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Importance of Residues Arg-167 and Gln-231 in both the Allosteric and Catalytic Mechanisms of *Escherichia coli* Aspartate Transcarbamoylase†

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ABSTRACT: Site-specific mutagenesis has been used to create two mutant versions of aspartate transcarbamoylase. Arg-167 and Gln-231, both previously identified as interacting with the portion of the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA) that corresponds to aspartate [Krause, K. L., Voltz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527-553], were replaced by glutamine and leucine, respectively. The Arg-167 → Gln and the Gln-231 → Leu enzymes show approximately 900-fold and 1500-fold reductions in the maximal observed specific activity, respectively. The aspartate concentration at half the maximal observed specific activity is increased 18-fold for the Gln-231 → Leu enzyme compared to the value for the wild-type enzyme, but is altered little in the case of the Arg-167 → Gln enzyme. The carbamoyl phosphate concentration at half the maximal activity is unchanged by either mutation, suggesting that these mutations result in only local changes to the aspartate binding site. Both mutations eliminate homotropic cooperativity; however, the Gln-231 → Leu enzyme also has altered heterotropic interactions and no longer exhibits substrate inhibition. At relatively low concentrations of aspartate and saturating carbamoyl phosphate, PALA is able to activate the Gln-231 → Leu enzyme, whereas the Arg-167 → Gln enzyme is inhibited at PALA concentrations that normally activate the wild-type enzyme. Equilibrium binding measurements indicate that the Gln-231 → Leu enzyme binds CTP approximately 10-fold more weakly than the wild-type enzyme, even though the mutation is some 70 Å from the regulatory binding site. The binding of carbamoyl phosphate, PALA, and succinate to the Gln-231 → Leu catalytic subunit indicates poor binding for PALA and succinate but normal binding for carbamoyl phosphate. For the Arg-167 → Gln enzyme, there is very little alteration in the binding of these substrates and substrate analogues as compared to the wild-type enzyme. These results indicate that Gln-231 in the wild-type enzyme is critical for the binding of aspartate both in productive and in nonproductive orientations. Gln-231 through its interactions with aspartate and the side chain of Arg-234 is important for both homotropic cooperativity and heterotropic interactions. The function of Gln-231 and Arg-234 in the allosteric and catalytic mechanisms is also discussed.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the committed step of the pyrimidine biosynthesis pathway, the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate. The enzyme exhibits positive cooperativity for both substrates (Bethell et al., 1968; Gerhart & Pardee, 1962), and its activity is feedback-inhibited by CTP and UTP, the end products of the pyrimidine biosynthesis pathway, and is activated by ATP, an end product of the purine biosynthesis pathway (Gerhart & Pardee, 1962; Wild et al., 1989).

The holoenzyme[1] is composed of six catalytic chains (M_r 33 000), which are grouped into two catalytic trimers (i.e., catalytic subunits), and six regulatory chains (M_r 17 000),

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[1] Abbreviations: PALA, *N*-(phosphonoacetyl)-L-aspartate; CP, carbamoyl phosphate; T and R states, tight and relaxed states of the enzyme having low and high affinity, respectively, for the substrate; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; $[S]_{0.5}^{CP}$, carbamoyl phosphate concentration at half the maximal observed specific activity; holoenzyme, entire aspartate transcarbamoylase molecule composed of two catalytic subunits and three regulatory subunits.

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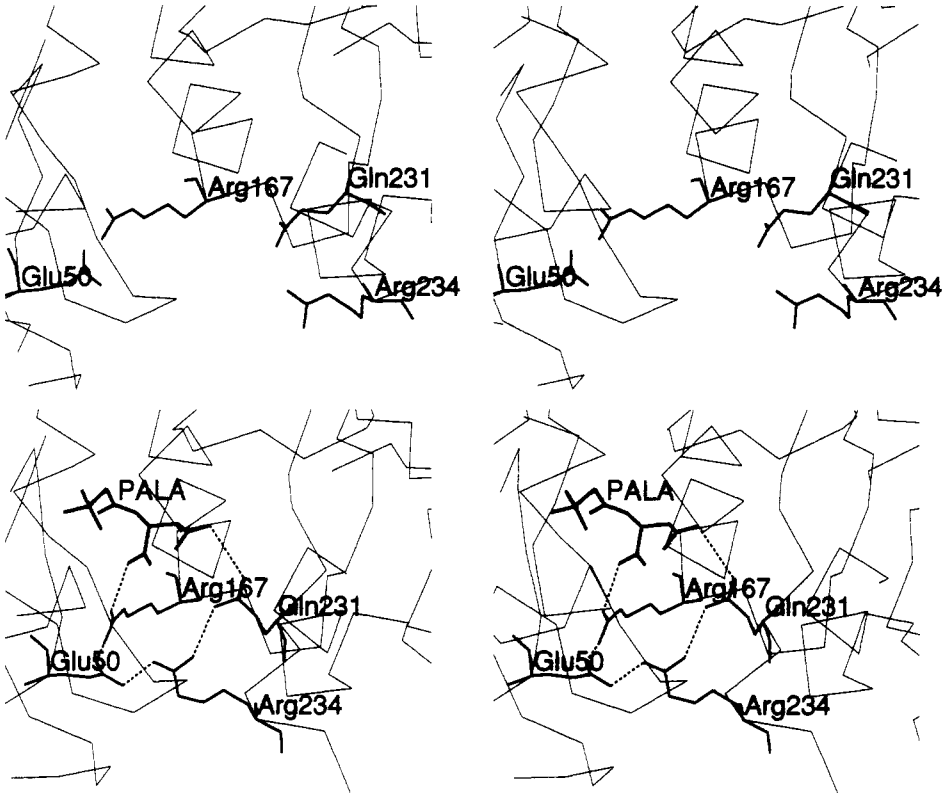

