransferase

(cooperativity/site-directed mutagenesis)

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ABSTRACT Site-directed mutagenesis has been used to replace tyrosine-240 with phenylalanine in each of the catalytic chains of aspartate carbamoyltransferase. Tyrosine-240 is part of a loop in the structure of the enzyme, between residues 230 and 245, which undergoes a substantial conformational change as the enzyme becomes ligated [Krause, K. L., Voltz, K. W. & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. USA* 82, 1643–1647]. The mutant enzyme with phenylalanine at position 240 has substantially reduced homotropic interactions and an increased affinity for the substrate aspartate but displays no alteration in maximal observed specific activity. The Hill coefficient decreases from 2.4 for the wild-type enzyme to 1.8 for the mutant, and the aspartate concentration at half the maximal observed velocity decreases from 11.9 mM to 4.7 mM at pH 8.3. Heterotropic interactions of the mutant enzyme are altered to a lesser extent. The catalytic subunit derived from the mutant enzyme exhibits kinetics identical to that of the wild-type catalytic subunit. Reactivity of the mutant enzyme with *p*-hydroxymercuribenzoate suggests that the unligated enzyme exists in an altered conformation. The properties of the mutant enzyme are explained in terms of the structure of the wild-type enzyme, and a model is proposed to account for the allosteric interactions of the wild-type enzyme in terms of specific interactions involving the 230–245 loop of the enzyme.

*Escherichia coli* aspartate carbamoyltransferase (EC 2.1.3.2) has been extensively studied as a model for allosteric regulation (1–5). This enzyme catalyzes the committed step in pyrimidine biosynthesis, the condensation of L-aspartate and carbamoyl phosphate to form N-carbamoylaspartate and inorganic phosphate. Homotropic cooperativity is induced by both substrates (6, 7), whereas CTP, a product of the pyrimidine pathway, acts as a heterotropic inhibitor and ATP, a product of the parallel purine pathway, acts as a heterotropic activator (6). The x-ray structure of the unligated holoenzyme has been determined (8), as well as the structure in the presence of CTP (9) and in the presence of N-phosphonacetyl-L-aspartate (PALA) (10), a potent inhibitor that combines into one molecule the binding loci of both of the natural substrates (11). The *M* subunit, 310,000 holoenzyme is composed of two *M* subunits, 100,000 and three *M* subunits, 34,000 subunits. The larger, catalytic subunits each contain three polypeptide chains on which the active sites are located, while the smaller, regulatory subunits each contain two polypeptide chains on which the effector binding sites are located.

A variety of physical studies, including sedimentation (12), circular dichroism (13), and x-ray solution scattering (14), indicate that there is a major reorganization of the quaternary structure upon binding of substrates. These structural changes have been confirmed by x-ray crystallography. A comparison of the three-dimensional structures in the presence and absence of PALA reveals both quaternary and tertiary structural changes. The two catalytic subunits move apart by 12 Å and mutually reorient by 10°, while the regulatory subunits reorient 15° about the 2-fold axes (10). Along with these quaternary changes, there are significant alterations in the tertiary structure of the catalytic chains. In particular, the loops involving residues 76–86 and 230–245, respectively, reorient, repositioning a number of residues necessary for the binding of PALA and, presumably, catalysis.

Previous studies by Landfear et al. (15, 16) have shown that the nitration of 1.6 tyrosine residues per catalytic chain causes loss of homotropic cooperativity, although the modified enzyme is still sensitive to the heterotropic effectors. Not all of the modified tyrosine residues were identified; however, tyrosine-240 was shown to be nitratated to a large extent (16). This work, in conjunction with the observed structural changes that the enzyme undergoes as substrate is bound, suggests that the 230–245 loop may be important for the allosteric interactions of the enzyme. To test this hypothesis, we used site-directed mutagenesis to introduce specific alterations in the primary structure of the 230–245 loop. Here we report the results of the substitution of phenylalanine for tyrosine at position 240.

**EXPERIMENTAL PROCEDURES**

Materials. ATP, CTP, carbamoyl phosphate, *N*-carbamoyl-L-aspartate, agar, agarose, ampicillin, L-aspartate, potassium dihydrogen phosphate, *p*-hydroxymercuribenzoate (pHMB), and Tris were purchased from Sigma. Enzyme-grade ammonium sulfate was purchased from Schwarz/Mann, and Casamino acids were purchased from Difco. The carbamoyl phosphate was purified by precipitation from 50% (vol/vol) ethanol and stored desiccated at −20°C (6).

