Articles

Site-Directed Alterations to the Geometry of the Aspartate Transcarbamoylase Zinc Domain: Selective Alteration to Regulation by Heterotropic Ligands, Isoelectric Point, and Stability in Urea†

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ABSTRACT: Structural aspects requisite for allosteric function in the regulatory chain of aspartate transcarbamoylase were explored by site-specific amino acid insertion or substitution within the zinc domain in the region of contact between the catalytic and regulatory chains. Amino acid substitution at two positions yielded enzymes which retained a maximum velocity similar to that of the wild-type enzyme but responded differently from the native enzyme in the presence of regulatory nucleoside triphosphates. A change of zinc coordinate amino acid C109 to histidine and a change of E119 to aspartic acid resulted in enzymes which demonstrated synergistic inhibition by CTP and UTP but not inhibition by CTP in either phosphate buffer or a morpholino-based tripartate (TP) buffer at pH 7. At pH 8.3, where there is a higher proportion of T-state conformers in the native enzyme, the mutants diverged from their similar kinetic behavior. C109H remained an enzyme which was not inhibited by CTP but was still inhibited by CTP+UTP. E119D was inhibited by both CTP and CTP+UTP. Activation of the mutants by ATP was found to vary either with pH or with phosphate as a buffer component. C109H was activated by ATP in phosphate, while in TP at either pH 7 or 8.3 its activation by ATP was diminished or absent. E119D was activated by ATP in phosphate at pH 7 or in TP at pH 8.3, but not in TP at pH 7. In TP at pH 7, where neither mutant was activated by ATP, the $S_{0.5}$ values and Hill coefficients of the unliganded mutant enzymes resembled those of the ATP-liganded wild-type enzyme. While neither mutation would be predicted to alter the net charge of the holoenzyme, differences in the isolectric point of the mutants were observed if phosphate was present. This result suggests that the isoelectric point of aspartate transcarbamoylase is conformationally dependent and that the mutants exist in an altered conformation. In addition, the stabilities of both mutant holoenzymes were reduced substantially from those of the wild-type enzyme in 4 M urea. C109H was more stable at pH 8.25 in a Tris buffer; E119D was more stable at pH 7 in the phosphate buffer. Potential effects of these mutations on the active site chemistry and geometry are discussed.

Aspartate transcarbamoylase of *Escherichia coli* (ATCase, carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) is an allosteric enzyme of long-standing investigation for its substrate induced cooperativity and the effect of heterotropic regulatory molecules on the kinetic properties [for reviews, see Stevens et al. (1991), Lipscomb (1991), Perutz (1990), Allewell (1989), Kantrowitz and Lipscomb (1988), Schachman (1988), Kantrowitz et al. (1980a,b)]. This enzyme catalyzes the condensation of carbamoyl phosphate onto the amine group of aspartic acid, to form *N*-carbamoyl-L-aspartic acid and inorganic phosphate (Gerhart & Pardee, 1962). Through further reactions, this metabolic intermediate is then converted to UTP and CTP, which are used for nucleic acid biosynthesis.

The nucleoside triphosphates, ATP, CTP, and UTP+CTP, change the enzyme's catalytic activity. At physiological concentrations of substrates, the effect is dramatic, as the range between ATP-activated and CTP+UTP-inhibited enzyme is a ~40-fold change in velocity. The effectors ATP and CTP act in a manner similar to a competitive inhibitor, in that only the $S_{0.5}$ and not V_m is altered (Gerhart & Pardee, 1962, 1963, 1964). The effector does not compete with the substrates for binding at the active site; it binds to a separate allosteric site on a distinct polypeptide chain, termed the regulatory chain (Gerhart & Pardee, 1962; Gerhart et al., 1965, 1968; Hammes et al., 1970; Honzatko et al., 1982). As these effector molecules are dissimilar to the substrates in structure and bind at a separate site, they are termed heterotropic ligands. The end product, CTP, alone inhibits the enzyme to ~40–60% the activity of unbound enzyme. UTP, the penultimate metabolite in the pathway, does not induce significant inhibition of the enzyme alone, but in conjunction with CTP it has been shown to reduce enzyme activity by 95% (Wild et al., 1989; Zhang & Kantrowitz, 1991).

Crystallographic, hydrodynamic, and spectroscopic data suggest that the enzyme has two isomeric conformations, tense (T) and relaxed (R), consistent with the concerted transition theory of Monod, Wyman, and Changeux (1963, 1965) for allosteric enzymes. The holoenzyme has the stoichiometry c_6r_6, and the subunit structure was found to be a set of two apposing trimers of catalytic chains (c_3) held slightly apart in staggered array by three pairs of adjoining regulatory dimers

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(r_2) (Wiley & Lipscomb, 1968). In the transition from the T to the R form, separation of the catalytic trimers by 12 Å is accompanied by a 15° rotation of the regulatory dimers resulting in a more open and less eclipsed enzyme (Ladner et al., 1984). In conjunction with the concerted expansion of the holoenzyme, disparate portions of the binding sites for CP and aspartic acid are brought into juxtaposition to complete formation of the active site in a process termed "domain closure" (Krause et al., 1987; Ladjimi & Kantrowitz, 1988; Kantrowitz & Lipscomb, 1988; Lesk & Chothia, 1984).