FIGURE 1: Stereoview of the region of the active site around Glu-50, Arg-167, Gln-231, and Arg-234 in the T (top) and R (bottom) states. The amino acid side chains and PALA are shown in bold type. In the T state, the active site is open as the 240s loop residues Gln-231 and Arg-234 are positioned out of the active site. In the R state, the active-site pocket (indicated by PALA) is closed as Arg-167 interacts with the α-carboxylate of PALA and the side chain of Glu-50. Gln-231 interacts with the β-carboxylate of PALA and the side chain of Arg-234, and the side chains of Glu-50 and Arg-234 form an interdomain bridging interaction between the carbamoyl phosphate and aspartate domains. Data for this diagram were from Kim et al. (1987) and Krause et al. (1987).

which are grouped into three regulatory dimers (i.e., regulatory subunits). The holoenzyme dissociates into the catalytic and regulatory subunits upon treatment with heat or mercurials; these can be separated by ion-exchange chromatography. The regulatory subunit binds UTP, CTP, and ATP but is devoid of catalytic activity, while the isolated catalytic subunit exhibits no homotropic cooperativity and is insensitive to the allosteric effectors. The active sites, three per catalytic subunit, are shared between adjacent catalytic chains within the trimer (Monaco et al., 1978; Krause et al., 1985; Robey & Schachman, 1985; Wente & Schachman, 1987). The biochemical properties of aspartate transcarbamoylase have been extensively reviewed (Allewell, 1989; Kantrowitz & Lipscomb, 1988; Schachman, 1974, 1988; Kantrowitz et al., 1980a,b; Jacobson & Stark, 1973; Gerhart, 1970).

The catalytic reaction follows an ordered mechanism with the binding of carbamoyl phosphate first (Porter et al., 1969; Wedler & Gasser, 1974; Hsuanyu & Wedler, 1987), inducing a conformational change that allows the binding of aspartate (Collins & Stark, 1969; Griffin et al., 1972; Stebbins et al., 1989). NMR data suggest that the carbonyl oxygen of carbamoyl phosphate is hydrogen bonded to a group on the enzyme, or is protonated by that group, activating the carbonyl carbon for nucleophilic attack by the amino group of aspartate (Roberts et al., 1976). Also a functional group in the enzyme–substrate complex has been identified with a pK_a of 7.2 that is involved in catalysis (Leger & Hervé, 1988). Gouaux et al. (1988) have proposed a mechanism involving a tetrahedral intermediate about the carbonyl carbon which decomposes upon intramolecular proton transfer between the amino group of aspartate and the leaving phosphate group.

Information concerning the specific groups on the enzyme which interact with aspartate has come from the analysis of X-ray structures of the enzyme with either PALA (Krause et al., 1987) or carbamoyl phosphate plus succinate bound (Gouaux & Lipscomb, 1988). The residues which interact with aspartate[2] are Arg-105, Arg-167, Arg-229, and Gln-231 from one catalytic chain plus Lys-84 from the adjacent catalytic chain. Site-specific mutagenesis has been used to determine if these interactions are functionally important. For example, Arg-105 and Arg-229 have been replaced by alanine. These substitutions cause a 1000-fold or greater reduction in activity, an increase in the substrate concentration at half the maximal velocity, and also a decrease in affinity for PALA (Stebbins et al., 1989; Middleton et al., 1989). Lys-84 has been replaced by both Gln and Arg (Robey et al., 1986). Neither substitution affects the aspartate affinity, but both mutations cause more than a 1000-fold loss of activity.

