Importance of Domain Closure for Homotropic Cooperativity in *Escherichia coli* Aspartate Transcarbamylase†

Colin J. Newton and Evan R. Kantrowitz*
Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167
Received August 21, 1989; Revised Manuscript Received October 13, 1989

ABSTRACT: The importance of the interdomain bridging interactions observed only in the R-state structure of *Escherichia coli* aspartate transcarbamylase between Glu-50 of the carbamoyl phosphate domain with both Arg-167 and Arg-234 of the aspartate domain has been investigated by using site-specific mutagenesis. Two mutant versions of aspartate transcarbamylase were constructed, one with alanine at position 50 (Glu-50 → Ala) and the other with aspartic acid at position 50 (Glu-50 → Asp). The alanine substitution totally prevents the interdomain bridging interactions, while the aspartic acid substitution was expected to weaken these interactions. The Glu-50 → Ala holoenzyme exhibits a 15-fold loss of activity, no substrate cooperativity, and a more than 6-fold increase in the aspartate concentration at half the maximal observed specific activity. The Glu-50 → Asp holoenzyme exhibits a less than 3-fold loss of activity, reduced cooperativity for substrates, and a 2-fold increase in the aspartate concentration at half the maximal observed specific activity. Although the Glu-50 → Ala enzyme exhibits no homotropic cooperativity, it is activated by *N*-(phosphonoacetyl)-L-aspartate (PALA). As opposed to the wild-type enzyme, the Glu-50 → Ala enzyme is activated by PALA at saturating concentrations of aspartate. At subsaturating concentrations of aspartate, both mutant enzymes are activated by ATP, but are inhibited less by CTP than is the wild-type enzyme. At saturating concentrations of aspartate, the Glu-50 → Ala enzyme is activated by ATP and inhibited by CTP to an even greater extent than at subsaturating concentrations of aspartate. Under these conditions, the wild-type enzyme is neither activated by ATP nor inhibited by CTP. The Glu-50 → Ala and the Glu-50 → Asp catalytic subunits exhibit losses of activity of 14-fold and 3.3-fold, respectively, compared to the wild-type catalytic subunit. The K_m values for aspartate of the Glu-50 → Ala and Glu-50 → Asp catalytic subunits are 69 and 40 mM, respectively, compared to 6 mM for the wild-type catalytic subunit. Analysis of these data suggests that the Glu-50 → Ala enzyme exists in a low-activity and low-affinity state even when saturated with substrates, implying that the interdomain bridging interactions between Glu-50 and both Arg-167 and Arg-234 in the wild-type enzyme are critical for the formation of the high-activity high-affinity state of the enzyme.

Aspartate transcarbamylase (EC 2.1.3.2), which catalyzes the formation of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and L-aspartate, plays a critical role in the control of pyrimidine metabolism in *Escherichia coli*. The amount of enzyme in the cell is regulated by an attenuation mechanism (Navre & Schachman, 1983; Roof et al., 1982; Turnbough et al., 1983), and the activity of the enzyme itself is regulated. The enzyme is heterotropically activated by ATP (Gerhart & Pardee, 1962), one of the products of the purine biosynthetic pathway, and is heterotropically inhibited by CTP and UTP (Gerhart & Pardee, 1962; Wild et al., 1989), the end products of the pyrimidine biosynthetic pathway. In addition, aspartate transcarbamylase is regulated by positive cooperative interactions involving both substrates (Bethell et al., 1968; Gerhart & Pardee, 1962). The enzyme has become a model system for the study of allosteric regulation and transmission of information between different protein subunits [see reviews by Allewell (1989), Gerhart (1970), Jacobson and Stark (1973), Kantrowitz and Lipscomb (1988), Kantrowitz et al. (1980a,b), and Schachman (1974, 1988)].

The holoenzyme (M_r 310000) is composed of two catalytic subunits (M_r 100000), each comprised of three identical chains, and three regulatory subunits (M_r 34000), each comprised of two identical polypeptide chains. The active sites are located on the catalytic subunit and are shared between two adjacent polypeptide chains (Krause et al., 1985; Monaco et al., 1978; Robey & Schachman, 1985; Wente & Schachman, 1987), while each regulatory chain has a binding site for the regulatory effectors (Gerhart & Schachman, 1965), all of which bind to the same site. The three-dimensional arrangement of the regulatory and catalytic subunits has been deduced by X-ray crystallography of the holoenzyme (Honzatko et al., 1982; Kim et al., 1987). In addition, X-ray crystallography has also been used to determine the alteration in quaternary structure that takes place upon the binding of the substrates and substrate analogues (Gouaux & Lipscomb, 1988; Ke et al., 1988; Krause et al., 1985, 1987).

According to the terminology of the Monod, Wyman, and Changeux model (Monod et al., 1965), the T to R transition involves an expansion of the enzyme by 12 Å along the 3-fold axis along with rotations of the catalytic subunits about the 3-fold axis, and rotations of the regulatory subunits about their respective 2-fold axis. These quaternary changes are accompanied by alterations in the tertiary structure of both the catalytic and regulatory subunits. The major changes involve movements of domains and reorientations of loops (Kantrowitz & Lipscomb, 1988; Ke et al., 1988). These tertiary conformational changes involve the breaking of a set of interactions that stabilize the T state[1] and are replaced by another set of

† This work was supported by Grants DK1429 and GM26237 from the National Institutes of Health.
* To whom correspondence should be addressed.

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

0006-2960/90/0429-1444$02.50/0

interactions that stabilize the R state.

In order to determine the importance of the interactions that are either formed or broken during the conversion between the T and R states, we have used site-directed mutagenesis to eliminate specific interactions and have determined the effect of the loss of each interaction on the homotropic and heterotropic properties of the enzyme (Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988; Middleton & Kantrowitz, 1986, 1988; Middleton et al., 1989). Using this approach, we have begun to map the interactions that are critical for the cooperativity of aspartate transcarbamylase and have determined why the allosteric transition is most probably concerted (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988). The movement of the aspartate domain toward the carbamoyl phosphate domain is of particular importance for homotropic cooperativity. In the R-state structure, the two domains are closed and are held together by interdomain bridging interactions involving Glu-50 of the carbamoyl phosphate domain and both Arg-167 and Arg-234 of the aspartate domain. In order for domain closure to take place, specific interactions between the upper and lower catalytic subunits must be broken, thus destabilizing the T state sufficiently to cause a concerted transition to the R state (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988).

