Three of the Six Possible Intersubunit Stabilizing Interactions Involving Glu-239 Are Sufficient for Restoration of the Homotropic and Heterotropic Properties of Escherichia coli Aspartate Transcarbamoylase*

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Jessica B. Sakash‡, Robin S. Chan‡, Hiro Tsuruta§, and Evan R. Kantrowitz¶

From the ‡Department of Chemistry, Boston College, Merkert Chemistry Center, Chestnut Hill, Massachusetts 02467 and §Stanford Synchrotron Radiation Laboratory, Stanford Linear Accelerator Center, MS69, Stanford, California 94309-0210

A hybrid version of Escherichia coli aspartate transcarbamoylase was investigated in which one catalytic subunit has the wild-type sequence, and the other catalytic subunit has Glu-239 replaced by Gln. Since Glu-239 is involved in intersubunit interactions, this hybrid could be used to evaluate the extent to which T state stabilization is required for homotropic cooperativity and for heterotropic effects. Reconstitution of the hybrid holoenzyme (two different catalytic subunits with three wild-type regulatory subunits) was followed by separation of the mixture by anion-exchange chromatography. To make possible the resolution of the three holoenzyme species formed by the reconstitution, the charge of one of the catalytic subunits was altered by the addition of six aspartic acid residues to the C terminus of each of the catalytic chains (AT-C catalytic subunit). Control experiments indicated that the AT-C catalytic subunit as well as the holoenzyme formed with AT-C and wild-type regulatory subunits had essentially the same homotropic and heterotropic properties as the native catalytic subunit and holoenzyme, indicating that the addition of the aspartate tail did not influence the function of either enzyme. The control reconstituted holoenzyme, in which both catalytic subunits have Glu-239 replaced by Gln, exhibited no cooperativity, an enhanced affinity for aspartate, and essentially no heterotropic response identical to the enzyme isolated without reconstitution. The hybrid containing one normal and one mutant catalytic subunit exhibited homotropic cooperativity with a Hill coefficient of 1.4 and responded to the nucleotide effectors at about 50% of the level of the wild-type enzyme. Small angle x-ray scattering experiments with the hybrid enzyme indicated that in the absence of ligands it was structurally similar, but not identical, to the T state of the wild-type enzyme. In contrast to the wild-type enzyme, addition of carbamoyl phosphate induced a significant alteration in the scattering pattern, whereas the bisubstrate analog N-phosphonooacetyl-L-aspartate induced a significant change in the scattering pattern indicating the transition to the R-structural state. These data indicate that in the hybrid enzyme only three of the usual six interchain interactions involving Glu-239 are sufficient to stabilize the enzyme in a low affinity, low activity state and allow an allosteric transition to occur.

The hallmark of allosteric regulation of enzymatic activity is the ability of the enzyme to exist in at least two different forms (1). On the functional level these two forms of the enzyme differ in catalytic activity and/or substrate affinity. Thus, the activity of the enzyme is modulated by the form in which it exists or by the relative proportions of the two forms in solution at any one time. On the structural level these two forms of the enzyme often differ on the quaternary level (2). These differences in quaternary structure are associated with the relative movements of subunits with respect to each other that may result in differences in subunit interface interactions. One set of interface contacts stabilizes one form of the enzyme, and another set of contacts stabilizes the other form of the enzyme. Here we set out to determine the contribution of specific interface contacts to the relative stability of the alternate conformations of the allosteric enzyme aspartate transcarbamoylase.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) has become a model system for the study of allosteric regulation. This enzyme catalyzes the committed step of pyrimidine biosynthesis, the condensation of carbamoyl phosphate and L-aspartate to form N-carbamoyl-L-aspartate (3). The enzyme shows homotropic cooperativity for the substrate L-aspartate and is heterotropically regulated by ATP, CTP (3), and UTP in the presence of CTP (4). The enzyme from E. coli is a dodecamer composed of six catalytic chains of M\textsubscript{r} 34,000 and six regulatory chains of M\textsubscript{r} 17,000. The catalytic chains are organized as two trimeric subunits, whereas the regulatory chains are organized as three dimeric subunits. The active sites are located at the interfaces between the catalytic chains, and the nucleotide effectors bind to the same site on each of the regulatory chains (5–9).

