Stabilization of the R Allosteric Structure of Escherichia coli Aspartate Transcarbamoylase by Disulfide Bond Formation*

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Here we report the first use of disulfide bond formation to stabilize the R allosteric structure of Escherichia coli aspartate transcarbamoylase. In the R allosteric state, residues in the 240s loop from two catalytic chains of different subunits are close together, whereas in the T allosteric state they are far apart. By substitution of Ala-241 in the 240s loop of the catalytic chain with cysteine, a disulfide bond was formed between two catalytic chains of different subunits. The cross-linked enzyme did not exhibit cooperativity for aspartate. The maximal velocity was increased, and the concentration of aspartate required to obtain one-half the maximal velocity, [Asp]0,5 was reduced substantially. Furthermore, the allosteric effectors ATP and CTP did not alter the activity of the cross-linked enzyme. When the disulfide bonds were reduced by the addition of 1,4-dithio-DL-threitol the resulting enzyme had kinetic parameters very similar to those observed for the wild-type enzyme and regained the ability to be activated by ATP and inhibited by CTP. Small-angle x-ray scattering was used to verify that the cross-linked enzyme was structurally locked in the R state and that this enzyme after reduction with 1,4-dithio-DL-threitol could undergo an allosteric transition similar to that of the wild-type enzyme. The complete abolition of homotropic and heterotropic regulation from stabilizing the 240s loop in its closed position in the R state, which forms the catalytically competent active site, demonstrates that the quaternary structural change and closure of the 240s loop has in the functional mechanism of aspartate transcarbamoylase.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) is one of the best characterized allosteric enzymes, so it serves an important role in the study of allosteric regulation. This enzyme catalyzes the committed step of pyrimidine biosynthesis, the car bamoylation of the amino group of L-aspartate by carbamoyl phosphate to form N-carbamoyl-L-aspartate (1). The enzyme demonstrates homotropic cooperativity for the substrate L-aspartate and is heterotropically regulated by effectors ATP, CTP (1), and UTP in the presence of CTP (2). Aspartate transcarbamoylase from E. coli is a dodecamer composed of six catalytic chains organized as two trimeric subunits and six regulatory chains organized as three dimeric subunits. Because aspartate transcarbamoylase is so well characterized and serves as a model for allosteric enzymes it is of particular interest to thoroughly understand the molecular mechanism of allostery of this enzyme.

Two different structural and functional states of aspartate transcarbamoylase have been characterized (3–6). The low activity, low affinity conformation of the enzyme is called the T state, and the high activity, high affinity conformation of the enzyme is called the R state. The conversion from the T to the R state occurs upon aspartate binding to the holoenzyme in the presence of carbamoyl phosphate, which is the source of the observed homotropic cooperativity. The most common method used to characterize the two functional states of aspartate transcarbamoylase has been kinetic studies. The use of site-directed mutagenesis has allowed the indirect kinetic characterization of the functional states. The substitution of key amino acids involved in hydrogen bonds stabilizing either the T or R state (7–10) has created T or R state destabilized enzymes. However, the only studies undertaken to characterize each quaternary structural state by locking the enzyme in a particular state used a bifunctional reagent that was reactive toward amines, resulting in nonspecific cross-linking (11–13).

Here we introduce a cysteine into the catalytic chain of aspartate transcarbamoylase at a site that in the R allosteric state can form a disulfide bond between two 240s loops in the final closed position and lock the enzyme in this state (see Fig. 1). The 240s loop has previously been shown to require saturation by substrate in order to complete its movement into the final closed position (14). Under oxidizing conditions the enzyme would be locked into the R state allowing us to study a mutant version of the enzyme that was otherwise completely wild type in composition. Under reducing conditions the enzyme would no longer be locked in the R state and would function much the same as the wild-type enzyme. Hence, this study gives insight into the relative importance that the switch between quaternary structural states and hindrance of movement of the 240s loop has on the homotropic and heterotropic regulation of this enzyme.