We have previously investigated the interdomain bridging interactions between Glu-50 and both Arg-167 and Arg-234 by replacing Glu-50 by glutamine (Ladjimi et al., 1988) and Arg-234 by serine (Middleton & Kantrowitz, 1988). Here we report the analysis of two mutant enzymes with either alanine or aspartic acid at position 50 in the catalytic chain of aspartate transcarbamylase to further evaluate the importance of these interdomain bridging interactions for the cooperativity of aspartate transcarbamylase.

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. Restriction endonucleases were obtained from either U.S. Biochemicals or New England Biolabs and used according to the supplier's recommendations. The Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, and T4 ligase were products of U.S. Biochemicals.

Methods

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

Construction of the Glu-50 → Ala[2] *and Glu-50 → Asp Mutations by Site-Specific Mutagenesis.* The replacement of glutamic acid by alanine or aspartic acid at position 50 in the catalytic chain of aspartate transcarbamylase was accomplished by site-specific mutagenesis using the method of Zoller and Smith (1982), with modifications as previously described (Ladjimi & Kantrowitz, 1987).

In order to overproduce the mutant enzymes, a small fragment of the mutant M13 RF was recloned into a plasmid as previously described (Ladjimi et al., 1988). The plasmids, pEK88 for Glu-50 → Asp and pEK91 for Glu-50 → Ala, were isolated and then transformed into strain EK1104 for the overproduction of enzyme (see below).

Wild-Type and Mutant Holoenzyme Purification. Wild-type and the mutant aspartate transcarbamylases were isolated as described by Nowlan and Kantrowitz (1985), from *E. coli* strain EK1104 [F⁻ *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *pyrF*⁻, *rpsL*], containing the plasmids pEK2 (Smith et al., 1986), pEK91, and pEK88 for the wild-type, the Glu-50 → Ala, and the Glu-50 → Asp enzymes, respectively.

Wild-Type and Mutant Catalytic Subunit Overproduction and Purification. The isolation of the catalytic subunit of the wild-type, the Glu-50 → Ala, and the Glu-50 → Asp aspartate transcarbamylases was accomplished after the in vivo overproduction of catalytic subunit using strain EK1104 harboring a plasmid which had a portion of the gene for the regulatory subunit deleted (Nowlan & Kantrowitz, 1985). The plasmids pEK17, pEK102, and pEK101 used for the production of the wild-type, the Glu-50 → Ala, and the Glu-50 → Asp catalytic subunits, respectively, were constructed as previously described (Nowlan & Kantrowitz, 1985). Purification of the catalytic subunit was accomplished by the procedure previously reported (Stebbins et al., 1989).

Aspartate Transcarbamylase Assay. The transcarbamylase activity was measured at 25 °C by either the colorimetric (Pastra-Landis et al., 1981) or the pH-stat method (Wu & Hammes, 1973). pH-stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburette. All colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

Determination of Protein Concentration. Concentrations of pure wild-type holoenzyme and catalytic subunit were determined by absorbance measurements at 280 nm using extinction coefficients of 0.59 and 0.72 cm^2/mg, respectively (Gerhart & Holoubek, 1967). The protein concentrations of the mutant holoenzyme and catalytic subunit were determined by the Bio-Rad version of Bradford's dye binding assay (Bradford, 1976).

Data Analysis. The analysis of the steady-state kinetic data was carried out as previously described by Silver et al. (1983). The analysis of the structural data, based on the three-dimensional coordinates of the CTP–enzyme complex (Kim et al., 1987), the PALA–enzyme complex (Krause et al., 1987), and the enzyme complexed with carbamoyl phosphate plus succinate (Gouaux & Lipscomb, 1988), was accomplished by using the program FRODO (Department of Biochemistry, Rice University) on an Evans & Sutherland PS390 interfaced to a MicroVAX Q5.

Results

Effects of Mutations on the Kinetic Properties of the Enzymes with Respect to Aspartate and Carbamoyl Phosphate. Figure 1 shows the aspartate saturation curves for the wild-type and mutant enzymes with aspartate, alanine, and glutamine at position 50 in the catalytic chain of aspartate transcarbamylase. As expected, the aspartate substitution at position 50 alters the properties of the enzyme to a smaller extent than either the alanine or the glutamine substitutions. The maximal observed specific activity of the Glu-50 → Asp enzyme is about half that of the wild-type enzyme, the Hill coefficient is reduced, and the $[S]_{0.5}^{Asp}$ is almost doubled (see Figure 1A and Table I). On the other hand, the Glu-50 →

[2] The notation used to name the mutant enzymes is, for example, the Glu-50 → Ala 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.

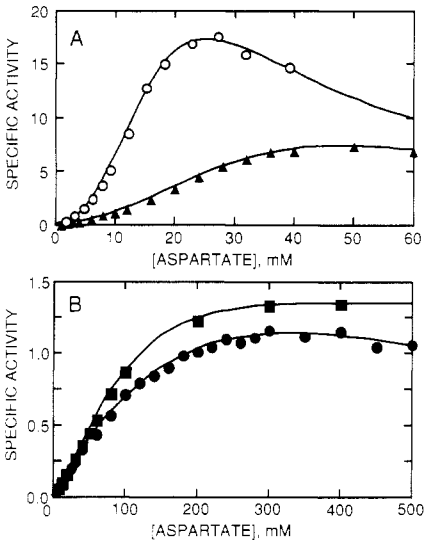

FIGURE 1: Aspartate saturation curves for the wild-type and the mutant holoenzymes carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM. (A) Data are shown for the wild-type (○) and the Glu-50 → Asp (▲) enzymes and (B) for the Glu-50 → Ala (■) and the Glu-50 → Gln (●) enzymes. The curves shown were determined by a nonlinear, least squares fit to the Hill equation incorporating a term for substrate inhibition (Pastra-Landis et al., 1978) (A) or to the Michaelis–Menten equation incorporating a term for substrate inhibition (B).

