240s Loop Interactions Stabilize the T State of Escherichia coli Aspartate Transcarbamoylase*

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Here the functional and structural importance of interactions involving the 240s loop of the catalytic chain for the stabilization of the T state of aspartate transcarbamoylase were tested by replacement of Lys-244 with Asn and Ala. For the K244A and K244N mutant enzymes, the aspartate concentration required to achieve half-maximal specific activity was reduced to 8.4 and 4.0 mM, respectively, as compared with 12.4 mM for the wild-type enzyme. Both mutant enzymes exhibited dramatic reductions in homotropic cooperativity and the ability of the heterotropic effectors to modulate activity. Small angle x-ray scattering studies showed that the unligated structure of the mutant enzymes, and the structure of the mutant enzymes ligated with N-phosphonacetyl-L-aspartate, were similar to that observed for the unligated and N-phosphonacetyl-L-aspartate-ligated wild-type enzyme. A saturating concentration of carbamoyl phosphate alone has little influence on the small angle x-ray scattering of the wild-type enzyme. However, carbamoyl phosphate was able to shift the structure of the two mutant enzymes dramatically toward R, establishing that the mutations had destabilized the T state of the enzyme. The x-ray crystal structure of K244N enzyme showed that numerous local T state stabilizing interactions involving 240s loop residues were lost. Furthermore, the structure established that the mutation induced additional alterations at the subunit interfaces, the active site, the relative position of the domains of the catalytic chains, and the allosteric domain of the regulatory chains. Most of these changes reflect motions toward the R state structure. However, the K244N mutation alone only changes local conformations of the enzyme to an R-like structure, without triggering the quaternary structural transition. These results suggest that loss of cooperativity and reduction in heterotropic effects is due to the dramatic destabilization of the T state of the enzyme by this mutation in the 240s loop of the catalytic chain.

Regulation of metabolic pathways via enzymes whose activity is dependent upon the concentration of various metabolites is of paramount importance for all living systems. As a response to a change in the concentration of certain metabolites, a regulatory enzyme can alter its catalytic activity thereby regulating the flux of metabolic intermediates. In particular, a change in function (such as the affinity for substrate) necessarily mandates a change in structure (1). One excellent example of a regulatory enzyme in which there are dramatic functional and structural changes dependent upon the concentrations of substrates and allosteric effectors is Escherichia coli aspartate transcarbamoylase. Aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the first reaction in pyrimidine biosynthesis, the reaction between carbamoyl phosphate and L-aspartate to form N-carbamoyl-L-aspartate and inorganic phosphate. The enzyme shows homotropic cooperativity for the substrate L-aspartate (2). Its activity is inhibited heterotropically by CTP (3), by UTP in the presence of CTP (4), and activated heterotropically by ATP (5–7), the product of the parallel purine biosynthetic pathway.

The holoenzyme consists of two trimeric catalytic subunits (Mr, 34,000/chain) and three dimeric regulatory subunits (Mr, 17,000/chain). Each of the catalytic subunits contains three active sites shared across the interface between two adjacent catalytic chains (8, 9). The catalytic subunit catalyzes the reaction, whereas the regulatory subunit binds the allosteric effectors and is catalytically inactive (10).

When substrates or suitable substrate analogues bind, the enzyme undergoes a transition from a low-activity low affinity conformation (T state) to a high activity high affinity conformation (R state). During the allosteric transition, the two catalytic subunits separate by 11 Å along the 3-fold axis (vertical expansion) and rotate ~15° about the 3-fold axis, while the three regulatory subunits rotate ~15° about their respective 2-fold axes. In addition, the two folding domains of each catalytic chain, the CP1 and ASP domains, close about the substrates to form the catalytically competent active site, and several subunit interfaces are restructured (11). An important functional consequence of this structural reorganization is to increase the affinity of the enzyme for aspartate and to significantly enhance catalytic activity. Although most of the structural rearrangements can be described by rigid body rotations and translations of the two domains of the catalytic chain, the loop composed of residues 73–88 in the CP domain (80’s loop)

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The atomic coordinates and structure factors (code ISKU) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: CP domain, carbamoyl phosphate binding domain of the catalytic chain; c, catalytic chain; r, regulatory chain; ASP domain, aspartate binding domain of the catalytic chain; AL domain, allosteric domain of the regulatory chain; ZN domain, zinc domain of the regulatory chain; SANS, small angle X-ray scattering; PEG, polyethylene glycol; r.m.s., root mean square; PALA, N-(phosphonacetyl)-L-aspartate; PDB, Protein Data Bank.
and residues 230–245 in the ASP domain (240s loop) undergo dramatic conformational changes in the T to R transition that cannot be explained as rigid body domain motions. When the 240s loop reorients it moves toward the CP domain helping to create the high activity high affinity active site (12).

