The Conserved Residues Glutamate-37, Aspartate-100, and Arginine-269 Are Important for the Structural Stabilization of *Escherichia coli* Aspartate Transcarbamoylase†

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ABSTRACT: Aspartate transcarbamoylase from *Escherichia coli* is a dodecameric enzyme consisting of two trimeric catalytic subunits and three dimeric regulatory subunits. The X-ray structure of this enzyme indicates that the side chains of His-41, Asp-100, and Asp-90 from one catalytic chain form interactions with the side chains of Glu-37, Arg-65, and Arg-269, respectively, from an adjacent catalytic chain. In order to determine whether these interactions are important for the structural stabilization of the enzyme and/or homotropic and heterotropic effects, four mutant versions of aspartate transcarbamoylase, Glu-37 → Ala, Asp-100 → Asn, Asp-100 → Ala, and Arg-269 → Ala, were created by site-specific mutagenesis. The Glu-37 → Ala holoenzyme exhibits essentially wild-type behavior with respect to homotropic cooperativity and heterotropic regulation by ATP and CTP. The Glu-37 → Ala catalytic subunit exhibits a half-life of inactivation at 69 ± 0.5 °C of 4.9 min, as compared to 5.8 min for the wild-type catalytic subunit. The Asp-100 → Asn and Asp-100 → Ala holoenzymes are slightly more active than the wild-type holoenzyme, exhibit 1.4-fold and 1.8-fold reductions in the aspartate concentration at half the maximal specific activity, respectively, and show increased affinities for ATP and CTP. Both the Asp-100 → Asn and Asp-100 → Ala catalytic subunits exhibit a 2-fold reduction in the half-life of inactivation at 69 ± 0.5 °C. The Arg-269 → Ala holoenzyme exhibits a 2-fold reduction in the maximal specific activity, normal homotropic cooperativity, but 12-fold and 44-fold increases in the aspartate and carbamoyl phosphate concentrations at half the maximal specific activity, respectively. Furthermore, the Arg-269 → Ala enzyme shows a 1.4-fold and 17-fold reduction in the affinity for ATP and CTP, respectively, as well as a 40-fold reduction in the maximal activation by ATP and a 3-fold reduction in the maximal inhibition by CTP. Replacement of Arg-269 by Ala also results in a dramatic loss of stability. At 50.25 ± 0.25 °C, a temperature at which the wild-type holoenzyme is stable, the Arg-269 → Ala holoenzyme is inactivated completely with a half-life of 2.0 min.

Escherichia coli aspartate transcarbamoylase (ATCase; EC 2.1.3.2) catalyzes the committed step in the biosynthesis of pyrimidine nucleotides: the reaction between carbamoyl phosphate and L-aspartate to form *N*-carbamoyl-L-aspartate and inorganic phosphate (Jones et al., 1955; Reichard & Hanshoff, 1956). The enzyme has been the focus of intensive research for almost 30 years, and it has served as a model system for the study of cooperativity and allosteric regulation of enzymatic activity [see reviews by Jacobson and Stark (1973), Schachman (1974), and Kantrowitz and Lipscomb (1988, 1990) and references cited therein]. The enzyme exhibits homotropic cooperativity for both its substrates (Bethell et al., 1968; Gerhart & Pardee, 1962), is heterotropically activated by ATP, the end product of the purine biosynthetic pathway (Gerhart & Pardee, 1962), and is heterotropically inhibited by CTP (Gerhart & Pardee, 1962) and UTP (in the presence of CTP) (Wild et al., 1989), the end products of the pyrimidine biosynthetic pathway. It is the ability of the enzyme to be regulated in such a manner that presumably allows it to sense the level of nucleotides in the cell and therefore maintain a balanced flux of intermediates through the pathway.

The dodecameric holoenzyme (M_r 310 000) is composed of six catalytic chains (M_r 33 000), which are associated as two trimers (catalytic subunits), and six regulatory chains (M_r 17 000), which are associated as three dimers. The active sites are located between adjacent chains within each catalytic subunit (Monaco et al., 1978; Robey & Schachman, 1985; Krause et al., 1987; Wente & Schachman, 1987), whereas the nucleotide-binding sites are located on the regulatory subunits some 60 Å from the active site. When treated with heat (Gerhart & Pardee, 1962, 1964) or mercurials (Gerhart & Schachman, 1965), the holoenzyme dissociates into its constitutive subunits. The isolated catalytic subunit exhibits a higher specific activity than the holoenzyme, shows Michaelis–Menten kinetics, and does not show heterotropic regulation. By contrast, the isolated regulatory subunits are inactive, but still bind the regulatory nucleotides (Changeux et al., 1968).

In the absence of substrates, the holoenzyme exists in the low-affinity, low-activity T state, but is converted into the high-affinity, high-activity R state upon the binding of carbamoyl phosphate and aspartate. The three-dimensional crystal structure of the holoenzyme in the T state has been determined in the absence of ligands (Honzatko et al., 1982; Ke et al., 1984; Stevens et al., 1990), in the presence of CTP (Honzatko et al., 1982; Stevens et al., 1990; Kim et al., 1987; Kosman et al., 1993), and in the presence of the carbamoyl phosphate analog phosphonoacetamide (Gouaux & Lipscomb, 1990). In addition, the structure of the R-state enzyme has

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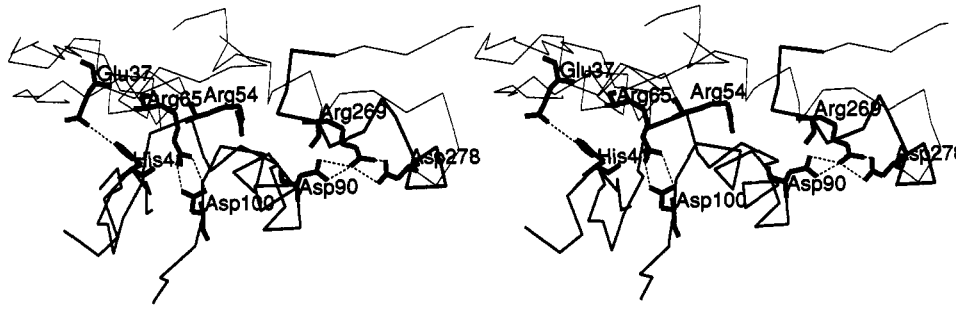

FIGURE 1: Stereoview of the C1–C2 interface of *E. coli* aspartate transcarbamoylase. The α-carbon trace of the upper C2 chain is shown as a thin line, whereas the α-carbon trace of the lower C1 chain is shown as a thicker line. The side chains of His-41, Asp-90, and Asp-100 from C1 and the side chains of Glu-37, Arg-65, Arg-269, and Asp-278 from C2 are shown in boldface. The hydrogen-bonding interactions are shown as dashed lines. The locations of the active-site residue, Arg-54, as well as the loop region (His-265–Asp-278) of C2 which contains the active-site residues Pro-266 and Leu-267 are shown in boldface.