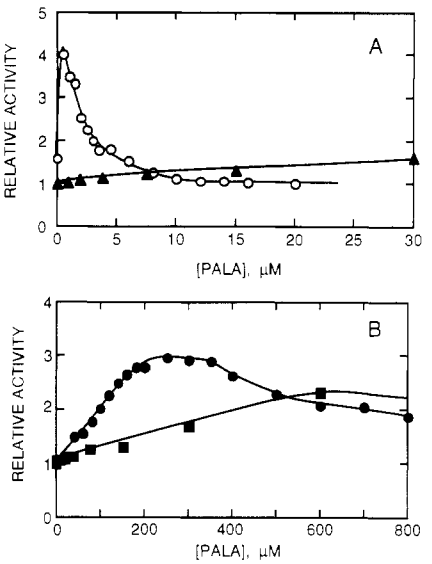

FIGURE 2: Effect of PALA concentration on the activity of the wild-type and mutant holoenzymes at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. Carbamoyl phosphate concentration was held constant at 4.8 mM, and the aspartate concentration used for each enzyme is shown in parentheses. (A) Data are shown for the wild-type (2 mM) (○) and the Glu-50 → Asp (1.7 mM) (▲) enzymes, and (B) data are shown for the Glu-50 → Ala (7 mM) (■) and the Glu-50 → Gln (7 mM) (●) enzymes.

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
Glu-50 → Ala	1.3	74.6	1.0	0.24	1.0
Glu-50 → Asp	7.3	21.0	1.8	0.13	1.3
Glu-50 → Gln	1.1	79.6	1.0	0.21	1.0

[a] The 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.

Table II: Influence of PALA on the Activity of the Wild-Type and Mutant Enzymes[a]

enzyme	$[PALA]^b$ (μM)	A^{PALA}/A at $[S]_{0.5}^{Asp}$	A^{PALA}/A at 2 × $[S]_{0.5}^{Asp}$
wild type	0.5	1.8	0.86
Glu-50 → Ala	600	1.9	1.7
Glu-50 → Asp	250	1.6	1.4

[a] Assays were performed at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. In all cases, the carbamoyl phosphate concentration was held constant at 4.8 mM. The $[S]_{0.5}^{Asp}$ values used for the three enzymes are listed in Table I. A^{PALA} corresponds to the specific activity of the respective enzyme in the presence of PALA, at the indicated concentration, and A corresponds to the specific activity of the respective enzyme in the absence of PALA. [b] The PALA concentration used corresponds to the concentration necessary to activate the enzyme maximally under the conditions used in Figure 2.

Gln and the Glu-50 → Ala enzymes both exhibit about a 10-fold reduction in the maximal observed specific activity, an elimination of cooperativity, and an elevation of the $[S]_{0.5}^{Asp}$ to approximately 80 mM (see Figure 1B and Table I).

The carbamoyl phosphate saturation curve of the Glu-50 → Asp enzyme shows a reduction in the maximal observed specific activity, a reduced $[S]_{0.5}^{CP}$, and a Hill coefficient similar to the wild-type enzyme (see Table I). The carbamoyl phosphate saturation curve of the Glu-50 → Ala enzyme is similar to that of the Glu-50 → Gln enzyme (Ladjimi et al., 1988) with about a 10-fold reduction in the maximal observed specific activity, a loss of cooperativity, and a $[S]_{0.5}^{CP}$ similar to the wild-type enzyme (see Table I).

Effects of PALA and Succinate on the Mutant Enzymes. Since the Glu-50 → Gln enzyme could be activated by PALA at subsaturating concentrations of aspartate (Ladjimi et al., 1988), it was of interest to determine whether the Glu-50 → Ala enzyme could be activated by PALA even though this enzyme shows no detectable aspartate cooperativity and a relatively high $[S]_{0.5}^{Asp}$. As seen in Figure 2B, at an aspartate concentration low compared to the $[S]_{0.5}^{Asp}$, the Glu-50 → Ala enzyme is activated by PALA. However, the affinity for PALA is even weaker than that of the Glu-50 → Gln enzyme, which is activated just under 3-fold at a PALA concentration of approximately 250 μM. By contrast, the Glu-50 → Ala enzyme requires a PALA concentration of 600 μM for maximal activation. However, the extent of the activation is similar to that of the Glu-50 → Gln enzyme (Ladjimi et al., 1988). The Glu-50 → Asp enzyme, which has a Hill coefficient of 1.8, was activated at a relatively lower concentration of PALA than the Glu-50 → Ala and Glu-50 → Gln enzymes, but did not show maximal stimulation even at 30 μM PALA, whereas the wild-type enzyme is 3-fold stimulated at 0.5 μM PALA (Figure 2A).

PALA is able to enhance the activity of the mutant enzymes even at saturating concentrations of aspartate and carbamoyl phosphate. As seen in Table II, the wild-type enzyme is activated only 2-fold by 0.5 μM PALA at an aspartate concentration corresponding to the $[S]_{0.5}^{Asp}$, while the wild-type enzyme is inhibited by the same concentration of PALA at an aspartate concentration corresponding to 2 × $[S]_{0.5}^{Asp}$. Although the Glu-50 → Ala and Glu-50 → Gln enzymes are also activated by PALA at an aspartate concentration corresponding to $[S]_{0.5}^{Asp}$, these same enzymes are activated almost to the same extent at an aspartate concentration corresponding to 2 × $[S]_{0.5}^{Asp}$ (see Table II). As opposed to PALA, succinate

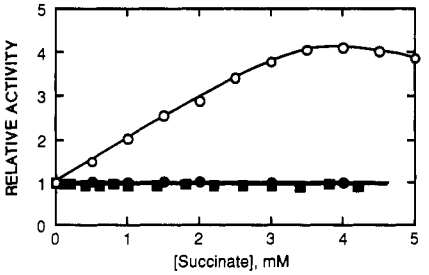

FIGURE 3: Effect of succinate concentration on the activity of the wild-type and mutant holoenzymes at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. Carbamoyl phosphate concentration was held constant at 4.8 mM, and the aspartate concentration is shown in parentheses. Data are shown for the wild-type (2 mM) (○), the Glu-50 → Ala (7 mM) (■), and the Glu-50 → Gln (7 mM) (●) enzymes.