Two functionally and structurally different states of aspartate transcarbamoylase have been characterized. The low affinity, low activity conformation of the enzyme is described as the “tense” or T state, and the high affinity, high activity conformation of the enzyme is described as the “relaxed” or R state. The conversion from the T to the R state occurs upon aspartate binding to the enzyme in the presence of carbamoyl phosphate. Structurally, the enzyme elongates by at least 11 Å along the 3-fold axis, the upper catalytic trimer rotates 10° relative to the lower trimer, and the regulatory dimers rotate 15° around the 2-fold axes (10, 11). In addition to these quaternary changes, several tertiary changes also occur during the T to R state transition. Specifically, the 80s loop and 240s loop become reoriented. The distinct interchain contacts of side chains of the 240s loop in the T and R states have been identified as being the major contributors to the concerted transition and the stabilization of the T and R states (12). In particular, site-specific mutagenesis has been used to determine that the interactions of Glu-239, Asp-236, and Tyr-240 in the T and...
R states are critical for the stabilization of these alternate conformations of the enzyme. In the T state Glu-239 of C1\(^1\) forms interchain interactions with Lys-164 and Tyr-165 of C4 (13). Conversion to the R state breaks these interactions and establishes new intrachain interactions between Lys-164 and Tyr-165 of the C1 chain, whereas Glu-239 of the C4 chain forms interchain interactions with Lys-164 and Tyr-165 of the C1 chain. In the R state (bottom), Glu-239 forms intrachain interactions with Lys-164 and Tyr-165. This figure was drawn with SETOR (36) using the Protein Data Bank codes 5at1 (top) and 8atc (bottom).

In addition, the E239Q mutant holoenzyme, in which all these interactions are eliminated, exhibits no cooperativity, has an increased affinity for aspartate, and the maximal velocity remains the same as that of the wild-type enzyme. On a functional level, the kinetic data suggest that the elimination of these interactions reduces the stability of the T state and shifts the enzyme into an R-functional state (12). On a structural level, small angle x-ray scattering studies have shown that the E239Q substitution induces a change in the quaternary structure of the unligated wild-type enzyme toward the R structural state. The addition of PALA\(^2\) resulted in a structure identical to that of the PALA-ligated wild-type enzyme. In contrast to the wild-type enzyme, the addition of carbamoyl phosphate alone shifts the scattering pattern of the E239Q holoenzyme to the R-structural state. Thus, the energy associated with the binding of carbamoyl phosphate is sufficient to convert the E239Q holoenzyme into the R-structural state. The conversion of the E239Q holoenzyme into the R state by carbamoyl phosphate explains the functional properties of this mutant enzyme. Since the kinetic experiments are performed in the presence of a saturating concentration of carbamoyl phosphate, the mutant holoenzyme is already converted to the R state and thus the addition of aspartate or PALA cannot cause any additional structural transition. Without the possibility of a structural transition, cooperativity as well as nucleotide effects would not be expected.

Because aspartate transcarbamoylase contains six identical catalytic chains, the wild-type enzyme has six interactions involving Glu-239 that stabilize the T state of the enzyme. Here we have created a hybrid holoenzyme in which one catalytic subunit has Glu-239 replaced by Gln. This hybrid enzyme will allow us to determine whether only three of the six possible interchain interactions are sufficient for stabilization of the R state of the enzyme and will provide insight into the catalytic chain; (E239Q-C)\(_1\)(AT-C)\(_3\)R\(_3\), mutant hybrid holoenzyme in which one catalytic subunit has Gln substituted in place of Glu-239 in each catalytic chain and the other catalytic subunit has 6 aspartate residues added to the C-terminus of each catalytic chain; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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\(^1\) C followed by a number, e.g. C1 or C4, refers to a particular polypeptide chain in aspartate transcarbamoylase.