been determined in the presence of phosphonoacetamide (PAM) + malonate (Gouaux & Lipscomb, 1990), in the presence of carbamoyl phosphate + succinate (Gouaux & Lipscomb, 1988), and in the presence of the bisubstrate analog *N*-(phosphonoacetyl)-L-aspartate (PALA)[1] (Krause et al., 1987; Ke et al., 1988). These X-ray structures have been used not only to identify those amino acid residues involved in substrate and nucleotide binding but also to analyze the conformational changes that occur upon the T to R transition. Changes in the quaternary structure that accompany the allosteric transition include an elongation of the molecule along its 3-fold axis of 12 Å, the relative reorientation of the catalytic subunits about their 3-fold axis by 10°, and the rotation of the regulatory dimers about their 2-fold axis by 15°(Ke et al., 1988; Gouaux & Lipscomb, 1990). Changes in the tertiary structure of the catalytic and regulatory subunits also occur upon the transition from the T to the R state. For the catalytic subunit, this includes closure of the aspartate and carbamoyl phosphate domains to form the active site, and substantial movement of the 80's and 240's loops (Ke et al., 1988; Gouaux & Lipscomb, 1990).

The C1–C2[2] interface, which corresponds to the interface between adjacent catalytic chains within a single trimer, is a complex network of polar and nonpolar interactions that forms part of the shared active site, and also serves to tether the chains of the catalytic subunit together. Comparison of the T and R state structures indicates that, with the exception of localized changes in the vicinity of residues 50–55 and 73–85, the C1–C2 interface does not undergo a significant conformational change during the T to R transition since the trimers move as a rigid unit (Krause et al., 1987; Ke et al., 1988). In the X-ray structure of the CTP-ligated, T-state holoenzyme, the side chains of His-41, Asp-100, and Asp-90 from C1 form interactions with the side chains of Glu-37, Arg-65, and Arg-269 from C2, respectively (Stevens et al., 1990). In addition, the side chain of Arg-269 forms an intrachain interaction with the side chain of Asp-278 also from C2 (Figure 1). In the X-ray structure of the (PAM + malonate)-ligated, R-state enzyme, the interactions between Asp-100 and Arg-65 and between Asp-90 and Arg-269 are maintained, although only half of the six possible interactions between Glu-37 and His-41 are observed (Gouaux & Lipscomb, 1990; Stevens et al., 1991a). In the PALA-ligated R-state structure, however, all the interactions between Glu-37 and His-41 are broken, although the two aspartate–arginine interactions are likewise maintained (Ke et al., 1988). It is not known whether this apparent discrepancy between the two R-state structures is significant, or whether the interaction between Glu-37 and His-41 is important for the T to R transition.

Interestingly, Glu-37 is conserved in the aspartate transcarbamoylases from *Serratia marcescens* (Beck et al., 1989), *Salmonella typhimurium* (Michaels et al., 1987), and hamster (Simmer et al., 1989), whereas Asp-100 and Arg-269 are conserved in these enzymes as well as the enzymes from *Bacillus subtilis* (Lerner & Switzer, 1986) and wheat (R. Yon, personal communication). Furthermore, Asp-100 is also conserved in the ornithine transcarbamoylases from *E. coli* (Bencini et al., 1983; Van Vliet et al., 1984), *Pseudomonas aeruginosa* (Baur et al., 1987; Itoh et al., 1988), *Pseudomonas syringae* (Mosqueda et al., 1990), *Neisseria gonorrhoeae* (Martin et al., 1990), rat (Takiguchi et al., 1984), and human (Horwich et al., 1984), enzymes which catalyze an analogous reaction between carbamoyl phosphate and L-ornithine.

As these residues are conserved, and since the interchain salt links between Asp-100 and Arg-65 and between Asp-90 and Arg-269 are maintained in both the T and R states, it was suspected that these interactions play an important role in the structural stabilization of the catalytic subunit.

In order to probe the function of Glu-37, Asp-100, and Arg-269 in aspartate transcarbamoylase, we have used site-specific mutagenesis to replace them with alternative residues. Here we report the characterization of the Glu-37 → Ala,[3] Asp-100 → Asn, Asp-100 → Ala, and Arg-269 → Ala mutant enzymes.

EXPERIMENTAL PROCEDURES

Materials

Q-Sepharose Fast Flow resin was purchased from Pharmacia. ATP, CTP, L-aspartate, *N*-carbamoyl-L-aspartate, ampicillin, potassium dihydrogen phosphate, and uracil were obtained from Sigma. Carbamoyl phosphate dilithium salt was obtained from Sigma and Fluka, was purified before use by precipitation from 50% (v/v) ethanol, and was stored

[1] Abbreviations: PALA, *N*-(phosphonoacetyl)-L-aspartate; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; $[S]_{0.5}^{CP}$, carbamoyl phosphate concentration at half the maximal observed specific activity; C_6R_6, entire holoenzyme consisting of two catalytic trimers and three regulatory dimers; C_6R_4, species of the holoenzyme lacking one of the regulatory dimers.

[2] The catalytic chains C1, C2, and C3 comprise the upper catalytic subunit while the C4, C5, and C6 chains comprise the lower catalytic subunit. Chain C4 is below C1, while C5 and C6 are below chains C2 and C3, respectively.

[3] The notation used to name the mutant enzymes is, for example, the Glu-37 → Ala enzyme. The wild-type amino acid and its location within the catalytic chain are indicated to the left of the arrow while the new amino acid is indicated to the right of the arrow.

desiccated at −20 °C (Gerhart & Pardee, 1962). Agarose, electrophoresis-grade acrylamide, enzyme-grade ammonium sulfate, and Tris were obtained from ICN Biomedicals. Casamino acids, yeast extract, and tryptone were obtained from Difco. Antipyrine was obtained from Kodak. Diacetyl monoxime was obtained from Fisher Scientific. Restriction endonucleases were obtained from either U.S. Biochemicals or New England Biolabs and used according to the manufacturers' recommendations. The Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, and T4 DNA ligase were obtained from U.S. Biochemicals.

Strains. The *E. coli* K12 strain MV1190 [Δ(*lac-proAB*), *supE*, *thi*, Δ(*sri-recA*) 306::Tn10(tet^r)/F′ *traD36*, *proAB*, *lacI*^{q}, *lacZ*ΔM15] and the M13 phage M13K07 were obtained from J. Messing. The *E. coli* strain CJ236 [*dut-1*, *ung-1*, *thi-1*, *relA-1*/pCJ105 (Cm^r)] was obtained from T. Kunkel.

Methods

Oligonucleotide Synthesis. The oligonucleotides used for site-specific mutagenesis and sequencing were obtained from Operon Technologies or synthesized "in-house" using an Applied Biosystems 381A DNA synthesizer and purified by HPLC employing a DuPont Zorbay Oligo ion-exchange column.

