Probing the Regulatory Site of *Escherichia coli* Aspartate Transcarbamoylase by Site-Specific Mutagenesis†

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ABSTRACT: The effector binding site of *Escherichia coli* aspartate transcarbamoylase, composed of the triphosphate and ribose–base subsites, is located on the regulatory (r) chains of the enzyme. In order to probe the function of amino acid side chains at this nucleotide triphosphate site, site-specific mutagenesis was used to create three mutant versions of the enzyme. On the basis of the three-dimensional structure of the enzyme with CTP bound, three residues were selected. Specifically, Arg-96r was replaced with Gln, and His-20r and Tyr-89r were both replaced with Ala. Analyses of these mutant enzymes indicate that none of these substitutions significantly alter the catalytic properties of the enzyme. However, the mutations at His-20r and Tyr-89r produced altered response to the regulatory nucleotides. For the His-20r→Ala enzyme, the affinities of the enzyme for ATP and CTP are reduced 40-fold and 10-fold, respectively, when compared with the wild-type enzyme. Furthermore, CTP is able to inhibit the His-20r→Ala enzyme 40% more than the wild-type enzyme. In the case of the Tyr-89r→Ala enzyme, ATP can increase the mutant enzyme's activity 181% compared to 157% for the wild-type enzyme, while simultaneously the affinity of this enzyme for ATP decreases about 70%. These results suggest that Tyr-89r does have an indirect role in the discrimination between ATP and CTP. The His-20r→Ala enzyme shows no UTP synergistic inhibition in the presence of CTP. UTP alone does not significantly affect the activity of the wild-type or the Arg-96r→Gln enzymes; however, UTP can activate both the His-20r→Ala and the Tyr-89r→Ala enzymes in the absence of CTP. The UTP activation of both of these enzymes can easily be reversed by addition of low concentrations of CTP. Analysis of the kinetic and binding data from these mutant enzymes suggests that His-20r and Tyr-89r in the wild-type enzyme are important for recognition of UTP. The reduced affinity of the His-20r→Ala enzyme for CTP and ATP suggests that His-20r in the wild-type enzyme is directly involved in effector binding.

Aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the committed step of the pyrimidine biosynthesis pathway, the carbamoylation of the amino group of aspartate by carbamoyl phosphate. In *Escherichia coli*, this enzyme is activated by ATP and feedback-inhibited by CTP, the end product of the pathway (Yates & Pardee, 1956; Gerhart & Pardee, 1962, 1963). At pH 7.0, the combination of CTP and UTP can inhibit the enzyme more than either one alone (Wild et al., 1989). The holoenzyme,[1] composed of two trimers of catalytic chains (M_r 33 000) and three dimers of regulatory chains (M_r 17 000), exhibits homotropic cooperative interactions for both substrates, aspartate and carbamoyl phosphate. The catalytic subunit, a trimer of catalytic chains, carries the active sites and is insensitive to the effectors ATP and CTP and shows Michaelis–Menten kinetics. The regulatory subunit, a dimer of regulatory chains, exhibits no catalytic activity but binds the nucleotide effectors.

Analysis of the structure of aspartate transcarbamoylase (Honzatko & Lipscomb, 1982; Gouaux et al., 1990; Stevens et al., 1990) along with kinetics (Gerhart & Pardee, 1963, 1964; Ladjimi et al., 1985; Thiry & Hervé, 1978) and binding measurements (Changeux et al., 1968) suggests that ATP and CTP bind competitively to the same site on the enzyme but they introduce opposite effects on the homotropic cooperativity of the enzyme and cause alterations in the $[S]_{0.5}$ for the substrates. The binding of CTP and ATP to the regulatory site is complex. Two classes of regulatory binding sites with different affinities for CTP can be distinguished (Gray et al., 1973; Matsumoto & Hammes, 1973; Tondre & Hammes, 1974; Winlund & Chamberlin, 1970; Suter & Rosenbusch, 1977). The binding of ATP follows a pattern similar to that of CTP with two classes of affinity sites, except that ATP binding to the regulatory site is about an order of magnitude weaker than that of CTP (Matsumoto & Hammes, 1973). In addition, like any other phosphate-containing compound, CTP and ATP directly compete with carbamoyl phosphate, at the active site (Porter et al., 1969). UTP binds preferentially to the three low-affinity CTP sites, resulting in enhanced affinity for CTP at the three high-affinity sites (Zhang & Kantrowitz, 1991). Previous amino acid replacements at the nucleotide binding site have shown changes in both homotropic (Wild & Corder, 1989; Zhang et al., 1988) and heterotropic (Zhang & Kantrowitz, 1989; Zhang et al., 1988; Wild & Corder, 1989) properties of the enzyme.

At least three models have been proposed to explain heterotropic regulation in aspartate transcarbamoylase. The first model suggests that the heterotropic effectors alter the T ⇌ R equilibrium by preferentially binding to either the T or the R state (Hensley & Schachman, 1979). The second model suggests that the effectors cause an alteration in substrate affinity and it is this alteration in substrate affinity that controls the allosteric transition (Thiry & Hervé, 1978; Tauc et al., 1982). The third model, based on crystallographic data

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[1] Abbreviations: T and R states, tense and relaxed states of the enzyme having low and high affinity, respectively, for the substrates; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; holoenzyme, entire aspartate transcarbamoylase molecule composed of two catalytic trimers and three regulatory dimers; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

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of the enzyme in both the T and R states complexed with the heterotropic effectors, suggests that the effectors alter the quaternary structure and it is this alteration in structure which alters substrate affinity (Stevens & Lipscomb, 1990).