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

enzyme	activity with ATP[b]	K_{ATP} (mM)	activity with CTP[c]	K_{CTP} (mM)
wild type	2.0	0.94	0.16	0.18
Glu-50 → Ala	1.8	2.3	0.65	0.21
Glu-50 → Asp	1.6	1.8	0.64	0.83
Glu-50 → Gln	2.0	0.4	0.14	0.15

[a] Assays were performed at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. In all cases, the carbamoyl phosphate concentration was held constant at 4.8 mM. The aspartate concentrations used were approximately the $[S]_{0.5}^{Asp}$, which corresponds to 12, 80, and 20 mM for the wild-type, the Glu-50 → Ala, and the Glu-50 → Asp enzymes, respectively. [b] Activity with ATP is defined as A^{ATP}/A where A^{ATP} is the activity extrapolated to an infinite concentration of ATP and A is the activity in the absence of ATP. [c] Activity with CTP is defined as A^{CTP}/A where A^{CTP} is the activity extrapolated to an infinite concentration of CTP and A is the activity in the absence of CTP.

is unable to activate either the Glu-50 → Ala or the Glu-50 → Gln enzymes (Figure 3).

Influence of the Allosteric Effectors. At half-saturating concentrations of aspartate, ATP activates and CTP inhibits the Glu-50 → Ala and Glu-50 → Asp enzymes, but the extent of the activation and inhibition is less than that observed for the wild-type enzyme (Table III). The affinity of the Glu-50 → Asp and the Glu-50 → Ala enzymes for ATP is decreased approximately 2-fold with respect to the wild-type enzyme. However, the affinity of the Glu-50 → Ala enzyme for CTP is influenced only slightly, whereas there is a 5-fold decrease in CTP affinity for the Glu-50 → Asp enzyme.

Although ATP activates and CTP inhibits these mutant enzymes, these nucleotides influence the activity of the mutant enzymes differently than the wild-type enzyme. Figure 4 compares the aspartate saturation curves of the wild-type and the Glu-50 → Ala enzymes in the absence and presence of ATP and CTP. For the wild-type enzyme (Figure 4A), the nucleotide effectors alter the activity more at low aspartate concentrations than at high aspartate concentrations. If the concentration of aspartate is increased further, the nucleotides have no effect whatsoever (data not shown). This behavior for the wild type is not observed for the Glu-50 → Ala enzyme, in which case the extent of inhibition by CTP and activation by ATP persists as the concentration of aspartate increases. Even at saturating concentrations of aspartate, inhibition by CTP and activation by ATP are still observed (see Figure 5). The activation by ATP and inhibition by CTP at saturating aspartate concentrations are also observed for the Glu-50 → Gln enzyme (Ladjimi et al., 1988).

Kinetic Properties of the Catalytic Subunits. The aspartate saturation curve of the Glu-50 → Ala catalytic subunit shows a relatively high K_m^{Asp} (Table IV) similar to the Glu-50 → Gln

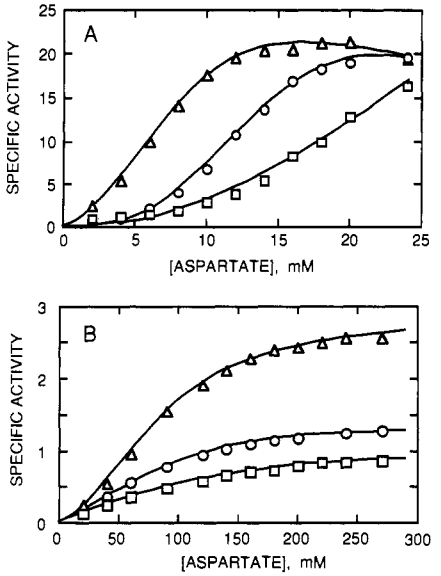

FIGURE 4: Aspartate saturation curves of the wild-type (A) and the Glu-50 → Ala (B) enzymes in the absence and presence of either ATP or CTP. Assays were carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM. Data are shown for the enzyme in the absence of effectors (○), in the presence of 6 mM ATP (△), and in the presence of 1 mM CTP (□). The curves were drawn by a nonlinear, least-squares fit to the Hill equation incorporating a term for substrate inhibition (Pastra-Landis et al., 1978) (A) or to the Michaelis–Menten equation incorporating a term for substrate inhibition (B).

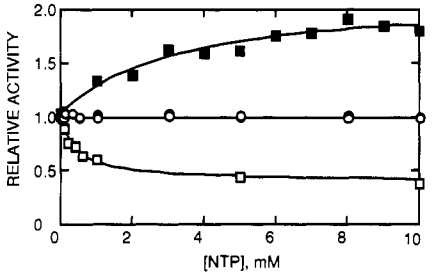

FIGURE 5: ATP activation and CTP inhibition of the Glu-50 → Ala and wild-type holoenzymes at concentrations of aspartate high relative to their $[S]_{0.5}^{Asp}$. Assays were carried out at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. The carbamoyl phosphate concentration was held constant at 4.8 mM. For the wild-type and the Glu-50 → Ala enzymes, the aspartate concentration was held constant at 2.5 and 4 times their $[S]_{0.5}^{Asp}$, respectively. ATP effect on the wild-type enzyme (●) and the Glu-50 → Ala enzyme (■). CTP effect on the wild-type enzyme (○) and the Glu-50 → Ala enzyme (□).

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

enzyme	maximal velocity[b] (mmol·h^{-1}·mg^{-1})	K_m^{Asp} (mM)
wild type	23.0	6
Glu-50 → Ala	2.4	69
Glu-50 → Asp	10.1	40
Glu-50 → Gln[c]	2.9	57

[a] Assays were performed at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. In all cases, the carbamoyl phosphate concentration was held constant at 4.8 mM. The maximal velocity was calculated by a nonlinear least-squares procedure using a modified Michaelis–Menten equation which incorporates a term for substrate inhibition (Pastra-Landis et al., 1978) when necessary. [b] Maximal observed specific activity. [c] Data for the Glu-50 → Gln catalytic subunit are from Ladjimi et al. (1988).

catalytic subunit and the holoenzymes from which they were derived. However, the Glu-50 → Asp catalytic subunit exhibits a 2-fold increase in $[S]_{0.5}^{Asp}$ relative to its holoenzyme. This is in contrast to the approximate 2-fold decrease in the $[S]_{0.5}^{Asp}$

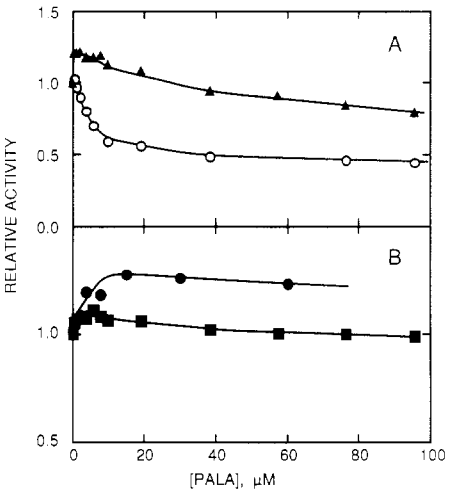

FIGURE 6: Effect of PALA concentration on the activity of the wild-type and mutant catalytic subunits. Assays were determined at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. In each case, the carbamoyl phosphate concentration was held constant at 4.8 mM, while the aspartate concentration used is shown in parentheses. Data are shown for the wild-type enzyme (2 mM) (○), the Glu-50 → Asp enzyme (7 mM) (▲), the Glu-50 → Ala enzyme (12 mM) (■), and the Glu-50 → Gln enzyme (9 mM) (●).

