Engineered Complementation in *Escherichia coli* Aspartate Transcarbamoylase

HETEROTROPIC REGULATION BY QUATERNARY STRUCTURE STABILIZATION*

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§ The abbreviations used are: PALA, N-(phosphonoacetyl)-L-aspartate; [Asp]d, aspartate concentration at one-half the maximal observed specific activity.

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*Escherichia coli* aspartate transcarbamoylase regulates pyrimidine biosynthesis by altering its activity homotropically in response to one of its substrates and heterotropically in response to nucleotide effectors. The mechanism of this regulation involves two structurally and functionally different forms of the enzyme, one with low activity and low affinity for substrates (T state) and the other with high activity and high affinity for substrates (R state). Heterotropic regulation may be due to the direct transmission of a regulatory “signal” between the regulatory site and the active site some 60 Å away and/or may involve altering the relative stability of the two forms of the enzyme. By combining a T state-stabilized mutant version of the enzyme, previously thought to have a defect in a heterotropic transmission pathway, with a known R state-stabilized mutant enzyme, we were able to restore some properties of the wild-type enzyme. These data imply that the relative stabilization of the T and R states of the enzyme is in part responsible for the homotropic and heterotropic properties of aspartate transcarbamoylase and that direct pathways for transmission of the heterotropic signals are unlikely.

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Site-specific mutagenesis studies have indicated that the closure of the domains of the catalytic chain is critical not only for the conversion of the enzyme from the T to the R state but also for the concerted allosteric transition (9–11). In addition, site-specific mutagenesis studies have identified certain side chain interactions that are important for the stabilization of either the T or R states of the enzyme (11). For example, the intersubunit interaction between Glu-239c with both Lys-164c and Tyr-165c has been shown to be critical for T state stabilization (9). When this T state link is weakened by the substitution of Glu for Glu-239c, carbamyl phosphate alone is capable of converting the enzyme into the R structure (12); thus, both the homotropic and heterotropic properties of the enzyme are eliminated (9). Mutations at the interface between the two domains of the catalytic chain have also been shown to be important for the stabilization of the R state of the enzyme. For example, the replacement of Glu-50c by Ala results in a mutant enzyme that is impaired in domain closure and thus cannot be converted to the R state by the natural substrates. This mutant enzyme catalyzes the reaction by a random mechanism rather than the ordered mechanism observed for the wild-type enzyme (13–15).

Mutations at the interface between the two domains of the regulatory chain also alter both the cooperativity and hetero-
tropic interactions of the enzyme (16). One particular mutation, Tyr-77r → Phe,\(^3\) causes loss of aspartate cooperativity, eliminates the ability of ATP to activate the enzyme, and actually converts ATP into an inhibitor of the enzyme. In addition, the extent of the ATP inhibition and the strength of its interaction with the enzyme are the same as that observed with CTP (17). These studies, involving mutants at the interface between the zinc and allosteric domains of the regulatory chains, have led to the proposal that the influence of the heterotropic effectors is transmitted from the regulatory binding site to the active site via a specific pathway involving helix H2 of the regulatory chain (16). Another possible interpretation of the effects of these mutations is that they alter the global stabilization of either the T or R states of the enzyme. For example, if a particular mutation has stabilized the T state, the mutant enzyme may not exhibit cooperativity and may or may not be influenced by the heterotropic effectors in the same manner as the wild-type enzyme. Here we report a series of experiments designed to test the hypothesis that global free energy changes are responsible for the manifestation of the homotropic and heterotropic properties of aspartate transcarbamoylase. In this work we attempt to complement the effect of the postulated regulatory pathway mutation Tyr-77r → Phe by adding a mutation in the catalytic chains, Glu-239c → Gln, that stabilizes the R-state of the enzyme. Such a complementation would suggest that direct pathways for transmission of the heterotropic signals are unlikely.