Wild-Type and Mutant Enzyme Overproduction and Purification. The wild-type and mutant aspartate transcarbamoylases were isolated from *E. coli* strain EK1104 [F$^-$ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *pyrF*$^{\pm}$, *rpsL*], containing the plasmids pEK54, pEK198, pEK214, pEK218, and pEK219 which code for the wild-type, Glu-37 → Ala, Asp-100 → Ala, Asp-100 → Asn, and Arg-269 → Ala enzymes, respectively. The holoenzymes were purified to electrophoretic homogeneity essentially as described by Nowlan and Kantrowitz (1985), except that an additional isoelectric precipitation step was employed.

Aspartate Transcarbamoylase Assay. The transcarbamoylase activity was measured at 25 °C by either a colorimetric (Prescott & Jones, 1969; Pastra-Landis et al., 1981) or a pH-stat method (Wu & Hammes, 1973). Colorimetric assays were performed in 50 mM Tris–acetate, pH 8.3, in duplicate, and the data points in the figures are the average values. pH-stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburette.

Data Analysis. The analysis of steady-state kinetic data was carried out as described previously (Silver et al., 1983). Data points were fit by a nonlinear least-squares procedure to the Hill equation incorporating a term for substrate inhibition when necessary (Pastra-Landis et al., 1978). The analysis of the structural data of the wild-type holoenzyme, based on the three-dimensional coordinates of the CTP-ligated complex (Stevens et al., 1990), the (phosphonoacetamide + malonate)-ligated complex (Gouaux & Lipscomb, 1990), and the PALA-ligated complex (Ke et al., 1988), was accomplished using the program QUANTA (Molecular Simulations Inc., Waltham, MA) on an IBM RISC/6000 computer.

Determination of Protein Concentration. Concentrations of pure wild-type holoenzyme were determined by absorbance measurements at 280 nm using the extinction coefficient of 0.59 cm^2 mg^{-1} (Gerhart & Holoubek, 1967). The protein concentrations of the mutant holoenzymes were determined using the Bio-Rad version of Bradford's dye-binding assay (Bradford, 1976) with wild-type holoenzyme as the standard.

RESULTS

Construction of Plasmid pEK152. The plasmid pEK152, which carries the *pyrBI* operon, was derived from the plasmid pEK54 (Xu et al., 1988) by deletion of a 1-kb *Sma*I–*Sna*BI fragment which lies downstream of the 3′ end of the *pyrI* gene. pEK54 was digested with *Sma*I and *Sna*BI, and the two product fragments were separated by agarose gel electrophoresis. The larger, 4.9 kb, fragment carrying the *pyrBI* operon was isolated, and the blunt ends were ligated with T4 DNA ligase. The construction was confirmed by restriction analysis.

Construction of the Glu-37 → Ala, Asp-100 → Asn, Asp-100 → Ala and Arg-269 → Ala Mutations by Site-Specific Mutagenesis. The mutational changes in the catalytic chain of aspartate transcarbamoylase were introduced using the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987). The uracil-containing single-stranded DNA template required for this procedure was obtained by infection of *E. coli* strain CJ236 containing the plasmid pEK152, with the helper phage M13K07 (Vieira & Messing, 1987). Mutants were selected directly by dideoxy sequencing (Sanger et al., 1977) with single-stranded DNA isolated from several candidates.

Recloning and Confirmation of the Mutations. After each mutation had been verified, a small fragment of the gene carrying the mutation was recloned into an unmutated vector. For the Glu-37 → Ala mutation, a 0.76-kb *Pst*I–*Bst*EII fragment was isolated and cloned into pEK54 which had the corresponding fragment removed with the same restriction enzymes. For the Asp-100 → Ala and Asp-100 → Asn mutations, a 0.62-kb *Pst*I–*Bss*HII fragment was isolated and cloned into pEK54 which had the corresponding fragment removed, and for the Arg-269 → Ala mutation, a 0.45-kb *Acc*I–*Bgl*II fragment was isolated and cloned into pEK152 which had the corresponding fragment removed. Verification of the plasmid construction was accomplished by restriction analysis. Each mutation was verified for a second time by dideoxy sequencing with single-stranded DNA isolated after coinfection with the helper phage M13K07. No mutations, other than the desired ones, were found within the sequence of the cloned fragments. In this fashion, plasmids pEK198, pEK214, pEK218, and pEK219 were constructed, carrying the Glu-37 → Ala, Asp-100 → Ala, Asp-100 → Asn, and Arg-269→ Ala mutations, respectively.

The Purified Arg-269 → Ala Enzyme Does Not Contain the Species Lacking One Regulatory Subunit. Nondenaturing polyacrylamide gels of purified wild-type holoenzyme invariably show a species of enzyme that lacks one of the three regulatory dimers (C_6R_4), migrating slightly ahead of C_6R_6 (Yang et al., 1974; Evans et al., 1974, 1975). The preparations of purified Glu-37 → Ala, Asp-100 → Asn, and Asp-100 → Ala enzymes show both the C_6R_6 holoenzyme and the C_6R_4 species. However, the preparation of the purified Arg-269 → Ala enzyme did not show the C_6R_4 species although the C_6R_6 holoenzyme, which comigrated with the wild-type C_6R_6 holoenzyme, was observed (data not shown).

Kinetic Properties of the Wild-Type and Mutant Holoenzymes with Respect to Aspartate and Carbamoyl Phosphate. The aspartate saturation curves of the wild-type, Glu-37 → Ala, Asp-100 → Asn, Asp-100 → Ala, and Arg-269 → Ala holoenzymes are shown in Figure 2, and a summary of the kinetic data is given in Table I. The aspartate saturation curves of the mutant holoenzymes are clearly sigmoidal, indicating the presence of cooperative interactions between the active sites. The Glu-37 → Ala mutation results in an enzyme that is very similar to the wild-type enzyme, and although there is a slight reduction in the $[S]_{0.5}^{Asp}$, the maximal activity and Hill coefficient remain essentially unchanged (Figure 2A and Table I). The Asp-100 → Asn mutation