The position of the 240s loop in the T and R states are stabilized by a series of salt links and hydrogen bonds. In the T state, these interactions seem to not only position the 240s loop, but to stabilize the entire T-state structure. When some of these interactions have been disrupted by amino acid substitution, the T state is destabilized relative to the R state (12–16).

The exact position of the 240s loop has been difficult to discern because of relatively weak electron density in this portion of the structure (17–19). In a mutant structure more recently determined (20), the quality of the electron density map of the 240s loop was sufficiently high to allow for the conformation of the loop to be more firmly established, including the presence of a short segment of well defined 3_10 helix at the end of the fragment. In addition, this revised conformation of the 240s loop shows a salt link between Lys-244 and Asp-271 that previously had not been observed. Here, we investigate the role of this interaction for the stabilization of the T state of aspartate transcarbamoylase by site-specific mutagenesis, kinetics, small angle x-ray scattering, and x-ray crystallography.

EXPERIMENTAL PROCEDURES

Materials—Agar, 1- aspartate, ampicillin, ATP, CTP, carbamoyl phosphate, N-carbamoyl-L-aspartate, 2-mercaptoethanol, polyethylene glycol 1450, potassium dihydrogen phosphate, Bis-Tris, malonic acid, sodium EDTA, sodium acetate, sodium azide, and uracil were obtained from Sigma. Q-Sepharose Fast Flow resin was obtained from Amersham Biosciences. The QuickChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Primers were supplied from Operon Technology (Alameda, CA). The QIAprep Miniprep kit from Qiagen (Valencia, CA) was used for plasmid preparation. Casamino acids, yeast extract and tryptone were obtained from Difco (Detroit, MI). Sodium dodecyl sulfate and protein assay dye were purchased from Bio-Rad. Enzyme grade ammonium sulfate, Tris, electrophoresis grade acrylamide, and agarose were purchased from ICN Biomedicals (Costa Mesa, CA). Carbamoyl phosphate diethyl salt, obtained from Sigma, was purified before use by precipitation from 50%(v/v) ethanol and was stored desiccated at −20 °C. Antipyrine was obtained from Fisher Chemical Co. Crystal cryo-mounting loops were obtained from Hampton Research (Laguna Niguel, CA).

Construction of Mutant Aspartate Transcarbamoylase—The mutant versions of E. coli aspartate transcarbamoylase were constructed by introducing site-specific base mutations in the pyrB gene using the QuickChange site-directed mutagenesis kit. DNA primers were designed to insert the mutations in both the forward and reverse directions. Site-specific mutagenesis was performed using plasmid pEK152 (21) as the template DNA. Candidates were screened for the mutation by DNA sequence analysis. Once candidates were identified, the entire pyrB gene was sequenced to verify that the only mutation was at the 244 codon. The final plasmids with the K244N and K244A mutations were identified as pEK582 and pEK584, respectively.

Protein Oxidation and Purification—Plasmin cleaved pEK152, pEK582, and pEK584 for the wild-type, K244N, and K244A enzymes, respectively, were transformed into the E. coli strain EK1104 for expression and purification as previously described (22). The enzyme purity was judged both by native PAGE (23, 24); as well as by SDS-PAGE (25) stained with Coomassie Blue.

Protein Determination—The concentration of pure wild-type enzyme was determined by absorbance measurements at 280 nm with an extinction coefficient of 0.59 cm2/mg−1 (26). The protein concentration of the mutant enzymes was determined by the Bio-Rad version of the Bradford Protein Determination—The concentration of pure wild-type enzyme was determined by absorbance measurements at 280 nm with an extinction coefficient of 0.59 cm2/mg−1 (26). The protein concentration of the mutant enzymes was determined by the Bio-Rad version of the Bradford method (26). Saturation kinetics were performed in duplicate, and data points shown are the average values. Assays were performed in 50 mM Tris acetate buffer, pH 8.3 in the presence of a saturating concentration of carbamoyl phosphate (4.8 mM). Analysis of the steady-state kinetics data was carried out as previously described (29). Fitting of the experimental data to theoretical equations was achieved by non-linear regression. When substrate inhibition was significant, data were analyzed using an extension of the Hill equation (30). The nucleotide saturation curves were fit to a hyperbolic binding isotherm by non-linear regression.

Small-Angle X-ray Scattering—The SAXS experiments were performed on Beamline 4-2 at the Stanford Synchrotron Radiation Laboratory. The experimental set up and procedures were performed as described previously (31).