The structural mechanism of nucleotide effector regulation, however, is more subtle than the T→R transition, and it is still refractory. The nucleoside triphosphate (NTP) binding site is separated from the active site by ~60 Å and is situated within the N-terminus of the regulatory chain, while it is the C-terminus of this chain which interacts with the catalytic chain. Binding of the NTP effectors results in altered $S_{0.5}$ values for aspartic acid. The binding constant for the transition-state analogue *N*-(phosphonoacetyl)-L-aspartic acid, or PALA, (Collins & Stark, 1971), decreased with the activator ATP, and increased with the inhibitor CTP, following the same line of logic as the changes in $S_{0.5}$ for the natural substrates (Newell et al., 1989).

As explanation for the heterotropic effect, at least five hypotheses have been proposed. One postulate suggests that the T and R forms have different affinities for the substrates and that the alteration is a result of a change in the equilibrium between the T and R forms of the molecule (Schachman, 1988, and references therein). A second model suggests that the data can be explained by a change in the "on" rates for substrates (Hsunya & Wedler, 1988). In crystallographic studies on the NTP-ligated enzyme, a global expansion (ATP-liganded T state) or contraction (CTP-liganded R state) was observed, while only a small reorientation of the active site residues was observed at the reported resolution of the structure (Gouaux et al., 1990; Stevens et al., 1990), leading to the formulation of the nucleotide perturbation theory (Stevens & Lipscomb, 1992). A fourth model, proposed by Herve and co-workers (Xi et al., 1991), describes a two-step process termed the "primary, secondary" effect in which NTP binding induces a directed conformational change in the regulatory chain. This model includes the (T→R independent) direct transmission of NTP-specific signals that are propagated through "channels" within the regulatory chain. Fifth, on the basis of solvent accessibility and proton uptakes studies, Allewell has suggested that NTP binding precipitates structural perturbations at the boundary of the zinc:allosteric domains and that these conformational changes lead to an enthalpy-driven energy change that is manifested in linked protonation reactions and changes in hydrogen bond formation with the enzyme and between the enzyme and water (Allewell, 1989).

These studies were undertaken to examine the role of the zinc domain in tne nucleotide effector response, as it is the bridge between the allosteric domain and the catalytic chain. Several residues at the zinc domain:catalytic chain interface were substituted by site-specific mutagenesis. As the interface amino acids of the regulatory chain are clustered in sets of 3–7 sequential amino acids, a set of insertion mutants also were made in which a single amino acid was added to either the amino or carboxy side of the interaction cluster.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Standard methods of site-directed mutagenesis using mismatched oligonucleotides (Zoller & Smith, 1983) and a uracil-enriched M13 template (Kunkel, 1985) were employed as described (Wales et al., 1988). Restriction endonucleases were purchased from Promega Corp. (Madison, WI).

In mutants where an insertion was made, the same methods were used, introducing an extra three nucleotides in the mutant oligonucleotide between two adjacent codons in the wild-type (WT) sequence. Mutation frequencies of 15–70% were observed for these mutations, in comparison to the observed frequency for standard point mutations of 50–80%. Dideoxynucleotide sequence analysis with Sequenase (USB, Cleveland, OH) according to Sanger et al. (1977) with additions as described (Sambrook et al., 1989) was used to confirm the altered DNA sequence. The entire *pyrLBI* coding region was transferred to PBR322 at the *Eco*RI and *Sal*I restriction sites as described (Wales et al., 1988). Plasmids were expressed in EK1104 cells (Nowlan & Kantrowitz, 1985), and large quantities of enzyme were obtained from growth in limiting uracil.

Enzyme Purification. Aspartate transcarbamoylase was purified as described by Gerhart and Holoubek (1967), except for substitution of Sephacryl S300 for Sephadex G200 and the inclusion of 20 mM zinc acetate in the chromatography buffers. In some cases, the enzyme was of sufficient purity (by gel electrophoresis, 280/260 nm, absorbance ratio and holoenzyme structure) that S300 gel filtration and DEAE chromatography were unnecessary. In the isoelectric precipitation step, the mutants were found to have different pH values for the optimal values for precipitation: the C109H enzyme was precipitated at pH 5.7, and the E119D enzyme was precipitated at pH 5.5. For the initial screening of a large number of enzymes, cell lysates were used; all other enzymes were of >90% purity when assayed as determined from densitometry on SDS–PAGE.