The X-ray structure of the PALA–enzyme complex (Krause et al., 1987) and the structure with carbamoyl phosphate and succinate bound (Gouaux & Lipscomb, 1988) suggest that Arg-167 may interact with the α-carboxylate of aspartate and Gln-231 may interact with the β-carboxylate of aspartate (see Figure 1). To determine the function of residues Arg-167 and Gln-231 in the aspartate binding site of aspartate transcarbamoylase, two mutant versions of the enzyme were constructed with glutamine and leucine substituted for Arg-167 and Gln-231, respectively. These mutations at positions 167 and 231 should allow us to further investigate the region of the enzyme near the aspartate binding site and determine if these residues are important for catalysis, cooperativity, or heterotropic interactions.

[2] The interactions with aspartate are based upon analogy with the structures of the enzyme with either PALA (Krause et al., 1987) or carbamoyl phosphate and succinate bound (Gouaux & Lipscomb, 1988).

Materials and Methods

Materials

CTP and ATP were purchased from Sigma Chemical Co., while [^{3}H]CTP was purchased from NEN Research Products. All other materials are as previously described (Stebbins et al., 1989).

Methods

Site-Specific Mutagenesis. The introduction of specific base changes in the *pyrB* gene to create altered versions of aspartate transcarbamoylase was accomplished by site-specific mutagenesis (Zoller & Smith, 1982), with the modifications previously described (Carter et al., 1985; Ladjimi et al., 1988). In the case of the Arg-167 → Gln[3] mutation, candidates were screened and identified by dot-blot hybridization (Carter et al., 1984). Single-stranded DNA from candidates showing dark spots was then isolated and sequenced by the dideoxy method (Sanger et al., 1977). In the case of the Gln-231 → Leu mutation, single-stranded DNA was isolated from 20 mutant candidates and sequenced directly by the dideoxy method, and 2 of these had the DNA sequence corresponding to the mutation.

In each case, after verification of the mutations, a small fragment of the gene was removed with restriction enzymes and inserted into a plasmid which had the corresponding section of the wild-type gene removed. For both mutants, the *Bst*EII to *Bgl*II fragment of 633 base pairs containing the desired mutation was isolated from the purified M13 RF after agarose gel electrophoresis, with NA45 paper. In addition, the plasmid pEK54 (Xu et al., 1988) was cut with the same two restriction enzymes, and the larger fragment was isolated in a similar fashion. The fragment, containing the remainder of the *pyrBI* operon, was combined with the fragment from the mutant M13 RF and treated with T4 DNA ligase. Selection was accomplished after transformation in U39a [F$^-$ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *rspL*], a strain which has a deletion in the *pyrBI* region.

CTP Binding Measurements. The binding of CTP to the Gln-231 → Leu enzyme was determined by the technique of equilibrium dialysis using Spectra/Pro-2 (Spectrum Medical Industries) dialysis tubing, which was pretreated as previously described (Jacobsberg et al., 1975). Dialysis experiments were carried out in microdialysis cells which hold 50 μL on each side of the dialysis membrane. 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 CTP was determined by liquid scintillation employing a LKB 1217 Rackbeta liquid scintillation counter. Complete equilibration was confirmed under the experimental conditions. Equilibrium dialysis experiments were preformed in 0.1 M imidazole acetate buffer, 0.2 m EDTA, and 2 mM 2-mercaptoethanol, pH 7, and the enzyme was dialyzed into this buffer before use.

Data Analysis. The analysis of the steady-state kinetic data was carried out as previously described (Silver et al., 1983). Data points were fit by a nonlinear least-squares procedure to either the Hill equation or the Michaelis–Menten equation, incorporating a term for substrate inhibition when necessary (Pastra-Landis et al., 1978). The analysis of the structural data, based on the three-dimensional coordinates of the PALA–enzyme complex (Krause et al., 1987), the enzyme with carbamoyl phosphate and succinate bound (Gouaux & Lipscomb, 1988), and the CTP–enzyme complex (Kim et al., 1987), was accomplished by using the program FRODO (Department of Biochemistry, Rice University) on an Evans & Sutherland PS390 interfaced to a MicroVAX Q5.

Other Methods. Oligonucleotide synthesis, enzyme purification, determination of protein concentration, and the assays for aspartate transcarbamoylase were as previously described (Stebbins et al., 1989).

[3] The notation used to name the mutant enzymes is, for example, the Arg-167 → Gln enzyme. The wild-type amino acid and 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.