Crystallography and Crystal Mounting—Crystals of the K244N mutant were obtained by the sitting drop method. Prior to crystallization the enzyme was dialyzed into 40 mM KH2PO4, 2.0 mM 2-mercaptoethanol, 0.2 mM EDTA, pH 7.0 for 24 h. Single crystals of the K244N mutant were obtained by mixing a 20 mg/ml filtered solution (0.22 μm) of the K244N enzyme with a solution of 17% (w/v) PEG 1450, 50 mM malonate, 0.2 mM EDTA, 1 mM sodium azide, and 20 mM Bis-Tris buffer, pH 7.0 in a 1:1 ratio (v/v). Rhombohedral-shaped crystals grew in ~1 week. In preparation for data collection, crystals of dimensions ~0.6 × 0.5 × 0.3 mm3 were sequentially transferred to a solution containing 20% PEG 1450, 20 mM Bis-Tris, pH 7.0, 50 mM malonate, 0.2 mM EDTA, 1 mM sodium azide, and 15% glycerol as cryoprotectant. Then the crystals were briefly dipped into a solution of 23% PEG 1450, 20 mM Bis-Tris, pH 7.0, 50 mM malonate, 0.2 mM EDTA, 1 mM sodium azide, and 26% glycerol as cryoprotectant. Crystals mounted in cryo-loops were frozen in liquid nitrogen and kept frozen until exposure to x-rays for data collection.

Data Collection and Structure Refinement—All the crystallographic data used for structure determination in this work were collected on beamline X12C at 1 Å wavelength using the Brandeis-2k detector at 100 K at the National Synchrotron Light Source, Brookhaven National Laboratory. Data were collected up to 2.6 Å resolution, and analyzed using DENZOO and SCALGPACK (32). The initial model used for refinement was the P38A mutant structure (PDB code: 1EZZ (33)). The crystals of the K244N mutant were of the same space group (R3) as the crystals of the P268A enzyme, there was a significant difference in the length of the α and β axes of the unit cell. After initial rigid body refinement and simulated annealing in CNS, it was apparent from an inflated Rfree value that the model bias was cumbersome. Therefore, a consensus model was built from overlaying several T state structures of aspartate transcarbamoylase (PDB codes 1EZZ, 1BNE, and 3AT1) using the program SEQUOIA (34) with all water molecules removed. Automated molecular replacement was performed using AmoRe in CCP4. This model was used for rigid body and simulated annealing refinement with initial bulk solvent correction with CNS (35). Initial model rebuilding was performed using XtaView (36). After several rounds of rebuilding and simulated annealing, the spread between the Rfactor and Rfree increased. Therefore, the TLS protocol utilizing tensor, libration and screw axis was employed in CCP4. All the crystallographic coordinating residue parameters were used to define a global parameterization of the asymmetric unit. This was done by defining the individual domains of the asymmetric unit as rigid bodies. This takes into account the tensor and libration parameters of the TLS refinement: both dynamic internal motions of the domains and static disorder, respectively. In addition, the screw axis parameter defined in the TLS matrices accounts for the screw axis of the dimer unit of the regulatory chains (of the asymmetric unit) not described by the crystallographic symmetry of R3. The non-crystallographic 2-fold axis of the regulatory dimer is also accounted for in the TLS matrices that were used. All of these factors were included in the calculation of the anisotropic displacement parameters and refinement statistics using REFMAC5 in CCP4. The TLS parameters were chosen to define the domains of both the upper and lower catalytic and regulatory chains as rigid bodies. For the catalytic chains, residues 101:250 of the ZN domain and residues 101:153 of the AL domain. For the regulatory chains, residues 1:100 of the AL domain and residues 101:153 of the ZN domain. Non-crystallographic symmetry describing the 2-fold axis of the regulatory dimers was also applied. After several rounds of refinement with REFMAC5 in CCP4, the refinement was continued with CNS. The positional refinement performed with the crystallographic symmetry of R3. The non-crystallographic 2-fold axis of the regulatory dimer was also accounted for in the TLS matrices that were used. All of these factors were included in the calculation of the anisotropic displacement parameters and refinement statistics using REFMAC5 in CCP4. The TLS parameters were chosen to define the domains of both the upper and lower catalytic and regulatory chains as rigid bodies. For the catalytic chains, residues 101:250 of the ZN domain and residues 101:153 of the ASP domain with the C-terminal helix of the CP domain. For the regulatory chains, residues 1:100 of the AL domain and residues 101:153 of the ZN domain. Non-crystallographic symmetry describing the 2-fold axis of the regulatory dimers was also applied. After several rounds of refinement with REFMAC5 in CCP4, the refinement was continued with CNS. The positional refinement performed with the TLS protocol was preserved by defining rigid body motions. For the catalytic chains, residues 101:250 of the ZN domain and residues 151:310 of the ASP domain with the C-terminal helix of the CP domain.
Table I