Restriction endonucleases were obtained from either Bethesda Research Laboratories or New England Biolabs and used according to the supplier’s recommendations. T4 DNA ligase, the Klenow fragment of DNA polymerase, and T4 polynucleotide kinase were products of United States Biochemicals.

Isolation of Holoenzyme and Catalytic Subunit. Wild-type aspartate carbamoyltransferase was isolated from *E. coli* strain KE1104 (F* ara, thi, Δpro-lac, ΔpyrB, pyrF* a, rpsL*), containing the plasmid pEK2, by procedures previously described (17). The plasmid pEK2 carries the entire wild-type pyrB operon. This strain/plasmid combination was used to produce the wild-type enzyme because the pyrB gene used for mutagenesis was originally derived from the plasmid pEK2 (18). Separation of regulatory and catalytic subunits of

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Abbreviations: PALA, N-phosphonacetyl-L-aspartate; pHMB, *p*-hydroxymercuribenzoate; [S]o,s, substrate concentration at half the maximal observed specific activity.

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wild-type and mutant aspartate carbamoyltransferases was carried out as described (19).

**Aspartate Carbamoyltransferase Assay.** The carbamoyltransferase activity was measured at 25°C by either a colorimetric (20) or a pH-stat method (21). pH-stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburette.

**Polycrylamide Gel Electrophoresis.** The Ornstein and Davis procedure was used for nondenaturing polycrylamide gel electrophoresis (22, 23); the Laemmli procedure was used for denaturing polycrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (24).

**Determination of Protein Concentration.** The concentration of pure wild-type aspartate carbamoyltransferase holoenzyme was determined by absorbance measurements at 280 nm, using an extinction coefficient of 0.59 cm²/mg (25). All other protein concentrations were determined either by the method of Lowry et al. (26) or by the Bio-Rad version of Bradford's dye-binding assay (27).

**Data Analysis.** The analysis of the kinetic data was carried out as previously described (28).

**Isolation of Plasmid.** Small-scale purification of plasmid was carried out by the alkaline-lysis procedure (29), and large-scale plasmid purification was performed by the method of Schleif and Wensink (30).

**Oligonucleotide Synthesis.** The oligonucleotides required for the site-directed mutagenesis were synthesized using the Applied Biosystems 381A DNA synthesizer. Purification was accomplished, after deblocking, by preparative polycrylamide gel electrophoresis and was checked by reversed-phase HPLC using a Beckman C3 Ultrapore column.

**RESULTS**

**Site-Directed Mutagenesis.** The replacement of tyrosine by phenylalanine at position 240 of the catalytic chain of aspartate carbamoyltransferase was accomplished by the procedure of Zoller and Smith (31), with two modifications. First, the standard M13 sequencing primer was used to provide a second priming site on the 5' side of the mutagen in order to reduce in vivo strand displacement of the mutagenic primer (32). Second, after the fill-out and ligation steps, the M13 replicative form (RF) was transformed into HB2154 [F' ara, thi, Δpro-lac, mutL::Tn10(Tet')/F' proAB, lacP, lacZΔM15] and plated onto a lawn of HB2151 (F' ara, thi, Δpro-lac/F' proAB, lacP, lacZΔM15). HB2154 is defective in DNA repair and therefore mismatch repair does not occur (33). Plating onto a lawn of HB2151, a repair-positive version of HB2154, reduced the time the M13 RF was in the repair-defective background. The construction of the M13 phage carrying the entire pyrBI operon has been described (18). Before use, the oligonucleotide mutagen was tested to determine whether it annealed at a single position on the M13-pyrBI, by using the mutagen as a primer in sequencing by the dideoxy chain-termination method (34). Excellent sequence was obtained, indicating that the oligonucleotide annealed to only the desired site. Following primer extension, ligation, and transformation, the mutant phage were screened by colony-blot hybridization using the same labeled oligonucleotide as probe (35). The sequence of the putative mutants was confirmed by dideoxy sequencing using a specific primer located 75 bases from the mutation site. Approximately 35% of the candidates were identified as having the desired single nucleotide change.

