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A Possible Model for the Concerted Allosteric Transition in *Escherichia coli* Aspartate Transcarbamylase As Deduced from Site-Directed Mutagenesis Studies†

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Received May 20, 1987; Revised Manuscript Received September 10, 1987

ABSTRACT: Aspartate transcarbamylase is stabilized in a low-affinity–low-activity state exhibiting no cooperativity by selective perturbation of the Glu-50–Arg-167 and Glu-50–Arg-234 interdomain salt bridges. Similarly, a high-affinity–high-activity state of the enzyme, retaining a significant amount of cooperativity, is obtained by perturbation of the interaction between Tyr-240 and Asp-271. In this work, we show that the rupture of the link between Tyr-240 and Asp-271 in the enzyme already lacking the interdomain salt bridges regenerates the homotropic cooperative interactions between the catalytic sites and substantially increases the activity and affinity of the enzyme for aspartate. These results suggest a possible relationship between these two sets of interactions for the establishment of the cooperative behavior of the enzyme. Another mutation, Glu-239 to Gln, introduced to perturb the Glu-239–Lys-164 and Glu-239–Tyr-165 interactions between the two catalytic subunits, is sufficient to "lock" the enzyme in the R state. These observations emphasize the importance of the interactions at the interface between the catalytic trimers in maintaining the T state of the enzyme and shed light on the role played by this pathway in the communication of homotropic cooperativity between the different sites. A model including all these findings, as well as the interactions stabilizing the T state or the R state in the presence of the natural substrates, is proposed. In this model, it is suggested that domain closure induced by aspartate binding to one active site triggers a concerted structural change that promotes the domain closure of the remaining active sites, primarily by rupture of the interactions between the catalytic subunits.

A major conclusion that has emerged from kinetic and physicochemical studies of *Escherichia coli* aspartate transcarbamylase (EC 2.1.3.2) is that a concerted transition occurs between two alternate conformational states upon the binding of asparate. In this fashion, the extent of the quaternary change during the conversion from the constrained to the relaxed state need not be directly linked to the degree of ligand saturation (Monod et al., 1965; Howlett & Schachman, 1977; Blackburn & Schachman, 1977; Howlett et al., 1977). However, the structural basis of this transition remained unknown until the structure of the enzyme complexed with the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA)[1] became available (Krause et al., 1985, 1987). The

†This work was supported by a grant from the National Institutes of Health (GM26237). E.R.K. thanks the Camille and Henry Dreyfus Foundation for a fellowship.
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0006-2960/88/0427-0276$01.50/0

Table I: A Summary of the Plasmids Used

name	vector	insert	mutation site	amino acid change	source
pUC119	pUC119				J. Messing
pEK2	pUC8	*pyrBI*			Smith et al. (1986)
pEK33	pUC8	*pyrBI*	240	Tyr → Phe	Middleton & Kantrowitz (1986)
pEK38	pUC119	*pyrBI*			this work
pEK42	pUC8	*pyrBI*	50	Glu → Gln	Ladjimi et al. (1988)
pEK56	pUC119	*pyrBI*	239	Glu → Gln	this work
pEK58	pUC119	*pyrBI*	50/240	Glu → Gln/Tyr → Phe	this work

comparison of this structure with that of the unliganded enzyme (Honzatko et al., 1982; Ke et al., 1984) led to a possible structural mechanism for the homotropic transition which assumes that the structure of the PALA-liganded enzyme is equivalent to the structure of the high-affinity–high-activity state induced by the binding of the natural substrates (Krause et al., 1987). Although this mechanism provides a good starting point, further investigation is required in order to assess the role played by specific residues in the stabilization of either the T state or R state and to identify the pathways by which the local changes are coupled to each other within the entire molecular structure. The use of site-directed mutagenesis allows us to address these issues.

Upon the binding of PALA a complex rearrangement takes place at the active site of the enzyme involving a hinge motion, extensive local reorganization, and movement of loops. The aspartate binding domain[2] moves roughly as a rigid body by 3 Å toward the carbamyl phosphate binding domain[2] around a hinge region located near residue 130, and a small region involving residues 230–245 (the 240s loop) undergoes an additional shift of about 3 Å as well as a major independent reorganization (Krause et al., 1987). As a consequence of these combined effects, Glu-50 and Arg-234 change from being more than 10 Å apart in the unliganded form to less than 3 Å apart in the liganded form, and Arg-167 is shifted by about 2 Å to become involved in a salt link with Glu-50. In turn, the motion of Arg-234 toward Glu-50 ruptures the interaction between Tyr-240 and Asp-271 and the intersubunit interactions between Glu-239 and both Lys-164 and Tyr-165 (Krause et al., 1987).

In the preceding paper (Ladjimi et al., 1988), it was shown that the site-specific replacement of Glu-50 by Gln, which causes the rupture of the Gln-50–Arg-167 and Gln-50–Arg-234 interdomain salt bridges, stabilizes the enzyme in a state having low affinity, low activity, and no cooperativity. On the other hand, Middleton and Kantrowitz (1986) have shown that the replacement of Tyr-240 by Phe, which affects the Tyr-240–Asp-271 hydrogen bond, results in an enzyme with high affinity, high activity, and a small amount of cooperativity. It was then suggested that the interdomain salt bridges were necessary to lock the active site in a configuration optimal for binding and catalysis and were involved in cooperativity (Ladjimi et al., 1988), whereas the Tyr-240–Asp-271 interaction constrains the 240s loop in such a position that an open active site with low affinity and low activity is maintained (Middleton & Kantrowitz, 1986). Since it has become clear that a specific pattern of interactions is associated with distinct affinity states exhibiting little or no cooperativity, it was of interest to determine how they contribute to the mechanism of homotropic cooperative interactions. A double mutant with Gln at position 50 and Phe at position 240 was therefore prepared by recombinant DNA methodology.