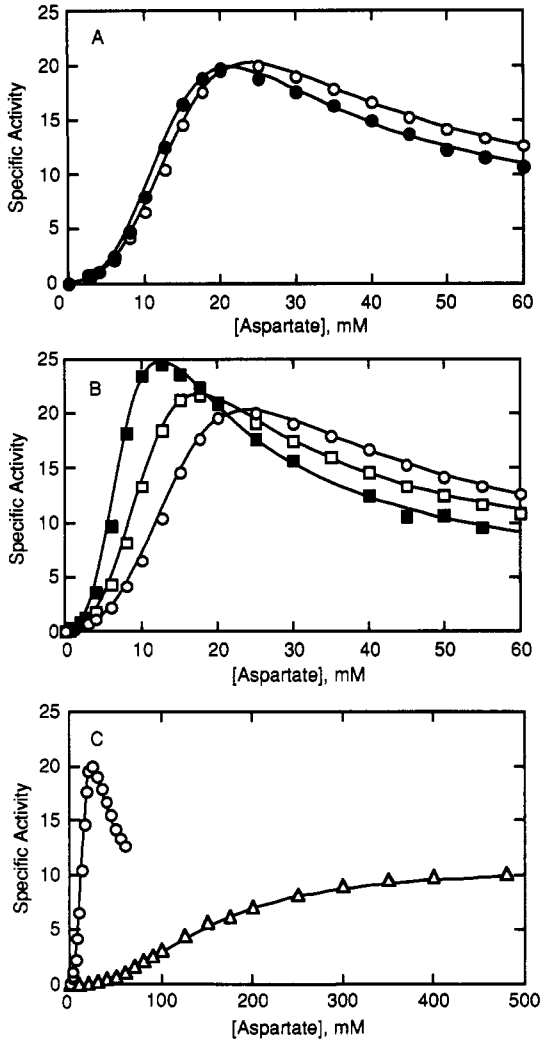

FIGURE 2: Aspartate saturation curves for the wild-type and the mutant holoenzymes were carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM for all enzymes except the Arg-269 → Ala enzyme which was assayed in the presence of 50 mM carbamoyl phosphate. (A) Data are shown for the wild-type (○) and Glu-37 → Ala (●) enzymes, (B) for the wild-type (○), Asp-100 → Asn (□), and Asp-100 → Ala (■) enzymes, and (C) for the wild-type (○) and Arg-269 → Ala (△) enzymes. The specific activity is reported in units of millimoles per hour per milligram of protein.

results in an enzyme that shows essentially wild-type activity, although the Asp-100 → Ala enzyme is slightly more active than the wild-type enzyme. Both mutant enzymes show small increases in their respective Hill coefficients. The most notable feature of these mutations, however, is an increase in the affinity for aspartate. For the Asp-100 → Ala enzyme, there is an approximate 2-fold decrease in the $[S]_{0.5}^{Asp}$, whereas the $[S]_{0.5}^{Asp}$ for the Asp-100 → Asn enzyme is intermediate between those for the wild-type and Asp-100 → Ala enzymes (Figure 2B and Table I). The mutations also lead to an increase in substrate inhibition. At 60 mM aspartate, the reduction in activity of the wild-type, Asp-100 → Asn, and Asp-100 → Ala enzymes, as a percentage of the maximal activity, is 39%, 49%, and 61%, respectively (Figure 2B). Mutation of Arg-269 → Ala results in the most dramatic effect on the kinetic parameters with respect to aspartate. While the enzyme retains homotropic cooperativity with only a slight reduction in the Hill coefficient, the maximal velocity and the affinity for aspartate are markedly different. The enzyme exhibits about a 2-fold reduction in the maximal specific activity and a 12-fold increase in the $[S]_{0.5}^{Asp}$ (Figure 2C and Table I). Unlike the wild-type enzyme, the Arg-269 → Ala enzyme does not exhibit substrate inhibition even at a concentration of 480 mM aspartate (Figure 2C).

The carbamoyl phosphate saturation curves of the wild-type, Glu-37 → Ala, Asp-100 → Asn, and Asp-100 → Ala holoenzymes are also sigmoidal, indicating that the mutations have not abolished cooperativity for carbamoyl phosphate. However, reduced cooperativity is observed for the Glu-37 → Ala enzyme, as well as a slight reduction in the $[S]_{0.5}^{CP}$ (Table I). The Asp-100 → Asn and Asp-100 → Ala enzymes show similar cooperativity to the wild-type enzyme and essentially identical affinities for carbamoyl phosphate (Table I). Mutation of Arg-269 → Ala also has the most dramatic effect on the kinetic parameters with respect to carbamoyl phosphate. While the enzyme retains homotropic cooperativity with only a slight increase in the Hill coefficient, the $[S]_{0.5}^{CP}$ increases approximately 44-fold as compared to the wild-type enzyme (Figure 3 and Table I).

Influence of the Allosteric Effectors on the Wild-Type and Mutant Holoenzymes. In order to determine whether the heterotropic effects induced by the regulatory nucleotides were altered by the mutations, the wild-type and mutant enzymes were assayed in the presence of ATP and CTP. The ATP activation and CTP inhibition curves of the wild-type, Glu-37 → Ala, Asp-100 → Asn, Asp-100 → Ala, and Arg-269 → Ala holoenzymes are shown in Figure 4, and a summary of the data is given in Table II. At an aspartate concentration corresponding to half the $[S]_{0.5}^{Asp}$, ATP activates and CTP inhibits the wild-type and mutant enzymes, although the patterns of activation and inhibition differ (Figure 4 and Table II).

In the presence of ATP, the wild-type, Glu-37 → Ala, Asp-100 → Asn, and Asp-100 → Ala enzymes are activated to about the same extent, although the concentration of ATP required for half-maximal activation (K_{ATP}) is increased 1.2-fold for the Glu-37 → Ala enzyme. By contrast, the K_{ATP} for the Asp-100 → Asn and Asp-100 → Ala enzymes is reduced 1.6-fold and 1.4-fold, respectively. Mutation of Arg-269 → Ala results in the most dramatic effect on ATP activation. While the K_{ATP} increases only 1.4-fold as compared to the wild-type enzyme, the maximal activation is reduced 40-fold (Figure 4 and Table II).

In the presence of CTP, the wild-type and Glu-37 → Ala enzymes show the same degree of inhibition, whereas the Asp-100 → Asn and Asp-100 → Ala enzymes exhibit an increase in the maximal inhibition. Mutation of Arg-269 → Ala results in the largest alteration, with a 3-fold decrease in maximal inhibition as compared to the wild-type enzyme. In addition, the enzymes also differ from the wild-type enzyme with respect to the concentration of CTP required for half-maximal inhibition (K_{CTP}). Most notable are the Asp-100 → Asn and Asp-100 → Ala enzymes, which both exhibit 3-fold reductions in K_{CTP}, and the Arg-269 → Ala enzyme which exhibits a 17-fold increase in K_{CTP} as compared to the wild-type enzyme.