Table I: Kinetic Parameters for Wild-Type and Mutant Holoenzymes[a]

enzyme	maximal velocity[b]	$[S]_{0.5}^{Asp}$ (mM)	n_H^{Asp}	$[S]_{0.5}^{CP}$ (mM)	n_H^{CP}
wild-type	17.2	11.8	2.2	0.21	2.0
Arg-167 → Gln	0.019	9.5	1.0	0.38	1.0
Gln-231 → Leu	0.011	216.0	1.0	0.11	1.0

[a] These data were determined from either the aspartate or the carbamoyl phosphate saturation curves. Colorimetric assays were performed in 0.05 M Tris–acetate buffer (pH 8.3), holding the concentration of the nonvaried substrate constant at saturating levels. [b] The maximal velocity represents the maximal observed specific activity of the aspartate saturation curves.

Results

Construction of the Mutant Aspartate Transcarbamoylases by Site-Specific Mutagenesis. The Arg-167 → Gln and the Gln-231 → Leu mutant versions of aspartate transcarbamoylase were constructed by site-specific mutagenesis employing the method of Zoller and Smith (1982) using strains incapable of mismatch repair (Carter et al., 1984) (see also Materials and Methods).

Saturation Kinetics of the Wild-Type and Mutant Holoenzymes. Both the Arg-167 → Gln and the Gln-231 → Leu holoenzymes have decreased maximal observed specific activity as compared to the wild-type enzyme. As seen in Table I, the Arg-167 → Gln and the Gln-231 → Leu enzymes show approximately 900-fold and 1500-fold reductions in the maximal observed specific activity, respectively. The aspartate concentration at half the maximal observed specific activity, $[S]_{0.5}^{Asp}$, of the Gln-231 → Leu enzyme is increased dramatically to 220 mM whereas the $[S]_{0.5}^{Asp}$ for the Arg-167 → Gln enzyme is 9.5 mM, close to the wild-type value of 11.2 mM. The mutant and wild-type enzymes have similar carbamoyl phosphate concentrations at half the maximal observed specific activity, $[S]_{0.5}^{CP}$ (Table I); however, the mutant enzymes are not cooperative for either aspartate or carbamoyl phosphate as indicated by their hyperbolic saturation curves (Figure 2). Finally, as seen in the aspartate saturation curves of Figure 2, significant substrate inhibition occurs for both the wild-type and the Arg-167 → Gln enzymes while no substrate inhibition is detectable for the Gln-231 → Leu enzyme.

Effects of PALA on the Mutant and Wild-Type Holoenzymes. Both the wild-type and the Gln-231 → Leu enzymes are activated by PALA, whereas the Arg-167 → Gln enzyme is inhibited at PALA concentrations that normally activate the wild-type enzyme (Figure 3). However, 10 mM PALA is required to activate the Gln-231 → Leu enzyme, approximately a 2500-fold increase over the value for the wild-type enzyme. These results are consistent with weak binding of PALA to the Gln-231 → Leu enzyme and strong binding of PALA to the Arg-167 → Gln enzyme.

Influence of ATP and CTP on the Mutant and Wild-Type Holoenzymes. As seen in Figure 4, both the mutant and wild-type enzymes are activated and inhibited by ATP and CTP, respectively. However, the concentration of effector at which the Gln-231 → Leu enzyme is half-activated, $K_{0.5}^{ATP}$, or

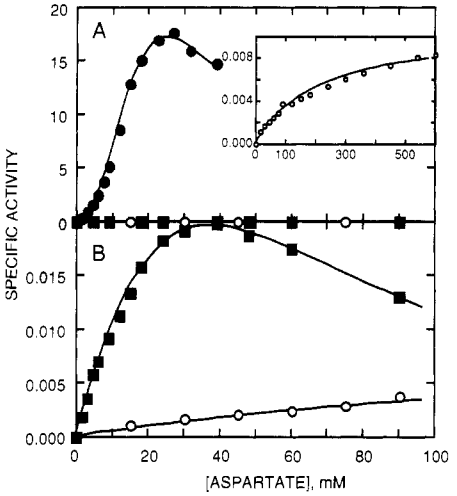

FIGURE 2: Aspartate saturation curves of the wild-type and mutant aspartate transcarbamoylases. Specific activity is reported in millimoles of *N*-carbamoyl-L-aspartate formed per hour per milligram of protein. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) at saturating concentrations of carbamoyl phosphate (4.8 mM). All data points are averages of duplicates. (A) Saturation curves for the wild-type (●), the Arg-167 → Gln (■), and the Gln-231 → Leu (○) enzymes. (Inset) Region corresponding to high aspartate concentrations and low specific activity for the Gln-231 → Leu enzyme (○). (B) Detail of low specific activity data for the Arg-167 → Gln (■) and Gln-231 → Leu (○) enzymes.