**Expression and Purification of the Mutant Aspartate Carbamoyltransferase.** A 925-base-pair fragment of the pyrBI operon containing the mutation at position 240 was removed from the recombinant M13 phage by cleavage with BsrEII and EcoRV. This fragment was then ligated into pEK2 (17) that had been cut previously with the same two enzymes. Selection was accomplished after transformation into U39a (F' ara, thi, Δpro-lac, ΔpyrB, rpsL), a strain with a deletion in the pyrBI region. A plasmid was isolated, pEK33, that carried the desired mutation. Overproduction of the mutant aspartate carbamoyltransferase (Phe240C) was accomplished using the derepression system of Nowlan and Kantrowitz (17).

A single species with exactly the same mobility as wild-type aspartate carbamoyltransferase holoenzyme was observed by polycrylamide gel electrophoresis of the purified Phe240C enzyme, under nondenaturing conditions.

**Cooperativity of the Phe240 Enzyme.** The allosteric properties of the Phe240C mutant enzyme are dramatically different than those of the wild-type enzyme. As seen in Fig. 1A, the homotropic cooperativity is substantially reduced. The Hill coefficient at pH 8.3 for the Phe240C enzyme is 1.8, as compared to 2.4 for the wild-type enzyme (see Table 1), and at pH 7.0, the cooperativity of the mutant is nearly abolished (data not shown).

The kinetic analyses of the mutant enzyme also revealed a drastic alteration in affinity for the substrate aspartate. The aspartate concentration at one-half of the maximal observed velocity, [S]₀.₅, decreases from 11.9 mM to 4.7 mM at pH 8.3 (see Fig. 1A). Although both the cooperativity and the affinity of the Phe240C enzyme for aspartate are altered, the maximal observed specific activity is almost identical to that of the wild-type enzyme.

**FIG. 1.** Aspartate saturation curves of wild-type and mutant aspartate carbamoyltransferase with phenylalanine at position 240 of the catalytic chain. Specific activity is reported in mmol of N-carbamoyl aspartate formed per hr per mg of protein. Colorimetric assays were performed at 25°C in 0.05 M Tris acetate buffer (pH 8.3) at saturating carbamoyl phosphate (4.8 mM). (A) Saturation curves for the wild-type (●) and Phe240C (○) holoenzyme. (B) Saturation curves for wild-type (●) and Phe240C (○) catalytic subunit. All data points are averages of duplicates, and the curves drawn are the best-fit curves calculated by a nonlinear least-squares procedure (28).
Table 1. Kinetic parameters for wild-type and Phe240C aspartate carbamoyltransferase in the presence and absence of ATP and CTP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ATP</th>
<th>Control</th>
<th>CTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill coefficient*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.7 (0.06)</td>
<td>2.4 (0.13)</td>
<td>2.5 (0.18)</td>
</tr>
<tr>
<td>Phe240C</td>
<td>1.0 (0.06)</td>
<td>1.8 (0.06)</td>
<td>2.0 (0.09)</td>
</tr>
<tr>
<td>[S]0.5, mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>6.5</td>
<td>11.9</td>
<td>12.6</td>
</tr>
<tr>
<td>Phe240C</td>
<td>2.5</td>
<td>4.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Maximal observed specific activity†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>17.4</td>
<td></td>
<td>13.5</td>
</tr>
<tr>
<td>Phe240C</td>
<td>15.7</td>
<td>16.6 (1.0)</td>
<td>15.8</td>
</tr>
<tr>
<td>Activation/inhibition§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>191%</td>
<td></td>
<td>73%</td>
</tr>
<tr>
<td>Phe240C</td>
<td>148%</td>
<td></td>
<td>67%</td>
</tr>
</tbody>
</table>

Data in the table are extracted from aspartate saturation curves determined in the presence of 0.3 mM CTP or 4.0 mM ATP or in the absence of nucleotide effector (control; see Fig. 1).

*Standard error in parentheses.
†mmol of N-carbamoylaspargate per hr per mg of protein.
‡Standard deviation of five trials including two independent purifications of the enzyme.
§Percent of the activity without effector, at the [S]0.5 of the wild-type or Phe240C enzyme.

Activation by ATP and Inhibition by CTP. Aspartate saturation curves in the presence and absence of the nucleotide effectors ATP and CTP were used to determine the effect of the substitution on the heterotropic interactions of the Phe240C enzyme. The Phe240C enzyme is activated to 148% of control by ATP, compared to 191% for the wild-type enzyme, and is inhibited to 67% of control by CTP, compared to 73% for the wild-type enzyme. In the presence of ATP, the Phe240C enzyme exhibits a Hill coefficient of 1.0 and a [S]0.5 of 2.5 mM; in the presence of CTP, the enzyme exhibits a Hill coefficient of 2.0 with a [S]0.5 of 5.8 mM. The kinetic data on the Phe240C enzyme are summarized in Table 1.