MATERIALS AND METHODS

Chemicals—Agarose, ATP, CTP, DTT, 1-L-aspartate, N-carbamoyl-L-aspartate, 2-mercaptoethanol, potassium dihydrogen phosphate, and uracil were obtained from Sigma. PALA was obtained from the NCI, (SSRL) is operated by the Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Biology Resource is supported by the National Institutes of Health, National Center for Research Resources and Environmental Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ This work was supported by Grant GM26237 from the National Institutes of Health, Stanford Synchrotron Radiation Laboratory (SSRL) is operated by the Department of Energy, Office of Basic Energy Sciences. The SSRL Structural Biology Resource is supported by the National Institutes of Health, National Center for Research Resources (P41RR01929), and by the Department of Energy, Office of Biological and Environmental Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: DTT, 1,4-dithio-DL-threitol; C, catalytic chain; R, regulatory chain; PALA, N-(phosphonacetyl)-L-aspartate; C47A/A241C holoenzyme, the double mutant aspartate transcarbamoylase catalytic subunit with Cys-47 replaced by Ala and Ala-241 replaced by Cys in each of the catalytic chains; C47A/A241C catalytic subunit, the cross-linked enzyme had kinetic parameters very similar to those observed for the wild-type enzyme. The maximal velocity was increased, and the concentration of aspartate required to obtain one-half the maximal velocity, [Asp]0.5 was reduced substantially. Furthermore, the allosteric effectors ATP and CTP did not alter the activity of the cross-linked enzyme. When the disulfide bonds were reduced by the addition of 1,4-dithio-DL-threitol the resulting enzyme had kinetic parameters very similar to those observed for the wild-type enzyme and regained the ability to be activated by ATP and inhibited by CTP. Small-angle x-ray scattering was used to verify that the cross-linked enzyme was structurally locked in the R state and that this enzyme after reduction with 1,4-dithio-DL-threitol could undergo an allosteric transition similar to that of the wild-type enzyme. The complete abolition of homotropic and heterotropic regulation from stabilizing the 240s loop in its closed position in the R state, which forms the catalytically competent active site, demonstrates that the quaternary structural change and closure of the 240s loop has in the functional mechanism of aspartate transcarbamoylase.

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National Institutes of Health. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). Sodium dodecyl sulfate and the protein assay dye 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 (1). The Qiagen gel extraction kit was used for isolation of DNA from agarose gels, and the Qiagen (Valencia, CA) miniplasmid prep kit was used for purification of plasmid DNA. Nucleotides were purchased from Operon Technologies (Alameda, CA).

Construction of Mutant Aspartate Transcarbamoylases—The C47A and A241C mutants of E. coli aspartate transcarbamoylase were constructed by introducing specific base changes in the pyrB gene using the Kunkel method (16). The uracil-containing single-stranded DNA required was obtained by infection of E. coli strain C7236, containing the phagemid pEK54 (17) for the C47A mutation and the phagemid pEK152 (18) for the A241C mutation, with the helper phage M13K07 (19). Selection of the mutations was performed by sequencing with single-stranded DNA isolated from phagemid candidates after coinfection with the helper phage M13K07 (19). After the mutations were verified the resulting plasmids with the C47A and A241C mutations, pEK184 and pEK612, respectively, were digested with the restriction enzymes BstEI and EcoRI. The large fragment of the pEK184 plasmid and small fragment of the pEK612 plasmid were removed from an agarose gel, combined, and treated with DNA ligase for 1 h at 16 °C. The resulting plasmid, pEK613, was sequenced directly by double-strand dideoxy sequencing to verify the mutations.

The C47A/A241C2 catalytic subunit was constructed by first digesting pEK184 with the restriction enzymes BstEI and BamHI and di-gesting pEK612 with the restriction enzymes BstEI and BglII. The large fragment of the pEK184 plasmid and small fragment of the pEK612 plasmid were removed from an agarose gel, combined, and treated with DNA ligase for 1 h at 16 °C. The resulting plasmid, pEK614, was sequenced directly by double-strand dideoxy sequencing to verify the mutations.

Overexpression and Purification of the Mutant Enzymes—The mutant holoenzyme and catalytic subunit of aspartate transcarbamoylase were overexpressed and purified to homogeneity as described previously (15). After concentration, the purity of the enzymes was checked by SDS-PAGE (20), non-reducing SDS-PAGE (in which mercaptoethanol is omitted from the buffer), and nondenaturing PAGE (21, 22).

The concentrations of the wild-type holoenzyme and catalytic subunit were determined from absorbance measurements at 280 nm using extinction coefficients of 0.59 and 0.72 cm2 mg–1, respectively (23). The concentrations of the mutants’ enzymes were determined by the Bio-Rad version of the Bradford dye binding assay (24).