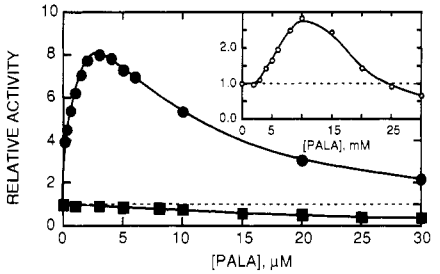

FIGURE 3: Influence of PALA concentration on the activity of the wild-type (●) and Arg-167 → Gln (○) holoenzymes. (Inset) Activation of the Gln-231 → Leu (○) holoenzyme by high concentrations of PALA. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) at saturating concentrations of carbamoyl phosphate (4.8 mM) and subsaturating concentrations of aspartate (1, 1.5, and 18 mM for the wild-type, the Arg-167 → Gln, and the Gln-231 → Leu enzymes, respectively). Relative activity represents the ratio of activity in the absence or presence of PALA to that in the absence.

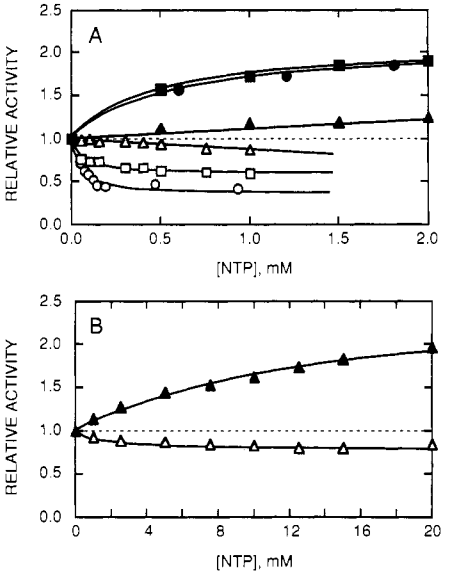

FIGURE 4: Influence of ATP and CTP concentrations on the activity of the wild-type and mutant holoenzymes. Colorimetric assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) in the absence or presence of increasing concentrations of either ATP or CTP (NTP). The aspartate concentration was held constant at the $[S]_{0.5}^{Asp}$ of the respective enzyme, and the carbamoyl phosphate concentration was saturating (4.8 mM). (A) The wild-type enzyme in the presence of ATP (●) and CTP (○); the Arg-167 → Gln enzyme in the presence of ATP (■) and CTP (□); the Gln-231 → Leu enzyme in the presence of ATP (▲) and CTP (△). (B) The effect of high concentrations of ATP (▲) and CTP (△) on the Gln-231 → Leu enzyme. For this experiment, the carbamoyl phosphate concentration was increased to 15 mM to prevent nucleotide binding at the active site.

Table II: Kinetic Parameters for Wild-Type and Mutant Catalytic Subunits[a]

enzyme	maximal velocity[b]	K_m^{Asp} (mM)	K_m^{CP} (mM)
wild-type	22.3	5.7	0.027
Arg-167 → Gln	0.14	3.73	0.022
Gln-231 → Leu	0.036	156.0	0.032

[a] These data were determined from either the aspartate or the carbamoyl phosphate saturation curves. Colorimetric assays were performed in 0.05 M Tris–acetate buffer (pH 8.3), holding the concentration of the nonvaried substrate constant at saturating levels. [b] The maximal velocity represents the maximal observed specific activity of the aspartate saturation curves.

inhibited, $K_{0.5}^{CTP}$, is dramatically increased to 17 and 2 mM, respectively, compared to 0.6 and 0.04 mM, respectively, for the wild-type enzyme. In the case of the Arg-167 → Gln enzyme, $K_{0.5}^{ATP}$ and $K_{0.5}^{CTP}$ are unaltered as compared to the values for the wild-type enzyme. These results are consistent with a decrease in affinity for ATP and CTP for the Gln-231 → Leu enzyme. Furthermore, the Gln-231 → Leu enzyme displays only a 20% decrease in activity at saturating concentrations of CTP whereas the activity of the wild-type enzyme decreases by 60% at saturating concentrations of CTP.

CTP Binding to the Gln-231 → Leu and the Wild-Type Holoenzyme. In order to directly determine if the binding at the regulatory site had been altered by the Gln-231 → Leu mutation, the binding of CTP was measured by equilibrium dialysis. As seen in Figure 4, the affinity of the Gln-231 → Leu enzyme for CTP is reduced by approximately 10-fold as compared to the wild-type enzyme.