Kinetics of the Catalytic Subunit. In the wild-type enzyme, Tyr-240 is approximately 20 Å from the active site, and therefore it is unlikely that this residue participates directly in catalysis. Since the catalytic subunit alone does not show any cooperativity, it was of interest to determine whether the substitution of phenylalanine for Tyr-240 has any effect on the kinetics of the isolated catalytic subunit. As seen in Fig. 1B, hyperbolic kinetics are observed for the catalytic subunit of both the wild-type and the Phe240C enzyme. Furthermore, the kinetic parameters of the mutant and wild-type catalytic subunits are almost identical. Thus, the substitution of phenylalanine at position 240 has no effect on the maximal velocity of the mutant catalytic subunit.

Reaction with pHMB. The rate of reaction of pHMB with aspartate carbamoyltransferase has been found to be indicative of the allosteric conformational state of the holoenzyme (12). Therefore, this technique was used to evaluate the conformational state of the Phe240C enzyme. As seen in Fig. 2, the reaction rate of pHMB with the Phe240C enzyme is approximately 7-fold faster than with the wild-type. When the experiment is repeated in the presence of a saturating concentration of PALA, the pHMB reaction rate of the Phe240C enzyme actually decreases by a factor of 2. Under identical conditions, the pHMB reaction rate for wild-type enzyme increases such that both the wild-type and the mutant enzymes have identical reaction rates (Fig. 2).

FIG. 2. Reaction of pHMB with wild-type and Phe240C aspartate carbamoyltransferase. The reaction was carried out at 25°C in 40 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM pHMB and was monitored by the absorbance increase at 250 nm. Data are shown for unligated wild-type (●) and Phe240C (○) holoenzyme and for wild-type (■) and Phe240C (▲) holoenzyme in the presence of 50 μM PALA.

FIG. 3. Stereoview of two catalytic chains of aspartate carbamoyltransferase in the unligated form (A) and in the form that has the bisubstrate analog PALA bound (B) (6, 7). The 230-245 loop is shown in black and the Asp-271 and Tyr-240 side chains are shown with heavy lines. View is from the side of the molecule perpendicular to the 3-fold axis.

DISCUSSION

The results of Landefear et al. (15, 16) suggested that the portion of aspartate carbamoyltransferase near Tyr-240 is important for the homotropic and heterotropic interactions of...
the enzyme. A comparison of the three-dimensional structures of the enzyme with (10) and without (8) PALA bound indicates that the loop between residues 230 and 245 of the catalytic chain undergoes a substantial rearrangement. To probe the functional role of the 230–245 loop, we have used the technique of site-directed mutagenesis (31) to introduce specific amino acid substitutions in this portion of the molecule.

The replacement of Tyr-240 by phenylalanine causes a substantial reduction in the cooperativity of the enzyme along with a marked increase in the affinity of the enzyme for aspartate. The $K_{d}$ of 4.7 mM for the Phe240C holoenzyme is even lower than the $K_m$ of 5.7 mM observed for the wild-type catalytic subunit. This substitution has significant effects on aspartate affinity and cooperativity; however, within experimental error, the maximal observed specific activity is not altered. This suggests that the change in aspartate affinity is due to changes in cooperativity and not in catalysis. Further support for this comes from a comparison of the kinetic data obtained for the isolated catalytic subunits. As seen in Fig. 1B, the Phe240C and wild-type catalytic subunits exhibit identical kinetics.

To probe the conformational state of the mutant enzyme, we have employed the reaction of the holoenzyme with pHMB. The rate of this reaction has been shown to be dependent on the conformational state of the enzyme (12). Carbamoyl phosphate and succinate, an analog of aspartate, or PALA cause a significant increase in reactivity of the holoenzyme toward pHMB. Presumably, this increased reactivity is due to the greater accessibility of the sulfhydryl groups when the enzyme is in the ligated form.

The rate of reaction of pHMB with the unligated Phe240C enzyme is approximately 7-fold higher than with the unligated wild-type enzyme. In the presence of PALA, the Phe240C reaction rate slows, whereas the reaction rate for the wild-type enzyme is enhanced, resulting in identical reactivities for both enzymes.