**EXPERIMENTAL PROCEDURES**

**Materials—** Agar, ampicillin, L-aspartate, carbamyl phosphate (lithium salt), N-carbamyl-L-aspartate, uracil, ATP (sodium salt), and CTP (sodium salt) were obtained from Sigma. The carbamyl phosphate was purified before use by precipitation from 50% (v/v) ethanol and was stored desiccated at −20°C (3). Electrophoresis grade acrylamide, enzyme grade ammonium sulfate, and Tris were obtained from ICN Biochemicals. Antipyrine and diacetyl monoxime were obtained from Eastman Kodak Co. and Fisher Scientific, respectively. Casamino acids were from Difco. Restriction endonucleases were obtained from either New England Biolabs, Inc., and used according to suppliers’ recommendations. T4 DNA ligase was obtained from Amer sham Corp., and calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. The plasmid carrying the Tyr-77r mutation in the ppyrI gene was a gift of G. Hervé. DNA fragments were isolated from agarose gels with the GeneClean II kit from BIORAD. The enzymes were purified before use by precipitation from 50% (v/v) ethanol and was stored desiccated at −20°C (3). Electrophoresis grade acrylamide, enzyme grade ammonium sulfate, and Tris were obtained from ICN Biochemicals. Antipyrine and diacetyl monoxime were obtained from Eastman Kodak Co. and Fisher Scientific, respectively. Casamino acids were from Difco. Restriction endonucleases were obtained from either New England Biolabs, Inc., and used according to suppliers’ recommendations. T4 DNA ligase was obtained from Amer sham Corp., and calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. The plasmid carrying the Tyr-77r mutation in the ppyrI gene was a gift of G. Hervé. DNA fragments were isolated from agarose gels with the GeneClean II kit from BIORAD. The enzymes were purified before use by precipitation from 50% (v/v) ethanol and was stored desiccated at −20°C (3). Electrophoresis grade acrylamide, enzyme grade ammonium sulfate, and Tris were obtained from ICN Biochemicals. Antipyrine and diacetyl monoxime were obtained from Eastman Kodak Co. and Fisher Scientific, respectively. Casamino acids were from Difco. Restriction endonucleases were obtained from either New England Biolabs, Inc., and used according to suppliers’ recommendations. T4 DNA ligase was obtained from Amer sham Corp., and calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. The plasmid carrying the Tyr-77r mutation in the ppyrI gene was a gift of G. Hervé.

**Aspartate Transcarbamoylase Assay—** All transcarbamoylase activities were measured by the colorimetric procedure (19). Assays were performed at 37°C in 50 mM Tris-Cl buffer, pH 8.0 (20). The carbamyl phosphate was held constant at a saturating concentration of 5 mM. All assays were performed in duplicate, and the data points in the figures are averages. The concentration of enzyme was kept low to ensure no more than 3% substrate utilization.

The influence of the nucleotides was determined by performing a series of experiments in which the extent of the ATP inhibition and the strength of its interaction with the enzyme are the same as that observed with CTP (17). A fragment of the ppyrI gene containing the Phe-77r mutation was cut from the plasmid with the restriction enzymes BamHI and BglII and was isolated after agarose gel electrophoresis.

**Bio-Rad version of Bradford’s dye-binding assay (22) using the wild-type enzyme as the standard.**

**Data Analysis—** Analysis of steady-state kinetic data was carried out as described previously (23). Data points were fit by a nonlinear least squares procedure either to the Hill equation or the Michaelis-Menten equation, incorporating a term for substrate inhibition (24).

**Plasmid Construction—** Construction of the plasmid containing the Gln-239c/Phe-77r double mutation was accomplished by the combination of appropriate DNA fragments from plasmids containing the single mutations (9, 17). A fragment of the ppyrI gene containing the Phe-77r mutation was cut from the plasmid with the restriction enzymes BamHI and BglII and was isolated after agarose gel electrophoresis.

This same fragment of the ppyrI gene was removed from the plasmid pEK56, which has the Glu-239c → Gln mutation in the ppyrI gene (16). The fragment containing the Gln-239c mutation was isolated as above and treated with calf intestinal alkaline phosphatase to prevent self-ligation. The fragments containing the Gln-239c and Phe-77r mutations were then mixed and treated with T4 DNA ligase overnight at 14°C, followed by transformation into E. coli strain MV1190 (Δlacs-proAB, supE, thi, Δsri-recA) 306-Tn5(tet)’F’ trdD36, proAB, lacI’, lacZAM15) (25). Ten candidates were selected and plasmid DNA was isolated. Each of the plasmid candidates was first checked for proper size by restriction analysis. The restriction analysis was used to verify both the insertion of the BamHI and BglII fragment and its orientation. Plasmid pEK276 was identified which contained the Gln-239c and Phe-77r mutations in the ppyrB and ppyrI genes, respectively.

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\(^3\) The notation used to name the mutant enzymes is, e.g., the Tyr-77r → Phe enzyme. The wild-type amino acid and its location within the catalytic (c) or regulatory (r) chains are indicated to the left of the arrow while the new amino acid is indicated to the right of the arrow.

\(^4\) Gln-239c/Phe-77r, the double mutant enzyme with Gln at position 239 in the catalytic chain and Phe at position 77 in the regulatory chain.
RESULTS AND DISCUSSION

The kinetic parameters of the wild-type, Glu-239c \( \rightarrow \) Gln, Tyr-77r \( \rightarrow \) Phe and Glu-239c/Phe-77r enzymes are summarized in Table I. The kinetic data in Table I were determined under the same conditions used previously for the Tyr-77r \( \rightarrow \) Phe enzyme (17). The data obtained here for the Tyr-77r \( \rightarrow \) Phe and the wild-type enzymes are in good agreement with those reported previously (17). Kinetic data were also measured under these conditions for the previously constructed Glu-239c \( \rightarrow \) Gln enzyme as well as for the Glu-239c/Phe-77r double mutant enzyme.

