Importance of a Conserved Residue, Aspartate-162, for the Function of *Escherichia coli* Aspartate Transcarbamoylase†

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ABSTRACT: Aspartate-162 in the catalytic chain of aspartate transcarbamoylase is conserved in all of the sequences determined to date. The X-ray structure of the *Escherichia coli* enzyme indicates that this residue is located in a loop region (160's loop) that is near the interface between two catalytic trimers and is also close to the active site. In order to test whether this conserved residue is important for support of the internal architecture of the enzyme and/or involved in transmitting homotropic and heterotropic effects, the function of this residue was studied using a mutant version of the enzyme with an alanine at this position (Asp-162→Ala) created by site-specific mutagenesis. The Asp-162→Ala enzyme exhibits a 400-fold reduction in the maximal observed specific activity, approximately 2-fold and 10-fold decreases in the aspartate and carbamoyl phosphate concentrations at half the maximal observed specific activity respectively, a loss of homotropic cooperativity, and loss of response to the regulatory nucleotides ATP and CTP. Furthermore, equilibrium binding studies indicate that the affinity of the mutant enzyme for CTP is reduced more than 10-fold. The isolated catalytic subunit exhibits a 660-fold reduction in maximal observed specific activity compared to the wild-type catalytic subunit. The K_m values for aspartate and carbamoyl phosphate for the Asp-162→Ala catalytic subunit were within 2-fold of the values observed for the wild-type catalytic subunit. Computer simulations of the energy-minimized mutant enzyme indicate that the space once occupied by the side chain of Asp-162 may be filled by other side chains, suggesting that Asp-162 is important for stabilizing the internal architecture of the wild-type enzyme.

Aspartate transcarbamoylase (EC 2.1.3.2) from *Escherichia coli* was one of the first discovered allosteric enzymes (Yates & Pardee, 1956) and because of its many interesting properties has been the subject of intensive investigation. The challenge exists to explain its catalytic and regulatory properties in molecular and mechanistic terms. The enzyme catalyzes the committed step in pyrimidine biosynthesis, the reaction between carbamoyl phosphate and L-aspartate to form *N*-carbamoyl L-aspartate and inorganic phosphate. Besides showing homotropic cooperativity with both substrates (Gerhart & Pardee, 1962; Bethell et al., 1968), the enzyme is activated by the end product of the purine pathway, ATP (Gerhart & Pardee, 1962), and inhibited by end products of the pyrimidine pathway, CTP (Gerhart & Pardee, 1962) and UTP (in the presence of CTP) (Wild et al., 1989).

Structurally, the holoenzyme (M_r 310 000) consists of two catalytic subunits (M_r 100 000) which are trimers and three regulatory subunits (M_r 34 000) which are dimers. The X-ray structure of the enzyme in the absence of ligands (Ke et al., 1984) is similar to that in the presence of CTP (Kim et al., 1987), and this structure is considered to correlate with the T functional state. The X-ray structure in the presence of the transition-state analogue PALA[1] (Krause et al., 1987; Ke et al., 1988) is considered to correlate with the R functional state. Differences between the T and R states includes an expansion by 10.8 Å along the molecular 3-fold axes, a 12° rotation of the two catalytic subunits relative to one another, and a 15° rotation of the regulatory subunits about their respective 2-fold axes. At the tertiary level there is a closure of the gap between the carbamoyl phosphate and aspartate domains (Kim et al., 1987; Krause et al., 1987; Ke et al., 1988). The 240's loop undergoes a major change in position between the T and R states, and several mutations made in this portion of the polypeptide chain have shown that the region is very important for the normal catalytic, homotropic cooperative, and allosteric properties of the enzyme (Middleton & Kantrowitz, 1986, 1988; Ladjimi & Kantrowitz, 1988; Middleton et al., 1989; Stebbins et al., 1990; Newton & Kantrowitz, 1990b).