In order to further probe the interactions between the nucleotide effectors and their binding site in aspartate transcarbamoylase and the mechanism by which the effectors alter the activity of the enzyme, we have prepared three mutant enzymes each with a single amino acid substitution in the effector binding site. Here we report the production and characterization of these mutant versions of aspartate transcarbamoylase in which Arg-96r[2] is replaced by Gln, and Tyr-89r and His-20r are both replaced by Ala.

Materials and Methods

ATP, CTP, UTP, carbamoyl phosphate, *N*-carbamoyl-L-aspartate, L-aspartate, potassium dihydrogen phosphate, imidazole, Tris, and HEPES[1] were purchased from Sigma Chemical Co. [^{3}H]CTP was purchased from NEN Research Products. The carbamoyl phosphate was purified by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C (Gerhart & Pardee, 1962). Enzyme-grade ammonium sulfate was purchased from ICN Biomedicals. The plasmid pUC119 and the phage M13K07 were obtained from J. Messing, Rutgers University, while the *E. coli* K12 strain U39a [F$^-$ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *rspL*] was obtained from J. R. Wild, Texas A&M University.

Oligonucleotide Synthesis. The oligonucleotides required for the site-specific mutagenesis as well as the sequencing primers were synthesized using an Applied Biosystems 381A DNA synthesizer.

Site-Specific Mutagenesis. The mutant versions of aspartate transcarbamoylase were made by introducing specific base changes in the *pyrI* gene by the method of site-specific mutagenesis (Zoller & Smith, 1982), with the modifications previously described (Ladjimi et al., 1988; Carter et al., 1985). To select for the Tyr-89r→Ala[3] mutation, candidates were screened by dot-blot hybridization (Carter et al., 1984). The DNA from candidates showing dark spots was then isolated and sequenced by the dideoxy method (Sanger et al., 1977). Three candidates with the correct mutation were identified out of total of 13. To select the Arg-96r→Gln and His-20r→Ala mutations, single-stranded DNA from a number of candidates was isolated and sequenced directly by the dideoxy method. The mutagenesis frequency for these mutations was approximately 10%.

After the mutations had been verified, a small fragment of the M13 RF carrying the mutation was removed with restriction enzymes and inserted into a plasmid which had the corresponding section of the wild-type gene removed. For all three mutants, the restriction enzymes *Bst*EII and *Eco*RV were used to remove a fragment of 864 base pairs containing the desired mutation from the M13 RF. The fragment was isolated with NA45 paper after agarose gel electrophoresis. At the same time, the plasmid pEK54 (Xu et al., 1988) was cut with the same two restriction enzymes and the larger fragment isolated in a similar fashion. This larger fragment was then treated with calf intestinal alkaline phosphatase to prevent self-ligation. The fragment, containing the remaining section of the *pyrBI* operon, was combined with the fragment from the mutant M13 RF and treated with T4 DNA ligase at 14 °C overnight. Selection was accomplished after transformation into U39a, a strain which carries a deletion in the *pyrBI* region.

Wild-Type and Mutant Enzyme Purification. The wild-type and all mutant versions of the aspartate transcarbamoylase were isolated as described by Nowlan and Kantrowitz (1985), from *E. coli* strain EK1104 [F$^-$ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *pyrF*$^{\pm}$, *rpsL*], containing the plasmid pEK2 (Smith et al., 1986) for wild-type enzyme, pEK80 for the Tyr-89r→Ala, pEK95 for the Arg-96r→Gln, and pEK121 for the His-20r→Ala enzymes.

Determination of Protein Concentration. The concentration of pure wild-type holoenzyme was determined by absorbance measurements at 280 nm with an extinction coefficient of 0.59 cm^2/mg (Gerhart & Holoubek, 1967). The protein concentration of the mutant holoenzymes was determined by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976).

Aspartate Transcarbamoylase Assay. The transcarbamoylase activity was measured at 25 °C by either the colorimetric (Pastra-Landis et al., 1981) or the pH-stat assay method (Wu & Hammes, 1973). The pH-stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburet. The saturation curves for aspartate, CTP, ATP, and UTP as well as the competition experiments were determined in duplicate using the colorimetric assay, and the data points shown in the figures are the average. All the colorimetric assays were performed in 0.1 M imidazole/acetate buffer at pH 7.0, unless otherwise indicated. Assays at pH 8.3 were performed in 0.05 M Tris/acetate buffer.

Binding Measurements. The binding of CTP to the wild-type and the His-20r→Ala enzymes was determined by the technique of equilibrium dialysis in 0.1 M HEPES/acetate, 0.2 mM EDTA, and 2 mM 2-mercaptoethanol, pH 7.0; the enzyme was dialyzed into this buffer before use. Dialysis experiments were carried out in microdialysis cells holding 50 μL on each side of the dialysis membrane (Spectra/Pro-2, Spectrum Medical Industries), which was pretreated as previously described (Jacobsberg et al., 1975). After equilibration for 18–20 h at 25 °C, 25-μL samples were removed from each side of the dialysis cell, and the concentration of UTP or CTP was determined by liquid scintillation employing a LKB 1217 Rackbeta liquid scintillation counter. Complete equilibration was confirmed under the experimental conditions.