| Data collection and refinement summary of the K244N structure |
|-------------------------------------------------------------|
| **Data collection**                                        |
| Space group                                               |
| $d_{max}$ (Å)                                             | R3 |
| Total reflections                                        | 230,763 |
| Unique reflections                                      | 34,988 |
| Redundancy                                               | 6.5 |
| Completeness (%) (all/outer shell)                       | 94.7/89.4 |
| Unit cell (Å)                                             |
| $a = b = 125.667$, $c = 198.204$ |
| Angles                                                   |
| $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$ |
| $R_{merge}$ (% (all/outer shell))                       | 5.3/9.6 |
| **Refinement**                                           |
| Resolution range (Å)                                     | 29.0–2.6 |
| Sigma cutoff ($\sigma$)                                  | 0 |
| Reflections                                              | 30,529 |
| Average ($I_{ave}$)                                      | 18.5 |
| Working $R$-factor                                        |
| Beginning                                                | 0.274 |
| End                                                       | 0.201 |
| Free $R$-factor                                           |
| Beginning                                                | 0.332 |
| End                                                       | 0.231 |
| Statistics (r.m.s. deviations)                           |
| Bonds (Å)                                                | 0.010 |
| Angles (degrees)                                         | 1.40 |
| Improper (degrees)                                       | 1.23 |
| Dihedrals (degrees)                                      | 25.6 |

$R_{merge} = \left( \sum_{hkl} \left( I_{obs} - I_{calc} \right)^2 / \sum_{hkl} I_{obs} \right)^{1/2}$.

$^a$ Statistics were calculated in CNS with domains presented as rigid bodies to preserve the TLS assignments.

RESULTS

Steady-state Kinetics—The kinetic parameters calculated from the aspartate saturation curves (shown in Fig. 1) are displayed in Table II. Although the substitutions at position 244 have little influence on the maximal catalytic activity, both mutations cause dramatic reductions in cooperativity and the $[\text{Asp}]_{0.5}$. The K244N enzyme exhibited a hyperbolic saturation curve with a maximal velocity of 18.7 mmol$h^{-1}$mg$^{-1}$, and an $[\text{Asp}]_{0.5}$ of 4.0 mM. In contrast, the wild-type enzyme exhibited a sigmoidal aspartate saturation curve with a Hill coefficient of 2.5, a maximal velocity of 18.9 mmol$h^{-1}$mg$^{-1}$ and an $[\text{Asp}]_{0.5}$ of 12.4 mM. The K244A enzyme displayed substantially reduced cooperativity, with a Hill coefficient of 1.2, a maximal velocity of 18.8 mmol$h^{-1}$mg$^{-1}$ and an $[\text{Asp}]_{0.5}$ of 8.6 mM.

Influence of the Allosteric Effectors—The influence of the heterotropic effectors ATP and CTP were determined for the wild type as well as the K244N and K244A enzymes at one-half the $[\text{Asp}]_{0.5}$. Nucleotide effects were determined at this aspartate concentration because the nucleotides exert a larger influence on the activity of the enzyme as the aspartate concentration is reduced (39). From these nucleotide saturation curves, the maximal extent of activation or inhibition at infinite nucleotide concentration was calculated. The parameters were derived from the curves (shown in Fig. 2) and are displayed in Table III.

As shown in Fig. 2, the K244N and K244A enzymes exhibited similar activation by ATP and inhibition by CTP. However, the nucleotide effects for both mutant enzymes were much reduced as compared with the wild-type enzyme. The K244N and K244A enzymes were activated by ATP approximately one-fourth that of the wild-type enzyme, and were inhibited by CTP approximately one-third that of the wild-type enzyme.

Effects of N-(phosphonoacetyl)-L-aspartate (PALA) on the K244N and K244A Enzymes—At low concentrations of aspartate, where the enzyme is predominately in the T state, low concentrations of PALA activate the wild-type enzyme (40) by shifting the population to the R state. At higher concentrations of PALA inhibition is observed as the remaining active sites are filled. For the K244N and K244A enzymes, there was no activation observed by PALA (data not shown).

Small Angle X-ray Scattering—Small-angle x-ray scattering (SAXS) in solution was used to evaluate the quaternary structures of the wild-type, K244A and K244N enzymes in the absence and presence of saturating concentrations of carbamoyl phosphate and PALA at pH 8.3. For the wild-type enzyme, shown in Fig. 3A, there is a significant change in the SAXS pattern upon addition of PALA, with both a change in the peak position and an increase in relative intensity (41). A saturating concentration of carbamoyl phosphate (5 mM) only shifted the SAXS curve slightly as compared with the unliganded curve for the wild-type enzyme, indicating that CP binding induces only a small quaternary structural change.

The SAXS scattering curves for the unliganded K244A and K244N enzymes, shown in Fig. 3, B and C, respectively, are shifted slightly toward the R state as compared with the T state SAXS pattern of the wild-type enzyme. In the presence of PALA, the SAXS curves of both the K244A and K244N enzymes are nearly identical to the SAXS curve of the wild-type R state. Unlike the wild-type enzyme, the addition of a saturating concentration of carbamoyl phosphate (5 mM) significantly shifted the SAXS curves for both the K244A and K244N enzymes. For the K244A enzyme, the addition of CP shifted the structure nearly all the way to the R state. However, for the K244N enzyme the addition of CP shifted the structure all the way to the R state.