The above-mentioned mutagenesis and X-ray crystallographic studies suggested that the conformational change involved in the transition between T and R states may be mediated primarily by the 240s loop. With this in mind, we decided to perturb another major intersubunit interaction involving this loop: in the T state, Glu-239 of C1 (belonging to one catalytic subunit) interacts with both Lys-164 and Tyr-165 of C4 (belonging to the other catalytic subunit), thereby maintaining the juxtaposition of the two catalytic subunits in a highly constrained structure (Krause et al., 1987; Ke et al., 1987) (see Figure 1). In order to evaluate the role played by this set of interactions in the information transfer between sites, Glu-239 was replaced by Gln with site-directed mutagenesis.

Following kinetic characterization of the Gln-50–Phe-240 and the Gln-239 enzymes, a model for a concerted allosteric transition is proposed in which the movements of the 240s loop plays a major role in the transmission of the conformational change.

Experimental Procedures

Materials. The plasmid pUC119, the M13 phage, M13K07, and the *E. coli* strain MV1190 were obtained from J. Messing. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim. Details concerning the plasmids used in this work are shown in Table I. All other materials were as previously described (Ladjimi & Kantrowitz, 1987; Ladjimi et al., 1988).

Methods. (*i*) *Construction of pEK38.* In order to allow the sequencing of mutants in the same plasmid as was used for the overproduction of the enzyme, a new plasmid was constructed on the basis of pUC119, which contains both an M13 and pBR322 origin of replication. Phage particles containing single-stranded copies of the plasmid DNA can be produced in the presence of a helper phage. A 2.8-kb *Pst*I–*Bam*HI fragment, containing the entire *pyrBI* operon, was isolated from the plasmid pEK2 (Table I) after agarose gel electrophoresis with NA45 paper. This fragment was mixed with pUC119 which had been previously cut with the same two enzymes, and the mixture was treated with T4 DNA ligase at 4 °C overnight, followed by transformation into U39a (F^- *ara*, *thi*, Δ*pro-lac*, Δ*pyrB*, *rpsL*), a strain which has a deletion in the *pyrBI* region. Ten transformants were selected, and

[1] Abbreviations: PALA, *N*-(phosphonoacetyl)-L-aspartate; T and R states, tight and relaxed states of the enzyme having low and high substrate affinity, respectively; $[S]_{0.5}$, substrate concentration at half the maximal observed specific activity; Tris, tris(hydroxymethyl)aminomethane; Gln-50 enzyme, mutant enzyme with glutamine substituted in place of glutamic acid at position 50 in the catalytic chain of aspartate transcarbamylase; Gln-239 enzyme, mutant enzyme with glutamine substituted in place of glutamic acid at position 239 in the catalytic chain of aspartate transcarbamylase; Gln-50–Phe-240 enzyme, mutant enzyme with Gln replacing Glu at position 50 and Phe replacing Tyr at position 240 in the catalytic chain of aspartate transcarbamylase; holoenzyme, entire aspartate transcarbamylase molecule composed of two catalytic subunits and three regulatory subunits. C, followed by a number, e.g., C1 or C4, refers to a particular polypeptide chain in aspartate transcarbamylase as specified in Figure 6 of Honzatko et al. (1982).

[2] The aspartate and carbamyl phosphate binding domains of the catalytic chain have been previously referred to as the equatorial and polar domains, respectively (Monaco et al., 1978; Honzatko et al., 1982; Krause et al., 1987).