For the dialysis experiments in which CTP binding was measured in the presence of UTP, the enzyme and UTP concentrations were kept constant at 17.1 mg/mL and 10 mM, respectively, on one side of the dialysis membrane while the concentration of [^{3}H]CTP was varied on the other. For the dialysis experiments in which the competition between CTP and UTP was investigated, the enzyme and [^{3}H]CTP concentrations were kept constant at 17.1 mg/mL and 0.24 mM (0.01 μCi/μL), respectively, on one side of the membrane while the concentration of UTP was varied on the other. For both of the experiments above, carbamoyl phosphate at an equilibrium concentration of about 19.2 mM was also present in order to prevent the binding of the nucleotide effectors at the active site.

Data Analysis. The analysis of the steady-state kinetic data was carried out as previously described by Silver et al. (1983). The equilibrium dialysis data were analyzed in the form of Scatchard plots (Scatchard, 1949). Analysis of the structural data, based on the three-dimensional coordinates of the enzyme without ligands (Ke et al., 1984; Stevens et al., 1990), and the

[2] The letter "r" after the number indicates that the residue is located in the regulatory chain of aspartate transcarbamoylase.

[3] The notation used to name the mutant enzymes shows the wild-type amino acid and location within the regulatory chain (r) to the left of the arrow and the new amino acid indicated to the right to the arrow, for example, Tyr-89r→Ala enzyme.

Table I: Kinetic Parameters for the Wild-Type and the Regulatory Chain Mutant Aspartate Transcarbamoylases in the Absence and Presence of ATP, CTP, and CTP plus UTP[a]

parameters	ATP	control	CTP	CTP + UTP
V_{max}[b]				
wild type	5.6	5.6	6.0	6.6
Arg-96r→Gln	5.9	5.9	5.9	5.9
Tyr-89r→Ala	6.3	5.5	5.8	6.0
His-20r→Ala	5.8	5.7	5.5	5.2
$[S]_{0.5}^{Asp}$ (mM)				
wild type	3.4	6.2	8.9	19.4
Arg-96r→Gln	4.6	6.8	11.0	22.9
Tyr-89r→Ala	4.8	7.4	11.4	29.3
His-20r→Ala	4.9	5.7	9.7	9.9
Hill coefficient				
wild type	1.3	2.3	2.7	2.7
Arg-96r→Gln	1.7	2.6	2.8	2.1
Tyr-89r-Ala	1.4	2.3	2.3	2.0
His-20r→Ala	2.3	2.5	2.4	2.2

[a] Data in this table are extracted from aspartate saturation curves determined in the absence of nucleotide effectors (control) or in the presence of 4 mM ATP, or 4 mM CTP, or 4 mM CTP plus 4 mM UTP. Colorimetric assays were performed at 25 °C in 0.1 M imidazole/acetate buffer (pH 7.0) at saturating concentrations of carbamoyl phosphate (19.2 mM). [b] Maximal observed specific activity, millimoles of *N*-carbamoylaspartate per hour per milligram of protein.

CTP- or ATP–enzyme complex (Kim et al., 1987; Stevens et al., 1990; Gouaux et al., 1990), was accomplished by using the program QUANTA (Polygen Corp., Waltham, MA) on an IBM/RISC 6000 computer.

RESULTS

Steady-State Kinetics of the Mutant Holoenzymes. At pH 7.0, without effectors, the Arg-96r→Gln, the Tyr-89r→Ala, and the His-20r→Ala enzymes all have similar maximal observed specific activities which are comparable to that of the wild-type enzyme (Table I). The aspartate concentration at half the maximal observed specific activity ($[S]_{0.5}^{Asp}$) and the Hill coefficient (n_H) for the mutant enzymes are also not significantly altered. However, the Tyr-89r→Ala enzyme shows a 20% increase in the $[S]_{0.5}^{Asp}$ compared to the wild-type enzyme.

In the presence of saturating concentrations of the effectors ATP or CTP, the aspartate saturation curves of the mutant enzymes are similar to the curves obtained for the wild-type enzyme. However, in the presence of 2 mM CTP plus 2 mM UTP, the aspartate saturation curves for all the mutant enzymes are different from the wild-type enzyme (Figure 1). In the presence of both CTP and UTP, the $[S]_{0.5}^{Asp}$ value of the His-20r→Ala enzyme is 9.9 mM, significantly lower than the 19.4 mM value for the wild-type enzyme, while the $[S]_{0.5}^{Asp}$ values of the Arg-96r→Gln and the Tyr-89r→Ala enzymes are 22.9 and 29.3 mM, respectively, both higher than the $[S]_{0.5}^{Asp}$ value of the wild-type enzyme. The addition of UTP to the CTP-saturated wild-type enzyme increases the $[S]_{0.5}^{Asp}$ value from 8.9 to 19.4 mM (Table I), while the addition of UTP to the CTP-saturated Arg-96r→Gln and Tyr-89r→Ala enzymes increases the $[S]_{0.5}^{Asp}$ values from 11.0 to 22.9 mM and from 11.4 to 29.3 mM, respectively. However, the addition of UTP to the CTP-saturated His-20r→Ala enzyme has almost no effect on the $[S]_{0.5}^{Asp}$ value.