Three-dimensional Structure of the K244N Enzyme—The K244N enzyme in the absence of ligands was crystallized in the R3 space group with unit cell dimensions of $a = b = 125.67$, $c = 198.2$ (Table I). The K244N enzyme had similar unit cell dimensions along the $c$ axis and the same space group as observed for crystals of the P268A aspartate transcarbamoylase that was crystallized under similar conditions (33). However, the length of the $a$ and $b$ axes were ~4 Å shorter than that observed for the crystal of the P268A enzyme. The crystals of the K244N enzyme diffracted to 2.6 Å resolution with completeness of ~95% (Table I), and the structure was refined to an $R_{factor}$ and $R_{free}$ of 20.1 and 23.1%, respectively, with 50 waters.

The vertical separation of the two catalytic trimers of aspartate transcarbamoylase is defined as the distance between the
mined at saturating levels of carbamoyl phosphate (4.8 mM) and aspar
acetate buffer, pH 8.3. ATP and CTP saturation curves were deter-
activity in the presence of ATP, and
A
ATP.

FIG. 2. Influence of ATP (top) and CTP (bottom) on the activity of the wild-type and mutant aspartate transcarbamoylases. Col-
ometric assays were performed at 25 °C in 50 mM Tris acetate buffer (pH 8.3) in the absence or presence of increasing concentrations of either ATP or CTP. The aspartate concentration was held constant at the one-half the \([\text{Asp}]_{\text{0.5}}\) of the respective enzyme, and the carbamoyl phosphate concentration was saturating (4.8 mM). Data are shown for the wild-type (■), the K244N (○), and the K244A (□) enzymes.

TABLE II
Kinetic parameters of the wild-type and mutant aspartate transcarbamoylases

| Enzyme     | \(V_{\text{max}}^a\) mmol h\(^{-1}\) mg\(^{-1}\) | \([\text{Asp}]_{\text{0.5}}\) mM | \(n_H\) |
|------------|---------------------------------------------|--------------------------------|---------|
| Wild type  | 18.9 ± 1.4\(^b\)                           | 12.4 ± 0.6                      | 2.4 ± 0.2 |
| K244N      | 18.7 ± 0.2                                 | 4.0 ± 0.2                      | 1       |
| K244A      | 18.8 ± 0.3                                 | 8.4 ± 0.2                      | 1.2     |

\(^a\) Maximal observed specific activity.
\(^b\) Errors are the average deviation of three determinations.

FIG. 3. Small-angle x-ray scattering curves for the wild-type (A), K244A (B), and K244N (C) enzymes. For each enzyme, curves are shown in the absence of ligands (●), in the presence of a saturating concentration of carbamoyl phosphate (5 mM) (○), and in presence of 10 mM PALA (■). The x-ray scattering experiments were performed in 50 mM Tris acetate buffer, 2 mM 2-mercaptoethanol, pH 8.3

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. ATP and CTP saturation curves were deter-
ated at saturating levels of carbamoyl phosphate (4.8 mM) and aspar-
tate concentrations at one-half the \([\text{Asp}]_{\text{0.5}}\) of the respective enzyme at pH 8.3.

TABLE III
ATP inhibition of the wild-type and mutant aspartate transcarbamoylases

| Enzyme   | ATP\(^a\) | \(K_{\text{ATP}}^b\) | CTP\(^a\) | \(K_{\text{CTP}}^b\) |
|----------|-----------|----------------------|-----------|----------------------|
| Wild type| 400       | 0.25                 | 30        | 0.52                 |
| K244N    | 120       | 0.63                 | 75        | 0.035               |
| K244A    | 140       | 0.61                 | 73        | 0.051               |

\(^a\) 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.

\(^b\) \(K\) is the nucleotide concentration required to activate or inhibit the enzyme by 50% of the maximal effect.

\(^c\) 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.

Details of how the upper and lower catalytic chains are positioned is more specifically described when the center of mass is calculated for each domain, omitting mobile elements as previously described (42). For example, the vertical separation of the CP domain between the C1 and C4 chains in the wild-type T structure (PDB code, 3AT1) (43) is 40.02 Å for ASP domain and 57.78 Å for CP domain. In the wild-type R structure (PDB code: 1DO9) (42) these values increase to 49.97 Å for the ASP domain and 68.97 Å for the CP domain.

The vertical separation observed in the K244N structure between the C1 and C4 CP domains is 57.15 Å, whereas the vertical separation between C1 and C4 ASP domains is 42.39 Å. This corresponds to a 2.0 Å shift in the ASP domain toward the R state.