Saturation Kinetics of the Mutant and Wild-Type Catalytic Subunits. As seen in Table II, the Arg-167 → Gln and the Gln-231 → Leu enzymes show approximately 160-fold and 600-fold reductions in the maximal observed specific activity, respectively. However, compared to their holoenzymes, the Arg-167 → Gln and the Gln-231 → Leu catalytic subunits show approximately a 7-fold and 3-fold increase in the maximal observed specific activity, respectively, whereas the wild-type catalytic subunit shows only an approximately 40% increase in activity over its holoenzyme (see Tables I and II). The K_m^{Asp} of the Gln-231 → Leu catalytic subunit is substantially increased to 160 mM as compared to the wild-type value of 5.7 mM, whereas the K_m^{Asp} of the Arg-167 → Gln catalytic subunit is 3.7 mM, similar to the values for the wild-type enzyme. The K_m^{CP} values of both mutant catalytic subunits are unaltered by the amino acid substitution. As was the case for the holoenzymes, there is significant substrate inhibition in the aspartate saturation curves of the wild-type and the Arg-167 → Gln catalytic subunits but no detectable substrate inhibition for the Gln-231 → Leu catalytic subunit (data not shown).

Binding of Carbamoyl Phosphate, PALA, and Succinate to the Mutant and Wild-Type Catalytic Subunits. To examine

Table III: Affinity Parameters for Wild-Type and Mutant Catalytic Subunits

enzyme	K_D for carbamoyl phosphate[a] (mM)	K_i for PALA[b] (μM)	K_i for succinate[c] (μM)
wild-type	0.022	0.024	0.21
Arg-167 → Gln	0.025	0.014	0.43
Gln-231 → Leu	0.0051	7.5	270.0

[a] The K_D for carbamoyl phosphate was determined kinetically by the method of Porter et al. (1969). Because of the low activity of the mutant enzymes, the K_D for all the catalytic subunits was determined at $0.2K_m^{Asp}$. [b] The K_i for PALA was determined kinetically by the method of Collins and Stark (1971). [c] The K_i for succinate was determined kinetically by the method of Porter et al. (1969).

the alterations in the binding of substrates and substrate analogues due to these single amino acid substitutions, the K_i values of PALA and succinate as well as the K_D^{CP} were determined for the wild-type and mutant catalytic subunits. As seen in Table III, the K_D^{CP} of the Gln-231 → Leu catalytic subunit is 5 μM compared to 22 μM for the wild-type catalytic subunit; in contrast, the K_D^{CP} for the Arg-167 → Gln catalytic subunit is not significantly altered. The K_i of PALA and the K_i of succinate for the Gln-231 → Leu catalytic subunit are dramatically increased to 7.5 μM and 270 mM, respectively, as compared to 0.024 μM and 0.21 mM for the wild-type catalytic subunit. In the case of the Arg-167 → Gln catalytic subunit, the K_i for PALA decreases by approximately 2-fold while the K_i of succinate increases by approximately 2-fold as compared to the corresponding values for the wild-type catalytic subunit (Table III).

pH Dependence of the Mutant and Catalytic Subunits. The profiles of activity versus pH for the wild-type and mutant catalytic subunits were determined (data not shown). The maxima for the Arg-167 → Gln and the Gln-231 → Leu mutant catalytic subunits shifted to 8.9 and 8.3, respectively, as compared to 8.1 for the wild-type enzyme.

Discussion

Glutamine-231 Is Critical for the Binding of Aspartate. When Gln-231 in the catalytic chain of aspartate transcarbamoylase is replaced by leucine, the $[S]_{0.5}^{Asp}$ values of the resulting mutant holoenzyme and catalytic subunit increase 20- and 30-fold, respectively; however, the $[S]_{0.5}^{CP}$ values of the mutant holoenzyme and catalytic subunit are unaltered, as compared to the values for the wild-type enzyme. These results suggest that the Gln-231 → Leu enzyme has low affinity for aspartate, and are consistent with the weak binding of PALA to the Gln-231 → Leu holoenzyme, and the absence of substrate inhibition. Furthermore, the K_i of the substrate analogues succinate and PALA for the mutant catalytic subunit also increases significantly whereas the dissociation constant of carbamoyl phosphate is unaltered as compared to the wild-type enzyme. From the analysis of X-ray crystallographic data of the holoenzyme bound with either carbamoyl phosphate and succinate (Gouaux & Lipscomb, 1988) or PALA (Krause et al., 1987), it is proposed that Gln-231 interacts with the β-carboxylate of aspartate. The results presented above establish that Gln-231 is critical for the binding of aspartate. Furthermore, the data also indicate that Gln-231 in the wild-type enzyme is involved in the binding of aspartate in a nonproductive orientation that results in substrate inhibition.

Both Arg-167 and Gln-231 Are Important for Catalysis. When Arg-167 is replaced by glutamine or Glu-231 is replaced by leucine, the maximal observed specific activity of the resulting mutant enzymes decreases by 900-fold and 1500-fold, respectively. However, the Arg-167 → Gln mutation has only a small effect on aspartate affinity whereas the Gln-231 → Leu mutation has a dramatic effect on aspartate affinity. In the crystal structures of the holoenzyme either with carbamoyl phosphate and succinate bound (Gouaux & Lipscomb, 1988) or with PALA bound (Krause et al., 1987), Arg-167 interacts with the α-carboxylates of either succinate or PALA (Figure 1). Since, these structures are thought to more closely resemble the transition state, they may not be a good representation of the unliganded high-affinity state of the enzyme (Gouaux & Lipscomb, 1988). Thus, Arg-167 may be involved in the binding of the transition state or in product release rather than the binding of aspartate. On the other hand, Gln-231 is most likely involved in the binding of aspartate and orientating it correctly for catalysis.