Enzyme Assays. Assay for the production of *N*-carbamoyl-L-aspartic acid was conducted according to Prescott and Jones (1969), with the following adaptations made for automated analysis on a Beckman Biomek 1000 Robotics apparatus: the total reaction volume was 250 μL, and components of the mixture were reduced proportionately to retain the same concentrations. The diluted enzyme samples were incubated in plastic tubes within an aluminum heating block and then mixed with acid (75 μL of enzyme mixed with 225 μL of acid solution) in a standard 96-well microtiter plate. The plate was covered with a plastic coversheet and incubated as usual in a 65 °C water bath. The plate was then cooled to room temperature to eliminate condensation; the cover was removed, and the color development was determined on the Biomek at 470 nm. Absorbance readings were manipulated as previously described (Corder & Wild, 1989). In parallel experiments with both the Biomek 1000 and hand assays, the absorbance readings on the Biomek 1000 were found to be ~15% lower than similar readings on a standard visible spectrophotometer. However, this difference was observed in all samples, including the CAA standards. Consequently, it was not due to adsorption of protein to the plastic labware or catalytic inhibition.

Saturation Studies with Nucleoside Triphosphates. Nucleotide titrations were performed at the respective $S_{0.5}$ (aspartic acid) for each mutant and the wild-type enzyme (see Table II for these values). For a single nucleoside triphosphate, the concentration was varied from 0 to 10 mM using stock solutions of 20 and 100 mM NTP. Duplicate samples were made, and an average of the results is presented. When two NTPs were used, the saturating NTP was added first to 2 mM from a 20 mM stock solution, followed by

dilutions of the second NTP, which were made exactly as the dilutions of the single NTP. Rates from samples with no added NTP were also determined, and the data were plotted as a ratio of the NTP-liganded rate to the unliganded rate.

Isoelectric Precipitation. Enzymes at ~10 mM (0.1 mL) were dialyzed into low-ionic-strength buffer of either 10 mM MES (Gouaux et al., 1987) or 10 mM phosphate (Gerhart & Holoubek, 1967). The final ratio of dialysate to protein solution was >30 000:1. Sample suspensions were then centrifuged for 5 min at 12 000 rpm, and the supernatant was carefully decanted. Supernatant and dissolved precipitate were adjusted to 1.0 mL with 40 mM potassium phosphate, pH 7, and protein was then determined either by absorbance at 280 nm, using an extinction coefficient for aspartate transcarbamoylase of 0.59 mol L^{-1} cm^{-1}, or by Bradford assay using the absorbance at 595 nm and a standard curve of BSA. For the wild-type and E119D enzymes, greater than 85% of the protein precipitated under conditions of maximal precipitation. For C109H, 70–75% of the available protein precipitated at maximal levels.

Urea-Induced Dissociation. Protein samples in buffer at 0 °C and 20 mM were diluted 1:2 with an appropriate stock urea solution at 4 °C, using a rapid but gentle vortex and dropwise addition. The time course of dissociation was dependent on both the protein concentration, and the ratio of protein to urea; consequently, the molarities of both components were kept constant to compare mutant enzymes to the native aspartate transcarbamoylase. Urea solutions were made as 2-fold-concentrated solutions in the same buffer as the protein solution. Protein samples in urea were then put into a heating block which had been preheated to 32 °C. Protein in the same buffer without added urea served as control; two control samples were maintained, one at 32 °C and a second at 0 °C.

Aliquots for electrophoretic analysis were taken at timed intervals, loaded onto a 7.5% nondenaturing acrylamide gel, and electrophoresed at 20 mA for 90–120 min. At the conclusion of the time course for complete dissociation in urea, an additional sample of protein was taken and electrophoresed on a 12.5% polyacrylamide gel, under denaturing conditions according to Laemmli (1970).

Gels were soaked in a prestaining buffer at 50% (v/v) methanol and 7% (v/v) acetic acid, stained in the same buffer with 0.5% (w/v) Coomassie Brilliant Blue R added, destained in the prestaining buffer, and scanned with a Molecular Dynamics 300A densitometer. Areas for the protein band were corrected for background by subtraction of the absorbance at the band edge, using two or three areas as an average. Commonly measured absorbance values were 1.5–2.1 for intense bands and 0.05–0.15 for background. The two samples with no urea were used for a 100% control, and the fraction of holoenzyme which remained at each time point was calculated. Using either the 0 °C or the 32 °C control yielded similar numbers for the fraction of holoenzyme remaining, suggesting that no concomitant thermal denaturation to the proteins occurred. The rate of dissociation was determined from the slope of the graph of the fraction of remaining holoenzyme vs time, in the time range where the dissociation was linear. A second value, the time for 50% dissociation, was also determined from the graph of the fraction of holoenzyme vs time by direct extrapolation. An error in time also was estimated from this same graph using a band density error of ~15%. As an example, for an error of 15% in band density, the times of dissociation for 42.5% and 57.5% dissociation of protein were estimated and used as error bars in this determination. Therefore, the error in time of dissociation is extrapolated, but the number for the variation in band density is determined directly for each sample from an average of 3–5 measurements.

Molecular Graphics. Distance measurements and figures on the coordinate sets of aspartate transcarbamoylase and its liganded derivatives were performed on an Evans and Sutherland workstation using FRODO, version 6.6.