**Comparison of the K244N and Wild-type Structures**—The K244N and wild-type T state structures (44) are quite similar, with an r.m.s. deviation of only 0.62 Å for α-carbons and 3.1 Å when all mobile elements were included in the comparison. In contrast, when this analysis was performed by domain subtle differences emerge. For example, the α-carbon r.m.s. deviation

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-R4, and R3-R5. A regulatory chain is in direct contact with the same numbered catalytic chain. In the crystal the asymmetric unit is composed of one catalytic chain from the top trimer, the regulatory dimer connected to it, and one catalytic chain from the bottom catalytic trimer (e.g. C1-R1-R6-C6). These four polypeptide chains are labeled A, B, D, C, respectively, in the coordinate data file.
between the CP and ASP domains of the wild-type T structure (43) and the same domains of K244N structure were 0.44 and 0.41 Å, respectively. When the same analysis was performed using the wild-type R (42) and K244N structures, the α-carbon r.m.s. deviations were 0.49 Å for the CP domain and 0.36 Å for the ASP domain.

In the K244N structure, despite disruption of local polar interactions between adjacent and 270° loops, the electron density in this region was of good quality as shown by Fig. 4A. The electron density for the AL domains of the regulatory chains was of lower quality than the electron density for the ZN domains of the regulatory chains and for both domains of the catalytic chains. The 50° loop region of the AL domain had particularly poor electron density. Although TLS parameterization in the refinement allowed an intermediate description of the AL domain, the inherent increased mobility of this region of the structure resulted in higher B factors in the AL domain as compared to the rest of the molecule. For example, average B factors for the α-carbons and side chains were 37.8 Å²/38.5 Å² (reported as C1/C6) for the CP domain, 39.5 Å²/41.5 Å² for the ASP domain, 45.8 Å²/50.2 Å² for the ZN domain, and 71.5 Å²/75.2 Å² for the AL domain. The average B factors for the AL domain were nearly 1.5-fold higher than the neighboring ZN domain. Increased mobility of this domain is mainly in the first 55 residues, which has an average B factor (α-carbons and side chain) 1.8-fold higher than the second half of the AL domain (residue 55–100). In addition to disrupting the allosteric domain of the regulatory chains, the K244N mutation also caused global shifts in the backbone from the wild-type T state at the C1-R4 interface due to disruption of local polar contacts of residues in the 240s loop (see Fig. 4B). Despite the shift from wild-type T conformation, the quality of electron density of large portions of the regulatory/catalytic chain interface is reasonably good at 2σ. In the structure of the K244N enzyme the contact between the Lys-143R4 3 of the ZN domain and Asp-236C1 of ASP domain are lost, again suggesting that the K244N enzyme has a structure shifted somewhat toward the R state. Fig. 4C shows a close-up of K244N C1-R4 interface. Loss of this critical T state stabilizing interaction between Asp-236C1 and Lys-143R4 was not due to higher mobility than observed in the wild-type structure in this region, as demonstrated by the 2Fo–Fc electron density map contoured at 1.8σ (Fig. 4C).

3 To distinguish amino acids in the catalytic and regulatory chains of aspartate transcarbamoylase, a c (catalytic) or r (regulatory) is appended to the residue number (e.g. Asp236c and Lys143r). To clarify a particular catalytic or regulatory chain, the chain number is also appended to the residue number (e.g. Asp236c4 and Lys143r1).
DISCUSSION

A comparison of the x-ray structures of aspartate transcarbamoylase in the T and R states demonstrates that a loop of the catalytic chain, the 240s loop, undergoes a significant conformational change between the T and R structures (17, 46, 47). The 240s loop is stabilized by one set of interactions in the T state structure, which is replaced by another set of interactions that stabilize the position of the 240s loop in the R state structure. The R state conformation of the 240s loop positions and stabilizes residues involved in substrate binding. Site-specific mutagenesis experiments have confirmed that many of these 240s loop interactions are critical for not only the stability of the T and R states, but also for substrate binding and catalysis (13, 15, 31, 48). Based upon a structure of the mutant aspartate transcarbamoylase (T82Ar) that had better electron density for the 240s loop (20) than the wild-type T state structure (17), a previously unobserved interaction between Lys-244 and Asp-271 was identified. This interaction appears to stabilize the T state conformation of the 240s loop. In this study, a multifaceted approach, employing site-specific mutagenesis, small angle x-ray scattering, and x-ray crystallography was used to better understand the role of the interaction between Lys-244 and Asp-271, in particular, and the role of the 240s loop, in general, for the function of aspartate transcarbamoylase.

Functional Behavior of the K244A and K244N Enzymes—

The K244A and K244N enzymes exhibit no reduction in maximal activity as compared with the wild-type enzyme (see Table II). However, the [Asp]0.5 for both the K244A and K244N enzymes were significantly lower than that observed for the wild-type enzyme. In addition, cooperativity for the mutant enzymes was reduced dramatically. No cooperativity toward aspartate was observed for the K244N enzyme, and the lack of cooperativity was verified by the inability of PALA to activate the enzyme at low concentrations of aspartate. The 240s loop seems to be important for not only homotropic, but also heterotropic interactions as well. Both the K244A and K244N enzymes show much reduced heterotropic effects, suggesting that the 240s loop directly or indirectly is involved in the heterotropic regulation of aspartate transcarbamoylase. The importance of the 240s loop for the homotropic regulation of the enzyme has recently been verified by variations in the fluorescence of a pyrene label site-specifically attached to the 240s loop induced by the binding of ATP or CTP (49).