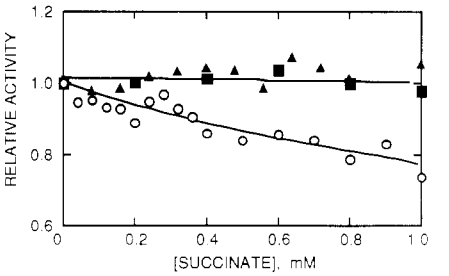

FIGURE 7: Effect of succinate concentration on the activity of the wild-type and mutant catalytic subunits. Assays were determined at 25 °C in 50 mM Tris–acetate buffer, pH 8.3. In each case, the carbamoyl phosphate concentration was held constant at 4.8 mM, while the aspartate concentration used is shown in parentheses. Data are shown for the wild-type enzyme (2 mM) (○), the Glu-50 → Asp enzyme (4 mM) (▲), and the Glu-50 → Ala enzyme (12 mM) (■).

for the wild-type catalytic subunit compared to the wild-type holoenzyme.

PALA is able to activate the catalytic subunit of these mutant enzymes while the wild-type catalytic subunit is only inhibited (Figure 6). Although PALA is able to activate the mutant catalytic subunits at subsaturating concentrations of aspartate, succinate was unable to activate them (Figure 7).

Discussion

X-ray crystallographic evidence indicates that upon the transition from the T to R states the aspartate transcarbamylase molecule undergoes both tertiary and quaternary conformational changes (Ke et al., 1988; Kim et al., 1987; Krause et al., 1987). On the tertiary level, the two domains of the catalytic chain move together along with major reorientations of the 80s and the 240s loops resulting in the repositioning of a number of side chains that are critical for catalysis (Ladjimi et al., 1988; Middleton & Kantrowitz, 1988; Middleton et al., 1989). The closure of the two catalytic chain domains is stabilized by interactions between Glu-50 of the carbamoyl phosphate domain with both Arg-167 and Arg-234 of the aspartate domain. The quaternary conformational change results in a 12-Å elongation of the molecule about the 3-fold axis, along with rotations of the catalytic and regulatory subunits about their 3-fold and 2-fold axes, respectively.

The homotropic cooperativity of the enzyme can be rationalized in terms of a concerted allosteric transition between a low-activity low-affinity functional state and a high-activity high-affinity functional state. The closure of the two domains of the catalytic chain is critical for the concerted allosteric transition since domain closure in one catalytic chain cannot take place without a quaternary conformational change that forces the remaining catalytic chains into their domain-closed high-affinity high-activity state (Kantrowitz & Lipscomb, 1988; Ladjimi & Kantrowitz, 1988; Middleton et al., 1989).

The importance of domain closure and particularly the interdomain bridging interactions between Glu-50 and both Arg-167 and Arg-234 that stabilize the R state have previously been tested by two amino acid substitutions. Glu-50 has been replaced by glutamine (Ladjimi et al., 1988), and Arg-234 has been replaced by serine (Middleton & Kantrowitz, 1988). In order to further evaluate the importance of these interdomain bridging interactions for homotropic cooperativity, two additional mutations have been made at position 50, Glu-50 → Ala and Glu-50 → Asp.

Glu-50 → Ala and Glu-50 → Gln Enzymes Exist in a Low-Activity Low-Affinity State Even When Saturated with Substrates. The X-ray structures of aspartate transcarbamylase in the presence of CTP (Kim et al., 1987) and PALA (Krause et al., 1987) provide structural data on the T and R states, respectively. The kinetic and physicochemical properties of the wild-type enzyme can be rationalized in terms of a transition between a T state, that has low activity and low affinity for substrates, and an R state, that has high activity and high affinity for substrates (Foote & Schachman, 1985; Gerhart & Schachman, 1968; Gibbons et al., 1976; Griffin et al., 1973; Hammes & Wu, 1971; Howlett & Schachman, 1977; Johnson & Schachman, 1980; Kirshner & Schachman, 1973). The replacement of Glu-50 with either alanine or glutamine results in a mutant enzyme that lacks homotropic cooperativity and has low activity and low affinity for aspartate. Thus, the Glu-50 → Ala and Glu-50 → Gln enzymes resemble in many respects an enzyme restrained in the T state by chemical cross-linking (Enns & Chan, 1979). In the case of the enzyme cross-linked in the T state, the R state is no longer accessible. For the Glu-50 → Ala and Glu-50 → Gln enzymes, the loss of the interdomain bridging interactions destablizes the R state in favor of the T state. When Glu-50 is replaced by aspartic acid, the interdomain bridging interactions are weakened, and the resulting enzyme exhibits properties intermediate between the wild type and the mutants with alanine or glutamine at position 50. As outlined below, the results reported here as well as previous work from this laboratory (Ladjimi & Kantrowitz, 1988; Ladjimi et al., 1988; Middleton & Kantrowitz, 1988; Middleton et al., 1989) suggest that the interdomain bridging interactions are important for cooperativity because they stabilize the high-activity high-affinity form of the enzyme.