\(^2\) The abbreviations used are: PALA, N-(phosphonoacetyl)-L-aspartate; [Asp]\(_{0.5}\), the aspartate concentration at half the maximal observed specific activity; C, catalytic subunit of aspartate transcarbamoylase composed of three catalytic chains; R, regulatory subunit of aspartate transcarbamoylase composed of two regulatory chains; AT-C, the mutant catalytic subunit in which 6 aspartate residues have been added to the C terminus of each catalytic chain; (AT-C)\(_1\)R\(_3\), the reconstituted mutant holoenzyme in which 6 aspartate residues have been added to the C terminus of each catalytic chain; (E239Q-C)\(_3\)R\(_3\), reconstituted mutant holoenzyme with Gln substituted in place of Glu-239 in the catalytic chain; (E239Q-C)\(_1\)(AT-C)\(_3\)R\(_3\), mutant hybrid holoenzyme in which one catalytic subunit has Gln substituted in place of Glu-239 in each catalytic chain and the other catalytic subunit has 6 aspartate residues added to the C-terminus of each catalytic chain; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.
strength of the interchain interactions necessary to stabilize the tense T state of aspartate transcarbamoylase.

**EXPERIMENTAL PROCEDURES**

**Materials**—agarose, ATP, CTP, L-aspartate, N-carbamoyl-L-aspartate, potassium dihydrogen phosphate, and uracil were obtained from Sigma. Q-Sepharose Fast Flow and Source Q resins, ampicillin, and Tris were obtained from Amersham Pharmacia Biotech. The UNO-Q 1 ion-exchange column, Protein Assay Dye, and sodium dodecyl sulfate were purchased from Bio-Rad. 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 (3). Casamino acids, yeast extract, and tryptone were obtained from Difco. Ammonium sulfate and electrophoresis grade acrylamide were purchased from ICN Biomedicals (Costa Mesa, CA). Antipyrine was obtained from Fisher. Oligonucleotides were purchased from Operon Technologies (Alameda, CA). The pGEM-T vector system was purchased from Promega (Madison, Wisconsin) and used according to their protocol.

**Strains**—The E. coli strain MV1190 (Δlac-proAB), supE, thi, Δ(sri-reca)306::Tn10(tet’’’’Fdrad36, proB, lacI, lacZAM15) was obtained from J. Messing. EK1104 (F ara, thi, Δ(pro lac), BstBII, pyrF, pyrG) was previously constructed in this laboratory (15).

**Site-directed Mutagenesis to Add an Aspartate Tail to the Catalytic Chain of Aspartate Transcarbamoylase**—Since the E239Q and wild-type holoenzymes cannot be resolved by anion-exchange chromatography, six aspartic acid residues were added to the wild-type catalytic chain to allow for chromatographic separation of the hybrid enzyme. The Asp residues were introduced by addition of six Asp codons to the pyrB gene by PCR (16). The PCR product was purified by agarose gel electrophoresis, and the DNA fragment was isolated from the agarose using glass beads (Bio101, Vista, CA). The DNA fragment containing the modified pyrB gene was mixed with the linear T-tail vector, pGEM-T, and treated with T4 DNA ligase for 16 h at 4 °C. Both a candidate with the PCR product inserted and the plasmid used for expression, pEK54 (17), were digested with the restriction enzymes BstEII and SacI. The insert and vector fragments were isolated from an agarose gel electrophoresis gel and treated with DNA ligase. The resulting plasmid, pEK357, was sequenced (18) to confirm that the mutation had been successful and that the PCR had introduced no other mutations.

**Construction of Plasmid to Express the E239Q Catalytic Subunit**—Plasmid pEK56 (12) carries the E239Q mutation in the pyrB gene and also carries the pyrG gene. In order to remove the pyrG gene so that this plasmid would only produce catalytic subunit, it was digested at the HindIII and BamHI sites. The larger fragment of 4543 base pairs, lacking most of the pyrG gene, was isolated from the agarose gel using glass beads. This DNA fragment with complementary ends was treated with T4 DNA ligase to form pEK316.

**Construction of pEK168**—A smaller version of the plasmid pEK164 (19) was digested at the restriction enzymes SmaI and SphI. The 3958-base pair fragment was isolated from agarose gel using glass beads. This DNA fragment with blunt ends was treated with T4 DNA ligase to form pEK168.