Materials. Carbamoyl phosphate, obtained as the dilithium salt from Sigma and freshly prepared, was used at a final concentration of 4.5 mM. Saturating levels of nucleotide (USB, Cleveland, OH) employed were 2 mM for each nucleoside triphosphate. Buffers used were either potassium phosphate or Tris-HCl. Reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphate buffer was made with a mixture of the monobasic and diabasic forms and used at 40 mM potassium phosphate, pH 7. The Tris buffer was made as a 10× stock using Trizma base and brought to pH with HCl. The tripartate buffer, which allowed retention of ionic strength and buffering capacity across the pH range 6–10, was used as described (Ellis & Morrison, 1982). The conductivities (in mS $m\Omega^{-1}$ cm^{-1}) of the buffers were similar and are reported here: 40 mM potassium phosphate, pH 7, 5.1; 0.1 Tris-HCl, 3.44; TP, pH 7, 3.7; and TP, pH 8.3, 4.3.

RESULTS

Within the C-terminal domain of the regulatory chain of aspartate transcarbamoylase is a bound Zn^{2+}, and the ~50 residues of the zinc domain comprise the structural bridge of communication between the allosteric binding site and the active sites of the catalytic chain. Zinc domain amino acids with side chains which interact with residues of the catalytic chain were chosen for site-specific substitution in this study. For the insertion mutants, glycine was inserted at all points except where the insertion point was found within an element of β-sheet. At those positions, alanine was inserted instead.

Screening of Mutants. Mutants were screened initially for holoenzyme assembly, and alterations in effector-induced kinetics were used as the key selection criteria. The results of an initial screening are presented in Table I. The results can be classified in three categories: enzymes which do not form holoenzyme, holoenzymes with no change in effector response, and holoenzymes with a selective change in effector response.

Amino acid substitutions which resulted in the lack of holoenzyme assembly were observed in all of the different types of mutation, including the insertion mutants within the region of residues 114 through 121 and changes to the zinc coordinate amino acid C114. Three mutant enzymes, A118S, E142D, and V149A, had kinetic parameters and regulatory properties similar to the wild-type enzyme. Finally, there were three positions which demonstrated differential enzyme stability specific to theamino acid type at the point of substitution. At cysteine 109, a change to aspartic acid resulted in the inability to form holoenzyme, whereas a change to histidine resulted in a holoenzyme with a selective difference in its allosteric response. At glutamic acid 119, a change to leucine resulted in the lack of holoenzyme assembly, while a change to aspartic acid resulted in a holoenzyme with a change in its inhibition by CTP. Mutations to A118 yielded holenzyme with substitution-specific changes in the effector responses as well. A change of alanine to serine caused little apparent change, while a change of alanine to phenylalanine caused alteration in the activation of this enzyme by ATP.

Table I: Mutations to the Zinc Domain of Aspartate Transcarbamoylase[a]

assembly/ mutant	$S_{0.5}$(aspartic acid) mM	effector response (% relative activity) ATP	CTP	CTP+UTP
catalytic trimer	3	100	94	98
Holoenzyme, Little or No Change in Effector Response				
WT	5	160	20	4
A118S	8	140	45	5
E142D	6.5	160	30	15
V149A	7	210	20	4
Holoenzyme, Altered Effector Response				
WT	5	160	20	4
C109H	10	140	80	4
A118F	6	100	60	18
E119D	10	130	100	9

[a] Site-specific substitutions are noted with the single-letter amino acid code for the wild-type residue, its position number, and the substituted amino acid. Where the change was an insertion, an "I" was used to note that an insertion was made, followed by the code for the amino acid which was inserted, followed by the position which occurs immediately prior to the insertion point; i.e., IG114 indicates an insertion of a glycine immediately after the amino acid at position 114 of the regulatory chain. $S_{0.5}$ was determined as the aspartic acid concentration at which the enzyme is at half-maximal velocity. Activity measurements were made at 5 mM aspartic acid for all effectors and with all enzymes. Assembly to holoenzyme was monitored by native gel electrophoresis. Enzymes which remained catalytic trimer were simply cataloged, since no change in the kinetic properties due to regulatory chain alterations could be measured with the simple catalytic trimer. These enzymes were C109D, C114D, C114H, E119L, IG114, IG117, and IA121. When cells were lysed with great care and electrophoresed immediately after lysis, a mixture of holoenzyme and catalytic trimer in approximately an equimolar ratio was observed for mutant IG117.

Kinetic Analysis of C109H and E119D in Phosphate Buffer, pH 7. Of the enzymes reported here, C109H and E119D alone separated CTP from CTP+UTP inhibition; they were examined in further detail relative to their effect on aspartate transcarbamoylase allostery and changes to their structural properties. A summary of the kinetic characterization for the enzymes E119D and C109H in comparison to wild-type aspartate transcarbamoylase holoenzyme and catalytic trimer is presented in Table IIA.