Alterations in the Response of the Mutant Enzymes to ATP and CTP. For the His-20r→Ala enzyme, as determined from the activation and inhibition curves, the affinities of the enzyme for ATP and CTP are reduced 40-fold and 10-fold, respectively, when compared with the wild-type enzyme. At saturating concentrations of ATP, the His-20r→Ala enzyme is activated to the same level as is the wild-type enzyme.

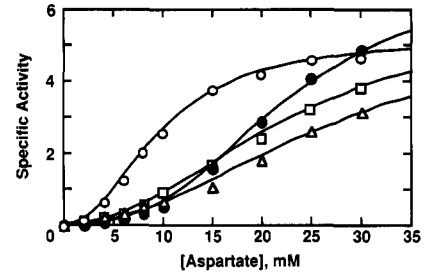

FIGURE 1: Aspartate saturation curves of the wild-type (●), the Arg-96r→Gln (□), the Tyr-89r→Ala (△), and the His-20r→Ala (○) aspartate transcarbamoylases in the presence of 2 mM CTP and 2 mM UTP. Specific activity is reported in millimoles of *N*-carbamoyl-L-aspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.1 M imidazole/acetate buffer (pH 7.0) at saturating concentrations of carbamoyl phosphate (19.2 mM).

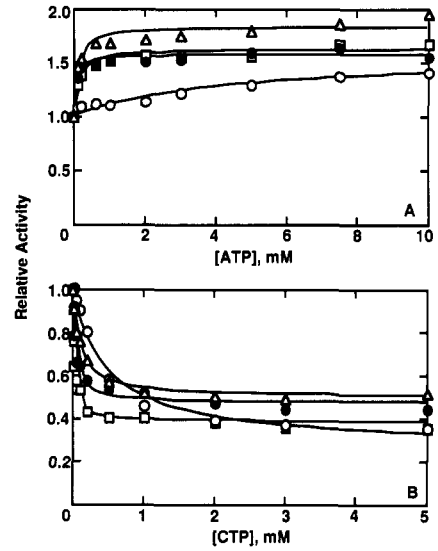

FIGURE 2: Influence of [ATP] (A) and [CTP] (B) on the activity of the wild-type (●), Arg-96r→Gln (□) Tyr-89r→Ala (△), and His-20r→Ala (○) enzymes. The aspartate concentration was held constant at the $[S]_{0.5}^{Asp}$ of the respective enzyme, and the experimental conditions were identical to those described in the legend to Figure 1.

However, CTP is able to inhibit the His-20r→Ala enzyme more than the wild-type enzyme, with 26% residual activity compared to 43% for the wild-type enzyme (Figure 2).

In the case of the Tyr-89r→Ala enzyme, ATP can increase the mutant enzyme's activity 181% compared to 157% for the wild-type enzyme, while the ATP affinity of this enzyme decreases about 70%. According to previous reports (Thiry & Hervé, 1978), for the complete manifestation of allosteric activation, the ATP triphosphate group must be fully ionized. At pH 8.3, the Tyr-89r→Ala enzyme shows a maximal ATP activation of 460% compared to 270% for the wild-type enzyme, even though the ATP affinity of the mutant enzyme is decreased by half (Figure 3). The Tyr-89r→Ala enzyme shows approximately the same affinity and maximal inhibition by CTP as does the wild-type enzyme.

The Arg-96r→Gln enzyme did not exhibit significant alterations in the maximal activation or inhibition caused by ATP or CTP, respectively. Furthermore, the affinity of these nucleotides for this enzyme was very similar to that of the wild-type enzyme.

UTP Does Not Synergistically Inhibit the His-20r→Ala Enzyme in the Presence of CTP. As reported by Wild et al. (1989), at pH 7.0, UTP and CTP synergistically inhibit wild-type aspartate transcarbamoylase more than either ef-

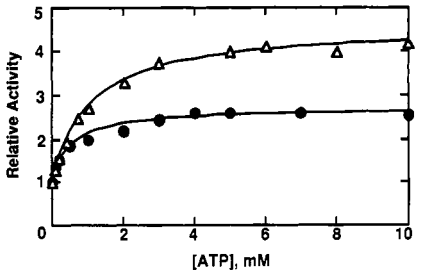

FIGURE 3: Influence of [ATP] on the activity of the wild-type (●) and the Tyr-89r→Ala (△) enzymes at pH 8.3. The activity of the enzymes was measured at 25 °C in 0.05 M Tris/acetate buffer. Carbamoyl phosphate concentration was held constant at 19.2 mM, and aspartate concentration was held constant at the $[S]_{0.5}^{Asp}$ of the respective enzyme.