The most compelling evidence for the existence of a low-activity low-affinity functional state of the Glu-50 → Gln and the Glu-50 → Ala enzymes in the presence of saturating concentrations of substrates is the fact that these mutant enzymes can be activated, under these conditions, by ATP (see Figure 4) and PALA (see Table II). The ATP activation of the Glu-50 → Gln, Glu-50 → Ala, and Glu-50 → Asp enzymes suggests that the binding of ATP stabilizes the domain-closed conformation of the enzyme. The PALA molecule itself is also able to stabilize the domain-closed conformation of these mutant enzymes. At saturating aspartate, PALA is able to activate the Glu-50 → Gln and Glu-50 → Ala enzymes

(see Table II), further supporting the notion that the mutant enzymes with alterations at Glu-50 cannot achieve the wild-type functional R state although PALA and ATP are able to stabilize a form of the enzyme that has *higher* activity than can be achieved by aspartate and carbamoyl phosphate. PALA alone, but not succinate in the presence of saturating carbamoyl phosphate, is able to activate the mutant enzymes because it can bridge across the two domains of the catalytic chain and thereby stabilize the domain-closed conformation. However, the influence of PALA is not at the active site to which it actually binds, since this site will be inactivated, but rather at the remaining active sites. The binding of ATP to the regulatory sites must cause conformational changes in the regulatory chains that propagate across the subunit boundary resulting in the stabilization of the domain-closed conformation. The effect of CTP can be explained in a similar fashion. The binding of CTP results in a stabilization of the domain-opened form that causes a lowering of the maximal velocity, even in the presence of saturating aspartate.

PALA Is Able To Convert the Mutant Enzymes into an R-like Structure. The T and R states of aspartate transcarbamylase observed by X-ray crystallography (Ke et al., 1988; Kim et al., 1987; Krause et al., 1987) are consistent with data obtained in solution by either sedimentation (Gerhart & Schachman, 1968; Kirshner & Schachman, 1971, 1973), X-ray scattering (Hervé et al., 1985), or analytical gel chromatography (Bromberg et al., 1990). The fact that the Glu-50 → Gln enzyme reacts significantly faster with *p*-(hydroxymercuri)benzoate in the presence of PALA than the unliganded enzyme (Ladjimi et al., 1988) suggests that PALA can induce the quaternary conformational change in this mutant enzyme, even though the interdomain bridging interactions are weakened by the replacement of Glu-50 by glutamine. The measurement of the fractional inclusion volume of the Glu-50 → Gln enzyme, in the absence and presence of PALA by analytical gel chromatography (Bromberg et al., 1990), more directly shows that PALA causes an alteration in the quaternary conformation of the Glu-50 → Gln enzyme. Furthermore, the alteration of the fractional inclusion volume due to the binding of PALA is almost identical with that observed for the binding of PALA to the wild-type enzyme. Therefore, the Glu-50 → Gln enzyme, and by analogy the Glu-50 → Ala enzyme, can undergo the structural transition from the T to R state.

In the presence of saturating concentrations of carbamoyl phosphate, the enzymes with mutations at position 50 can be activated by PALA at low concentrations of aspartate, but not by succinate, suggesting that PALA can induce a conformational change that the two substrates when bound simultaneously cannot. Since PALA can form a bridge between the carbamoyl phosphate and aspartate domains, this is additional evidence for the importance of the interdomain bridging interactions for cooperativity in aspartate transcarbamylase.

Importance of Domain Closure for the Creation of the Aspartate Binding Site. The Glu-50 → Gln and Glu-50 → Ala enzymes exhibit substantial increases in $[S]_{0.5}^{Asp}$, even though the mutation is in the carbamoyl phosphate and not the aspartate domain of the enzyme. The fact that the Arg-234 → Ser enzyme, which has a modification at the opposite side of this interdomain bridging interaction, exhibits a similar increase in $[S]_{0.5}^{Asp}$ implies that the interdomain bridging interactions are partially responsible for the creation of the high-activity high-affinity aspartate binding site.

The ordered binding of carbamoyl phosphate before aspartate (Hsuanyu & Wedler, 1987; Porter et al., 1969; Wedler & Gasser, 1974) indicates that the aspartate binding site does not exist until carbamoyl phosphate binds. This is supported by the fact that for the wild-type enzyme the binding of carbamoyl phosphate induces a conformational change in the carbamoyl phosphate domain which has been observed by UV (Collins & Stark, 1969) and circular dichroism difference spectroscopy (Griffin et al., 1972). The importance of carbamoyl phosphate binding for the creation of the high-activity high-affinity aspartate binding site is also seen from site-specific mutagenesis experiments. Substitution of amino acid side chains that interact directly with carbamoyl phosphate has resulted in large alterations in not only carbamoyl phosphate but also aspartate affinity as well (Stebbins et al., 1989). The structural basis of the conformational changes observed by spectroscopy (Collins & Stark, 1969; Griffin et al., 1972) has been investigated by examination of the X-ray structure of aspartate transcarbamylase in the presence of phosphonoacetamide (Gouaux & Lipscomb, 1990). Analysis of this structure reveals that the most dramatic change upon the binding of phosphonoacetamide to the T-state enzyme is the relocation of the 50s loop to its R-state position. These data suggest that the binding of phosphonoacetamide, and by analogy carbamoyl phosphate, repositions the 50s loop so that the interdomain bridging interaction between Glu-50 of the 50s loop and both Arg-167 and Arg-234 of the aspartate domain can more easily take place, perhaps induced by the binding of aspartate. The fact that the mutations at Glu-50 and Arg-234 have little or no effect on carbamoyl phosphate affinity but have a large effect on aspartate affinity further establishes the importance of this interdomain bridging interaction for the formation of the aspartate binding site.

Regulatory Subunits Act To Restrain the Position of the Aspartate and Carbamoyl Phosphate Domains. Gerhart and Pardee (1962) observed that heating wild-type aspartate transcarbamylase caused an increase in activity, due to the dissociation of the holoenzyme into the more active catalytic subunits as the regulatory subunits denature. In addition to an increase in activity, the catalytic subunits also exhibit a higher affinity for the substrates than the holoenzyme. Therefore, in the wild-type enzyme, the regulatory subunits must function to restrain the catalytic subunits in a lower affinity lower activity form relative to the isolated catalytic subunits. The manner of restraint may involve the positioning of the carbamoyl phosphate and aspartate domains relative to each other or by altering the energetics of the closure of the two catalytic chain domains. Since the intersubunit links between Glu-239 and both Lys-164 and Tyr-165 can no longer form in the isolated catalytic subunit, the high-activity high-affinity wild-type catalytic subunit may form as a result of a reorientation of the 240s loop that in turn would favor the formation of the interdomain bridging interactions between Glu-50 and both Arg-167 and Arg-234. For the Glu-50 → Gln and Glu-50 → Ala catalytic subunits, the affinity for aspartate is approximately the same for the holoenzymes and the catalytic subunits, suggesting that these enzymes are in a low-affinity low-activity state both as the holoenzyme and as the catalytic subunit. The situation is different for the cooperative Glu-50 → Asp enzyme. In this case, the aspartate affinity of the catalytic subunit decreases by 2-fold compared to the holoenzyme. This result suggests the Glu-50 → Asp catalytic subunit cannot attain the high-affinity conformation because of the weakening of the interdomain bridging interactions and suggests that in the Glu-50 → Asp holoenzyme as in the wild-type holoenzyme the regulatory subunits influence the energetics of domain closure. For the Glu-50 →

Asp holoenzyme, the weakening of the interdomain bridging interactions is partially compensated for by the influence of the regulatory subunits, resulting in a more or less normal Glu-50 → Asp holoenzyme. However, without the influence of the regulatory subunits, the Glu-50 → Asp catalytic subunit exists in a state with relatively low affinity for aspartate.