In 40 mM potassium phosphate, pH 7, the specific activities, in mmol of CAA min^{-1} mg of protein) 10^{-3}, for the enzymes were found to be 63 ± 7 for wild type, 62 ± 6 for C109H, and 74 ± 9 for E119D. Therefore, within experimental error, the mutant enzymes have the same maximal velocity as the wild-type enzyme. However, the apparent $S_{0.5}$ is slightly increased for each mutant from the $S_{0.5}$ observed for wild type.

In the presence of certain effectors, the altered enzymes again demonstrated similar responses which were distinct from wild type. Each mutant was activated by ATP, as demonstrated by a reduced Hill coefficient, a lower $S_{0.5}$ (aspartic acid), and a higher relative activity at the $S_{0.5}$ for the unliganded enzyme (WT, 180%; C109H, 160%; E119D, 170%). This suggests that the mutants still bound ATP and underwent the conversion to the more active R form of the enzyme. Both of the mutants differed significantly from the wild-type enzyme when assayed for activity in the presence of CTP. E119D and C109H demonstrated a substantive decrease in the inhibition typical of this pyrimidine triphosphate and exhibited only a slight change in $S_{0.5}$(aspartic acid). Since the lack of inhibition by CTP could be explained either by abrogation of transmission of the CTP effect to the active site or a change in the binding affinity for CTP at the nucleotide site, the effect of CTP up to 10 mM was measured to discriminate these possibilities. There was no change from 2 to 10 mM; the titration data for 0–2 mM are shown (Figure 1A). For C109H, there was no demonstrated inhibition at any CTP concentration that was examined, nor was E119D inhibited by CTP concentrations up to 10 mM.

Table II: Kinetic Parameters for Mutant and Control Enzymes in Potassium Phosphate or Tripartate Buffer[a]

enzyme	effector(s) none	ATP	CTP	CTP+UTP
A. Potassium Phosphate, pH 7				
n_{app}				
WT	2.6	1.8	3.4	2.3
cat. trimer	1.0	0.9	1.3	1.0
C109H	3.0	2.0	2.7	3.4
E119D	3.2	2.1	3.8	2.5
$S_{0.5}$(mM Asp)				
WT	6	3	10.5	23
cat. trimer	6.7	5.5	7	6
C109H	10	6.5	12	24
E119D	9	5	13	23
B. Tripartate, pH 7				
n_{app}				
WT	2.2	1.6	2.5	3.1
cat. trimer	1.0	1.0	1.1	1.0
C109H	1.4	1.4	1.4	3.7
E119D	2.2	2.2	2.3	4.2
$S_{0.5}$(mM Asp)				
WT	8	6	16	20
cat. trimer	5	5.5	6	6
C109H	5	5	5	13
E119D	6	6	6	13
C. Tripartate, pH 8.3				
n_{app}				
WT	2.2	1.3	4.2	2.1
cat. trimer	1.0	0.9	0.9	1.0
C109H	3.0	2.7	2.8	4.1
E119D	3.7	3.4	2.8	4.9
$S_{0.5}$(mM Asp)				
WT	11	9	24	50
cat. trimer	8	6	7	6
C109H	6	6	7	44
E119D	9	6	22	46

[a] Reaction velocity was plotted as a function of aspartic acid concentration; this plot was used to determine the concentration of aspartic acid at which the enzyme operates at half-maximal velocity ($S_{0.5}$). From an assay in which 12–15 points were in the range 30–70% maximal velocity, the Hill coefficient (n_{app}) was determined from the slope of the logarithmic plot of velocity/(maximal velocity − velocity) vs substrate concentration. In (A), where a Hill coefficient of 1.3 was obtained for the catalytic trimer in the presence of CTP, the error in that measurement is ±0.2; therefore, we interpret this result as meaning that there is little or no cooperativity under these conditions.

Titration curves for the inhibition by CTP+UTP (Figure 1B,C) demonstrated that these enzymes were similar in the extent of inhibition (60%, allowing for 40% residual activity) and the NTP concentration at which maximal inhibition first occurred (~1 mM of the second NTP). Similar inhibition of each enzyme was observed with either NTP in excess, as is also the case for the wild-type enzyme [Figure 1B,C; also in Wild et al. (1989)].

Two aspects of the above titrations are of particular importance. The mutants clearly were inhibited by CTP and UTP, while they were not inhibited by CTP alone. Furthermore, the net change in inhibition between the unliganded and CTP+UTP liganded states for the mutants resembled the net change in inhibition between the CTP liganded and the CTP+UTP liganded states for the native enzyme. In titrations with 2 NTPs, parallel curve shapes were found for both mutants and the wild-type enzyme. There was a sharp decrease in activity at low concentrations of the second NTP,