The enhanced reactivity of the Phe240C enzyme toward pHMB suggests that the unligated conformation of the mutant enzyme is distinctly different from the unligated conformation of the wild-type enzyme. The accelerated reaction rate is reminiscent of a number of mutant versions of aspartate carbamoyltransferase, which have been thought to be frozen in the state of the enzyme that has a high affinity for aspartate (19, 36). The Phe240C enzyme cannot be frozen in the high-affinity state, because this enzyme still shows cooperativity and an alteration in pHMB reactivity upon PALA binding. If the Phe240C enzyme were frozen in the high-affinity state, hyperbolic kinetics and a pHMB reactivity unaffected by PALA would be expected. However, the conformation of the unligated Phe240C mutant is different from the wild type, since the reactivity of the sulfhydryl groups is enhanced.

The reactivity of pHMB toward the Phe240C enzyme, in the presence and absence of PALA, is similar to that observed for the aspartate carbamoyltransferase mutant which has Gly-128 replaced by aspartic acid (Asp128C) (37). Based on pHMB reactivity, the accessibility of the sulfhydryl groups in these two mutants is similar; however, the active site conformation of the Asp128C mutant does not allow catalysis to occur.

Fig. 3 shows the difference in conformation between the unligated (A) and ligated (B) states of the wild-type enzyme (8, 10). The large alteration in quaternary structure between the two forms of the enzyme is sufficient to explain the enhanced pHMB reactivity of the wild-type enzyme in the presence of PALA.

Examination of the structure reveals that the link between Asp-271 and Tyr-240, which is observed in the unligated structure, is lost when the molecule binds PALA. When phenylalanine is substituted for Tyr-240, the interaction between Asp-271 and Tyr-240 is no longer possible. This results in a destabilization of the unligated conformation of the enzyme, leading to a new conformation that is more reactive toward pHMB. When the pHMB-reactivity experi-

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**Fig. 4.** Closeup stereoview of the 230–245 loop of two aspartate carbamoyltransferase catalytic chains in the unligated form (A) and in the form with PALA bound (B) (8, 10). Asp-271, Tyr-165, and Tyr-240 to the top and left are from one polypeptide chain, whereas the same residues to the bottom and right are from the other polypeptide chain. For clarity, the side chains of Lys-164 and Glu-239 are shown but not labeled. Orientation of the molecule is identical to that in Fig. 3.
ment is carried out in the presence of PALA, both the Phe240C and the wild-type enzymes react identically. These data suggest that PALA induces a structural change in the mutant enzyme resulting in a conformation identical to that observed for the ligated wild-type enzyme.

The data reported here suggest that the 230–245 loop is critical for the cooperative interactions of aspartate carbamoyltransferase. In particular, the interaction between Asp-271 and Tyr-240 helps stabilize the enzyme in the conformation that has low affinity for substrate.

As seen in Fig. 4A, in addition to the link between Asp-271 and Tyr-240, there are a number of other interactions involving the 230–245 loop (10). For example, there is an intersubunit interaction between Glu-239 and Tyr-165. Upon PALA binding, this intersubunit interaction is lost and a new intersubunit interaction forms between Glu-239 and both Lys-164 and Tyr-165 (Fig. 4B). One of these interactions has been examined using site-directed mutagenesis (38). A mutant with Tyr-165 replaced with serine exhibits no cooperativity, a maximal velocity reduced by 66%, and a $K_{m}$ increased from 6.6 to 160 mM. These results, along with the fact that residues near 165 are known to interact with PALA (10), suggest that the substitution of serine at position 165 prevents the interaction with Glu-239 of the 230–245 loop. In the ligated state, the loss of this interaction alters the active site conformation resulting in reduced activity and weaker substrate binding.

The data on the Phe240C mutant suggest a possible model for the allosteric interactions of the enzyme. In the unliganded enzyme, the 230–245 loop serves to destabilize the active site. Specifically, intersubunit interactions involving the 230–245 loop, such as the one between Glu-239 and Tyr-165, promote the low-affinity state at the active site. The intrasubunit interaction between Asp-271 and Tyr-240 re-strains the unligated molecule in the low-affinity conformation by stabilizing the position of the 230–245 loop. The binding of substrate induces a conformation change that disrupts these interactions, leading to the quaternary-structure change. The resulting rearrangement at the active site, to the high-affinity form, is now stabilized by an intrasubunit interaction between Glu-239 and both Lys-164 and Tyr-165, along with a variety of other interactions that have been observed in the three-dimensional structure (10). To test this model, additional amino acid substitutions in the 230–245 loop of aspartate carbamoyltransferase need to be examined.

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