**Overexpression and Purification of the Aspartate Transcarbamoylase Wild-type and Mutant Catalytic Subunit**—The aspartate transcarbamoylase wild-type, AT-C, and E239Q-C catalytic subunits were overexpressed utilizing strain EK1104 containing plasmids pEK17, pEK357, and pEK316, respectively. Bacteria were cultured at 37 °C with agitation in M9 media (20) containing 0.5% casamino acids, 12 μg/ml uracil, and 150 μg/ml ampicillin. Cells were harvested and resuspended in 0.1 M Tris-Cl buffer, pH 9.2, followed by sonication to lyse the cells. Two 65% ammonium sulfate fractionation steps were performed. Ion-exchange chromatography using Q-Sepharose Fast Flow resin was used to purify the enzyme further (21). After concentration, the purity of the enzymes was checked by SDS-PAGE (22) and nondenaturing PAGE (23, 24).

**Formation and Purification of Reconstituted Mutant Holoenzymes**—Equal amounts of purified wild-type catalytic subunit and AT-C catalytic subunit were mixed with excess regulatory subunit and then dialyzed against 50 mM Tris acetate buffer, pH 8.3, 2 mM mercaptoethanol, and 0.1 mM zinc acetate overnight. The mixture was examined by nondenaturing PAGE to confirm the existence of the three holoenzyme species, C2R3, (C)(AT-C)R3, and (AT-C)2R3. Separation of the species was performed using the UNO-Q 1 column as indicated above.

**Determination of Protein Concentration**—The concentration of the wild-type holoenzyme, catalytic subunit, and regulatory subunit were determined from absorbance measurements at 280 nm using extinction coefficients of 0.59, 0.72, and 0.32 cm² mg⁻¹, respectively (26). The concentrations of the mutant enzymes were determined by the Bio-Rad version of the Bradford dye-binding assay (27). The concentration of the nucleotides was determined from absorbance measurements at the λmax at pH 7.0 using the respective molar extinction coefficients of the nucleotides.

**Aspartate Transcarbamoylase Assay**—The aspartate transcarbamoylase activity was measured at 25 °C by the colorimetric method (28). Saturation curves were performed in duplicate, and data points shown in Figs. 1 and 2 are the average of duplicate determinations. The enzyme was assayed with 50 mM Tris acetate buffer, pH 8.3, 2 mM mercaptoethanol, and 0.1 mM zinc acetate (25). The mixture was examined by nondenaturing PAGE to confirm the existence of the three holoenzyme species, E239Q-C2R3, E239Q-C1(CT-C)R, and (AT-C)2R3. Separation of the species was performed using the UNO-Q 1 column as described above. The nucleotide saturation curves were fit to a hyperbolic binding isotherm.

**Small Angle X-ray Scattering**—The small angle x-ray scattering experiments were performed on the Beam Line 4-2 at the Stanford Synchrotron Radiation Laboratory. A significantly upgraded version of the small angle scattering instrument was used. The specimen-to-detector distance was 95 cm, and the x-ray wavelength was tuned to 1.381 Å using the Si(111) double-crystal monochromator (31). A linear gas chamber detector filled with a Xe/CO₂ mixture was used in the experiment. The counting rate on the detector was between 30,000 and 90,000 counts/s. The scattering curves are expressed as the momentum transfer h = 4π(α/sin(α)), where α is the scattering angle and the wavelength of the x-ray beam, respectively, which was calibrated using the (100) reflection from a cholesterol myristate powder sample held at the specimen position. Sample solutions were maintained at 25 °C. All scattering curves were normalized to incident beam intensity integrated over exposure time, and the corresponding solvent scattering was subtracted. The enzyme solution was diluted so all the scattering curves were performed at the identical protein concentration.
These data were determined from the aspartate saturation curves. Colorimetric assays were performed at 25 °C in 50 mM Tris acetate buffer, pH 8.3, and saturating levels of carbamoyl phosphate (4.8 mM).