Effect of Heat on the Stability of the Wild-Type and Mutant Enzymes. Gerhart and Pardee (1962) showed that heating wild-type aspartate transcarbamoylase in a low ionic strength buffer causes an increase in activity as the regulatory subunits dissociate from the more active catalytic subunits. Incubation of the wild-type enzyme for 2 min at temperatures of 50 °C and below does not induce dissociation of the regulatory subunits, whereas incubation at 60 °C for the same time period produces complete dissociation. As the temperature is increased from 50 to 60 °C, there is a concomitant

Table I: Kinetic Parameters of the Wild-Type and Mutant Holoenzymes[a,b]

enzyme	max velocity[c] (mmol·h^{-1}·mg^{-1})	$[S]_{0.5}^{Asp}$ (mM)	n_H^{Asp}	$[S]_{0.5}^{CP}$ (mM)	n_H^{CP}
wild-type	20.7 ± 0.4	12.2 ± 0.2	2.6 ± 0.0	0.26 ± 0.00	1.6 ± 0.1
Glu-37 → Ala	20.2 ± 0.3	10.9 ± 0.0	2.7 ± 0.0	0.17 ± 0.02	1.3 ± 0.0
Asp-100 → Asn	21.3 ± 0.4	8.9 ± 0.0	2.8 ± 0.0	0.27 ± 0.00	1.5 ± 0.1
Asp-100 → Ala	24.2 ± 0.5	6.6 ± 0.1	3.0 ± 0.0	0.26 ± 0.00	1.7 ± 0.1
Arg-269 → Ala	10.9 ± 0.2	148 ± 2.0	2.2 ± 0.0	11.4 ± 1.1	1.9 ± 0.1

[a] The data are the average of two independent experiments, except the maximal velocity, $[S]_{0.5}^{Asp}$, and n_H^{Asp} values of the wild-type and Asp-100 → Ala enzymes, which are the average of three independent experiments. [b] For the enzymes that exhibit cooperativity, the V_{max} and Hill coefficient (n_H) were calculated by a nonlinear least-squares procedure using the Hill equation. For the enzymes that also exhibit substrate inhibition, a modified Hill equation which incorporates a term for substrate inhibition was used (Pastra-Landis et al., 1978). [c] For the enzymes which exhibit substrate inhibition, the V_{max} represents the maximal observed specific activity from the aspartate saturation curve.

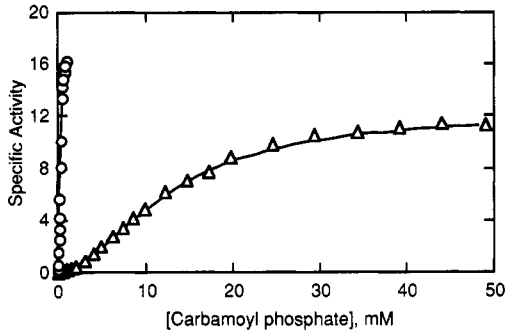

FIGURE 3: Carbamoyl phosphate saturation curves for the wild-type and Arg-269 → Ala mutant holoenzymes were carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The aspartate concentration was held constant at 24.5 mM for the wild-type enzyme, and 500 mM for the Arg-269 → Ala enzyme. Data are shown for the wild-type (○) and the Arg-269 → Ala (△) enzymes. The specific activity is reported in units of millimoles per hour per milligram of protein.

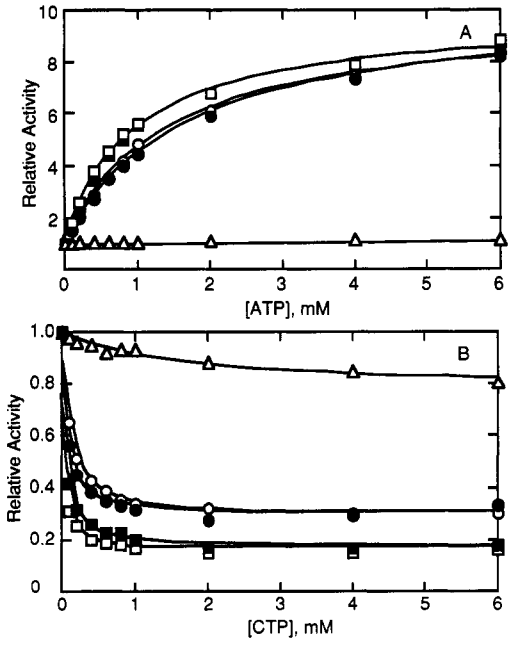

FIGURE 4: ATP activation (A) and CTP inhibition (B) of the wild-type and mutant holoenzymes were carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The aspartate concentration was held constant at half the $[S]_{0.5}^{Asp}$. The carbamoyl phosphate concentration was held constant at 4.8 mM for all the enzymes except the Arg-269 → Ala enzyme which was assayed in the presence of 50 mM carbamoyl phosphate. Data are shown for the wild-type (○), Glu-37 → Ala (●), Asp-100 → Asn (□), Asp-100 → Ala (■) and Arg-269 → Ala (△) enzymes.

increase in activity as the regulatory subunits dissociate from the catalytic subunits (Ladjimi & Kantrowitz, 1987; Xu et al., 1988). Therefore, if the regulatory subunits are first

Table II: ATP Activation and CTP Inhibition of the Wild-Type and Mutant Holoenzymes[a]

enzyme	act. with ATP[b]	K_{ATP} (mM)	act. with CTP[c]	K_{CTP} (mM)
wild-type	8.9	1.4	0.28	0.10
Glu-37 → Ala	9.2	1.7	0.29	0.06
Asp-100 → Asn	8.7	0.9	0.15	0.03
Asp-100 → Ala	8.8	1.0	0.17	0.04
Arg-269 → Ala	1.2	1.9	0.77	1.70

[a] Assays were performed at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM for all enzymes except for the Arg-269 → Ala enzyme which was assayed in the presence of 50 mM carbamoyl phosphate. The concentration of aspartate used was half the $[S]_{0.5}^{Asp}$, which corresponds to 6.1, 5.4, 4.4, 3.4, and 74 mM for the wild-type, Glu-37 → Ala, Asp-100 → Asn, Asp-100 → Ala, and Arg-269 → Ala enzymes, respectively. [b] Activity with ATP is defined as A^{ATP}/A where A^{ATP} is the activity extroplated to an infinite concentration of ATP and A is the activity in the absence of ATP. [c] Activity with CTP is defined as A^{CTP}/A where A^{CTP} is the activity extrapolated to an infinite concentration of CTP and A is the activity in the absence of CTP.

dissociated from the holoenzyme by incubation at temperatures above 60 °C, any subsequent loss in activity must be due to the dissociation and/or denaturation of the catalytic subunits, since free regulatory subunits denature irreversibly at 52 °C (Edge et al., 1988). Therefore, an experiment was devised to determine whether the mutations introduced at the C1–C2 interface of aspartate transcarbamoylase affected the stability of the catalytic subunit. When the wild-type holoenzyme was incubated at 59 and 65 °C, the activity increased to a maximal level after 2 min, but then remained constant throughout a further 28 min incubation. By contrast, incubation at 69 °C led to an exponential decrease in activity which followed the initial increase (data not shown). The higher temperature was therefore chosen to investigate the thermal stability of the wild-type and mutant catalytic subunits. The heat-induced activity profiles of the wild-type, Glu-37 → Ala, Asp-100 → Asn, and Asp-100 → Ala enzymes are shown in Figure 5. Analysis of the exponential decay yields a half-life of inactivation ($t_{1/2}$) for the wild-type enzyme of 5.8 min, whereas the Glu-37 → Ala, Asp-100 → Asn, and Asp-100 → Ala enzymes exhibit a half-life of inactivation of 4.9, 2.5, and 2.6 min, respectively. Much more pronounced, however, was the effect of the Arg-269 → Ala mutation on the stability of the enzyme. After 15 s incubation at 69 ± 0.5 °C, only 6% of the initial activity remained, and after 30 s, the enzyme was inactivated completely. A lower temperature was therefore sought at which to investigate the Arg-269 → Ala enzyme. At 50.25 ± 0.25 °C, the activity of the wild-type holoenzyme remained essentially unchanged over a 30-min incubation, whereas the activity of the Arg-269 → Ala enzyme decayed rapidly with a $t_{1/2}$ of 2.0 min (Figure 6). Unlike the wild-type