The notion that the mutant catalytic subunits are not in the same functional state as the wild-type catalytic subunit is clear from the PALA activation experiments. PALA is able to activate the mutant catalytic subunits to a small extent (see Figure 6) although the wild-type catalytic subunit cannot be activated at all. The reason that the mutant catalytic subunits do not exhibit homotropic cooperativity is probably because the affinities of the T and R states are not sufficiently different. Cooperativity in the catalytic subunit of aspartate transcarbamylase has been previously observed with a mutant version of the enzyme in which case the mutation was at the carbamoyl phosphate binding site and had a direct influence on activity (Stebbins et al., 1989).

Domain Closure and the Function of Aspartate Transcarbamylase. The X-ray structural analysis of aspartate transcarbamylase indicates that the two catalytic chain domains of the enzyme undergo a domain closure upon the T to R transition (Ke et al., 1988; Kim et al., 1987; Krause et al., 1987). Furthermore, the alteration in the sedimentation coefficient of the isolated catalytic subunit in the presence of carbamoyl phosphate and succinate (Gerhart & Schachman, 1968) suggests that the catalytic subunits also undergo a domain closure. Although aspartate transcarbamylase has been studied by a variety of methods, recent experiments using site-specific mutagenesis have begun to determine on a microscopic rather than a macroscopic level the interactions important for the allosteric transition which are independent of the model one selects to explain the macroscopic properties of the enzyme. The modifications of Glu-50 in this and previous work (Ladjimi et al., 1988) as well as the modification at Arg-234 (Middleton & Kantrowitz, 1988) have established that the reorientation of the carbamoyl phosphate and aspartate domains plays a critical part in homotropic cooperativity. Not until carbamoyl phosphate binds is the high-affinity aspartate binding site created, and not until aspartate begins to bind to the wild-type holoenzyme does the domain closure of the catalytic chain take place. Furthermore, it is this domain closure that induces the quaternary conformational change resulting in the allosteric transition. Additional site-specific mutagenesis studies are currently in progress to better establish the molecular level details of the allosteric transition and the importance of domain closure to that transition.

ACKNOWLEDGMENTS

We thank W. N. Lipscomb for providing the X-ray coordinates and M. M. Ladjimi for the Glu-50 → Gln enzyme.

REFERENCES

Allewell, N. M. (1989) *Annu. Rev. Biophys. Chem. 18*, 71–92.
Bethell, M. R., Smith, K. E., White, J. S., & Jones, M. E. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 1442–1449.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bromberg, S., Burz, D. S., & Allewell, N. M. (1990) *J. Biochem. Biophys. Methods* (in press).
Collins, K. D., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1869–1877.
Enns, C. A., & Chan, W.-C. (1979) *J. Biol. Chem. 254*, 6180–6186.
Foote, J., & Schachman, H. K. (1985) *J. Mol. Biol. 186*, 175–184.
Gerhart, J. C. (1970) *Curr. Top. Cell. Regul. 2*, 275–325.
Gerhart, J. C., & Pardee, A. B. (1962) *J. Biol. Chem. 237*, 891–896.
Gerhart, J. C., & Schachman, H. K. (1965) *Biochemistry 4*, 1054–1062.
Gerhart, J. C., & Holoubek, H. (1967) *J. Biol. Chem. 242*, 2886–2892.
Gerhart, J. C., & Schachman, H. K. (1968) *Biochemistry 7*, 538–552.
Gibbons, I., Ritchey, J. M., & Schachman, H. K. (1976) *Biochemistry 15*, 1324–1330.
Gouaux, J. E., & Lipscomb, W. N. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4205–4208.
Gouaux, J. E., & Lipscomb, W. N. (1990) *Biochemistry 29*, 389–402.
Griffin, J. H., Rosenbusch, J. P., Weber, K. K., & Blout, E. R. (1972) *J. Biol. Chem. 247*, 6482–6490.
Griffin, J. H., Rosenbusch, J. P., Blout, E. R., & Weber, K. (1973) *J. Biol. Chem. 248*, 5057–5062.
Hammes, G. G., & Wu, C. W. (1971) *Biochemistry 10*, 1051–1057.
Hervé, G., Moody, M. F., Tauc, P., Vachette, P., & Jones, P. T. (1985) *J. Mol. Biol. 185*, 189–199.
Honzatko, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Edwards, B. F. P., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C., & Lipscomb, W. N. (1982) *J. Mol. Biol. 160*, 219–263.
Howlett, G. J., & Schachman, H. K. (1977) *Biochemistry 16*, 5077–5083.
Hsuanyu, Y., & Wedler, F. C. (1987) *Arch. Biochem. Biophys. 259*, 316–330.
Jacobson, G. R., & Stark, G. R. (1973) *Enzymes (3rd Ed.) 9*, 225–308.
Johnson, R. S., & Schachman, H. K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1995–1999.
Kantrowitz, E. R., & Lipscomb, W. N. (1988) *Science 241*, 669–674.
Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980a) *Trends Biochem. Sci. 5*, 124–128.
Kantrowitz, E. R., Pastra-Landis, S. C., & Lipscomb, W. N. (1980b) *Trends Biochem. Sci. 5*, 150–153.
Ke, H.-M., Lipscomb, W. N., Cho, Y., & Honzatko, R. B. (1988) *J. Mol. Biol. 204*, 725–747.
Kim, K. H., Pan, Z., Honzatko, R. B., Ke, H.-M., & Lipscomb, W. N. (1987) *J. Mol. Biol. 196*, 853–875.
Kirshner, M. W., & Schachman, H. K. (1971) *Biochemistry 10*, 1919–1925.
Kirshner, M. W., & Schachman, H. K. (1973) *Biochemistry 12*, 2997–3004.
Krause, K. L., Voltz, K. W., & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1643–1647.
Krause, K. L., Voltz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527–553.
Ladjimi, M. M., & Kantrowitz, E. R. (1987) *J. Biol. Chem. 262*, 312–318.
Ladjimi, M. M., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 276–283.
Ladjimi, M. M., Middleton, S. A., Kelleher, K. S., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 268–276.
Middleton, S. A., & Kantrowitz, E. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5866–5870.
Middleton, S. A., & Kantrowitz, E. R. (1988) *Biochemistry 27*, 8653–8660.
Middleton, S. A., Stebbins, J. W., & Kantrowitz, E. R. (1989) *Biochemistry 28*, 1617–1626.