Aspartate Transcarbamoylase Assay—The aspartate transcarbamoylase activity was measured at 25 °C by the colorimetric method (25) or by the radioactive method for assays containing DTT at a concentration of 10 mM, as DTT at this concentration interferes with the development of color in the colorimetric method (26). The radiolabeled substrate was prepared by the radioactive method for assays containing DTT at a concentration of 10 mM, as DTT at this concentration interferes with the development of color in the colorimetric method (26). The aspartate saturation curves were performed in duplicate. Assays were performed in 50 mM Tris acetate buffer, pH 8.3, or in 50 mM Tris acetate buffer, pH 8.3, 10 mM DTT (for reducing conditions) and in the presence of saturating carbamoyl phosphate (4.8 mM). Data analysis of the steady-state kinetics was carried out as described previously (27). Fitting of the experimental data to theoretical equations was accomplished by non-linear regression. The data were analyzed using an extension of the Hill equation that included a term for substrate inhibition. If the fit to the Hill equation gave a Hill coefficient of 1 or less, the experimental data were fit to the Michaelis-Menten equation with an additional term for substrate inhibition (28). The nucleotide saturation curves were fit to a hyperbolic binding isotherm by non-linear regression.

Small Angle X-ray Scattering—The small-angle x-ray scattering experiments were performed at beam line 4–2 at the Stanford Synchrotron Radiation Laboratory (3.0 GeV, 50–100 mA). The experimental setup and procedures were performed as described by Sakash et al. (29).

RESULTS AND DISCUSSION

Rationale for Substitution of Ala-241 with Cysteine—Molecular modeling of the T and R states of aspartate transcarbamoylase protein data bank codes 6AT1 and 1D09, respectively) (30, 31) was performed with QUANTA. This modeling revealed that the distance between identical residues in the 240s loop in the C1 and C4 chains is much closer in the R state than in the T state. Therefore, the 240s loop was chosen as the location to insert a cysteine for putative disulfide bond formation in the R state. Of the residues available for mutation, Asp-236 and Glu-239 were eliminated because previously studies (32, 33) showed that these positions were important for the normal (wild-type) function of the enzyme. Based upon the location, the similar sizes of alanine and cysteine, and the lack of any known function, Ala-241 was chosen as the best candidate for substitution with cysteine. Molecular modeling in QUANTA of the R state showed that the distance between sulfur atoms in the two Cys-241 residues of C1 and C4 is less than 3 Å (represented with a disulfide bond in Fig. 1). To ensure specific disulfide bond formation, the one naturally occurring Cys in the catalytic chain, Cys-47, was replaced by Ala in the final construct (C47A/A241C).

Within the E. coli holoenzyme, the catalytic chains of the top catalytic trimer are numbered C1, C2, and C3, whereas the catalytic chains of the bottom catalytic trimer are numbered C4, C5, and C6, with C4 under C1. The regulatory dimers contain chains R1–R6, R2–R5, and R3–R4. A regulatory chain is in direct contact with the same numbered catalytic chain.
Colorimetric and radiometric assays were performed at 25 °C at saturating concentrations of carbamoyl phosphate (4.8 mM) in 50 mM Tris acetate buffer, pH 8.3, or in 50 mM Tris acetate buffer, pH 8.3, 10 mM DTT (for reducing conditions).

**Kinetic parameters of the wild-type and mutant forms of aspartate transcarbamoylase**

These data were determined from the aspartate saturation curves (Fig. 3). Colorimetric and radiometric assays were performed at 25 °C in 50 mM Tris acetate buffer, pH 8.3, or in 50 mM Tris acetate buffer, pH 8.3, 10 mM DTT (for reducing conditions) and saturating levels of carbamoyl phosphate (4.8 mM). The values reported are the average deviation of three determinations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( V_{\text{max}} ) [mmol-h⁻¹mg⁻¹]</th>
<th>([\text{Asp}]_{0.5})</th>
<th>( n_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>17.5 ± 0.5</td>
<td>13.0 ± 0.9</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>C47A</td>
<td>17.0 ± 0.5</td>
<td>13.5 ± 1.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>A241C</td>
<td>23.5 ± 1.1</td>
<td>3.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>C47A/A241C</td>
<td>25.6 ± 0.9</td>
<td>2.3 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>C47A/A241C (reducing conditions)</td>
<td>25.4 ± 2.3</td>
<td>10.8 ± 0.8</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Wild-type C(_C),(^b)</td>
<td>27.0 ± 1.5</td>
<td>7.5 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>C47A/A241C C(_C),(^b)</td>
<td>28.1 ± 1.2</td>
<td>6.6 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

\(^a\) Experimental data exhibited no cooperativity. Data were fit to the Michaelis-Menten equation with an additional term for substrate inhibition.