Analysis of the X-ray structure of *E. coli* aspartate transcarbamoylase has revealed a loop region (161–166) near the 240's loop (C1–C4 interface[2]) and the active site. The 160's loop structurally supports the internal architecture of the enzyme and may be important in transmitting the homotropic and heterotropic effects. Residues 161–166 form a loop connecting β-strand S6 and α-helix H6. Specifically, Asp-162 makes contacts with residues Lys-164 and Tyr-165 which have been shown to be important in stabilizing the C1–C4 interface

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[1] Abbreviations: PALA, *N*-(phosphonoacetyl)-L-aspartate; PAM, phosphonoacetamide; T and R states, tense and relaxed states of the enzyme having low and high affinity, respectively, for the substrates; $[S]_{0.5}^{CP}$, the carbamoyl phosphate concentration at half the maximal observed specific activity; $[S]_{0.5}^{Asp}$, the aspartate concentration at half the maximal observed specific activity; holoenzyme, the entire aspartate transcarbamoylase molecule composed of two catalytic subunits and three regulatory subunits; 160's loop, a loop in the catalytic chain corresponding to residues 160–166; 240's loop, a loop in the catalytic chain corresponding to residues 230–245.

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

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in both the T and R states (Kantrowitz & Lipscomb, 1988). The side chain of Asp-162 also makes an interaction with the side chain of Gln-231 in the T state and an interaction with the backbone nitrogen of Gln-231 in the R state. Gln-231 also interacts with the β-carboxylate of aspartate in the active site. Interestingly, many residues in the 160's loop are conserved between different species of aspartate transcarbamoylase. In *Serratia marcescens* (Beck et al., 1989), *Salmonella typhimurium* (Michaels et al., 1987), *Proteus vulgaris* (Wild & Wales, 1990), and *E. coli* (Hoover et al., 1983) aspartate transcarbamoylase residues 159–170 are conserved. The hamster (Shigesada et al., 1985) enzyme, which only contains the catalytic chains, conserves similar residues with the exception that Tyr-165 is replaced by a histidine. The *Bacillus subtilis* aspartate transcarbamoylase (Lerner & Switzer, 1986) conserves fewer residues, but Gly-161 and Asp-162 are conserved. The same two residues are conserved in ornithine transcarbamoylase (Bencini et al., 1983; Houghton et al., 1984; Van Vliet et al., 1984), an enzyme which catalyzes an analogous reaction between ornithine and carbamoyl phosphate. It is therefore suspected that Asp-162 may play a critical role in determining the dynamic structure, upon which the regulatory control of the catalytic rate depends, of the wild-type enzyme. In order to probe the function of Asp-162 in aspartate transcarbamoylase, we used site-specific mutagenesis to replace it with alanine. Here we report the characterization of this mutant enzyme.

Experimental Procedures

Materials

Agar, ampicillin, L-aspartate, *N*-carbamoyl-L-aspartate, carbamoyl phosphate, and potassium dihydrogen phosphate were purchased from Sigma Chemical Co. The carbamoyl phosphate was purified before use by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C (Gerhart & Pardee, 1962). Electrophoresis-grade acrylamide, agarose, urea, Tris, and enzyme-grade ammonium sulfate were obtained from ICN Biomedicals. The restriction enzymes *Pst*I and *Bgl*II and *Thermus aquaticus* (Taq) polymerase were obtained from U.S. Biochemicals. The M13 phage M13K07 was obtained from J. Messing, Rutgers University.

Methods

Construction of the Asp-162→Ala[3] *Enzyme by Site-Specific Mutagenesis.* The substitution of alanine for Asp-162 of the catalytic chain of aspartate transcarbamoylase was accomplished by site-specific mutagenesis using the method of Zoller and Smith (1982) with the modifications previously described (Ladjimi et al., 1988; Carter et al., 1985). Single-stranded DNA from 10 candidates was isolated and sequenced by the dideoxy method (Sanger et al., 1977) using Taq DNA polymerase. Three of the candidates gave the sequence corresponding to the mutation. A fragment of the gene (*Pst*I–*Bgl*II) containing the mutation was removed with restriction enzymes and inserted into a plasmid which had the corresponding section of the wild-type gene removed (Stebbins et al., 1989). The mutation was verified a second time, after construction of the plasmid, employing single-stranded DNA copied from the plasmid using the helper phage M13K07 (Vieira & Messing, 1987). The entire cloned fragment was sequenced to confirm that none but the intended mutation had taken place.