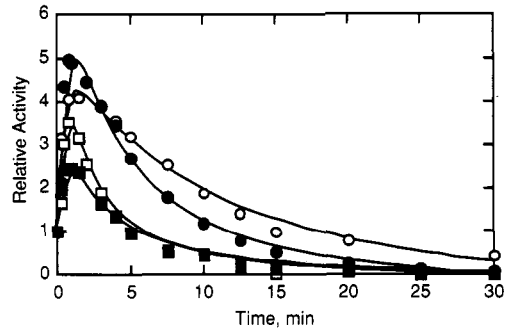

FIGURE 5: Heat inactivation of the wild-type and mutant enzymes at 69 ● 0.5 °C. The enzymes were diluted to 0.4 μg·mL^{-1} and incubated in 5 mM potassium phosphate buffer, pH 7.0. Aliquots of these solutions were removed at different times and chilled quickly on ice followed by determination of aspartate transcarbamoylase activity. Data are shown for the wild-type (○), Glu-37 → Ala (●), Asp-100 → Asn (□), and Asp-100 → Ala (■) enzymes.

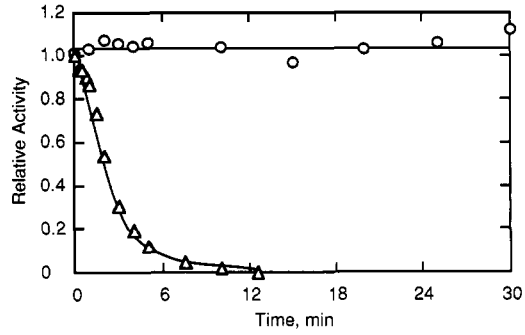

FIGURE 6: Heat inactivation of the Arg-269 → Ala enzyme at 50.25 ± 0.25 °C. The wild-type and Arg-269 → Ala enzymes were diluted to 2 μg·mL^{-1} and incubated in 5 mM potassium phosphate buffer, pH 7.0. Aliquots of these solutions were removed at different times and chilled quickly on ice followed by determination of aspartate transcarbamoylase activity. Data are shown for the wild-type (○) and Arg-269 → Ala (△) enzymes.

enzyme, the Arg-269 → Ala enzyme does not exhibit an increase in activity prior to the exponential decay.

DISCUSSION

Glu-37 Is Not Essential for Catalysis, Homotropic Cooperativity, or Heterotropic Regulation by ATP and CTP, but Is Involved in Stabilizing the Catalytic Subunit. In the X-ray structure of the T-state, wild-type holoenzyme (ligated to CTP), the carboxylate group of Glu-37 from C2 forms a hydrogen bond with the imidazole nitrogen of His-41 from C1 (Stevens et al., 1990). However, in the PALA-ligated R-state structure, this bond is broken (Krause et al., 1987; Ke et al., 1988), although in the (phosphonoacetamide + malonate)-ligated R-state structure, only half of the six possible interactions are lost (Gouaux & Lipscomb, 1990; Stevens et al., 1991a). When Glu-37 is replaced by Ala, no alteration in the maximal specific activity is observed, which indicates that Glu-37 is not essential for catalysis. Furthermore, the loss of the interaction between Glu-37 and His-41 observed in the R state seems to play an insignificant role in the T to R transition, since the mutant enzyme exhibits essentially normal cooperative behavior and allosteric responses to ATP and CTP. This is, perhaps, not too surprising since the C1–C2 interface undergoes only localized changes during the quaternary change, and these do not occur in the vicinity of Glu-37 (Krause et al., 1987; Ke et al., 1988). On the other hand, the fact that the half-life of inactivation at 69 ± 0.5 °C decreases from 5.8 min for the wild-type enzyme to 4.9 min for the mutant enzyme indicates that in the T state the interaction between Glu-37 and His-41 is involved in minor structural stabilization of the catalytic subunit.

Interchain Interaction between Asp-100 and Arg-65 Is Important for the Stability of the Catalytic Subunit. Mitchell et al. (1992) have shown that inter- and intramolecular arginine–aspartate interactions in proteins favor twin "end-on" and "side-on" N—H···O=C hydrogen bonds, respectively, and although these side chains may form only a single hydrogen bond, theoretical investigation of the energetics of the interaction, based on the methyl guanidinium cation and acetate anion, indicates that these doubly hydrogen-bonded interactions are the most stable configurations. Analysis of the X-ray structure of aspartate transcarbamoylase in both the T and R states reveals that the carboxylate of Asp-100 forms an "end-on" doubly hydrogen-bonded interaction with the guanidinium of Arg-65 (Stevens et al., 1990; Gouaux & Lipscomb, 1990; Ke et al., 1988). Since the interaction between Asp-100 and Arg-65 is maintained in both the T and R states, it was suspected that this interaction plays an important role in the stability of the catalytic subunit. Results from the heat inactivation experiments clearly demonstrate that this is the case. When Asp-100 is replaced by Asn and Ala, the half-life of inactivation of the catalytic subunit ($t_{1/2}$) at 69 ± 0.5 °C is reduced from 5.8 min for the wild-type enzyme to 2.5 and 2.6 min for the Asp-100 → Asn and Asp-100 → Ala enzymes, respectively. Furthermore, the fact that both mutant enzymes exhibit essentially identical $t_{1/2}$ values underlies the importance of the charged nature of this interaction, since even weakening the interaction in the Asp-100 → Asn enzyme results in essentially the same loss of stability as for the Asp-100 → Ala enzyme in which there is no hydrogen-bonding potential. Evidence in support of a stabilizing role for the interaction between Asp-100 and Arg-65 comes from differential scanning calorimetry of the related Asp-100 → Gly enzyme. Unlike the wild-type holoenzyme, which exhibits a thermal transition at 65.8 °C, corresponding to the denaturation of the regulatory subunits, and a thermal transition at 72.7 °C, corresponding to the denaturation of the catalytic subunits, the Asp-100 → Gly enzyme shows only a single transition at 65.6 °C (Burz et al., 1990). Since melting of the catalytic subunits can no longer be resolved from the melting of the regulatory subunits, then the stability of the Asp-100 → Gly catalytic subunit must be dependent upon its association with the regulatory subunits. Similarly, association of the regulatory subunits seems essential for the stability of the Asp-100 → Ala catalytic subunit. Attempts at purifying the Asp-100 → Ala catalytic subunit after dissociation of the holoenzyme with 4-(hydroxymercuri)benzoate (Gerhart & Holoubek, 1967) proved unsuccessful as the trimer precipitated once separated from the regulatory subunits (this study).