Monaco, H. L., Crawford, J. L., & Lipscomb, W. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5276–5280.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.
Navre, M., & Schachman, H. K. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1207–1211.
Nowlan, S. F., & Kantrowitz, E. R. (1985) *J. Biol. Chem. 260*, 14712–14716.
Pastra-Landis, S. C., Evans, D. R., & Lipscomb, W. N. (1978) *J. Biol. Chem. 253*, 4624–4630.
Pastra-Landis, S. C., Foote, J., & Kantrowitz, E. R. (1981) *Anal. Biochem. 118*, 358–363.
Porter, R. W., Modebe, M. O., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1846–1559.
Robey, E. A., & Schachman, H. K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 361–365.
Roof, W. D., Foltermann, K. F., & Wild, J. R. (1982) *Mol. Gen. Genet. 187*, 391–400.
Schachman, H. K. (1974) *Harvey Lect. 68*, 67–113.
Schachman, H. K. (1988) *J. Biol. Chem. 263*, 18583–18586.
Silver, R. S., Daigneault, J. P., Teague, P. D., & Kantrowitz, E. R. (1983) *J. Mol. Biol. 168*, 729–745.
Smith, K. A., Nowlan, S. F., Middleton, S. A., O'Donovan, C., & Kantrowitz, E. R. (1986) *J. Mol. Biol. 189*, 227–238.
Stebbins, J. W., Xu, W., & Kantrowitz, E. R. (1989) *Biochemistry 28*, 2592–2600.
Turnbough, C. L., Hicks, K. L., & Donahue, J. P. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 368–372.
Wedler, F. C., & Gasser, F. J. (1974) *Arch. Biochem. Biophys. 163*, 57–68.
Wente, S. R., & Schachman, H. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 31–35.
Wild, J. R., Loughrey-Chen, S. J., & Corder, T. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 46–50.
Wu, C. W., & Hammes, G. G. (1973) *Biochemistry 12*, 1400–1408.
Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487–6500.

Spectroscopic and Equilibrium Studies of Ligand and Organic Substrate Binding to Indolamine 2,3-Dioxygenase†

Masanori Sono
Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208
Received July 10, 1989; Revised Manuscript Received October 12, 1989

ABSTRACT: The binding of a number of ligands to the heme protein indolamine 2,3-dioxygenase has been examined with UV–visible absorption and with natural and magnetic circular dichroism spectroscopy. Relatively large ligands (e.g., norharman) which do not readily form complexes with myoglobin and horseradish peroxidase (HRP) can bind to the dioxygenase. Except for only a few cases (e.g., 4-phenylimidazole) for the ferric dioxygenase, a direct competition for the enzyme rarely occurs between the substrate L-tryptophan (Trp) and the ligands examined. L-Trp and small heme ligands (CN^-, N_3^-, F^-) markedly enhance the affinity of each other for the ferric enzyme in a reciprocal manner, exhibiting positive cooperativity. For the ferrous enzyme, L-Trp exerts negative cooperativity with some ligands such as imidazoles, alkyl isocyanides, and CO binding to the enzyme. This likely reflects the proximity of the Trp binding site to the heme iron. Other indolamine substrates also exert similar but smaller cooperative effects on the binding of azide or ethyl isocyanide. The pH dependence of the ligand affinity of the dioxygenase is similar to that of myoglobin rather than that of HRP. These results suggest that indolamine 2,3-dioxygenase has the active-site heme pocket whose environmental structure is similar to, but whose size is considerably larger than, that of myoglobin, a typical O_2-binding heme protein. Although the L-Trp affinity of the ferric cyanide and ferrous CO enzyme varies only slightly between pH 5.5 and 9.5, the unligated ferric and ferrous enzymes have considerably higher affinity for L-Trp at alkaline pH than at acidic pH. L-Trp binding to the ferrous dioxygenase is affected by an ionizable residue with a pK_a value of 7.3.

Despite the spectral similarities among the three protoheme IX containing monomeric proteins indolamine 2,3-dioxygenase, myoglobin, and horseradish peroxidase (HRP)[1] in their various oxidation states and ligand-bound forms (Sono & Dawson, 1984), the functions of these proteins are different from each other. In particular, although indolamine 2,3-dioxygenase and myoglobin bind O_2 reversibly in their normal functions, only the former can catalyze the insertion of molecular oxygen into the organic substrate L-tryptophan (Trp) to yield the product *N*-formylkynurenine (Hayaishi et al., 1975; Hayaishi, 1976). The existence of a binding site for Trp at the active site in the dioxygenase might be a prerequisite for its catalytic capability. Beyond this point, however, little information is available about either the conformational or the electronic structural difference of their active sites, which is directly related to their functional distinctions.

Ligand binding properties of heme proteins have often been shown to provide a useful clue to the understanding of the active-site structure which can be correlated to the functionality of the proteins. Typical examples are (a) myoglobin (O_2 binding) and HRP (H_2O_2 activation) and (b) cytochrome P-450_{cam} (O_2 activation) and chloroperoxidase (H_2O_2 and Cl^-

†Supported by the U.S. Public Health Service Grant GM 37806. A part of this work was performed at Kyoto University, Kyoto, Japan, while the author was in Professor Hayaishi's former laboratory.

[1] Abbreviations: Trp, tryptophan; CD, circular dichroism; MCD, magnetic circular dichroism; HRP, horseradish peroxidase; $O_2^{\cdot-}$, superoxide anion radical.

0006-2960/90/0429-1451$02.50/0