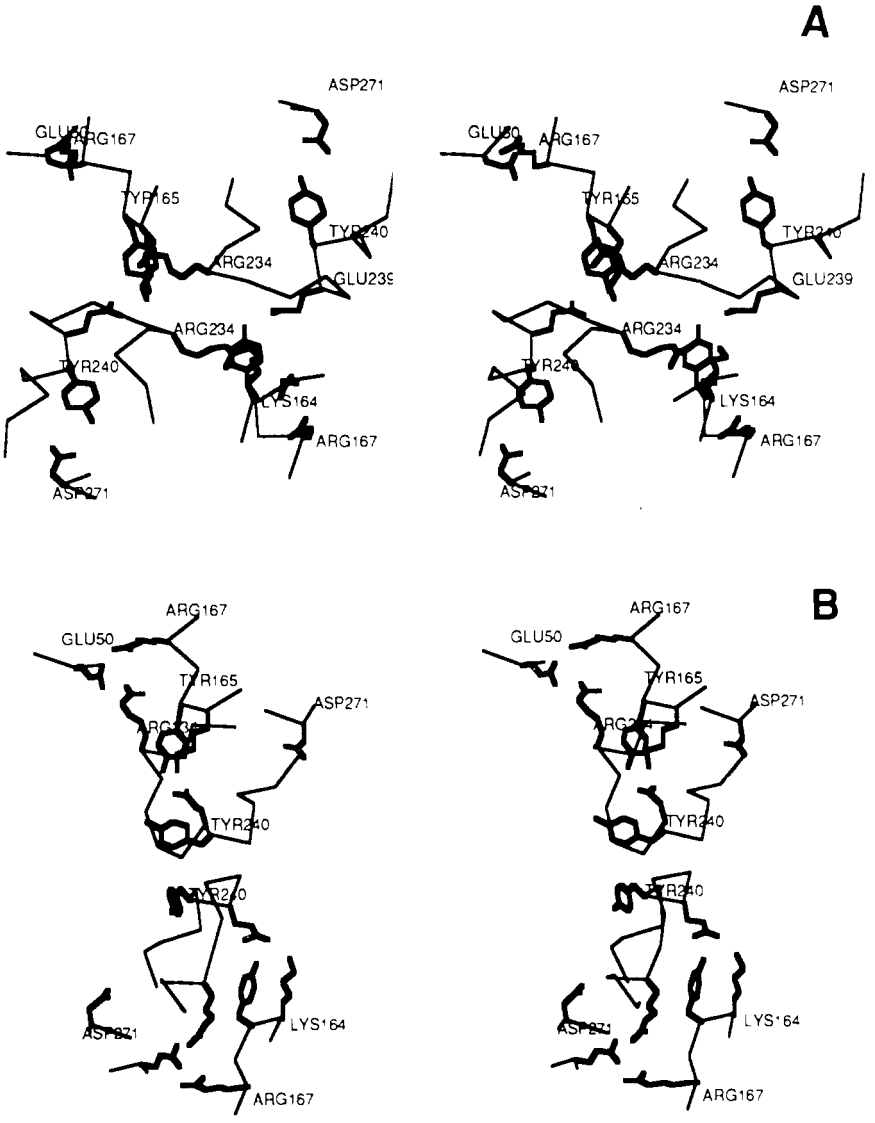

FIGURE 1: Stereoview showing key residues in the carbamyl phosphate binding domain (Glu-50) and the aspartate binding domain (Tyr-165, Arg-234, Glu-239, Tyr-240, and Asp-271) from two opposite catalytic chains (C1, top, and C4, bottom). In unliganded form (A), the 240s loops are side by side and stabilized by Glu-239–Lys-164 and Glu-239–Tyr-165 interchain interactions and by the Tyr-240–Asp-271 link. In this state, no interactions exist between Glu-50–Arg-167 and Glu-50–Argy-234. In the PALA-liganded form (B), the Tyr-240–Asp-271, Glu-239–Lys-164, and Glu-239–Tyr-165 interactions are lost while the Glu-50–Arg-167 and Glu-50–Arg-234 interdomain salt bridges are formed. For clarity, the labels of the following side chains have been omitted. In the unliganded form (A): Lys-164 of C1; Glu-50, Tyr-165, and Glu-239 of C4. In the PALA-liganded form (B): Lys-164 and Glu-239 of C1; Glu-50, Lys-164, Tyr-165, Arg-234, and Glu-239 of C4.

plasmid DNA was isolated by the alkaline lysis procedure (Maniatis et al., 1982). Each of the plasmid candidates was first checked for proper size and then by restriction analysis. A plasmid was isolated, pEK38, which contained the *pyrBI* operon in the vector pUC119.

(ii) Isolation of Single-Stranded DNA from pUC119-Based Plasmids. The appropriate plasmid was transformed into MV1190 [Δ(*lac-proAB*), *supE*, Δ(*sri-recA*)306::Tn10(tet^r)/F′ *traD36*, *proAB*, *lacI*^{q}ΔM15], and a single colony was used to inoculate 2 × YT media supplemented with 5 μg of thymine/mL and 100 μg of ampicillin/mL. M13K07, a kanamycin-resistant helper phage, was added at a phage to cell ratio greater than 20, and the culture was incubated for 2–3 h at 37 °C. At this point, 70 μg of kanamycin/mL was added, and the culture was incubated overnight at 37 °C. Single-stranded DNA was then isolated from the phage present in the supernatant (Messing, 1983).

(iii) Other Methods. Oligonucleotide synthesis, enzyme purification, determination of protein concentration, aspartate transcarbamylase assay, and data analysis were as previously described (Ladjimi & Kantrowitz, 1987; Ladjimi et al., 1988).

RESULTS

Construction of Gln-50–Phe-240 Double Mutant. The construction of the Gln-50–Phe-240 double mutant was accomplished by the combination of appropriate DNA fragments from plasmids containing the single mutants (Ladjimi et al., 1988; Middleton & Kantrowitz, 1986). The fragment of the *pyrB* gene containing the Gln-50 mutation was cut from plasmid pEK42 with the restriction enzymes *Pst*I and *Bst*EII, while the fragment of the *pyrB* gene containing the Phe-240 mutation was cut from plasmid pEK33 with the restriction enzymes *Bst*EII and *Bam*HI. The appropriate fragments were isolated after agarose gel electrophoresis with NA45 paper. Simultaneously, the vector to be used in the final construction was prepared by cutting the plasmid pUC119 with *Pst*I and *Bam*HI followed by treatment with calf intestine alkaline phosphatase. This vector, isolated as described above, was mixed with the fragments containing the Gln-50 and Phe-240 mutations, and the mixture was treated with T4 DNA ligase

overnight at 4 °C followed by transformation into U39a. Ten possible candidates were selected, and plasmid DNA was isolated by an alkaline lysis procedure (Maniatis et al., 1982). Each of the plasmid candidates was first checked for proper size and then by restriction analysis. A plasmid was isolated, pEK58, which contained only one copy of each of the three initial fragments.