Aspartate Transcarbamoylase Assay. The transcarbamoylase activity was measured at 25 °C by either the colorimetric (Pastra-Landis et al., 1981) or the pH-stat method (Wu & Hammes, 1973). All colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

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 of the wild-type enzyme, based on the three-dimensional coordinates of the T-state unligated, CTP-ligated, ATP-ligated (Stevens et al., 1990), PAM-ligated (Gouaux & Lipscomb, 1990) and the R-state (PAM + malonate)-ligated (Gouaux & Lipscomb, 1990), (ATP + PAM + malonate)-, (CTP + PAM + malonate)-ligated (Gouaux et al., 1990) enzyme complex, was accomplished using the program FRODO, Version 6.6 (Pflugrath et al., 1984), on an Evans & Sutherland PS390 interfaced to a MicroVax 3100.

Computer Simulations of the Energy-Minimized Asp-162→Ala Enzyme. Model building of the Asp-162→Ala enzyme was completed in both the unligated T-state (Stevens et al., 1990) and (PAM + malonate)-ligated R-state (Gouaux et al., 1990) X-ray crystal structures by removal of the aspartic acid side chain and replacing it with an alanine. Energy minimization and slow cooling molecular dynamics simulations (slow cooling protocol starting at 3000 K; (Brünger, 1990) were computed on the T- and R-state structures with the program X-PLOR, Version 2.1, on a DECstation 3100.

CTP Binding Measurements. The binding of CTP to the Asp-162→Ala 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 an LKB 1217 Rackbeta liquid scintillation counter. Complete equilibration was confirmed under the experimental conditions. Equilibrium dialysis experiments were performed in 0.1 M imidazole–acetate buffer, 0.2 mM EDTA, and 2 mM 2-mercaptoethanol, pH 7, and the enzyme was dialyzed into this buffer before use.

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

Results

Effects of the Mutation on the Kinetic Properties of the Holoenzyme with Respect to Aspartate and Carbamoyl Phosphate. Comparison of the aspartate saturation curves of the mutant and wild-type enzymes allows an understanding of the relative equilibrium between the T and R states in the mutant enzyme and whether the two forms can be interconverted with increasing aspartate concentration. A reduction in the maximal observed specific activity may reflect both a destabilization of the R state of the enzyme and a direct effect on the catalytic mechanism. Figure 1 shows the aspartate saturation curves for the wild-type and Asp-162→Ala holoenzymes. The mutant enzyme shows a 400-fold decrease in the maximal observed specific activity, a low $[S]_{0.5}^{Asp}$ of 5.6 mM,

[3] The notation used to name the mutant enzymes is, for example, the Asp-162→Ala enzyme. The wild-type amino acid and location within the catalytic chain is indicated to the left of the arrow while the new amino acid is indicated to the right of the arrow.

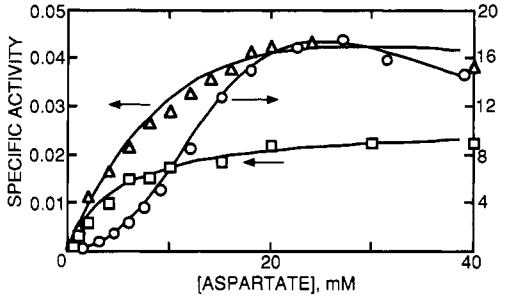

FIGURE 1: Aspartate saturation curves are shown for the wild-type (○) and Asp-162→Ala (△) holoenzymes and the mutant catalytic subunit (□). The activity was determined at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM. Specific activity is reported in units of millimoles of *N*-carbamoyl-L-aspartate formed per hour per milligram of protein.

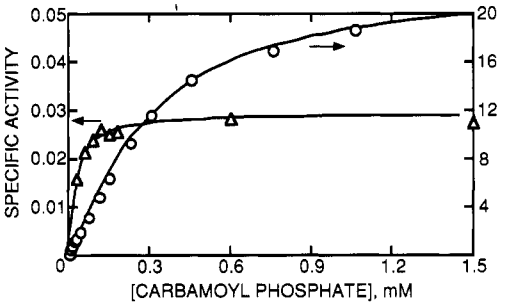

FIGURE 2: Carbamoyl phosphate saturation curves for the wild-type (○) and the Asp-162→Ala (△) holoenzymes. The aspartate concentration was held constant at 25 and 24 mM for wild-type and the Asp-162→Ala enzymes, respectively. The activity was determined at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. Specific activity is reported in units of millimoles of *N*-carbamoyl-L-aspartate formed per hour per milligram of protein.