Table I

| Enzyme               | Maximal velocity a | K_m | n_H |
|----------------------|--------------------|-----|-----|
| Wild-type C          | 27.4 ± 1.2         | 6.9 ± 1.3 |    |
| AT-C                 | 26.8 ± 3.5         | 5.0 ± 0.4 |    |
| E239Q-C              | 26.6 ± 0.6         | 27.0 ± 2.4 |    |

| Subunit Combination  | Maximal velocity a | [Asp] 0.5 | n_H |
|----------------------|--------------------|-----------|-----|
| (C)(AT-C)R_3         | 18.4 ± 1.5         | 12.0 ± 0.6 | 2.4 ± 0.3 |
| (C)(AT-CT)R_3        | 16.5 ± 0.6         | 12.8 ± 0.1 | 3.5 ± 0.2 |
| (AT-C)R_3            | 19.3 ± 1.8         | 9.6 ± 0.8  | 2.6 ± 0.5 |
| (E239Q-C)(AT-C)R_3   | 20.0 ± 0.6         | 8.4 ± 0.2  | 1.0 |
| (E239Q-C)(AT-CT)R_3  | 21.1 ± 1.8         | 6.4 ± 0.3  | 1.4 ± 0.09 |

* Maximal velocity represents the maximal observed specific activity from the aspartate saturation curve.
* a Average deviation of three determinations.
* Data from Sakash and Kantrowitz (25).

**RESULTS**

A Catalytic Subunit of Aspartate Transcarbamoylase with Six Asp Residues as a C-terminal Extension—A hybrid aspartate transcarbamoylase holoenzyme composed of two different catalytic subunits (i.e. one wild-type and one mutant) can be formed by reconstitution of the two different catalytic subunits with excess regulatory subunits. However, this result is a mixture containing pure wild-type holoenzyme, pure mutant holoenzyme, and the desired hybrid holoenzyme. It has been reported that hybrid species of this type can be separated after chemical modification of one of the subunits such that the charge on that subunit is altered (32). Although such an approach was attempted here, it was found unreliable so we altered the charge on one of the subunits by adding a C-terminal extension of six aspartic acid residues in an approach similar to that used by Onuffer and Kirsch (33) with aspartate aminotransferase employing Glu residues.

As a first control, the catalytic subunit containing the Asp extension (AT-C) was purified, and a kinetic characterization was performed to determine whether the addition of the six Asp tail had any significant influence on catalysis. Aspartate saturation curves were determined for the wild-type, the AT-C, and the E239Q catalytic subunits, and the kinetic parameters were calculated from these curves (Table I). The kinetic parameters of the AT-C were very similar to those of the wild-type catalytic subunit. AT-C had a specific activity of 26.3 mmol/h/mg and a K_m of 5.0 mM compared with the wild-type catalytic subunit parameters of 27.4 mmol/h/mg and 6.9 mM. E239Q catalytic subunit had a specific activity of 26.6 mmol/h/mg and a K_m of 27.0 mM, similar to the previously published kinetic data (12).

**Formation, Purification, and Steady-state Kinetics of Catalytic Subunit—**As a second control, the hybrid holoenzyme containing one wild-type catalytic subunit and one AT-C catalytic subunit, C((AT-C))R_3, was kinetically characterized to confirm that this hybrid has virtually the same kinetic parameters as the wild-type holoenzyme. Similar kinetic characterization of the reconstituted C(AR)_3 and (AT-C)R_3 holoenzymes verified that the actual reconstitution procedure does not alter the intrinsic properties of these enzymes. The reconstituted holoenzymes were formed by mixing wild-type catalytic subunit (C) and AT-C catalytic subunit in the presence of excess regulatory subunit (R). This mixture, resulting in the three possible reconstituted holoenzyme species C(AR)_3, C(AT-C)R_3, and (AT-C)R_3, was separated by anion-exchange chromatography. The purity of the individual species was verified by non-denaturing polyacrylamide gel electrophoresis, the major band in each lane (see Fig. 2). As is the case of wild-type aspartate transcarbamoylase holoenzyme, the hybrids also show a small contamination of (C)(AR)_3. Kinetic characterization indicated that all three of the reconstituted holoenzymes had similar maximal observed specific activities, [Asp] 0.5 and Hill coefficients, confirming that the addition of the Asp tail and reconstitution procedure do not significantly alter the holoenzyme (see Table I).