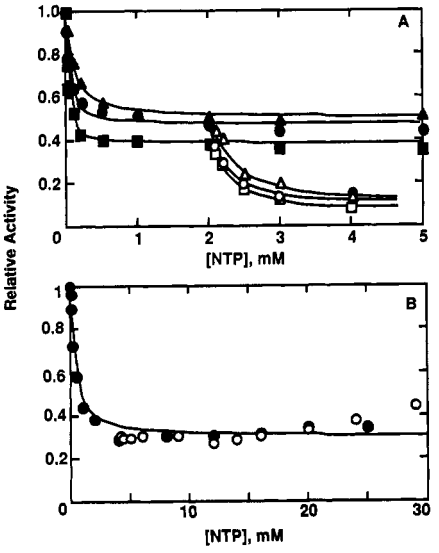

FIGURE 4: Influence of [CTP] and [CTP] plus [UTP] on the activity of the wild-type and the mutant aspartate transcarbamoylases. Colorimetric assays were performed in 0.1 M imidazole/acetate buffer (pH 7.0). Carbamoyl phosphate concentration was held constant at 19.2 mM, while aspartate concentration was held constant at the $[S]_{0.5}^{Asp}$ of the respective enzyme. (A) The influence of [CTP] alone (0–2 mM) on the activity of the enzymes in the absence of UTP was determined, as well as the influence of [UTP] while CTP concentration was held constant at 2 mM. The influence of [CTP] on the activity of the wild-type enzyme in the absence (●) and presence (○) of UTP; the influence of [CTP] on the activity of the Arg-96r→Gln enzyme in the absence (■) and presence (□) of UTP; the influence of [CTP] on the activity of the Tyr-89r→Ala enzyme in the absence (▲) and presence (△) of UTP. (B) The influence of [CTP] alone (0–4 mM) on the activity of the His-20r→Ala enzyme in the absence of UTP was determined, as well as the influence of [UTP] while CTP concentration was held constant at 4 mM. The influence of [CTP] on the activity of the His-20r→Ala enzyme in the absence (●) and presence (0–25 mM) (○) of UTP.

fector alone. The CTP/UTP synergistic inhibition of the Arg-96r→Gln and the Tyr-89r→Ala enzymes resembles the synergistic inhibition of the wild-type enzyme (Figure 4A), while the His-20r→Ala enzyme shows no synergistic inhibition by UTP in the presence of 2 mM CTP and 2 mM UTP. Since the His-20r→Ala enzyme has reduced affinity for ATP and CTP, the experiment was repeated with higher concentrations of CTP and UTP (4 mM CTP and 25 mM UTP). Even under elevated effector concentrations, no synergistic inhibition occurred for the His-20r→Ala enzyme (Figure 4B).

UTP Alone Is an Activator of the His-20r→Ala and the Tyr-89r→Ala Enzymes. UTP alone does not significantly affect the activity of the wild-type or the Arg-96r→Gln enzymes (data not shown). However, UTP can activate both the His-20r→Ala and the Tyr-89r→Ala enzymes in the absence of CTP. UTP increases the activity of the His-20r→Ala and the Tyr-89r→Ala enzymes more than 20% (Figure 5). The UTP concentrations required to reach half of the maximal activation for the His-20r→Ala and the Tyr-89r→Ala enzymes are 0.02 and 0.08 mM, respectively. These values are comparable to the affinity of the wild-type enzyme for ATP (0.1–0.2 mM). The UTP activation of both of these enzymes can easily be reversed by low concentrations of CTP (0.01 mM) (data not shown).

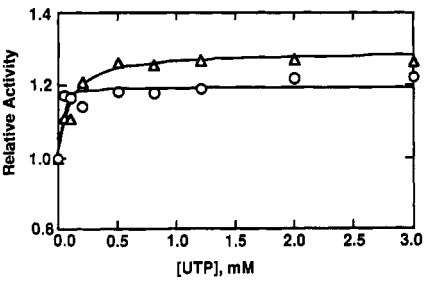

FIGURE 5: Influence of [UTP] on the activity of the Tyr-89r→Ala enzyme (△) and His-20r→Ala enzyme (○) in the absence of CTP. The assays were performed at 25 °C, in 0.1 M imidazole/acetate buffer (pH 7.0). The carbamoyl phosphate concentration was held constant at 19.2 mM, and the aspartate concentration was held constant at the $[S]_{0.5}^{Asp}$ of the respective enzyme.

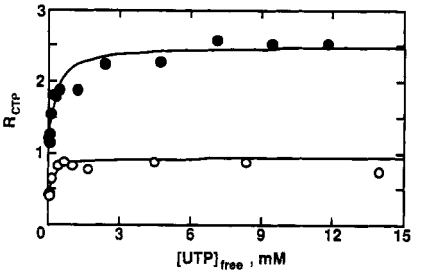

FIGURE 6: Influence of [UTP] on the binding of CTP to the wild-type (●) and the His-20r→Ala (○) enzymes. The binding of CTP, R_{CTP}, the moles of CTP bound per mole of aspartate transcarbamoylase, was measured by equilibrium dialysis as a function of free [UTP]. Equilibrium dialysis experiments were performed in 0.1 M HEPES, 2.0 mM 2-mercaptoethanol, and 0.2 mM EDTA (pH 7.0) following the protocol reported previously (Zhang & Kantrowitz, 1989). Carbamoyl phosphate was present at an equilibrium concentration of 19.2 mM to prevent nucleotide binding at the active site.