Construction of Gln-239 Mutant Aspartate Transcarbamylase by Site-Directed Mutagenesis. The replacement of glutamic acid at position 239 in the catalytic chain by glutamine was accomplished by site-directed mutagenesis according to the method of Zoller and Smith (1982) with the modification previously described (Ladjimi et al., 1988).

A 20-mer oligonucleotide was synthesized to introduce the specific G to C base change necessary for the replacement of Glu-239 by Gln. Before synthesis, the sequence of the oligonucleotide was compared to the sequence of the entire *pyrBI* operon to ensure that there were no secondary hybridization sites. Following primer extension, ligation, and transformation, ten plaques were selected and used to produce additional phage from which single-stranded DNA was isolated. Each candidate was sequenced (Sanger et al., 1977), by use of a specific primer located 85 bases from the mutation site, to confirm the mutation. Two of the ten candidates showed the expected GAG → CAG change.

Recloning the Gln-239 Mutation. In order to overproduce the Gln-239 mutant enzyme, it was necessary to reclone the mutated *pyrB* gene onto a plasmid. After agarose gel electrphoresis, a *Bst*EII–*Eco*RV fragment of 864 base pairs containing the desired mutation was isolated from the gel with NA45 paper. In addition, the plasmid pEK38 was cut with the same two restriction enzymes, and the larger fragment was isolated in a similar fashion. This fragment contained the vector pUC119 along with the rest of the *pyrBI* operon. The fragment from the mutant M13 RF was then combined with the fragment from the pEK38 plasmid and treated with T4 DNA ligase. Selection was accomplished after transformation in U39a. A plasmid was isolated, pEK56, which carried the desired mutation.

Confirmation of Mutations. (*i*) *The Gln-50–Phe-240 Double Mutant.* For the double mutant, dideoxy sequencing (Sanger et al., 1977) was utilized to verify the Gln-50 and Phe-240 mutations and the sequence around the *Bst*EII site, since this site was located within the *pyrB* gene.

(*ii*) *The Gln-239 Mutant.* The region of pEK56 between *Bst*EII–*Eco*RV was sequenced to ensure that no other mutations had occurred during the mutagenesis and subsequent recloning steps. Dideoxy sequencing (Sanger et al., 1977) was carried out with five oligonucleotide primers spaced along the fragment. Analysis of the sequence data revealed no mutations other than the desired change.

Expression and Purification of Mutant Aspartate Transcarbamylase. Overproduction of the Gln-50–Phe-240 and the Gln-239 mutant aspartate transcarbamylases was accomplished as described by Nowlan and Kantrowitz (1985) with plasmids pEK58 and pEK56, respectively.

Kinetic Properties of Gln-50–Phe-240 and Gln-239 Mutant Enzymes. The effect of the Phe-240 substitution on the aspartate saturation kinetics of the Gln-50 enzyme is shown in Figure 2. For comparison, the saturation curves of the previously described mutant enzymes in which Glu-50 was replaced by Gln (Ladjimi et al., 1988) and Tyr-240 by Phe (Middleton & Kantrowitz, 1986) are also shown. The replacement of Tyr-240 by Phe in the Gln-50 enzyme (double mutant) results in the regeneration of the homotropic cooperativity and increased catalytic activity relative to the Gln-50 enzyme (single mutant). The aspartate saturation curve of the Gln-239 enzyme is also shown in Figure 2, along with that of the wild-type enzyme. This change leads to a complete loss of cooperativity without altering the maximal specific activity.

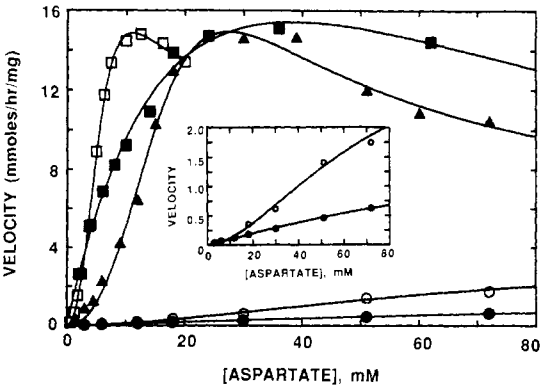

FIGURE 2: Aspartate saturation curves of the wild-type and mutant enzymes. The reactions were carried at 25 °C in 50 mM Tris–acetate (pH 8.3) at a saturating concentration of carbamyl phosphate (4.8 mM). (●) Gln-50; (○) Gln-50–Phe-240; (▲) wild type; (■) Gln-239; (□) Phe-240. (Inset) Closeup of the saturation curves of the Gln-50 (●) and Gln-50–Phe-240 (○) enzymes.

Table II: Kinetic Parameters for Wild-Type and Mutant Holoenzymes

	V_{max} (mmol·h^{-1}·mg^{-1})[a]	$[S]_{0.5}$ (mM)	n_H
wild type[b]	15.0	12.3	2.2
Glu-50			
Glu-239			
Tyr-240			
Phe-240[c]	16.6	4.7	1.8
Gln-50[b]	2.6	245.0	1.0
Gln-50–Phe-240[d]	3.4	63.8	1.6
Gln-239[d]	14.9	6.7	1.0

[a] Maximal specific activity. [b] Data from preceeding paper (Ladjimi et al., 1988). [c] Data from Middleton and Kantrowitz (1986). [d] These parameters for the Gln-50–Phe-240 and Gln-239 enzymes were obtained as described in the preceding paper (Ladjimi et al., 1988).