**Formation, Purification, and Steady-state Kinetics of Catalytic Subunit (C)(AT-C)R_3 and (AT-C)(AT-C)R_3—**The hybrid holoenzyme containing one wild-type catalytic subunit and one E239Q-C catalytic subunit was generated by mixing E239Q-C, AT-C, and regulatory subunit in excess. The three species were identified by non-denaturing PAGE and purified by anion-exchange chromatography. Fig. 3 shows the aspartate saturation curves produced by the reconstituted mutant enzymes, and the kinetic parameters calculated from these curves are given in Table I. The (E239Q-C)(AT-C)R_3 holoenzyme exhibited a hyperbolic saturation curve similar to the saturation curve of the native E239Q holoenzyme (12). The maximal velocity of the (E239Q-C)(AT-C)R_3 holoenzyme was 20.0 mmol/h/mg and the K_m was 6.4 mM (Table I). (E239Q-C)(AT-C)R_3 had a sigmoidal aspartate saturation curve with a Hill coefficient of 1.4, and the [Asp] 0.5 was 6.4 mM. The observed maximal specific activity, 21.1 mmol/h/mg, of the hybrid enzyme was similar to both the (AT-C)R_3 and (E239Q-C)(AT-C)R_3 holoenzymes.

**Influence of the Allosteric Effectors—**Nucleotide saturation curves with CTP and ATP were determined with the (E239Q-C)(AT-C)R_3, (E239Q-C)(AT-C)R_3, and (AT-C)R_3 holoenzymes at one-half the [Asp] 0.5. This concentration of aspartate was selected since the nucleotides exert a larger influence on the activity of the enzyme as the aspartate concentration is reduced (34). Based upon the nucleotide saturation curves, the maximal extent of activation or inhibition at infinite nucleotide concentration was calculated. As seen in Fig. 4, ATP activates the (E239Q-C)(AT-C)R_3 and (AT-C)R_3 holoenzymes and CTP inhibits, whereas both nucleotides have little or no effect on the (E239Q-C)(AT-C)R_3 holoenzyme. The (E239Q-C)(AT-C)R_3 holoenzyme is activated by ATP approximately one-half as much as...
the reconstituted wild-type holoenzyme control. In an analogous behavior, the residual activity in the presence of CTP is 37% for the (E239Q-C)(AT-C)R3 holoenzyme compared with 20% for the (E239Q-C)2R3 holoenzyme. This shift is even more significant when compared with the scattering curve of the unliganded wild-type holoenzyme. PALA significantly changes the first subsidiary minima and maxima of both (E239Q-C)(AT-C)R3 and the wild-type holoenzyme. The PALA curves do not overlay perfectly, but this may be the result of a difference in the amount of high molecular weight and (E239Q-C)(AT-C)R2 species present in the (E239Q-C)(AT-C)R3 preparation.

**DISCUSSION**

The addition of six aspartate residues to the C terminus of aspartate transcarbamoylase catalytic chain has proven to be an excellent chromatographic handle for the easy separation of the hybrid mixture, allowing the isolation of aspartate transcarbamoylases with two different catalytic subunits. The C-terminal extension did not result in any substantial alterations in the kinetic parameters of the controls with either one or two catalytic subunits containing such C-terminal extensions, (C)(AT-C)R3 or (AT-C)2R3. In the work reported here, this chromatographic handle has been utilized to create a hybrid enzyme in which one subunit was wild type and the other was mutant with Glu-239 replaced by Gln. This hybrid enzyme has three fewer interchain interactions than the wild-type enzyme and three more than the E239Q holoenzyme. By using this hybrid enzyme we could address the question whether three of these specific interchain interactions are sufficient to stabilize the T state of the enzyme and to provide sufficient T state stabilization to allow homotropic cooperativity to be observed.

Studies have demonstrated that the interchain interactions involving Glu-239 are crucial for the stabilization of the low activity, low affinity form of the enzyme (12, 14, 35). The replacement of Glu-239 by Gln results in a holoenzyme that exhibits no homotropic or heterotropic properties yet has full catalytic activity and enhanced substrate affinity (12). The kinetic studies on the native E239Q holoenzyme suggest that the T state of this mutant enzyme is destabilized.

Small angle x-ray scattering experiments have indicated that the E239Q enzyme, in the absence of ligands, is no longer in the T-structural state but rather in a new conformation, shifted toward the R-structural state of the wild-type enzyme, R'. In contrast to the wild-type enzyme, carbamoyl phosphate alone can shift the unliganded E239Q holoenzyme to the R state (14). These results suggest that the interchain interac-

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**Fig. 3.** Aspartate saturation curves of (E239Q-C)R3 holoenzyme (C), (E239Q-C)(AT-C)R3 hybrid ( ), and (AT-C)2R3 holoenzyme ( ). Specific activity is reported in millimoles of N-carbamoyl-L-aspartate formed per h per mg of protein. Colorimetric assays were performed at 25°C at saturating concentrations of carbamoyl phosphate (4.8 mM) in 50 mM Tris acetate buffer, pH 8.3.