As seen in Table I, the maximal observed specific activity for the Tyr-77r \( \rightarrow \) Phe enzyme (7.9 \( \pm \) 0.3 mmol h\(^{-1}\) mg\(^{-1}\)) was significantly lower than the values for the wild-type (21.8 \( \pm \) 0.8 mmol h\(^{-1}\) mg\(^{-1}\)) and the Glu-239c \( \rightarrow \) Gln (20.2 \( \pm \) 1.7 mmol h\(^{-1}\) mg\(^{-1}\)) enzymes. However, the reduced activity of the Tyr-77r \( \rightarrow \) Phe enzyme was restored to almost wild-type levels by the introduction of the Glu-239c \( \rightarrow \) Gln mutation; the specific activity of the Glu-239c/Phe-77r double mutant enzyme was 20.5 \( \pm \) 1.0 mmol h\(^{-1}\) mg\(^{-1}\). These data support previous conclusions that the Glu-239c \( \rightarrow \) Gln mutation stabilizes the R state and the Tyr-77r \( \rightarrow \) Phe mutation stabilizes the T state.

For aspartate transcarbamoylase, the concentration of aspartate required to attain one-half the maximal observed velocity [Asp]\(_{0.5}\) is a good measure of the affinity of the enzyme for the substrate. The affinity of the Glu-239c \( \rightarrow \) Gln enzyme for aspartate was slightly higher than for the wild-type enzyme, while the Tyr-77r \( \rightarrow \) Phe enzyme had a significantly lower aspartate affinity than for the wild-type enzyme. For the double mutant enzyme, 5 \( \mu \)M PALA was required to maximally activate the enzyme compared to about 2 \( \mu \)M for the wild-type enzyme. The lower affinity for PALA suggests a weaker affinity for substrate, which is in agreement with the change in the [Asp]\(_{0.5}\). Thus, the Tyr-77r \( \rightarrow \) Phe mutation stabilizes the T state and, in the presence of the Glu-239c \( \rightarrow \) Gln mutation, allows the enzyme to undergo the allosteric transition with carbamyl phosphate and aspartate; this phenomenon is not observed with the R state-stabilized enzyme.

### Table I

| Enzyme                | Maximal velocity \( \text{mmol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \) | [Asp]\(_{0.5}\) \( \text{ms} \) | Hill coefficient |
|-----------------------|---------------------------------------------------------------|-----------------|-----------------|
| Wild-type             | Wild-type \( \rightarrow \) Gln 21.8 \( \pm \) 0.8          | 20 \( \pm \) 1  | 1.9 \( \pm \) 0.1 |
| Glu-239c \( \rightarrow \) Phe 20.2 \( \pm \) 1.7          | 18.7 \( \pm \) 0.1 | 1.0            |                 |
| Tyr-77r \( \rightarrow \) Phe 7.9 \( \pm \) 0.3            | 47 \( \pm \) 3    | 1.0            |                 |
| Glu-239c/Phe-77r 20.5 \( \pm \) 1.0                       | 59 \( \pm \) 2    | 1.14 \( \pm \) 0.04 |

*The data were fit by a nonlinear least squares procedure using the Hill or Michaelis-Menten equation, incorporating a term for substrate inhibition (24).

### Table II

| Enzyme                | Activity with ATP \( \text{mA} \) | \( K_{\text{ATP}} \) \( \text{ms} \) | Activity with CTP \( \text{mA} \) | \( K_{\text{CTP}} \) \( \text{ms} \) |
|-----------------------|---------------------------------|-----------------|---------------------------------|-----------------|
| Wild-type             | 5.6 \( \pm \) 0.9                | 1.4 \( \pm \) 0.3 | 0.23 \( \pm \) 0.02             | 0.19 \( \pm \) 0.02 |
| Tyr-77r \( \rightarrow \) Phe 0.5 \( \pm \) 0.2            | 2.0 \( \pm \) 0.4    | 0.47 \( \pm \) 0.02             | 2.2 \( \pm \) 0.2 |
| Glu-239c \( \rightarrow \) Gln 1.2 \( \pm \) 0.03           | 0.16 \( \pm \) 0.1 | 1.0                             |                 |
| Glu-239c/Phe-77r 2.2 \( \pm \) 0.04                        | 1.4 \( \pm \) 0.2    | 0.59 \( \pm \) 0.01             | 0.06 \( \pm \) 0.01 |