UTP Enhances the Binding of CTP to the His-20r→Ala Enzyme. As seen in Figure 4, the addition of UTP to the CTP/wild-type enzyme complex further inhibits the activity, while the addition of UTP to the CTP/His-20r→Ala enzyme complex produces no further inhibition. For the wild-type enzyme, UTP causes a reduction in the number of CTP binding sites from six to three, and a 2–3-fold increase in the affinity of the remaining three sites (Zhang & Kantrowitz, 1991). In order to determine whether UTP and CTP compete for the same site on the His-20r→Ala enzyme, and whether the binding of UTP influences the affinity of CTP, a binding competition experiment was performed. In this experiment, the binding of a constant amount of CTP was measured as a function of increasing concentrations of UTP (Figure 6). The CTP concentration was chosen to give a reasonably high value of R_{CTP} (moles of CTP bound per mole of enzyme). If CTP and UTP bind to the same site, the R_{CTP} value should decrease as the UTP concentration increases. If UTP does not bind to the same site as CTP or if it does not bind at all, then the CTP binding should remain constant. The result for the His-20r→Ala enzyme is similar to that of the wild-type enzyme; increasing concentrations of UTP cause enhanced CTP binding (increased R_{CTP}), not the expected decrease (Figure 6). This result indicates that UTP does not compete

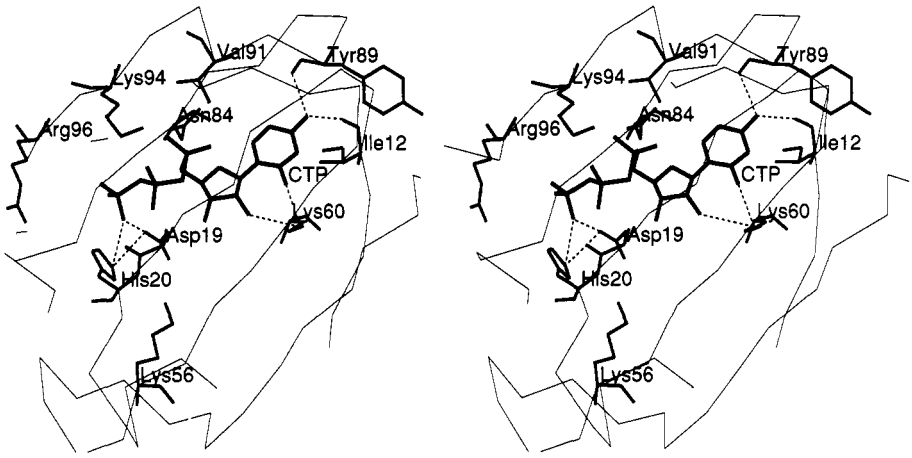

FIGURE 7: Illustration of selected side chains in the binding site of CTP. The cytidine 5′-triphosphate molecule (dark) bound to the regulatory site in chain R1 as well as nearby amino acid residues are shown. Asp-19r, Lys-60r, and Tyr-89r interact with the pyrimidine ribose moiety, whereas His-20r, Lys-56r, Lys-94r, and Arg-96r are closer to the triphosphate moiety. The data used to draw this figure are from Gouaux et al. (1990).

with CTP; rather, it enhances the binding of CTP. In addition, the lower R_{CTP} value of the His-20r→Ala enzyme compared to that of the wild-type, under identical conditions, indicates that the overall binding of CTP to the His-20r→Ala enzyme is considerably weaker than to the wild-type enzyme as expected from the kinetic study.

In the Presence of UTP, Three Relatively Strong CTP Binding Sites Exist in the His-20r→Ala Enzyme. CTP binding to the His-20r→Ala enzyme was measured by equilibrium dialysis both in the presence and in the absence of UTP. In the presence of 10 mM UTP, the binding of CTP is about 40 times weaker when compared to the binding of CTP to the wild-type enzyme under identical conditions ($K_d = 3.5 \times 10^{-5}$ M), and only three relatively strong CTP binding sites were observed. In the absence of UTP, CTP binding to His-20r→Ala enzyme is only slightly weaker than CTP binding in the presence of UTP (data not shown).

DISCUSSION

E. coli aspartate transcarbamoylase provides a useful model for allosteric regulation. Important for heterotropic regulation is the understanding of how the nucleotide effectors bind to the regulatory site, and how this binding signals the activation or inhibition of the enzyme activity at the active site some 60 Å away from the regulatory site. The effector binding site on each of the regulatory chains of aspartate transcarbamoylase has been subdivided into the ribose triphosphate subsite which binds the ribose triphosphate moiety of the nucleotide and the base subsite which binds the base moiety of the nucleotides (London & Schmidt, 1972). Previous site-specific mutagenesis studies have revealed the function of three residues in the nucleotide binding site. Lys-94r, located in the triphosphate subsite, is involved in the binding of both ATP and CTP (Zhang et al., 1988). Lys-60r, located in the area between the ribose and base subsites, is involved in the discrimination between ATP and CTP (Zhang & Kantrowitz, 1989). Asp-19r, located in the ribose triphosphate subsite, interacts with the ribose 3′-OH group of ATP and CTP, as well as the ϵ-amino group of Lys-56r (Zhang & Kantrowitz, 1991). Lys-56r, located at the beginning of the S3′ β-strand, does not directly interact with the nucleotides but is involved in both homotropic and heterotropic responses of the enzyme (Wild & Corder, 1989). In order to further understand the mode of action of the nucleotide effectors, we have constructed and analyzed three single amino acid substitutions of aspartate transcarbamoylase in the effector binding site at positions not previously investigated. These residues were selected for mutagenesis based on the available crystallographic data (Kim et al., 1987; Gouaux et al., 1990; Stevens et al., 1990). The interactions between CTP and the side chains of the wild-type enzyme are shown in Figure 7. Arg-96r[2] and His-20r are both located in the portion of the nucleotide binding site that interacts with the triphosphate moiety. Arg-96r is near the α-phosphate, and His-20r binds the γ-phosphate group of the nucleotides. Tyr-89r is located near the portion of the nucleotide binding site that interacts with the bases, and its backbone carbonyl oxygen interacts with N_{10} of ATP or N_4 of CTP. The kinetic parameters of these three mutant enzymes suggest that the amino acid substitutions at the allosteric effector domain do not substantially alter the ability of the enzyme to catalyze the transcarbamoylase reaction. However, two out of three mutant enzymes show substantial alterations in the response to the allosteric effectors, indicating that these sites are involved in the heterotropic cooperativity of the enzyme.