**Fig. 4.** Influence of ATP (A) and CTP (B) on the activity of (E239Q-C)R3 holoenzyme ( ), (E239Q-C)(AT-C)R3 hybrid ( ), and (AT-C)2R3 holoenzyme ( ). The colorimetric assays were performed at 25°C in 50 mM Tris acetate buffer, pH 8.3, at saturating concentrations of carbamoyl phosphate (4.8 mM). The aspartate concentration was held constant at half the [Asp]0.5.

The reconstituted wild-type holoenzyme control. In an analogous behavior, the residual activity in the presence of CTP is 37% for the (E239Q-C)(AT-C)R3 holoenzyme compared with 20% for the reconstituted wild-type holoenzyme control (Table II).
tions of Glu-239 are necessary for the stabilization of the enzyme in the T state. Weakening these interactions creates a new structural state of the enzyme that is presumably along the path between the wild-type T- and R-structural states. The loss of these interactions may allow the 240s loop greater flexibility toward moving into the active site that would promote closing of the active site and thereby promoting the allosteric transition.

**TABLE II**

| Enzyme                | CTP parameters | ATP parameters |
|-----------------------|----------------|----------------|
|                       | Residual activity<sup>a</sup> | $K_{\text{CTP}}$ | % activation<sup>c</sup> | $K_{\text{ATP}}$ |
| (AT-C)$_2$R$_3$       | 19.5 ± 1.6<sup>d</sup> | 0.8 ± 0.04 | 503.9 ± 80.4 | 0.58 ± 0.17 |
| (E239Q-C)(AT-C)R$_3$  | 90.0 ± 7.0 | NA<sup>e</sup> | None | NA<sup>e</sup> |
| (E239Q-C)(AT-C)R$_3$  | 36.9 ± 0.3 | 0.14 ± 0.011 | 258 ± 13 | 0.37 ± 0.07 |
| **Average deviation** | NA<sup>e</sup> | NA<sup>e</sup> | NA<sup>e</sup> | NA<sup>e</sup> |

<sup>a</sup> Percent residual activity is defined as 100 (A$_{\text{CTP}}$/A) where A$_{\text{CTP}}$ is the activity in the presence of CTP and A is the activity in the absence of CTP.

<sup>b</sup> $K$ is the nucleotide concentration required to activate or inhibit the enzyme by 50% of the maximal effect.

<sup>c</sup> Percent activation is defined as 100 (A$_{\text{ATP}}$/A) where A$_{\text{ATP}}$ is the activity in the presence of ATP and A is the activity in the absence of ATP.

<sup>d</sup> Average deviation of three determinations.

<sup>e</sup> NA, not applicable.

**FIG. 5.** The effect of PALA on the activity of (E239Q-C)(AT-C)R$_3$ holoenzyme (∆), (E239Q-C)(AT-C)R$_3$ hybrid ( ), and (AT-C)R$_3$ holoenzyme ( ) at 25 °C in 50 mM Tris acetate buffer, pH 8.3. Carbamoyl phosphate concentrations were saturating (4.8 mM), and the aspartate concentration was held constant at one-fifth the [Asp]$_{0.5}$ of the respective holoenzyme at pH 8.3.

**FIG. 6.** Solution low angle x-ray scattering curves for the wild-type (A) and the (E239Q-C)(AT-C)R$_3$ holoenzymes (B). For each enzyme curves are shown in the absence of ligands ( ), in the presence of 25 mM carbamoyl phosphate (Δ), and in the presence of 1 mM PALA ( ), B, the curve for the wild-type enzyme in the absence of ligands is also shown ( ). The x-ray scattering experiments were performed in 50 mM Tris acetate buffer, 2 mM 2-mercaptoethanol at pH 8.3.