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

enzyme	maximal velocity[b] (mmol·h^{-1}·mg^{-1})	$[S]_{0.5}^{Asp}$ (mM)	n_H^{Asp}	$[S]_{0.5}^{CP}$ (mM)	n_H^{CP}
wild type	17.2	11.8	2.2	0.29	1.3
Asp-162→Ala	0.042	5.6	1.0	0.025	1.0

[a] The data and experimental conditions used to determine the parameters in this table are presented in the legend to Figure 1. The maximal velocity and Hill coefficients (n_H) were calculated by a nonlinear least-squares procedure using a modified Hill equation which incorporates a term for substrate inhibition (Pastra-Landis et al., 1978). [b] Maximal observed specific activity.

compared to the wild-type enzyme value of 11.8 mM, and loss of cooperativity (Table I). The $[S]_{0.5}^{CP}$ is reduced by approximately 12-fold compared to the wild-type enzyme, and the carbamoyl phosphate saturation kinetics of the mutant enzyme do not exhibit cooperativity (Figure 2 and Table I).

Influence of the Allosteric Effectors. ATP and CTP affect the activity of the wild-type aspartate transcarbamoylase, at subsaturating concentrations of aspartate, by binding to the regulatory subunits at a site some 60 Å away from the active site. It is thought that ATP stabilizes the aspartate and carbamoyl phosphate domains in a closed conformation, and CTP stabilizes the enzyme in a domain-open conformation (Ladjimi et al., 1988; Newton & Kantrowitz, 1990a). A change in the ATP or CTP effect may reflect a change in binding of these effectors, a change in the ability of the enzyme to transmit the signal to the active site, and/or the inability of the enzyme to exist in alternate states. Neither ATP nor

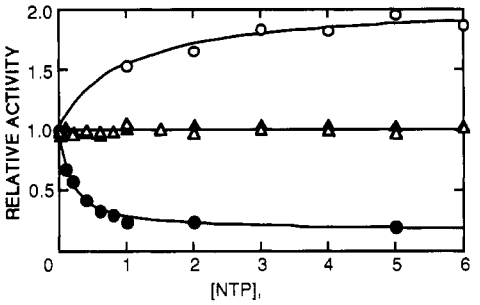

FIGURE 3: Influence of ATP and CTP on the activity of wild-type and the Asp-162→Ala holoenzymes at concentrations of aspartate equal to their $[S]_{0.5}^{Asp}$. Assays were carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM. For the wild-type and the Asp-162→Ala enzymes, the aspartate concentration was held constant at 12 and 6 mM, respectively. ATP effect on the wild-type (○) and the Asp-162→Ala (▲) enzymes. CTP effect on the wild-type (●) and the Asp-162→Ala (△) enzymes.

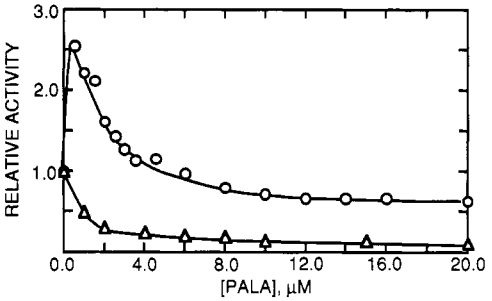

FIGURE 4: Effect of PALA on the activity of the wild-type and Asp-162→Ala holoenzymes at 25 °C in 50 mM Tris–acetate, pH 8.3. Carbamoyl phosphate was held constant 4.8 mM, and the aspartate concentration used for each enzyme is shown in parentheses. Data are shown for the wild-type (2 mM) (○) and the Asp-162→Ala (2 mM) (△) enzymes.

CTP exerted any effect on the rate of the enzyme-catalyzed reaction at an aspartate concentration equivalent to the $[S]_{0.5}^{Asp}$ (Figure 3). Equilibrium binding studies failed to detect the binding of CTP.[4] From this experiment it is estimated that the binding of CTP must be reduced more than 10-fold.