Arg-96r Is Not Significantly Involved in the Heterotropic Response. Kim et al. (1987) reported that Arg-96r binds to the α-phosphate group of the nucleotide effectors. On the basis of this report, we designed a substitution at this position to test the functional importance of Arg-96r. Our analysis of the Arg-96r→Gln enzyme revealed that the mutant enzyme is very much like the wild type, except for a minor alteration in the $[S]_{0.5}^{Asp}$ when both CTP and UTP are present. This small alteration in the $[S]_{0.5}^{Asp}$ suggests that the replacement of Arg-96r by Gln may cause small structural alterations in the effector binding domain. Recent refinement of the X-ray structural data at pH 7.0 indicates that neither ATP nor CTP interacts directly with Arg-96r (Gouaux et al., 1990; Stevens et al., 1990), although Arg-96r is close to the triphosphate. These recent structural studies are in agreement with our conclusion of a limited involvement of Arg-96r in the heterotropic response of aspartate transcarbamoylase.

Replacement of Tyr-89r by Ala Amplifies the ATP Activation. Although the X-ray crystal structures of aspartate transcarbamoylase in the absence and presence of ATP do not show any significant alterations in the enzyme structure, the side chain of Tyr-89r does shift position at the effector binding site when ATP is bound (Gouaux et al., 1990; Stevens et al., 1990). The replacement of Tyr-89r by alanine amplifies the ATP activation of the mutant enzyme relative to the wild-type enzyme. A possible explanation for the enhanced ATP activation of the Tyr-89r→Ala enzyme is that the position of

the side chain of Tyr-89r influences the structural conformation of the nucleotide binding site more when ATP is bound than when CTP is bound. In support of this argument, the X-ray structural data show that the backbone of residues Val-9r and Gln-10r interacts differently with ATP and CTP. These two residues move into place for interactions with ATP, but they do not interact with CTP (Gouaux et al., 1990; Stevens et al., 1990, 1991). The structural data also indicate that the binding of ATP and CTP results in different local conformations of the nucleotide binding site. The side chain of Tyr-89r moves more toward the nucleotide when ATP binds compared to its position when CTP binds [Figure 5 of Gouaux et al. (1990)]. Tyr-89r is located in the loop between the β-sheets S4′ and S5′ with Lys-13r and Arg-14r in the neighborhood, which are in the loop between β-sheet S1′ (15r–19r) and the N-terminus. The binding of ATP may be altered because the phenyl ring of Tyr-89r could influence the orientation of the backbone in the region of Lys-13r and Arg-14r. Any backbone alteration in this region would be transmitted to the flexible backbone of Val-9r and Gln-10r that directly interacts with the nucleotides. Therefore, the replacement of Tyr-89r by Ala may open the base subsite, allowing ATP to activate the mutant enzyme more than the wild-type enzyme.

Tyr-89r Is Indirectly Involved in the Discrimination between the Nucleotides. The X-ray crystallographic structure of the wild-type enzyme in the presence of phosphonoacetamide and malonate shows that when this R-state enzyme binds ATP or CTP, the largest displacement in the protein occurs in the vicinity of the base (Gouaux et al., 1990). Our results indicate that the Tyr-89→Ala enzyme has similar maximal inhibition and slightly decreased CTP affinity when compared to the wild-type enzyme. This normal CTP inhibition of the Tyr-89r→Ala enzyme implies that Tyr-89r does not play a key role in the inhibition of the enzyme by CTP. Since the amino acid replacement at position 89 mostly influences how ATP activates the enzyme activity, it suggests that Tyr-89r does have an indirect role in the discrimination between ATP and CTP.

UTP May Activate the Tyr-89r→Ala Enzyme by a Mechanism Similar to That of ATP. In addition to the enhanced ATP activation of the Tyr-89r→Ala enzyme, this mutant is also activated by UTP. The UTP affinity is also much enhanced for the Tyr-89r→Ala enzyme. This behavior is opposite from that of the wild-type enzyme, in which UTP alone inhibits slightly and binds weakly. As mentioned above, the base subsite of the effector binding region may be more open after replacement of the Tyr-89r side chain by Ala, and therefore the mutant enzyme may discriminate less efficiently between ATP and UTP. The elimination of the aromatic side chain of Tyr-89r could allow UTP to bind in a mode analogous to that of ATP, resulting in an increase in enzyme activity. The enhanced affinity of UTP for the Tyr-89r→Ala enzyme also supports this conclusion.