Kinetic analysis of the Asp-100 → Asn and Asp-100 → Ala enzymes indicates that although these enzymes exhibit almost normal maximal specific activity and cooperativity for aspartate, the $[S]_{0.5}^{Asp}$ values decrease to 8.9 and 6.6 mM, respectively, as compared to 12.2 mM for the wild-type enzyme (Figure 2B and Table I). It is clear, therefore, that although Asp-100 and Arg-65 play no direct role in catalysis or substrate binding, the interaction has an indirect effect on substrate binding, since removal of the carboxylate group of Asp-100 enhances the affinity for aspartate. Although the structural basis for this phenomenon is as yet unknown, attempts are currently in progress to crystallize the Asp-100 → Ala enzyme in order to determine how the mutation affects the C1–C2 interface and the structure of the holoenzyme.

Arg-269 May Affect Substrate Binding by Stabilization of a Loop Region That Contains the Active-Site Residues Pro-266 and Leu-267. X-ray structural data show that Arg-269 is located in a loop region (His-265–Asp-278) that contains the active site residues Pro-266 and Leu-267. These two residues, along with Pro-268, form a reverse turn in the polypeptide chain that defines the surface with which carbamoyl phosphate interacts. More specifically, the amino group of carbamoyl phosphate (or phosphonoacetamide) forms hydrogen bonds with the backbone carbonyl oxygens of Pro-266 and Leu-267 (Gouaux & Lipscomb, 1988, 1990). The guanidinium group of Arg-269 forms an intrachain salt link with the carboxylate of Asp-278, as well as a salt link with the carboxylate of Asp-90 from an adjacent, 3-fold-related catalytic chain. Upon binding of phosphonoacetamide to the wild-type holoenzyme, residues Pro-266–Pro-268 move up to 0.7 Å nearer their R-state positions, which brings residues Asp-271–Asp-278 to a location intermediate between their T- and R-state positions. The movement of residues Asp-271–Asp-278 is coupled to the movement of Arg-269 by main-chain covalent bonds and by the salt link between Arg-269 and Asp-278 (Gouaux & Lipscomb, 1990). If the salt bridge between Arg-269 and Asp-278 is critical for stabilizing Pro-266 and Leu-267 in an optimal position for binding carbamoyl phosphate, then it might be expected that mutation of Arg-269 → Ala would affect the affinity of the enzyme for this substrate. Indeed, replacement of Arg-269 by Ala results in a 44-fold increase in the $[S]_{0.5}^{CP}$, while the enzyme retains almost normal cooperativity and exhibits a maximal specific activity which is only 50% lower than the wild-type enzyme (Table I and Figure 3). Furthermore, the marked decrease in the affinity for carbamoyl phosphate may prevent the conformational changes that lead to the formation of the high-affinity aspartate-binding site, and thereby account for the decrease in affinity for aspartate. In addition to the loss of the interaction between Arg-269 and Asp-278, replacement of Arg-269 by Ala also results in the loss of the interchain interaction between Arg-269 and Asp-90. However, in the Asp-90 → Ala enzyme, the $[S]_{0.5}^{CP}$ and $[S]_{0.5}^{Asp}$ values are increased only 6-fold and 3-fold, respectively (N. Dembowski, unpublished observations), compared to 44-fold and 12-fold, respectively, for the Arg-269 → Ala enzyme. Since the loss of both inter- and intrachain interactions in the Arg-269 → Ala enzyme results in a more pronounced decrease in substrate affinity than the loss of the interchain interaction alone, then it is likely that an important function of the salt link between Arg-269 and Asp-278 is to stabilize the loop containing residues 265–278 and, more specifically, to position Pro-266 and Leu-267 at the active site.

Arg-269 Is Important for the Structural Stability of the Holoenzyme. Analysis of the X-ray structure of the wild-type holoenzyme in the CTP-ligated T state shows that Arg-269 from C2 forms an interchain salt link with Asp-90 from C1 (Stevens et al., 1990). Furthermore, this interaction is maintained in the (phosphonoacetamide + malonate)-ligated and PALA-ligated R-state structures (Gouaux & Lipscomb, 1990; Ke et al., 1988). Although arginine–aspartate interactions across subunit interfaces have been shown to favor "end-on" twin N—H··· O=C hydrogen bonds (Mitchell et al., 1992), the interaction of Arg-269 with Asp-278 from the same chain precludes the possibility of this kind of interaction between Arg-269 and Asp-90. It is likely, therefore, that Arg-269 has a dual function in stabilizing the position of Pro-266 and Leu-267 at the active site as well as stabilizing the C1–C2 interface. Results from the heat inactivation experiments clearly demonstrate that replacement of Arg-269 by Ala produces a substantial weakening of the mutant holoenzyme. Even at 50.25 ± 0.25 °C, a temperature at which the wild-type holoenzyme undergoes no significant change in activity, the Arg-269 → Ala enzyme is rapidly inactivated (Figure 6). Furthermore, the inactivation is not preceded by a rise in activity which, in the wild-type enzyme, is associated with the dissociation of the regulatory subunits from the more active catalytic trimers (Gerhart & Pardee, 1962). These results suggest, therefore, that replacement of Arg-269 by Ala substantially weakens the C1–C2 interface, since there is no rise in activity upon dissociation of the regulatory subunits, and that the stability of the Arg-269 → Ala catalytic subunit is dependent upon association with the regulatory dimers. Evidence supporting this notion comes from differential scanning calorimetry of the related Arg-269 → Gly enzyme. Whereas the wild-type holoenzyme exhibits a thermal transition at 65.8 °C, corresponding to the denaturation of the regulatory subunits, and a thermal transition at 72.7 °C, corresponding to the denaturation of the catalytic subunits, the Arg-269 → Gly enzyme shows only a single transition at 60.6 °C (Burz et al., 1990). Therefore, as with the Arg-269 → Ala enzyme, the stability of the Arg-269 → Gly enzyme seems dependent upon the presence of the regulatory subunits since a thermal transition corresponding to the denaturation of the catalytic subunits is not observed. Further evidence in support of this comes from the observation that the C_6R_4 species of the enzyme is not found in the preparation of the purified Arg-269 → Ala holoenzyme. Presumably, once one of the three regulatory dimers dissociates from the C_6R_6 holoenzyme, sufficient stabilization energy is lost so that the C_6R_4 species can no longer exist. The results from the heat inactivation experiments of the Arg-269 → Ala enzyme also indicate that the mutant is inactivated at a temperature which does not cause significant dissociation of the regulatory subunits from the wild-type holoenzyme (Figure 6). This suggests that replacement of Arg-269 by Ala has also weakened the interfaces between the regulatory and catalytic subunits. A similar effect is observed for the Arg-269 → Gly enzyme which denatures completely 5.2 °C below the temperature required to denature the regulatory chains from the wild-type holoenzyme (Burz et al., 1990). While replacement of Arg-269 by Ala would be expected to weaken the interface between adjacent chains within the catalytic subunit, it is evident that this residue plays a more complex role than simply stabilizing the C1–C2 interface.