Effect of PALA on the Wild-Type and Asp-162→Ala Holoenzyme. PALA activates the wild-type holoenzyme at low concentrations of PALA while higher concentrations inhibit the enzyme (Collins & Stark, 1971). The binding of PALA to the active site of the enzyme involves both the carbamoyl phosphate and aspartate binding sites (Ke et al., 1988), and PALA is thought to be an analogue of the transition state of the reaction (Collins & Stark, 1971). Upon PALA binding at one or so active sites all the other active sites are converted into the high-activity, high-affinity form, thus leading to activation (Foote & Schachman, 1985). However, as additional PALA molecules bind, activity is lost because substrate binding is blocked. In mutant enzymes that do not exhibit PALA activation, the R state may be unobtainable or the enzyme may be already in the R state.

At an aspartate concentration equivalent to one-sixth of the $[S]_{0.5}^{Asp}$, PALA does not activate the Asp-162→Ala holoenzyme but inhibits it (Figure 4). The wild-type enzyme is, in contrast, activated at approximately 0.5 μM PALA and inhibited at 10 μM PALA.

Effects of the Mutation on the Kinetic Properties of the Catalytic Subunit with Respect to Aspartate and Carbamoyl Phosphate. The mutant catalytic subunit shows a 660-fold

[4] The normally weaker ATP binding was not measured.

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

enzyme	maximal velocity[b] (mmol·h^{-1}·mg^{-1})	K_m^{Asp} (mM)	K_m^{CP} (mM)
wild type	23	6.0	0.020
Asp-162→Ala	0.026	5.3	0.046

[a] Saturation curves 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 the aspartate saturation curves, and the aspartate concentration was held constant at 30 mM for the carbamoyl phosphate saturation curves. [b] Maximal observed specific activity.

reduction in the maximal observed specific activity relative to the wild-type catalytic subunit (Table II). The K_m^{Asp} was not significantly changed for the Asp-162→Ala enzyme, but the K_m^{CP} was increased 2-fold relative to the value for the wild-type enzyme.

Discussion

Asp-162 in the catalytic chain of aspartate transcarbamoylase interacts with Lys-164 and Tyr-165. These two residues make stabilizing intertrimer interactions with Glu-239 in the T state and stabilizing intratrimer interactions with Glu-239 in the R state. Asp-162 makes another interaction with Gln-231, a residue involved in the binding of aspartate. Furthermore, Asp-162 is a conserved residue in aspartate transcarbamoylases from five different species (Lerner & Switzer, 1986; Beck et al., 1989; Wild & Wales, 1990) and in four different species of ornithine transcarbamoylase (Bencini et al., 1983; Houghton et al., 1984; Van Vliet et al., 1984). Ornithine transcarbamoylase shows sequence homology with aspartate transcarbamoylase, and the main differences between the enzymes occur in the amino acid binding domain. In fact, the carbamoyl phosphate domain of aspartate transcarbamoylase has been successfully interchanged with that of ornithine transcarbamoylase (Houghton et al., 1989). Since Asp-162 has no obvious catalytic role in aspartate transcarbamoylase, the most likely explanation for its conservation in different species of aspartate and ornithine transcarbamoylase is that Asp-162 plays a structural role. The present study investigates the functional role of Asp-162 in the wild-type enzyme by deleting this side chain and replacing it with an alanine residue by site-specific mutagenesis. Although a conservative mutation of Asp-162 to Asn would prove interesting, molecular modeling studies show that to investigate the stabilizing role of this conserved amino acid, mutation of Asp-162 to Ala would likely yield the most interesting results.