His-20r in the Wild-Type Enzyme Is Important for Effector Binding. The kinetic and equilibrium binding results indicate that the substitution of His-20r by Ala causes a large reduction in the affinity of the enzyme for both ATP and CTP. The reduced affinity for CTP and ATP indicates that the His-20r residue is directly involved in effector binding as suggested by its possible position in the three-dimensional structure (Gouaux et al., 1990; Stevens et al., 1990). The greatly reduced ATP affinity agrees with results for previous mutations at residues that interact directly with the triphosphate portion of the nucleotides (Zhang et al., 1988; Wild & Corder, 1989).

The CTP/UTP synergism observed in the wild-type enzyme [see Wild and Corder (1989) and Figure 4] is also diminished by the His-20r→Ala substitution. Although the loss of the CTP/UTP synergism may be due to the reduced binding affinity for CTP, no synergistic inhibition is observed even at high concentrations of CTP and UTP. CTP binding measured in the presence of UTP indicates that the affinity of CTP for the His-20r→Ala enzyme is not enhanced significantly, as is the case of the wild-type enzyme. Furthermore, the binding of CTP in the absence of UTP is significantly reduced as compared to the wild-type enzyme. These results indicate that although UTP can enhance the binding of CTP to the His-20r→Ala enzyme, the addition of UTP to the CTP–mutant enzyme complex does not result in further inhibition as is the case for the wild-type enzyme.

His-20r Is Important for the Synergistic Inhibition of the Wild-Type Enzyme by UTP in the Presence of CTP. The binding competition experiment shown in Figure 6 indicates that for the His-20r→Ala enzyme, UTP still binds to the enzyme and, furthermore, UTP still enhances the binding of CTP although the extent of the enhanced binding is much reduced compared to the wild-type enzyme. The increased binding of CTP either could result from the enzyme's increased affinity for CTP or could result from an increase in the number of binding sites. However, when the binding of CTP is directly measured in the presence of UTP for the His-20r→Ala enzyme, no detectable increase in the number of CTP binding sites is observed. Therefore, the increased binding of CTP must be due to increased CTP affinity.

Even though equilibrium dialysis experiments show that UTP still binds to the His-20r→Ala enzyme, kinetic experiments do not detect any alteration in activity when UTP is added to the enzyme–CTP complex (Figures 1 and 4B). Together, the kinetic and binding data suggest either the mutation at His-20r results in an alteration in the mode in which UTP binds or His-20r in the wild-type enzyme is involved in the transmission of the UTP binding signal to the active site. The structural data (Gouaux et al., 1990; Stevens et al., 1990) do indicate that His-20r is located in a position that may be important for transmission of the regulatory signal. His-20r not only interacts with the γ-phosphate group of the nucleotides but also interacts with adjacent residues on the regulatory chain that have no direct contacts with the effectors, but have been shown to be important for both the heterotropic and homotropic interactions of the enzyme (Wild & Corder, 1989).

UTP Activation of the His-20r→Ala Enzyme. The activation of the His-20r→Ala enzyme by UTP in the absence of CTP most likely involves UTP binding to the high-affinity nucleotide sites. The enhanced affinity of UTP in the absence of CTP supports this proposal. In the presence of CTP, the high-affinity nucleotide sites are occupied, and therefore UTP binds to the low-affinity sites. As opposed to the wild-type enzyme, the binding of UTP to the low-affinity sites does not cause any additional inhibition of the His-20r→Ala enzyme. The activation of the His-20r→Ala enzyme by UTP in the absence of CTP would occur by a mechanism analogous to the UTP activation of the Tyr-89r→Ala enzyme and the inhibition of the Asp-19r→Ala enzyme (Zhang & Kantrowitz, 1991).

Region Defined by His-20r, Asp-19r, and the 50r's Loop May Be Important for the Heterotropic Interactions of Aspartate Transcarbamoylase. The X-ray structure of the enzyme suggests that the 50's loop (residues 52–56) in the regulatory chain has a great deal of flexibility based on elevated atomic temperature factors. It has been previously suggested (Gouaux et al., 1990; Stevens et al., 1990) that the

flexibility of the 50r's loop, which is located very close to Asp-19r and His-20r, may mediate signal transduction (Gouaux et al., 1990; Stevens et al., 1990). Furthermore, Lys-56r, located at the end of the loop, interacts with both His-20r and Asp-19r, while Ser-50r, in the middle of the loop, is nearby (Stevens et al., 1990). The substitution of either His-20r or Asp-19r by alanine causes the enzyme to lose UTP/CTP synergism, while the elimination of the Lys-56r side chain also causes the enzyme to lose UTP/CTP synergism, even though it does not interact with the effectors (Wild & Corder, 1989; Zhang & Kantrowitz, 1991; Gouaux et al., 1990; Stevens et al., 1990). It would appear then that CTP/UTP synergism is very sensitive to the amino acid alterations in the general region defined by the His-20r, Asp-19r, and the 50r's loop. Any modification in this area of effector binding results in the loss of enzyme sensitivity to UTP when CTP is present. The above observations imply that this area is important for the heterotropic mechanism of aspartate transcarbamoylase. Additional studies including X-ray crystallography of the mutant enzymes and the creation of mutations at different positions of the allosteric domain of the regulatory chain will be necessary to further unravel the mechanism of heterotropic cooperativity in aspartate transcarbamoylase.

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Registry No. His, 71-00-1; Tyr, 60-18-4; ATP, 56-65-5; CTP, 65-47-4; UTP, 63-39-8; Asp, 56-84-8; aspartate transcarbamoylase, 9012-49-1.

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