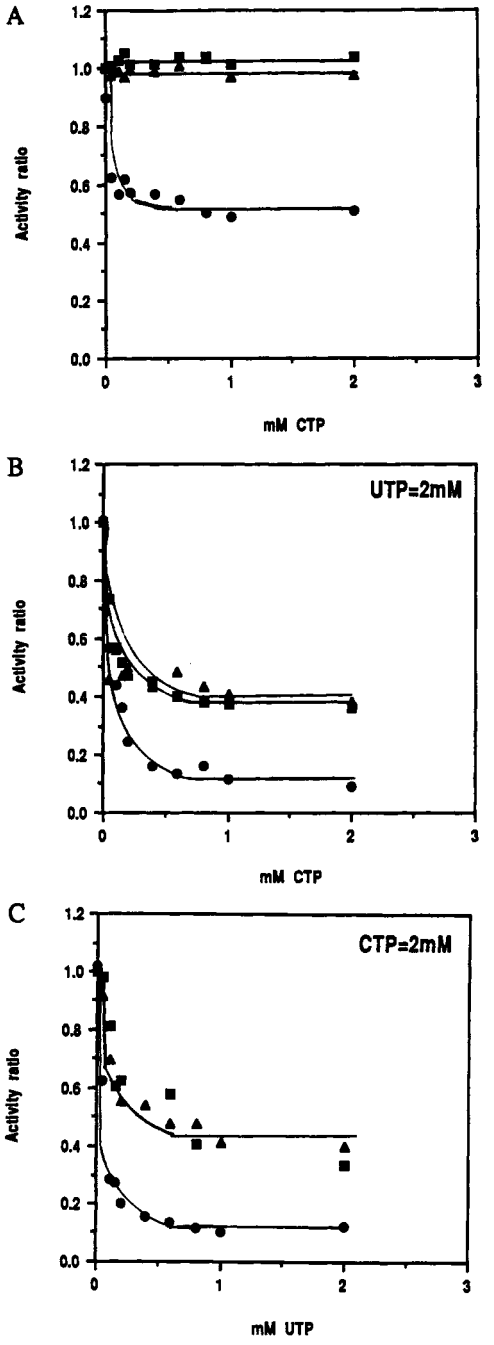

FIGURE 1: Nucleotide saturation curves for mutant and wild-type enzymes in 40 mM potassium phosphate. The enzymatic rate at 4.5 mM CP and the appropriate $S_{0.5}$(aspartic acid) was measured in the presence and absence of nucleoside triphosphates (NTPs). The ratio of activities was measured and plotted vs NTP concentration. Symbols: ●, WT; ■, C109H; and ▲, E119D. (A) Titration with CTP. (B) Titration with CTP in the presence of 2 mM UTP. (C) Titration with UTP in the presence of 2 mM CTP.

and the point of maximal inhibition was similar.

The occurrence of CTP+UTP inhibition in the absence of any inhibition induced by CTP alone suggests that it is a unique regulatory response in aspartate transcarbamoylase. It is notable, however, that although the V_m and the percentage of activation induced by ATP for all three enzymes were comparable, the $S_{0.5}$(aspartic acid) for either mutant was higher than that found for the wild-type enzyme, and the Hill coefficients of the mutants were slightly increased as well. In contrast, the $S_{0.5}$ for all three enzymes was found to be the same in the presence of CTP and UTP in combination, even though the enzymes were not inhibited to the full extent that is observed for the wild-type enzyme (95%).

Kinetic Analysis of C109H and E119D in Tripartate Buffer (TP), pH 7. Phosphate is a product of the reaction and therefore binds to the active site, although with lower affinity than carbamoyl phosphate (CP) (~200 fold). Consequently, the presence of phosphate at a concentration ~10 times that of CP might account for effects to the parameters reported here. Effector responses were calculated in a tripartate buffer of aliphatic amines first described by Ellis and Morrison (1987). The wild-type enzyme again was inhibited at millimolar concentrations of CTP, although the maximal inhibition was only ~20%. At pH 7, the respective maximal velocities, in mmol of CAA min^{-1} (mg of enzyme)10^{-3}, were 84 ± 7 for the native enzyme, 74 ± 9 for C109H, and 93 ± 8 for E119D and so within experimental error were found to be the same in this buffer as well. Other kinetic parameters for the enzymes under study are summarized in Table IIB. In the presence of ATP, the wild-type enzyme was activated, while C109H and E119D showed little activation. In the presence of CTP, no inhibition was observed in either mutant, while the activity of the wild-type enzyme was inhibited. CTP saturation curves for the three enzymes are shown in Figure 2A. Data to 2 mM NTP are shown; data were taken to 10 mM, and in no case was there a change in activity with NTP from 2 to 10 mM.

The inhibition by CTP+UTP which was previously observed in phosphate for all three enzymes was similar in TP, pH 7, and the activity curves for inhibition by CTP+UTP with CTP at 2 mM and UTP from 0 to 2 mM for the three enzymes are shown in Figure 2C.

A subtle difference in the kinetics of C109H and E119D relative to the kinetics of the native enzyme at pH 7 was observed. In the phosphate buffer, ATP activated all enzymes, but the apparent affinities for aspartic acid in C109H and E119D did not approach the value found for the native enzyme. In contrast, CTP+UTP effected a transition to strikingly similar values for $S_{0.5}$ for each enzyme. In the tripartate buffer, the opposite result was observed. The $S_{0.5}$ values for C109H, E119D, and the ATP-liganded wild-type enzyme were in near quantitative agreement, and while the CTP+UTP combination clearly inhibited both enzymes, the $S_{0.5}$ values indicated that the inhibition to C109H and E119D was diminished.