The kinetic parameters of the mutant and wild-type enzymes are summarized in Table II. It can be seen from the comparison of the various $[S]_{0.5}$ values that while the Gln-50 enzyme exhibits a dramatically low affinity for aspartate and no cooperativity, the Phe-240 enzyme shows the opposite. When the Phe-240 mutation was introduced into the Gln-50 enzyme, the $[S]_{0.5}$ decreased by a factor of 4 and the Hill coefficient increases from 1 to 1.6 as compared to the Gln-50 enzyme. The maximal velocity of the double mutant was also slightly improved although it still represents only 20% of that of the wild-type enzyme. The kinetic analysis of the Gln-239 enzyme shows an absence of cooperativity and an increased affinity for aspartate compared to that of the wild-type enzyme. However, the maximal specific activity is nearly identical with that of the wild-type enzyme.

Kinetic Properties of the Gln-50–Phe-240 and the Gln-239 Mutant Isolated Catalytic Subunits. In order to evaluate the effect of the various amino acid replacements on aspartate affinity, saturation curves were also performed with the isolated catalytic subunit. In this case, changes in the apparent K_m reflect changes in the intrinsic environment of the active site only and not secondary changes in cooperativity. Table III shows that the K_m for aspartate and the maximal velocity of the Gln-50–Phe-240 catalytic subunit are unchanged with respect to those of the Gln-50 catalytic subunit. The maximal velocity of the Gln-239 catalytic subunit is almost identical

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

	V_{max} (mmol·h^{-1}·mg^{-1})	k_{cat} (s^{-1})[a]	K_m (mM)	k_{cat}/K_m (s^{-1}·mM^{-1})
wild type[b]	27.6	253	5.9	42.8
Glu-50				
Glu-239				
Tyr-240				
Phe-240[c]	27.6	255	5.7	44.7
Gln-50[b]	2.9	27	57.2	0.47
Gln-50–Phe-240[d]	2.8	26	52.9	0.49
Gln-239[d]	24.4	225	21.9	10.3

[a] The k_{cat} values were calculated per active site and were obtained as described in the preceeding paper (Ladjimi et al., 1988). [b] Data from the preceeding paper (Ladjimi et al., 1988). [c] Data from Middleton and Kantrowitz (1986). [d] Parameters for the Gln-50–Phe-240 and Gln-239 enzymes were obtained as described in the preceding paper (Ladjimi et al., 1988).

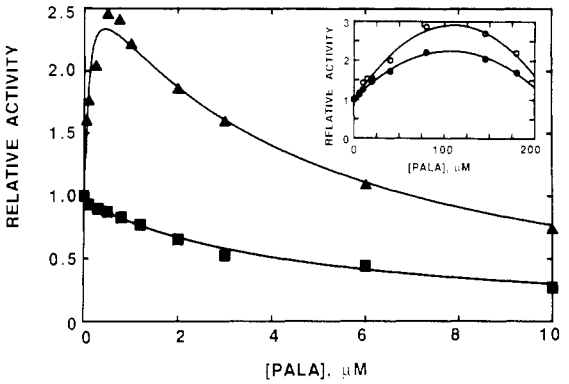

FIGURE 3: Activation of the wild-type (▲) and the Gln-239 mutant (■) enzymes by PALA at low concentrations of aspartate. The aspartate transcarbamylase activity was measured as described in the legend to Figure 2 but at an aspartate concentration corresponding to $(1/6)K_m$ (see Table I). The relative activity represents the ratio of the activity in the absence or in the presence of PALA to that in its absence. (Inset) Stimulation of the activity of the Gln-50 (●) and the Gln-50–Phe-240 (○) enzymes by PALA.

while the K_m for aspartate is increased by a factor of 4 with respect to the wild-type catalytic subunit.

Influence of PALA on Activity of Gln-50–Phe-240 and Gln-239 Mutant Enzymes. As shown in Figure 3 (inset), the Gln-50–Phe-240 mutant enzyme shows a slight increase in the maximal relative activity, as compared to that of the Gln-50 enzyme. However, the concentration of PALA required by the double mutant to attain the maximal stimulation is similar to that required by the Gln-50 single mutant. The activity of the Gln-239 enzyme, however, is inhibited even at low concentrations of PALA.

Discussion

Effect of Introduction of the Phe-240 Mutation on the Properties of Gln-50 Mutant Enzyme. As shown in the preceding paper (Ladjimi et al., 1988), the replacement of Glu-50 by Gln results in an enzyme lacking cooperativity and having both low affinity for aspartate and low catalytic activity. Since identical results, in terms of affinity for aspartate and activity, were obtained with the Michaelian catalytic subunit, we concluded that the altered properties of the Gln-50 enzyme must be related to the intrinsic structure of the active site and not to the absence of cooperativity. Correlating these changes with the structure of the enzyme, we then concluded that the Glu-50–Arg-167 and Glu-50–Arg-234 interdomain salt bridges were an absolute requirement for the establishment of the high-affinity–high-activity state of the active site. Since PALA stimulated the activity of the Gln-50 enzyme, it was clear that these salt bridges were not critical for the existence of interacting sites. In the present study, we show that the rupture of the Tyr-240–Asp-271 interaction at a site remote from the active site is sufficient to regenerate cooperativity in the Gln-50 enzyme. Examination of the crystallographic data indicates that the interdomain salt bridges and the Tyr-240–Asp-271 interaction do not occur simultaneously in the same form of the enzyme. In the unliganded form, the Tyr-240–Asp-271 link is present, and no interaction occurs between Glu-50–Arg-167 and Glu-50–Arg-234, whereas the opposite pattern of interactions is observed upon the binding of PALA (Krause et al., 1987). Thus, the lack of cooperativity in the Gln-50 enzyme is not due solely to the absence of the interdomain salt links in the R state but also to the presence of the Tyr-240–Asp-271 interaction in the T state. The rupture of the Tyr-240–Asp-271 interaction would give more stability to the Gln-50 R state, leading to the reestablishment of cooperativity. Since the K_m values for aspartate of the Gln-50–Phe-240 and Gln-50 catalytic subunits are almost identical, it would appear that the 4-fold decrease in the $[S]_{0.5}$ for aspartate of the Gln-50–Phe-240 holoenzyme relative to the Gln-50 holoenzyme is due to the increase in the Hill coefficient. The observation that the Gln-50–Phe-240 enzyme requires as much PALA to achieve maximal activation (Figure 3, inset) as the Gln-50 enzyme suggests that an intrinsic structural change at the active site is an unlikely explanation of the effects of the second mutation.