Asp-162 interacts with approximately the same residues in the T state as it does in the R state, at pH 5.8 and pH 7.0 according to X-ray data (Gouaux et al., 1990; Stevens et al., 1990) and as illustrated in Figure 5. The only side chain to side chain interaction that Asp-162 makes is with Gln-231 in the T state. Interactions between Asp-162 and both Lys-164 and Tyr-165 are from the side-chain oxygen of Asp-162 to the nitrogen atoms of the peptide backbone of Lys-164 and Tyr-165. There is no evidence that Asp-162 directly interacts with the substrates. The lack of cooperativity and reduced $[S]_{0.5}^{Asp}$ of the Asp-162→Ala holoenzyme are similar to those of the wild-type catalytic subunit except that the maximal observed specific activity is reduced 400-fold. Three significant pieces of evidence indicate that the Asp-162→Ala enzyme exists as the holoenzyme and not as the catalytic subunit. First, nondenaturing gel electrophoresis (data not shown) at protein concentrations at and below that used in the assay indicates that the mutant holoenzyme has a mobility almost identical to that of the wild-type holoenzyme and significantly different from that of the catalytic subunit; second, the pattern of substrate inhibition observed in the aspartate saturation curve with the mutant holoenzyme is not observed with the wild-type or mutant catalytic subunit (Figure 1); and finally, both the maximal observed specific activity and the $[S]_{0.5}^{CP}$ are different for the two forms of the enzyme. If the mutant holoenzyme had dissociated into the catalytic and regulatory subunits, the apparent specific activity of the mixture of catalytic and regulatory subunits would be expected to appear lower than that of the pure catalytic subunit because of the contribution that the R subunits make to the protein concentration. However, the actual observed specific activity of the mutant holoenzyme is higher than that of the catalytic subunit.

The kinetic properties of the Asp-162→Ala enzyme, the low $[S]_{0.5}^{Asp}$, loss of cooperativity, and loss of the ability to be activated by PALA at subsaturating concentrations of aspartate, are consistent with those of an enzyme in an R functional state, except that the Asp-162→Ala enzyme exhibits low activity. However, additional structural data are needed to establish if the R functional state observed for the Asp-162→Ala enzyme correlates with an R structural state.[5] Assuming that the enzyme is in an R structural state, why does the replacement of Asp-162 by Ala result in this state, and why is the activity of the enzyme greatly diminished? Without structural data on the mutant enzyme it is not possible to make a firm conclusion; however, a tentative explanation of the data may be derived from an examination of the properties of mutant enzymes in which residues interacting with Asp-162 have been modified. The possibility of a perturbation of the secondary structure in the 160's region by the Asp-162→Ala substitution is almost a certainty. However, the net effect of the mutation is still disruption of its interactions, whatever the primary effect. The data are consistent with the hypothesis that removal of the aspartate side chain leads to destabilization of the crucial interactions that these other (Lys-164, Tyr-165, Gln-231) side chains make. One or more of these interactions could have been lost in the Asp-162→Ala enzyme and/or a more serious loss of structure could have taken place. For instance, Asp-162 in the catalytic chain is spatially interposed between the sequence of residues 229–231 and the sequence from 163 to 166 and may regulate the distance that these parts of the polypeptide chain are apart. Here we can only mention some of the possibilities that could lead to the properties of the Asp-162→Ala enzyme through the loss of these second-sphere interactions.

Gln-231 interacts with PALA and probably the β-carboxylate of aspartate according to the X-ray data (Krause et al., 1987). Thus the R-state interactions of Gln-231 are expected to be important for the normal function of the enzyme. The Gln-231→Leu enzyme has a 1500-fold reduction in the maximal observed specific activity, an 18-fold increase in the $[S]_{0.5}^{Asp}$, and a relative loss in sensitivity to the allosteric effectors (Stebbins et al., 1990). In fact, the association constant of CTP from this enzyme is decreased approximately 10-fold. The Asp-162→Ala enzyme shows a 400-fold loss in activity and a decrease in CTP binding at least equivalent to that of the Gln-231→Leu enzyme. The reduction in maximal observed specific activity in the catalytic subunits of the Gln-231→Leu and Asp-162→Ala enzymes is 600-fold. This similarity in properties could arise by the perturbation of the position of Gln-231 when the side chain of Asp-162 is removed. Computer simulations of the energy-minimized Asp-162→Ala

[5] Many attempts have been made to crystallize the Asp-162→Ala enzyme; however, as yet none have succeeded in producing data-quality crystals.