Kinetic Analysis of C109H and E119D in Tripartate Buffer, pH 8.3. The kinetic response and nucleotide saturation curves for all three enzymes were examined also at pH 8.3. At this pH, both the Hill coefficient and the $S_{0.5}$ are typically increased in the native enzyme (Pastra-Landis et al., 1968; Lauritzen & Lipscomb, 1970; Stevens et al., 1991). In the TP buffer used here, the native enzyme also demonstrated elevated values for these parameters (Table IIC). The mutants had maximal velocities which were not different from the native enzyme while still possessing unlike regulatory parameters. C109H was neither activated by ATP nor inhibited by CTP. In the absence of inhibition by CTP or UTP singly, C109H was nonetheless inhibited by the two pyrimidines in combination (Table IIC, Figure 2B,D). There is a primary difference in the structure of C109H from pH 7 to 8.3, as reflected in its altered degree of cooperativity. At pH 7 its Hill index and $S_{0.5}$ values reflect an R-like structure, whereas at pH 8.3 there is a strong degree of cooperativity that is retained in the absence of much NTP response.

On the other hand, E119D responded in a fashion similar to the wild-type enzyme in the absence and presence of CTP, as well as with CTP in combination with UTP. E119D was

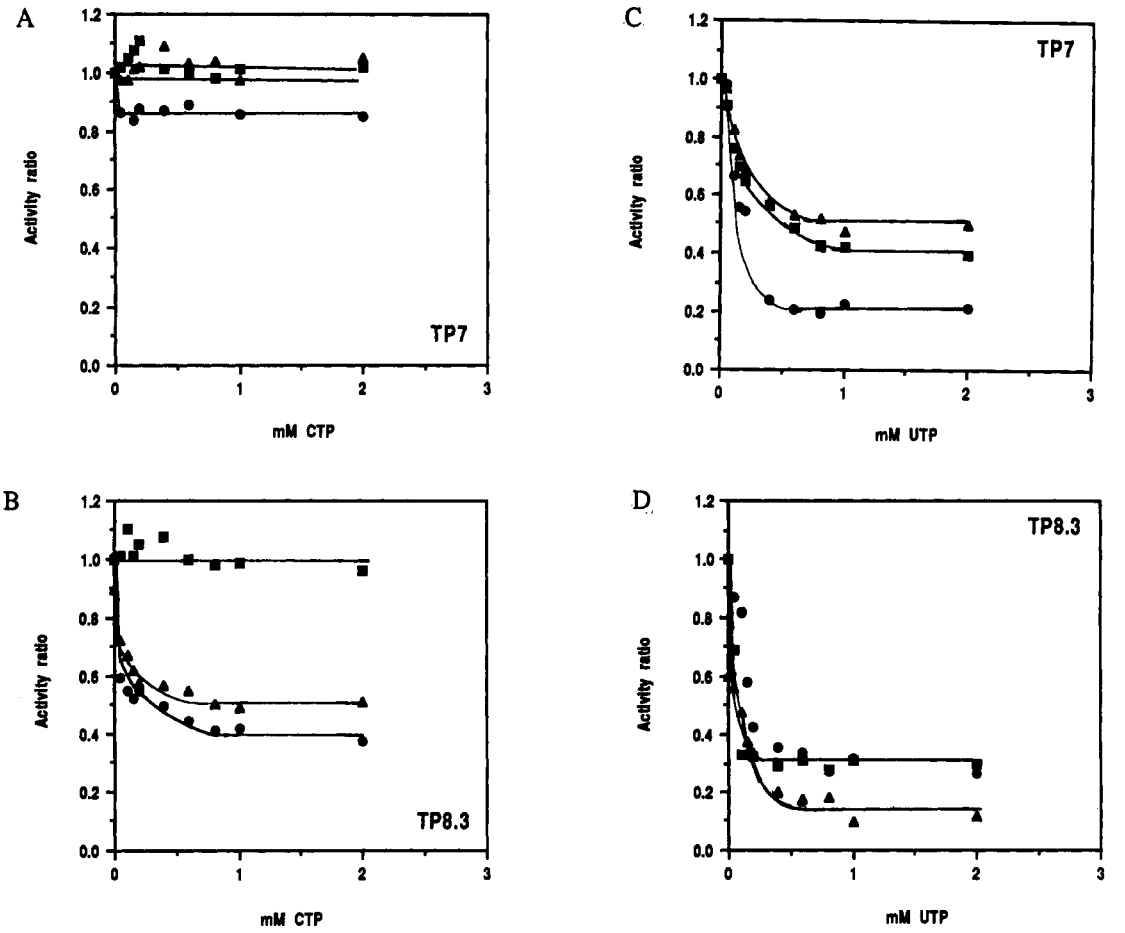

FIGURE 2: Nucleotide saturation curves for native aspartate transcarbamoylase, E119D, and C109H in TP 7 and TP 8.3. Symbols: ●, WT; ■, C109H; and ▲, E119D. Shown are curves for titration of CTP at pH 7 (A) and pH 8.3 (B) and titration of UTP in the presence of 2 mM CTP at pH 7 (C) and pH 8.3 (D).