It is interesting to note that the $[S]_{0.5}$ for aspartate of the Gln-50–Phe-240 holoenzyme is similar to that of the Gln-50 catalytic subunit, suggesting that the introduction of the Phe-240 mutation in the Gln-50 holoenzyme allows the active site to regain a configuration similar to that of the Gln-50 catalytic subunit. Likewise, the substitution of Phe for Tyr-240 in wild-type holoenzyme decreases the $[S]_{0.5}$ for aspartate to the value observed for the wild-type catalytic subunit. By assuming that the $[S]_{0.5}$ values are indicative of the affinity of both enzymes for aspartate, it is tempting to speculate that the role of the Tyr-240–Asp-271 interaction is to maintain the constraints that decrease the affinity of the catalytic site for aspartate and that the relief of this constraint, in the course of the T to R transition, restores the maximal affinity for aspartate.

Effects of Glu-239 to Gln Change on Binding, Catalytic, and Regulatory Properties of the Enzyme. The replacement of Glu-239 by Gln in the catalytic chain, which results in the perturbation of the Glu-239–Lys-164 and Glu-239–Tyr-165 intersubunit interactions, leads to a complete loss of cooperativity and decreased $[S]_{0.5}$ for aspartate. Although several interactions appear to participate in the stabilization of the unliganded form and a rather complex reorganization at the tertiary level takes place when the enzyme binds PALA, the single Glu to Gln change is sufficient to completely destabilize the T state and "freeze" the enzyme in the R state. The observation that the activity of the Gln-239 enzyme is inhibited by very low concentrations of PALA at very low aspartate concentrations is strongly indicative of the absence of interacting sites. Thus, the intersubunit interactions between Glu-239 of one chain (C1) and Lys-164 and Tyr-165 of the other chain (C4), and their symmetry-related counterparts (Ke et al., 1984; Krause et al., 1987), are critical for the stability of the T state.

Since the specific change involves the C1–C4 interface, it can be concluded that the interaction between the catalytic trimers is an important avenue for the communication of the homotropic cooperative interactions between the catalytic sites,