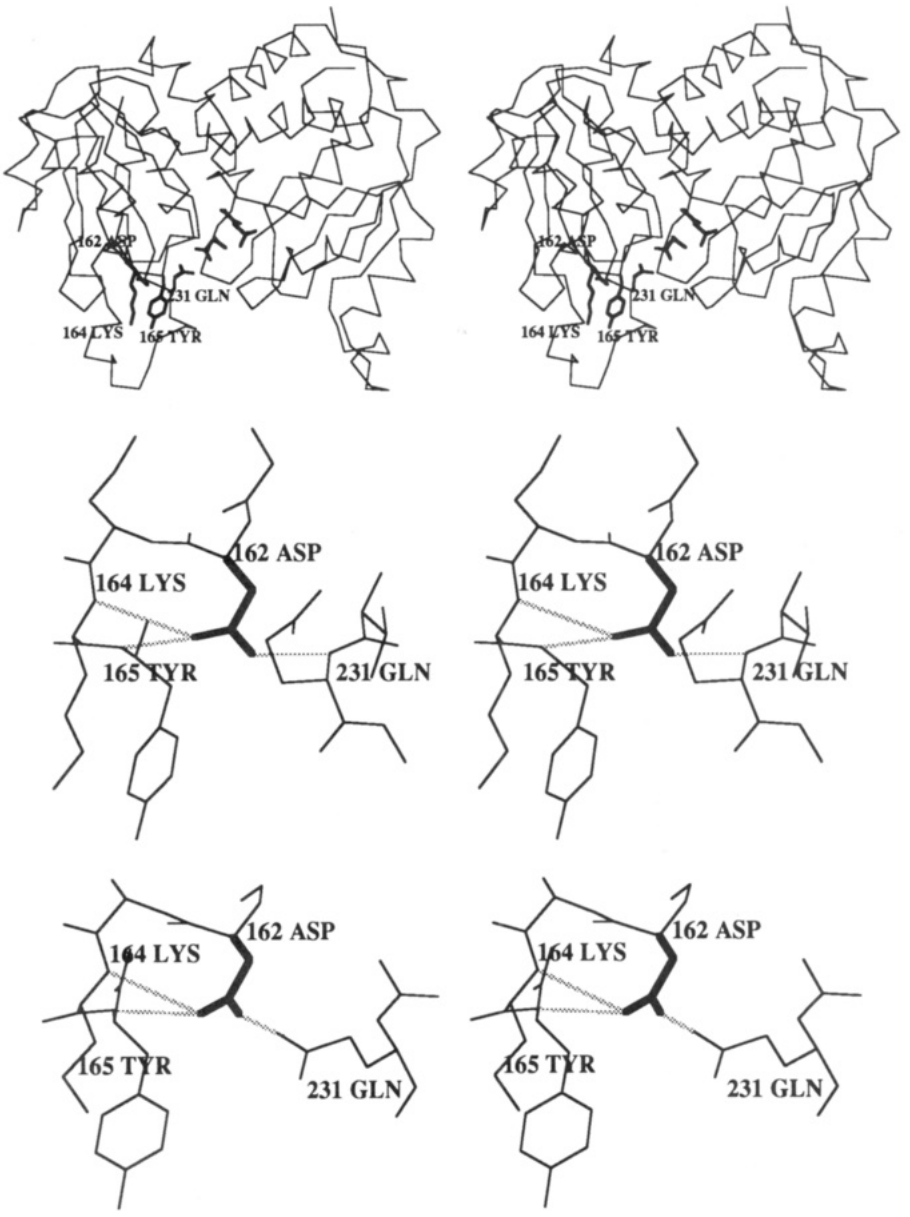

FIGURE 5: (Top) Stereoview showing the α-carbon trace of the catalytic chain R state [from the (PAM + malonate)-bound structure; PAM and malonate are shown in the active site]. Asp-162 is located in a loop region near the active site and the 240's loop. (Middle) Stereoview of the R-state Asp-162 region showing interactions between Asp-162 and the backbone nitrogens of Lys-164, Tyr-165, and Gln-231. (Bottom) Stereoview of the T-state Asp-162 region showing the interactions between Asp-162 and the backbone nitrogens of Lys-164, Tyr-165, and the side chain of Gln-231. Once the side chain of Asp-162 is mutated to alanine, Gln-231 is no longer locked into the wild-type conformation and the side chain of Gln-231 may form new interactions that are more difficult to break in the T to R transition, thus making the side chain of Gln-231 unable to participate in substrate binding.