One of the interactions that stabilizes the T state is the hydrogen bond between the hydroxyl group of Tyr-240 and the carboxylate of Asp-271 (13, 15, 50). In the R state structure of the wild-type enzyme in the presence of PALA (42) this interaction is broken. The importance of this interaction for the stabilization of the T state was verified by kinetics experiments on the Y240F (13) and the D271N enzymes (14). These two mutant enzymes exhibit almost identical kinetic properties, with normal maximal activity, reduced cooperativity, and lower [Asp]0.5, which are comparable with the kinetic parameters for the K244A and K244N enzymes.

Structural Alterations Due to Mutations at 244 in Solution—

The SAXS experiments indicate that in the absence of ligands
the structure of K244A and K244N enzymes were similar but not identical to the unliganded wild-type enzyme. The SAXS patterns of the wild-type and the K244A and K244N enzymes in the presence of PALA, the bisubstrate analogue that converts the wild-type enzyme to the R state, were almost identical, indicating that, at this resolution, the R state structures of the three enzymes in solution were virtually identical. Howver, the SAXS patterns of the wild-type and the two mutant enzymes were different in the presence of carbamoyl phosphate. As has been previously reported, carbamoyl phosphate alone has little influence on the SAXS pattern of the wild-type enzyme (51). For the K244A enzyme, the SAXS pattern is dramatically shifted toward the R structure, while for the K244N enzyme, the SAXS pattern in the presence of carbamoyl phosphate is identical to that observed for this mutant in the presence of PALA. In the case of the K244A enzyme, linear combinations of the wild-type T and R state curves, as well as the wild-type T and K244N R state curves, were not able to provide a suitable fit to the SAXS curve of the K244A enzyme in the presence of carbamoyl phosphate. This analysis suggests that the K244A enzyme, in the presence of carbamoyl phosphate, exists in a unique structure different from either the wild-type T or R structures. Furthermore, the fact that carbamoyl phosphate alone causes a dramatic shift in the SAXS pattern toward the R state indicates a dramatic destabilization of the T state structure by the mutation, and emphasizes the importance of 240s loop interactions for the stabilization of the T state of aspartate transcarbamoylase.

**Structural Alterations Because of K244N Mutation Based upon the Crystal Structure**—The determination of the three-dimensional structure of the K244N enzyme has allowed us to obtain molecular level details of the structural changes induced by this mutation, and has provided us the opportunity to correlate these structural changes to the observed functional and structural changes observed in solution. When Lys-244 was replaced by Asn, structural alternations were observed not only at the site of the amino acid replacement, but also at remote sites. Subtle alterations occurred at the active site, the subunit interfaces as well as in the AL domain where the nucleotide effectors bind. Correlations can be made between these subtle structural alterations and the observed functional changes.

**Local Structural Changes at the Site of the Mutation**—The direct consequence of the replacement of Lys-244 by Asn is the loss of the T state interaction observed in the wild-type enzyme between Asp-271 and Lys-244 as well as the interaction between Asp-271 and Tyr-240. In the wild-type T state structure (17, 19), the hydroxyl group of Tyr-240 forms a hydrogen bond to the carboxylate of Asp-271, stabilizing the T state. In the PALA-ligated R state structure (42, 47), the reorientation of the 240s loop prevents the hydroxyl of Tyr-240 for interaction with any other residues, rather the rings of Tyr-240 in the C1 and C4 chains stack against each other. The replacement of Lys-244 by Asn in the K244N enzyme has two local effects on polar interactions that contribute to destabilization of the T state. Mutation of Lys-244 to Asn allows Asn-244 to interact with Tyr-240 (3.75 Å between their side chains), which in turn disrupts the interaction between Tyr-240 and Asp-271 (13, 14, 48) by shifting the position of Tyr-240 and Asp-271. The mutation also disrupts the T state stabilizing interaction between Lys-244 and Asp-271 that was observed in 1NBE structure, and the Lys-232-Asp-271 interaction that was observed in the 3AT1 structure (Fig. 6 A and B). This local shift induced by the K244N mutant is more than a torsional motion of side chains and is reflected in a backbone shift of the 240s loop region of the ASP domain (Fig. 4B). A comparison of the 240s loop region of the K244N structure with several other T state structures reveals a loss of interactions that occurs in a stepwise progression as the enzyme moves out of the T state. The over-rotated T82Ar T state structure (PDB code: 1NBE) is stabilized by both interactions between Tyr-240-Asp271 and Lys-244—Asp-271, while the substrate-ligated or substrate analogue-ligated T state structures are stabilized by only an interaction between Lys-232 and Asp-271. However, the K244N structure exhibits a 2 Å shift of the ASP domain, away from the T state, and has neither the Asp-271—Lys-232 nor Asp-271—Lys-244 interactions. The loss of these interactions may be a requirement for the initial movement of the enzyme out of T before the global rearrangement of the 240s loop.