Glu-37, Asp-100, and Arg-269 May Play Similar Roles in Other Aspartate Transcarbamoylases. Glu-37 and His-41 are conserved in the aspartate transcarbamoylases from *S. marcescens* (Beck et al., 1989) and *S. typhimurium* (Michaels et al., 1987). Both these enzymes form dodecameric holoenzymes consisting of two catalytic trimers and three regulatory dimers (Wild et al., 1980). Furthermore, the catalytic chains show 88 and 91% identity, respectively, with the *E. coli* catalytic chain (Beck et al., 1989; Michaels et al., 1987). Since these enzymes show such striking homology, it is likely that the interaction between Glu-37 and His-41 is maintained in the *S. marcescens* and *S. typhimurium* enzymes. Glu-37, but not His-41, is also conserved in the aspartate transcarbamoylase from hamster (Simmer et al., 1989). Unlike the *E. coli* enzyme, the mammalian aspartate transcarbamoylase is part of a multifunctional polypeptide, CAD, which has glutamine-dependent carbamoyl-phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase activities (Coleman et al., 1977). Although the protein is a mosaic of

domains, with each associated with a separate enzymatic activity (Mally et al., 1981; Davidson et al., 1981), isolation of the aspartate transcarbamoylase domain has shown it to be a trimer of M_r 34 000 subunits (Grayson & Evans, 1983; Simmer et al., 1989). Moreover, the amino acid sequence shows 44% identity with the *E. coli* catalytic chain (Simmer et al., 1989). On the basis of this sequence homology, Scully and Evans (1991) used the X-ray coordinates of the *E. coli* enzyme to model the structure of the mammalian enzyme. In the refined model, the backbone carbonyl oxygen of Glu-37 from C2 clearly forms a salt link with the side chain of Arg-38 from C1, the first residue of an insert (Arg-Ser-Leu-Asp) that lies between the amino acids corresponding to Glu-37 and Leu-38 of the *E. coli* sequence (Scully & Evans, 1991). Therefore, Glu-37 may play a role in stabilizing the C1–C2 interface in the hamster enzyme, although it apparently involves a backbone rather than a side chain interaction.

More striking, however, is the conservation of Asp-100. Asp-100 is conserved not only in the aspartate transcarbamoylases from *S. marcescens* (Beck et al., 1989), *S. typhimurium* (Michaels et al., 1987), and hamster (Simmer et al., 1989) but also in the trimeric enzymes from wheat (R. Yon, personal communication) and *B. subtilis*, which exhibits 35% sequence identity with the *E. coli* catalytic chain and has a very similar tertiary fold (Lerner & Switzer, 1986; Stevens et al., 1991b). Furthermore, Arg-65, with which Asp-100 interacts in the *E. coli* enzyme, is conserved in the *S. marcescens*, *S. typhimurium*, and hamster enzymes although a Lys residue replaces Arg-65 in the *B. subtilis* enzyme. It is likely, therefore, that the interaction between Asp-100 from C1 and Arg-65 from C2 (and possibly between Asp-100 and the corresponding Lys residue in the *B. subtilis* enzyme) is maintained in these aspartate transcarbamoylases and that the interaction plays a similar role in stabilizing the C1–C2 interface. Unfortunately, only a partial sequence of the wheat enzyme is currently available, and it is not known whether Arg-65 is conserved in this plant enzyme (R. Yon, personal communication).

Arg-269 is conserved in the aspartate transcarbamoylases from *S. marcescens* (Beck et al., 1989), *S. typhimurium* (Michaels et al., 1987), *B. subtilis* (Lerner & Switzer, 1986), hamster (Simmer et al., 1989), and wheat (R. Yon, personal communication), as is Asp-90 with which it interacts in the *E. coli* enzyme. Therefore, the salt link between Arg-269 and Asp-90 is likely to be maintained in these enzymes and play a similar role in stabilizing the C1–C2 interface. In the *E. coli* enzyme, Arg-269 also forms an intrachain salt link with the side chain of Asp-278, and this interaction probably stabilizes the position of Pro-266 and Leu-267 at the active site. Asp-278 is conserved in the enzymes from *S. marcescens* (Beck et al., 1989), *S. typhimurium* (Michaels et al., 1987), hamster (Simmer et al., 1989), and wheat (R. Yon, personal communication), but not in the *B. subtilis* enzyme (Lerner & Switzer, 1986). It is highly likely that the interaction between Arg-269 and Asp-278 is maintained in the *S. marcescens*, *S. typhimurium*, and wheat enzymes since 13, 12, and 11 of the 14 residues, respectively, in the loop region containing residues His-265–Asp-278 are absolutely conserved. It is also probable that the interaction between Arg-269 and Asp-278 is conserved in the hamster enzyme since 9 of the 14 residues, including Pro-266 and Pro-268, are conserved in the hamster sequence (Simmer et al., 1989). However, a similar role for Arg-269 in stabilizing the position of the active-site residues is less likely in the *B. subtilis* enzyme since 8 of the 14 residues in this loop, including Asp-278, are not conserved. Furthermore, the tertiary structure of this loop may be significantly different from its *E. coli* counterpart since a two residue insert lies between the amino acids corresponding to Pro-268 and Arg-269 of the *E. coli* sequence (Lerner & Switzer, 1986).

In summary, replacement of Glu-37 by Ala in the aspartate transcarbamoylase from *E. coli* results in an enzyme that is essentially wild-type in nature, but which shows a slight decrease in thermal stability. Therefore, the interaction between Glu-37 and His-41 is not critical for catalysis or homotropic coooperativity but may play a minor role in stabilizing the C1–C2 interface. On the other hand, Asp-100 seems to play a more significant role in stabilizing this interface. Replacement of Asp-100 by Asn or Ala results in a 2-fold decrease in the stability of the catalytic trimer as well as increased affinity of the holoenzyme for aspartate. Replacement of Arg-269 by Ala has the most dramatic effect on the properties of the holoenzyme. Not only is the enzyme significantly less stable than the wild-type holoenzyme, but the affinities for carbamoyl phosphate and aspartate are markedly decreased. Arg-269 is likely to play a dual role in aspartate transcarbamoylase: stabilization of the C1–C2 interface by means of its interaction with Asp-90, as well as positioning of the active-site residues Pro-266 and Leu-267 by means of its interaction with Asp-278.

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