By using the C-terminal Asp tail methodology reported here, we have been able to create and purify a hybrid enzyme with one catalytic subunit that has Glu at 239 and the other that has Gln at 239, the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme. This hybrid enzyme, with only three of the possible six interchain interactions, exhibits cooperativity ($n_H = 1.4$) and retains essentially the same maximal velocity and [Asp]$_{0.5}$ as the parent molecules. PALA activates the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme at concentrations close to those of (AT-C)R$_3$; however, the activation is reduced, consistent with the observed lower Hill coefficient. The observed cooperativity as well as the ability of PALA to activate this hybrid suggests that the hybrid can undergo an allosteric transition from a low activity, low affinity state to a high activity, high affinity state. The lower value of the Hill coefficient can be directly related to the extent of T state stabilization afforded by the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme with only three of the six possible interchain interactions involving Glu-239.

To obtain direct structural data on the conformation of the (E239Q-C)(AT-C)R$_3$ hybrid enzyme, small angle x-ray scattering experiments were performed. As a control, the scattering curve was also recorded for the wild-type enzyme (Fig. 6). The scattering curves of the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme indicate that in the absence of ligands it has a conformation similar to but not identical with the T state structure of the wild-type enzyme. These data suggest that the three existing Glu-239 interactions are sufficient to stabilize the enzyme in a structural state that is similar to the T-structural state of the wild-type enzyme that contains six of these stabilizing interactions. However, carbamoyl phosphate alone induces a significant conformational change in the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme, suggesting that the three existing Glu-239 interactions do not provide adequate stability to maintain the enzyme in the T state upon carbamoyl phosphate binding, as is the case for the wild-type enzyme. An alternative possibility is that the weakening of the three interchain interactions destabilizes the T state and thereby shifts the T $\rightleftharpoons$ R equilibrium more toward the R state. Upon addition of carbamoyl phosphate to the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme, the T $\rightleftharpoons$ R equilibrium is shifted even more toward the R state. However, the binding of carbamoyl phosphate alone is not sufficient to convert the enzyme into either the R* or R-structural states that were observed for the (E239Q-C)(AT-C)R$_3$ holoenzyme.

For the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme the binding of aspartate, in the presence of saturating carbamoyl phosphate, is cooperative indicating that the enzyme can undergo a conformational change to the R state. The small angle x-ray scattering results for the (E239Q-C)(AT-C)R$_3$ hybrid holoenzyme in the presence of PALA supports the notion that this enzyme can be converted to a high activity, high affinity R state similar to that of the wild-type holoenzyme. The reduced coop-
erativity of the (E239Q-C)(AT-C)R₃ hybrid holoenzyme can be simply explained by the destabilization of the T state, especially in the presence of carbamoyl phosphate, resulting in the shift in the T ⇔ R equilibrium mentioned above.

The influence of ATP and CTP on the (E239Q-C)(AT-C)R₃ hybrid holoenzyme establishes a direct link between stabilization of the T state of the enzyme and the ability of the enzyme to be heterotropically regulated by the nucleotide effectors. The Monod et al. model (1) suggests that a change in the T ⇔ R equilibrium should not in itself alter the ability of the heterotropic effectors to activate or inhibit the enzyme on a percentage basis. The (E239Q-C)₂R₃ holoenzyme exhibits almost no alteration in activity in the presence of saturating amounts of the nucleotide effectors. This can be rationalized as due to the conversion of this enzyme into the R state by the addition of carbamoyl phosphate alone. Even at subsaturating concentrations of aspartate the enzyme is already completely shifted in the R state, thus ATP cannot shift the enzyme further toward the R state and CTP can only shift the enzyme slightly toward the T state. The same situation exists for the wild-type enzyme conversion of this enzyme into the R state by the addition of carbamoyl phosphate alone. Even at subsaturating concentrations of aspartate the enzyme is already completely shifted in the R state, thus ATP cannot shift the enzyme further toward the R state and CTP can only shift the enzyme slightly toward the T state. The same situation exists for the wild-type enzyme conversion of this enzyme into the R state by the addition of carbamoyl phosphate alone. Even at subsaturating concentrations of aspartate the enzyme is already completely shifted in the R state, thus ATP cannot shift the enzyme further toward the R state and CTP can only shift the enzyme slightly toward the T state.

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