**Local Changes at the Site of the Mutation Are Propagated to the Subunit Interfaces and the Active Sites**—The alterations to the structure of the K244N enzyme are not restricted to just the

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**Fig. 6. Comparison of stabilizing interactions between the 240s and 270s loops.** A, shown is the K244N structure (thick) overlaid with T82Ar (thin) T state structure (PDB code 1NBE). The Lys-244—Asp-271 interaction is shown (2.9 Å) as well as novel Asn-244—Tyr-240 interaction observed only in the K244N structure. Also shown are the different orientations of Lys-232 in the two structures. B, shown is the overlay of the K244N structure (thick) and the wild-type PAM-ligated T state structure (thin) (PDB code 3AT1). In the wild-type structure Asp-271 interacts with both Lys-232 and Tyr-240, whereas in the K244N structure Asp-271 exhibits no interactions.
immediate area of the mutation. A comparison of the structures of the wild-type enzyme in the T and R state (11) reveals that during the global conformational change of the enzyme local conformational changes occur within the 80s and 240s loops. These specific loop reorientations are responsible for the creation of the high activity high affinity active site. Furthermore, specific interface interactions between the C1 and R4 chains that are necessary to stabilize the enzyme in the T state are broken during the allosteric transition. In fact, no C1-R4 interactions exist in the R state. A particularly critical salt link is between Asp-256c1 and Lys-143r4. When this interaction is broken by mutation of either Lys-143r5 (52) or Asp-256c1 (53, 54) the enzyme no longer exists in the T state. This interaction is lost in the K244N mutant (Fig. 4C). Another consequence of the K244N mutation is the weakening of the C1-R4 interface because polar contacts of Tyr-240 and Asp-271 disrupted by the mutation are adjacent to Asn-242, which is shifted toward Phe-141r in the K244N structure (Fig. 4, B and C).

The SAXS experiments with the K244N enzyme reveal only subtle differences in the structure of the unliganded K244A and K244N enzymes as compared with the structure of the unliganded wild-type enzyme. A detailed analysis of the three-dimensional structure of the K244N enzyme revealed that this enzyme is in a quaternary structure very similar to other T state structures of the wild-type enzyme (19, 45) with a vertical expansion of 10.5 Å between C1 and C4 catalytic chains relative to the wild-type R structure (PDB code: 1D09). However, the ASP domains have shifted 2 Å out of the T state conformation, decreasing the difference between upper and lower domains relative to wild-type R state structures, while the CP domains shift by only 0.6 Å. The shift of the ASP domain is significant because it structurally describes the mechanical uncoupling of the ASP and CP domains suggesting a destabilization of the T state relative to the R state. The destabilization of the T state by the K244N mutation is confirmed by the SAXS experiments in the presence of carbamoyl phosphate. The loss of the salt link at the site of the amino acid substitution in the K244N enzyme along with alterations at the C1-R4 interface are sufficient to allow the binding of carbamoyl phosphate to convert the enzyme to the R structure. This has profound effects on the function of the enzyme. The lost of cooperativity is due to the inability of aspartate to alter the global conformation of the enzyme. The enzyme is already in the R state during the determination of the aspartate saturation curve, since a saturating concentration of carbamoyl phosphate is used. The dramatic reduction in heterotropic effects can be explained similarly. The stabilization of the enzyme in the R state by carbamoyl phosphate means that nucleotides cannot as easily alter the quaternary structure of the enzyme.

The analysis of the structure of the K244N enzyme also reveals that the amino acid substitution causes subtle but significant alterations in residues at the active site. When the active site of the K244N enzyme is compared with the wild-type active sites in the T and R states, the active site residues in the K244N structure are displaced toward the R state active site structure. For example, residues such as Glu-50 and Arg-167 in the K244N structure are nearly in the positions that they exhibit in R state structures. This alteration in active site conformation of the K244N enzyme may explain the lower [Asp]0.5 of this mutant enzyme.

Structural Alterations in the Regulatory Chains Caused by K244N Mutation.—The structure of the K244N enzyme exhibited unexpectedly high B factors for the first 55 residues of the regulatory chain. Other structures of aspartate transcarbamoylase in the R3 space group have also shown elevated B factors in the AL domain (33), and enzyme crystallized at pH 7 seems to have higher B factors than those crystallized at pH 5.9. Molecular dynamics simulations have previously shown that the high B factors in the AL domain are possibly due to the mechanical uncoupling of this domain from the rest of the enzyme (55). In the case with the K244N structure, the destabilization of the T state induced by the mutation seems to increase the uncoupling of the AL domains due to altered contacts at the C1-R4 interface (Fig. 4).
T State Stabilization of Aspartate Transcarbamoylase

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240s Loop Interactions Stabilize the T State of *Escherichia coli* Aspartate Transcarbamoylase

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