enzyme in the T and R states show that the side chain of Gln-231 is able to move into the space once occupied by the side chain of Asp-162. It is possible that once the side chain of Gln-231 moves into this region, it forms interactions that do not break in the T to R transition, thus making the side chain of Gln-231 unable to interact with aspartate. The Asp-162→Ala enzyme does not show an increase in $[S]_{0.5}^{Asp}$ as does the Gln-231→Leu enzyme. Thus at any aspartate concentration the degree of saturation of its active site is improved relative to that of the Gln-231→Leu enzyme. Possibly a change in the orientation of Gln-231 and thus aspartate in the active site is responsible for the reduction in the maximal observed specific activity.

The second set of interactions that Asp-162 makes is with residues Lys-164 and Tyr-165. The reduction in $[S]_{0.5}^{Asp}$ of the Asp-162→Ala enzyme is possibly related to the perturbation of the T-state interactions of Glu-239. This residue interacts with Lys-164 and Tyr-165 in both the T and R states, but the interactions change from being interchain (C1–C4) in the T state to intrachain in the R state (Kim et al., 1987; Krause et al., 1987). The kinetic properties of the Glu-239→Gln enzyme, carried out in the presence of saturating carbamoyl phosphate, suggest that the enzyme is in an R functional state (Ladjimi & Kantrowitz, 1988). It does not exhibit aspartate cooperativity and has a normal maximal observed specific activity and a reduced $[S]_{0.5}^{Asp}$ of 6.7 mM. In the holoenzyme, the loss of the T-state interactions seems to be the dominant disruptive effect and the R-state properties of the Asp-162→Ala enzyme could be related to perturbation of the T-state interactions of Glu-239. Interestingly, in the catalytic subunit, where there is no possibility of the T-state interactions that restrain the enzyme, the Glu-239→Gln enzyme shows an elevated K_m^{Asp} (22 mM) whereas the Asp-162→Ala enzyme shows a normal K_m^{Asp} (5.3 mM). Apparently, the interactions

of Glu-239 are important in the catalytic subunit. There is no evidence that these interactions are not intact in the Asp-162→Ala enzyme since the catalytic subunit shows no abnormality in the K_m^{Asp}. Like the Asp-162→Ala enzyme the Glu-239→Gln enzyme shows no kinetic response to ATP and CTP. So the loss of effector response could be explained by the disruption of either the interactions of Gln-231 or the disruption of the T-state interactions of Lys-164 and Tyr-165 with Glu-239, or a combination of both sets of interactions.

Tyr-165 has been mutated to serine (Robey & Schachman, 1984) and to phenylalanine (Wales et al., 1988). The properties of the Tyr-165→Phe holoenzyme are similar to the wild-type enzyme except that the $[S]_{0.5}^{Asp}$ is increased about 16-fold. The K_m^{Asp} of the mutant catalytic subunit is also increased 2-fold. Recently, the properties of the Lys-164→Glu enzyme have been reported (Newell & Schachman, 1990): a reduced maximal velocity, an increased K_m^{Asp} (20-fold), and no cooperativity. The dominant influence on these mutations seems to be the loss of the R-state interactions. One explanation is that the Asp-162→Ala mutation causes the disruption of the interaction of both Lys-164 and Tyr-165 with Glu-239, since both the Asp-162→Ala and Glu-239→Gln enzymes have mostly R-state kinetic properties. In either the T- or R-state computer simulations, Lys-164 and Tyr-165 did not move substantially.

In summary, the Asp-162→Ala enzyme has the kinetic properties of a 400-fold inactive but R-like enzyme. Its properties are like that of the isolated catalytic subunit in terms of the Michaelis–Menten constants, lack of homotropic cooperativity with aspartate, and absence of activation by ATP and inhibition by CTP. The most likely explanation for the altered properties of the Asp-162→Ala enzyme are due to repositioning of side chains in the immediate vicinity of position 162 such as the side chains of residue 164, 165, 231, and 239, suggesting that Asp-162 in the wild-type enzyme is important for stabilizing the internal architecture of the enzyme. Mutations at positions such as 164, 165, 231, and 239 support these conclusions; however, this explanation awaits clarification from structural information on the Asp-162→Ala holoenzyme.

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