\(^b\) C\(_C\), catalytic subunit.

Reducing and Non-reducing SDS-PAGE—The C47A/A241C holoenzyme and C47A/A241C catalytic subunit were evaluated by SDS-PAGE under both reducing and non-reducing conditions (Fig. 2). Lane 2 of Fig. 2, A and B, shows two bands corresponding to the catalytic and regulatory chains of wild-type aspartate transcarbamoylase migrating at 34 and 17 kDa, respectively. However, lane 4 (C47A/A241C holoenzyme) of the non-reducing SDS-PAGE (Fig. 2A) has a band at 17 kDa, a faint band at 34 kDa, and a band migrating at ~65–70 kDa. In contrast, in lane 4 (C47A/A241C holoenzyme) of the reducing SDS-PAGE (Fig. 2B) there are two bands at 34 and 17 kDa and no band visible in the 65–70 kDa range. Lane 3 (C47A/A241C catalytic trimer) in Fig. 2, A and B, shows just a single band corresponding to the catalytic chain of aspartate transcarbamoylase migrating at 34 kDa. Based upon the difference in the migration of the catalytic chain for the C47A/A241C holoenzyme under non-reducing and reducing conditions, we believed that a disulfide bond was forming between two catalytic chains. The identical migration of the catalytic chain for the C47A/A241C catalytic subunit under non-reducing and reducing conditions demonstrated that the disulfide bond formation was between catalytic chains on opposing catalytic subunits and not between catalytic chains on the same catalytic subunit.

**Steady-state Kinetics**—The kinetic parameters calculated from the aspartate saturation curves (Fig. 3) are shown in Table I. Most significantly, under non-reducing conditions the C47A/A241C holoenzyme displayed a hyperbolic aspartate saturation curve. No activation of this enzyme by PALA at low concentrations of aspartate verified that the C47A/A241C holoenzyme, under non-reducing conditions, was not cooperative toward aspartate (data not shown). Under these non-reducing conditions the C47A/A241C holoenzyme has a maximal ob-
modes (E and G) and presence of saturating concentrations of PALA (Fig. 5). The x-ray scattering experiments were performed in 50 mM Tris acetate buffer, pH 8.3, or in 50 mM Tris acetate buffer, pH 8.3, 10 mM DTT (for reducing conditions).

erved specific activity of 25.6 mmol h⁻¹ mg⁻¹ and a Kₘ of 2.3 mM compared with the wild-type holoenzyme parameters of 17.5 mmol h⁻¹ mg⁻¹, an [Asp]₀.₅ of 13.0 mM, and Hill coefficient of 2.4.

The kinetic parameters of the C47A/A241C catalytic subunit were very similar to those of the wild-type catalytic subunit. The C47A/A241C catalytic subunit has a specific activity of 28.1 mmol h⁻¹ mg⁻¹ and a Kₘ of 6.6 mM compared with the wild-type catalytic subunit parameters of 27.0 mmol h⁻¹ mg⁻¹ and 7.5 mM.

Under reducing conditions the kinetic parameters of the C47A/A241C holoenzyme changed dramatically. Under these reducing conditions the C47A/A241C holoenzyme has a sigmoidal aspartate saturation curve with a Hill coefficient of 1.3, specific activity of 25.4 mmol h⁻¹ mg⁻¹, and an [Asp]₀.₅ of 10.8 mM. Additional evidence that cooperativity was restored is demonstrated by the PALA saturation curves, which show activation by PALA although the activation is only approximately one-half as much as that observed for the wild-type enzyme (data not shown).

**Influence of the Allosteric Effectors**—Nucleotide saturation curves with CTP and ATP were determined for the wild-type and C47A/A241C holoenzymes at one-half the [Asp]₀.₅. The kinetic parameters calculated from the nucleotide saturation curves (Fig. 4) are shown in Table II. This concentration of aspartate was selected because the nucleotides exert a larger influence on the activity of the enzyme as the aspartate concentration is reduced (34). Under non-reducing conditions neither nucleotide caused any significant alteration in activity of the C47A/A241C holoenzyme.