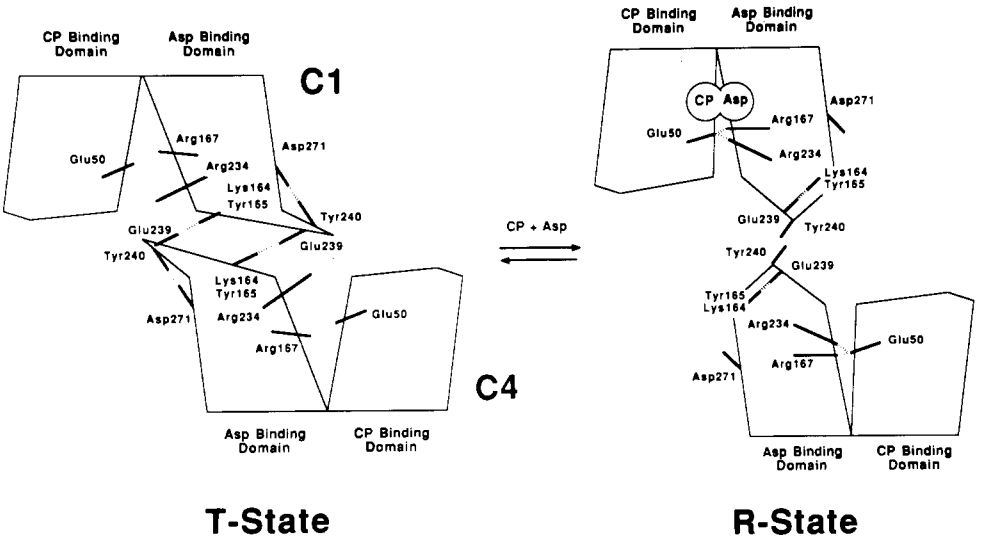

FIGURE 4: Model for the mechanism of cooperativity in aspartate transcarbamylase. Shown schematically are the two extreme conformations of a C1–C4 pair in the T state (left) and the R state (right), as deduced from X-ray crystallography (Honzatko et al., 1982; Ke et al., 1984; Krause et al., 1985, 1987). For clarity, only one catalytic chain from each of the upper (C1) and lower (C4) catalytic subunits is shown. Because of the molecular 3-fold axis, the various interactions shown here are repeated in the C2–C5 and C3–C6 pairs. Each catalytic chain is composed of two structural domains. The N-terminal portion of the catalytic chain comprises the carbamyl phosphate binding domain while the C-terminal portion comprises the aspartate binding domain. Upon aspartate binding (in the presence of carbamyl phosphate), the aspartate binding domain moves toward the carbamyl phosphate binding domain resulting in the closure of the active site. The 240s loops of C1 and C4 undergo a large alteration in position and change from being side by side in the T state to almost one on top of the other in the R state. On the quaternary level, the catalytic subunits move apart resulting in an elongation of the molecule. There is also a rotation about 3-fold axes of the enzyme, which is omitted in this diagram for clarity. The interactions necessary for the stability of either the T or R state in terms of affinity and activity were deduced from site-directed mutagenesis on the basis of the crystallographic structures of the unliganded and PALA-liganded enzymes.

as suggested previously (Krause et al., 1985; Ladjimi & Kantrowitz, 1987). However, the change at position 239 does not necessarily lead to a selective perturbation of the interface between the trimers. In fact, Glu-239 is located in the vicinity of the catalytic–regulatory interface (C–R) in the unliganded form (Honzatko et al., 1982; Ke et al., 1984), and the Glu-239 to Gln change could equally well disturb this interface, thereby destabilizing the T state. This is consistent with several reports emphasizing the involvement of the C–R interface in the homotropic cooperativity (Subranami et al., 1977; Bothwell & Schachman, 1980; Ladjimi et al., 1985; Chan, 1975a,b; Chan & Enns, 1981; Ladner et al., 1982).

The replacement of Glu-239 by Gln has opposite effects on the K_m for aspartate in the holoenzyme and the catalytic subunit. The K_m of aspartate decreases in the holoenzyme but increases in the catalytic subunit with respect to their wild-type counterparts. A comparison of the unliganded and PALA-liganded enzymes shows that the Glu-239–Lys-164 and Glu-239–Tyr-165 links exist in both forms. However, the interactions change from being interchain, between two differently catalytic chains (C1–C4) belonging to two distinct trimers, to intrachain within the same trimer (Krause et al., 1987). Since the perturbation of these interactions increases the K_m for aspartate of the catalytic subunit, it can be concluded that the Glu-239–Lys-164 and Glu-239–Tyr-165 interactions are involved in maintaining the optimal configuration of the active site in the catalytic subunit. However, when the Gln-239 catalytic subunits are complexed with the regulatory subunits to form the holoenzyme, the $[S]_{0.5}$ for aspartate becomes very close to that of wild-type catalytic subunit (6.7 and 5.9 mM, respectively). Provided that the $[S]_{0.5}$ values reflect the true affinities, this can be explained by assuming that the association of the catalytic with the regulatory subunit in the quaternary structure not only introduces constraints that lower the affinity of the active site in the T state but helps to maintain the optimal configuration of the active site in the R state as well, whether the Glu-239–Lys-164 and Glu-239–Tyr-165 interactions are present or not.

A Model for Cooperative Interactions in Aspartate Transcarbamylase. On the basis of the information from biochemical, crystallographic, and mutagenesis studies, it is now possible to propose a molecular mechanism for a concerted allosteric transition in aspartate transcarbamylase.

(*i*) *Structural Basis for the Model.* Previous studies have shown that the binding of the active site ligands to either the catalytic subunit or the holoenzyme causes a conformational change which can be detected by ultraviolet difference spectroscopy (Collins & Stark, 1969, 1971), circular dichroism (Griffin et al., 1972), and difference sedimentation (Kirshner & Schachman, 1971, 1973b; Howlett et al., 1977) and in a more direct way by X-ray solution scattering (Moody et al., 1979) and X-ray crystallography (Ladner et al., 1982; Krause et al., 1985). For example, upon succinate binding (in the presence of carbamyl phosphate) the isolated catalytic subunit exhibits an increase while the holoenzyme shows a decrease of the sedimentation coefficient. Thus, the isolated catalytic subunit seems to compress while the holoenzyme expands (Kirshner & Schachman, 1971, 1973b). Even more important, it is the compression of the catalytic subunit within the entire molecule that induces the expansion of the holoenzyme (Kirshner & Schachman, 1973a,b; Schachman, 1974). From X-ray crystallography, it has become clear that the compression of the catalytic subunit occurs as the two domains of each chain move together, whereas the expansion of the holoenzyme is due to a rotation and elongation of the molecule at the quaternary level.

Figure 4 represents schematically the major interactions that stabilize the two extreme conformations of aspartate transcarbamylase. In the T state, the 240s loop is stabilized by the interchain link between Tyr-240 and Asp-271 as well as intersubunit interactions between Glu-239 and both Lys-164 and Tyr-165. In the R state, the interaction between Tyr-240 and

Asp-271 is abolished, whereas the interdomain salt bridges Gln-50–Arg-167 and Glu-50–Arg-234 are formed. In addition, the Glu-239–Lys-164 and Glu-239–Tyr-165 interchain interactions are lost and replaced by new intrachain interactions between Glu-239 and both Lys-164 and Tyr-165.

(*ii*) *Functional Aspects of the Model As Deduced by Site-Directed Mutagenesis.* (*a*) *Replacement of Glu-50 by Glutamine.* The closure of the two domains of the catalytic chain upon the binding of the substrates (or PALA) results in the formation of the high-affinity–high-activity state of the enzyme. Specifically, salt bridging interactions between Glu-50 of the carbamyl phosphate domain and both Arg-167 and Arg-234 of the aspartate binding domain are formed. When the interdomain salt links cannot form, the R state is disfavored, and an enzyme that exhibits no homotropic cooperativity, and a dramatic reduction of affinity for aspartate and activity is obtained (Ladjimi et al., 1988).