Insights into the Catalytic Mechanism. Previous experiments utilizing site-specific mutagenesis have determined that the following active-site residues are essential for catalysis: Arg-54, Lys-84, Arg-105, and Arg-229 (Stebbins et al., 1989; Robey et al., 1986; Middleton et al., 1989). Since there is no covalent enzyme–substrate intermediate (Porter et al., 1969), these positively charged residues must be involved in ionic interactions in the enzyme–substrate complex that are essential for the transformation to products. Arg-54, which interacts with a terminal oxygen and the mixed anhydride oxygen of carbamoyl phosphate, is proposed to stabilize the phosphate leaving group (Stebbins et al., 1989). Arg-105, which interacts with the carbonyl oxygen of carbamoyl phosphate, polarizes the carbonyl group of carbamoyl phosphate, thereby facilitating nucleophilic attack by the amino group of aspartate (Stebbins et al., 1989). Both Lys-84 and Arg-105 interact with a phosphate oxygen of carbamoyl phosphate and the α-carboxylate of aspartate, and may be involved in bringing the two substrates into close proximity for reaction (Stebbins et al., 1989; Robey et al., 1986). Arg-229, which is exclusive to the aspartate binding site and interacts with the β-carboxylate of aspartate, is most likely involved in the binding and stabilization of the transition state (Middleton et al., 1989).

From the results of site-specific mutagenesis experiments, active-site residues can be divided into three classes: first, those that affect the affinity of substrates only; second, those that affect catalysis only; and third, those that affect both catalysis and the affinity of the substrates. Identification of the residues critical for catalysis (Arg-54, Lsy-84, Arg-105, Arg-167, Arg-229, and Gln-231) suggests that rate enhancement of the enzyme relies on the orientation and stabilization of substrates, intermediates, and products rather than the actual bond breaking and/or bond formation [for a review, see Jencks (1987)].

During catalysis, the amino group of aspartate must lose at least one proton. Studies of the isolated catalytic subunit have indicated a functional group in the enzyme–substrate complex with a pK_a of 7.2 that is involved in catalysis, which must be deprotonated to ensure maximal activity. This group is a likely candidate to act as the general base (Leger & Hervé, 1988). Although there has been much speculation about His-134, its pK_a has been recently determined to be less than 6 (Kleanthous et al., 1988). Furthermore, when His-134 is replaced by alanine by site-specific mutagenesis, the resulting mutant enzyme has considerable catalytic activity (Robey et al., 1986). Gouaux et al. (1987) have proposed a mechanism involving intramolecular proton transfer. In their mechanism, the carbonyl carbon of carbamoyl phosphate forms a tetrahedral intermediate, which collapses upon the transfer of a proton from the amino group of aspartate to the phosphate

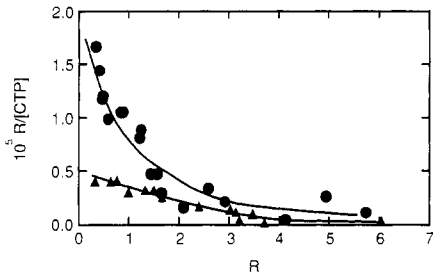

FIGURE 5: Binding of CTP to the wild-type (●) and the Gln-231 → Leu (▲) enzymes. Equilibrium dialysis experiments were performed at 25 °C for 18–20 h in 0.1 M imidazole acetate buffer, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.0. The binding data are represented as a Scatchard plot (Scatchard, 1949), $R/[CTP]$ versus R, where R is the number of moles of CTP bound per mole of aspartate transcarbamoylase (M_r 310 000) and [CTP] is the concentration of free CTP. The binding experiments were carried out in the presence of an equilibrium concentration of 15 mM carbamoyl phosphate to prevent CTP binding at the active sites (Matsumoto & Hammes, 1973).

leaving group. The results presented above are in support of this mechanism as no catalytically essential residue is a likely candidate to act as a proton acceptor.