Nucleotide saturation curves of the C47A/A241C holoenzyme were also performed under reducing conditions by the addition of 10 mM DTT. Under reducing conditions for the C47A/A241C holoenzyme the normal effect of the nucleotides on the activity of the enzyme was restored. The C47A/A241C holoenzyme under reducing conditions is activated by ATP approximately one-half as much as the wild-type holoenzyme and inhibited by CTP approximately one-half as much as the wild-type holoenzyme.

**Small Angle X-ray Scattering**—Small-angle x-ray scattering was used to evaluate the quaternary structures of the wild-type and C47A/A241C holoenzymes in the absence and presence of saturating concentrations of PALA (Fig. 5). The data for the wild-type holoenzyme shown in Fig. 5A display the characteristic change in the scattering pattern upon addition of PALA as noted by the change in the peak position and increase in relative intensity (35). The scattering pattern in Fig. 5B of the unliganded C47A/A241C holoenzyme displays a peak position and relative intensity that is nearly identical to the PALA-ligated wild-type holoenzyme scattering pattern. Addition of PALA to the C47A/A241C holoenzyme, also shown in Fig. 5B, caused little change to the scattering pattern. Addition of 10 mM DTT to the unliganded C47A/A241C holoenzyme, shown in Fig. 5C, caused a large change in the scattering pattern so that the peak position and relative intensity was very similar to the unliganded wild-type holoenzyme. Addition of PALA to the C47A/A241C holoenzyme containing 10 mM DTT caused a nearly identical change to the scattering pattern that was observed in the addition of PALA to the wild-type holoenzyme.

**CONCLUSIONS**

We believe that the 65–70-kDa band observed only on the non-reducing SDS-PAGE was a C₂ catalytic chain dimer formed from two catalytic chains of aspartate trans carbamoylase linked together by a disulfide bond between Cys-241 of C1 and Cys-241 of C4. The disulfide bond between Cys-241 and Cys-241 links opposing catalytic chains at the C1:C4 interface and locks the enzyme in an R structural state. This is supported by our modeling of the T and R states of the C47A/A241C holoenzyme, where the distance between sulfur atoms in the two Cys-241 residues of C1 and C4 is less than 3 Å in the R state and more than 20 Å in the T state (30, 36). In addition the small-angle x-ray scattering pattern under non-reducing conditions and with no ligands present was indicative of an R structural state.

The aspartate saturation curves of the C47A/A241C catalytic subunit and holoenzyme (non-reducing conditions) are both hyperbolic and show high activity. However, the [Asp]₀.₅ of the holoenzyme is significantly lower than the catalytic subunit (2.3 versus 6.6 mM Asp, respectively). Because cooperativity for aspartate returns for the C47A/A241C holoenzyme under reducing conditions, cooperative binding of aspartate is a function of the switch from the low affinity T to high affinity R state. The maximal activity of the C47A/A241C holoenzyme was greater under non-reducing versus reducing conditions (25.6 versus 21 mmol h⁻¹ mg⁻¹ respectively), suggesting that the switch from the T to R state is somewhat rate-limiting in the holoenzyme. This is supported by work done on the wild-type enzyme and D236A, another enzyme with enhanced maximal activity, which suggested that domain closure or “compression,” the final step in the T to R transition, is rate-limiting (37, 38). There was a significant difference in the nucleotide saturation curves under reducing and non-reducing conditions corresponding to a wild-type-like and R state enzyme, respectively. ATP caused activation and CTP caused inhibition under reducing conditions, whereas ATP and CTP had no effect on the non-reduced R state enzyme. This suggests that the heterotropic mechanism may be a function of the perturbation of the T/R state equilibrium and/or the tertiary structural position of the 240s loop, which is fixed in the closed position in this disulfide-stabilized enzyme.

In this work we have been able to use disulfide bond formation as a means to lock aspartate trans carbamoylase in the R structural state. This modified enzyme locked in the R structural state has allowed us to directly correlate the structural states observed by x-ray scattering and crystallography to the functional states defined by the allosteric model of Monod, Wyman, and Changeux (39). Crystallization and structure determination for the C47A/A241C holoenzyme is currently in progress to explore the high activity, high affinity conformation of aspartate trans carbamoylase in the absence of substrates.

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