(*b*) *Replacement of Glu-239 by Glutamine.* The interactions between the catalytic chains of the upper and lower trimer are critical for the stability of the T state. When these links are lost, the enzyme is incapable of remaining in the T state and flips into the R state. The replacement of Glu-239 by Gln abolishes homotropic cooperativity in the enzyme and allows the active site to reach the maximal affinity for aspartate without altering the maximal observed specific activity.

(*c*) *Replacement of Tyr-240 by Phenylalanine.* The interaction between Tyr-240 and Asp-271 is important but not critical for the stability of the T state and is unnecessary for the stability of the R state. When Phe was substituted for Tyr-240, a substantial reduction in cooperativity and a marked increase in the affinity for aspartate but no alteration in the specific activity was observed (Middleton & Kantrowitz, 1986).

(*iii*) *Evidence for Communication between Active Sites and a Concerted Quaternary Change.* Communication between all the catalytic chains of one subunit as well as the catalytic chains in different subunits was demonstrated in several studies [see review by Schachman (1974)]. Hybrids containing all the different combinations of active and inactive chains always exhibit the full change in the quaternary structure, which indicates that there are no discrete cooperative units within the enzyme (Gibbons et al., 1974, 1976). The evidence that the quaternary change is concerted comes from the fact that the maximal change in the quaternary structure is reached when only one out of six binding sites is occupied (Kirschner & Schachman, 1973b). The binding of one PALA molecule to a T-state active site is sufficient to convert all of the remaining sites to the R conformation (Foote & Schachman, 1985).

(*iv*) *A Possible Mechanism for the T to R Concerted Allosteric Transition.* For the wild-type enzyme, there is ample evidence to suggest that the allosteric change is induced by the binding of substrates. Substrate binding not only causes tertiary conformational changes within a catalytic chain resulting in the closure of the two domains but also results in an expansion of the holoenzyme. The domain closure is a complex structural rearrangement that involves more than a simple hinge motion. In particular, the 240s loop of the aspartate binding domain undergoes an additional shift in position, which is stabilized by new intradomain and intrachain interactions.

The binding of aspartate (in the presence of carbamyl phosphate) causes two structural changes in the enzyme. First, a closure of the domains within a catalytic chain occurs, thus establishing the high-affinity–high-activity active site. Second, this local conformational change induces the quaternary change in the enzyme which results in the domain closure of all the other active sites.

In the T state, the 240s loop of C1 is maintained far apart from the carbamyl phosphate binding domain by both the Tyr-240–Asp-271 interaction and the Glu-239–Lys-164 and Glu-239–Tyr-165 links between neighboring 240s loops. These interactions prevent the movement of the 240s loop toward the carbamyl phosphate binding domain, which would result, upon aspartate binding, in a closure of the domains and the formation of the high-affinity–high-activity state through the Glu-50–Arg-167 and Glu-50–Arg-234 salt bridges. In order for the domain closure to occur, not only do the interactions which stabilize the 240s loop in the T state have to be broken, but steric constraints have to be overcome as well. This steric hindrance is relieved by a structural rearrangement of the 240s loop which ruptures the Tyr-240–Asp-271 interaction and the two symmetrically related links between Glu-239 and Lys-164 and Tyr-165 (C1–C4) resulting in domain closure. However, this change cannot occur only in one C1–C4 pair, since the different catalytic chains of the upper and lower subunits are in fact forced together in a strained conformation by the favorable interactions between their respective 240s loops. Any change in the interchain interactions of one pair, say C1–C4, is transmitted to the C2–C5 and C3–C6 pairs as well. This leads to the conversion of the molecule into the unstrained R conformation through rotation and elongation of the enzyme.

Thus, in the mechanism presented here, it is suggested that the *tertiary changes* involving the movement of the 240s loop are sufficient to trigger a *concerted* allosteric transition via the rupture of the interactions at the C1–C4 interface. Therefore, the effects of ligand binding need only to extend to this interface in order for the enzyme to cross the energy barrier and reach to the R state. This is in agreement with the studies of Howlett et al. (1977) indicating that only 3.3 kcal/mol separate the two states in the absence of substrates.

The analysis of the double mutant enzyme with mutations at both Glu-50 and Tyr-240 supports this model. The mutant enzyme with only Glu-50 replaced by Gln is stabilized in the T state, and aspartate is not able to induce the activation of the enzyme. This is due to the lack of the interdomain salt bridges which destabilize the domain closure (Ladjimi et al., 1988). However, when this enzyme lacks the Tyr-240–Asp-271 link, which allows the 240s loop to attain its conformation in the R state, communication between the sites is restored and cooperativity reappears.

The major role given by this model to the interface between the catalytic subunits in propagating the conformational changes does not minimize the role of the C–R interface in the mechanism. Indeed, changes in the C–C interactions are likely to be accompanied by changes in the C–R interactions due to the rotation and elongation of the enzyme.

Although the model presented here seems to be consistent with an important body of experimental data, further physical and biochemical studies are necessary to test its validity.

ACKNOWLEDGMENTS

We are indebted to Steven A. Middleton for all his assistance and thank W. N. Lipscomb for providing the X-ray coordinates.

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