Gln-231 Is Involved in Homotropic Cooperativity and Heterotropic Interactions. The substitution of leucine at position 231 causes a dramatic decrease in affinity for substrates and substrate analogues and a loss of cooperativity. As the R state of the wild-type enzyme has high affinity for the substrates (Newell et al., 1989) and cooperativity is the manifestation of the shift in the T ⇌ R equilibrium toward R (Monod et al., 1965), these results indicate that Gln-231 is important for the stabilization of the R state and for homotropic cooperativity. How does Gln-231 function to stabilize the R state of aspartate transcarbamoylase and thus promote homotropic cooperativity? Analysis of the X-ray structures of the enzyme with PALA bound (Krause et al., 1987) or in the presence of both carbamoyl phosphate and succinate (Gouaux & Lipscomb, 1988) suggests that Gln-231 interacts with β-carboxylate of aspartate and with Arg-234 (see Figure 1). The loss of aspartate affinity upon the Gln-231 → Leu substitution indicates that Gln-231 does interact with aspartate in the wild-type enzyme and this interaction is critical for aspartate binding. Furthermore, the interaction between Gln-231 and Arg-234 may be involved in cooperativity since it has previously been established that the movement of the 240s loop and the formation of the interdomain bridging interactions between Glu-50 and Arg-234 are important for the formation of the high-affinity high-activity state of the enzyme (Ladjimi et al., 1988; Newton & Kantrowitz, 1990; Middleton & Kantrowitz, 1988). When this interdomain bridging interaction is lost, cooperativity is abolished. Thus, the interaction between Gln-231 and Arg-234 may be involved in domain closure by positioning Arg-234 correctly to interaction with Glu-50.

The kinetic properties of the Gln-231 → Leu enzyme are similar to the results of previous experiments using the antibiotic L-alanosine as an alternative substrate for aspartate transcarbamoylase. L-Alanosine, first found to inhibit the enzyme by its competition with L-aspartate (Gale et al., 1968), is also carbamoylated by the enzyme (Baillon et al., 1985). When L-alanosine is used as a substrate, the wild-type enzyme displays Michaelis–Menten saturation kinetics with high concentrations of L-alanosine required for half-saturation as compared to that for aspartate. The only difference between L-alanosine and aspartate is that L-alanosine has a *N*-nitrosohydroxylamino group instead of a carboxylate group at the β-position. The loss of cooperativity in the L-alanosine saturation curves confirms that the link between the β-carboxylate of aspartate and Gln-231 is important for cooperativity.

Why is the Gln-231 → Leu enzyme still activated by PALA? The kinetic activation by PALA of the Gln-231 → Leu enzyme suggests that homotropic allosteric effects can still occur; however, the mutant enzyme does not display kinetic cooperativity for either substrate. This rate enhancement may be due to the stabilization by PALA of a higher activity form of the mutant enzyme than can be stabilized by the substrates. Since PALA binds to both the carbamoyl phosphate and aspartate domains (Krause et al., 1987), it may cause a conformational change thereby stabilizing the enzyme in a more active form.

The active-site mutation at position 231 of the catalytic chain also causes an alteration in heterotropic regulation of the mutant enzyme. Although the mutation is approximately 70 Å from the nucleotide binding site, there is a decrease in affinity for both ATP and CTP in the Gln-231 → Leu enzyme as compared to the wild-type enzyme (Figures 4 and 5). Furthermore, the inhibition of the mutant enzyme at saturating concentrations of CTP is only 20% compared to 60% for the wild-type enzyme (Figure 4). The decrease in affinity for ATP and the decrease in inhibitory effects of CTP indicate a shift in the T ⇌ R equilibrium toward T and are consistent with the alterations of the homotropic properties of the enzyme.

The analysis of X-ray crystallographic data suggests that Gln-231 interacts with the β-carboxylate of aspartate and the side chain of Arg-234. Which of these two interactions are important for heterotropic regulation? The studies of the wild-type enzyme using L-alanosine instead of aspartate as substrate, mentioned above, show a normal heterotropic response to both ATP and CTP (Baillon et al., 1985). However, the Arg-234 → Ser enzyme exhibits substantially altered heterotropic properties (Middleton & Kantrowitz, 1988). These results imply that the interaction between the side chains of Gln-231 and Arg-234, which has been implicated as being important for domain closure, may also be involved heterotropic regulation.

The closure of the two catalytic chain domains and the movement of the 240s loop have been previously reported to be important for homotropic cooperativity in aspartate transcarbamoylase by alterating the affinity and activity of the active site for aspartate (Middleton & Kantrowitz, 1986; Ladjimi & Kantrowitz, 1988). Here we have identified the side chain of Gln-231 and its interaction with Arg-234, of the 240s loop, as critical for creation of the high-affinity aspartate binding site. These results emphasize the importance of the movement of the 240s loop, that repositions side chains of the loop to interact and position active-site residues thereby modulating the activity of the enzyme, as the molecular basis not only of homotropic cooperativity but also of the heterotropic interactions in aspartate transcarbamoylase as well.

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