*Assays were performed at 37°C in 50 mM Tris-Cl buffer, pH 8.0. The carcinobol phosphate concentration was held constant at 10 mM. The concentration of aspartate used was one-tenth the respective [Asp]\(_{0.5}\). Activity with ATP is defined as \( A^{\text{CTP}} / A \) where \( A^{\text{ATP}} \) is the activity extrapolated to an infinite concentration of ATP and A is the activity in the absence of ATP. Activity with CTP is defined as \( A^{\text{CTP}} / A \) where \( A^{\text{CTP}} \) is the activity extrapolated to an infinite concentration of CTP and A is the activity in the absence of CTP.
would expect that even small alterations to the enzyme in areas involved in the quaternary conformational change could dramatically influence the stabilization of the T and/or R states of the enzyme. This expectation is strengthened further by the fact that a single amino acid substitution in fact results in a total of six substitutions for the entire molecule. Tyr-77r, located in the interdomain region between the allosteric and zinc domains of the regulatory chain, lies directly on the path between the regulatory binding site and the active site (Fig. 3). However, alterations in the separation of the allosteric and zinc domains of the regulatory chain induced by the replacement of Tyr-77r by Phe could also act in a more global sense to stabilize either the T or R states of the enzyme.

The Glu-239c → Gln substitution stabilizes the R state of the enzyme (9, 12) and is located well away from the direct path between the regulatory binding site and the active site (see Fig. 3). If global free energy changes, rather than the transmission of a direct signal from the regulatory binding site to the active site along a distinct pathway, are responsible for the homotropic and heterotropic properties of aspartate transcarbamoylase, then the introduction of the Glu-239c → Gln mutation into the Tyr-77r → Phe background would be expected to result in a double mutant enzyme with properties more like wild-type than either of the individual mutant enzymes. As we have shown (Table I), the Glu-239c/Phe-77r double mutant enzyme has wild-type specific activity, is more cooperative than either of the single mutant enzymes, and is activated by ATP, as opposed to the single mutants, which were either unaffected or inhibited by ATP. Thus, the R state stabilization contributed by the Glu-239c → Gln substitution has compensated for the extra T state stabilization of the Tyr-77r → Phe substitution, supporting the notion that these mutations shift the global free energy state of the molecule. The model of global free energy changes also suggests a structural mechanism to describe how the regulatory nucleotides function. As seen in Fig. 3, the regulatory binding site is located on the periphery of the molecule near the interface between each pair of regulatory chains. CTP could function simply by binding and adding stabilization to the compressed form of the enzyme (T state), while ATP could function simply by binding and adding stabilization to the open form of the enzyme (R state). X-ray crystallographic studies of the enzyme with nucleotide present support this hypothesis (26).

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FIG. 3. A schematic of one-third of the aspartate transcarbamoylase enzyme showing one catalytic chain (C1) from the upper trimer, one catalytic chain (C6) from the lower trimer, and one regulatory dimer (R1–R6). Each catalytic chain is composed of the carbamyl phosphate (CP) and aspartate domains, and each regulatory chain is composed of the allosteric (AI) and zinc domains. The active site is indicated by the locations of carbamyl phosphate and aspartate and the regulatory binding site is indicated (NTP). The approximate position of residue 77 in the regulatory chain (77r) and of residue 239 in the catalytic chain (239c) are also shown.

Glu-239c → Gln enzyme.

In contrast to the wild-type enzyme, the R state-stabilized Glu-239c → Gln enzyme is neither activated nor inhibited significantly by either ATP or CTP (Table II), as would be expected since the enzyme shifts entirely to the R state in the presence of a saturating concentration of carbamyl phosphate (9, 12). However, the Tyr-77r → Phe enzyme is inhibited by both ATP and CTP (Table II) (17), and the effect of either one was similar with respect to apparent nucleotide affinity as well as the maximal inhibition (Table II). Both the apparent nucleotide affinity and the maximal inhibition of either nucleotide was less than that observed for the wild-type enzyme with CTP, although the introduction of the R-stabilizing Glu-239c → Gln mutation into the Tyr-77r → Phe enzyme resulted in a double mutant enzyme that was activated 2.2-fold by ATP, instead of being inhibited like the Tyr-77 → Phe enzyme (see Table II).

Previously, the unusual properties of the Tyr-77r → Phe mutant enzyme, the loss of apparent homotropic cooperativity and the conversion of ATP from a heterotropic activator to an inhibitor, have been explained on the basis of a possible movement of the H2' helix of the regulatory chain, which would disrupt the pathway of communication for ATP between the regulatory binding site and the active site (16). The activation of the Tyr-77r → Phe enzyme by PALA (Fig. 2) indicates that this mutant retains residual cooperativity, although it is not observed in steady-state kinetic experiments. The reduced affinity for aspartate, reduced catalytic activity, and reduced cooperativity all suggest that replacement of Phe for Tyr-77r results in a mutant enzyme that is more stabilized in the T state than the wild-type enzyme. Since the free energy difference between the T and R states is small, 3.3 kcal/mol (5), one
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