inhibited by UTP alone, while the native enzyme and C109H were not similarly inhibited. As measured by $S_{0.5}$(aspartic acid), the $S_{0.5}$ for E119D was changed from 10 to 20 mM in the presence of UTP, whereas the value for the wild-type enzyme was 11 mM with or without UTP. This fact notwithstanding, CTP+UTP resulted in a greater inhibition on E119D than that caused by either single nucleotide triphosphate, as the $S_{0.5}$(aspartic acid) under these conditions was well above 40 mM.

Isoelectric Precipitation. The amino acid substitutions described here were conservative and would not have been predicted to induce a change in net charge to the protein. Unexpectedly, then, neither protein precipitated in the isoelectric precipitation step at pH 5.9 that is a standard part of the purification procedure. A quantitative assessment of the pH dependence on isoelectric precipitation was made for the mutants and the wild-type enzyme over the pH range 5.5–6.6 (Figure 3). Two buffers were used. In 10 mM MES (Figure 3A), E119D and the wild type enzyme had very similar precipitation patterns which were spread over a wide range of pH values from 5.5 to 6.0. The same pattern was observed with C109H in a qualitative sense, although the proportion of precipitate in the two peaks was different than for E119D or wild-type enzyme. In 10 mM potassium phosphate (Figure 3B), however, the mutants precipitated maximally at pH values distinct from that for wild type. Each enzyme precipitated over a narrow range of only 0.3 pH unit, and the pH of maximal precipitation was 5.9 and 6.0 for C109H and E119D, respectively. Under these same conditions, less than 35% of the wild-type enzyme was precipitated. The wild-type enzyme, which also precipitated over a narrow range of ~0.4 pH unit, precipitated maximally at pH 5.7. At this same pH less than 10% of either mutant was precipitated.

Measurement of Holoenzyme Stability in Phosphate Buffer, pH 7, or Tris Buffer, pH 8.25. The stability of the interface between the regulatory and catalytic chains was measured by the kinetics of holoenzyme dissociation in buffered urea. The dissociation was measured in the assay buffer and a Tris buffer, and it was found to be dependent on the ratio of urea to protein, the protein concentration, and the type of buffer, as well as the protein under study. With the urea: protein ratio and the protein concentration held constant, the effects of each amino acid substitution and buffer type on protein stability were compared.

The time courses for the dissociation of C109H and E119D in either 40 mM phosphate or 100 mM Tris, pH 8.25, are shown in Figure 4A,B. The time course of dissociation in urea had two phases, an early first phase in which the protein remained holoenzyme and a second phase of rapid and irreversible dissociation in which the enzyme degenerated into separate polypeptides too small to be observed on this gel. Times for both the early and dissociative phases were variable. As shown here, for C109H the early phase of retained holoenzyme assembly was buffer dependent and lasted from ~20 to ~50 min, whereas the lag time for E119D was quite short. During this early lag period, some disruption of structure did occur, as enzyme activity in the wild-type enzyme was unregulated by NTP and had an $S_{0.5}$(aspartic acid) similar to that of the catalytic trimer (data not shown). The second dissociative phase of the denaturation could be approximated

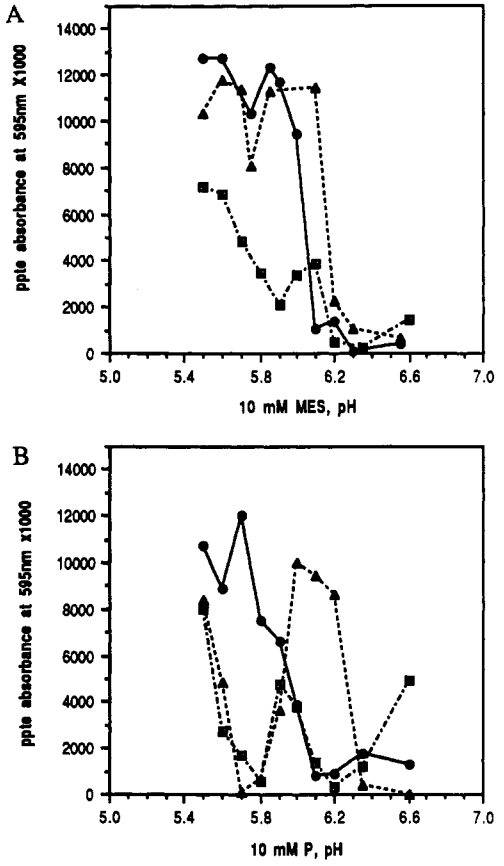

FIGURE 3: Isoelectric precipitation of aspartate transcarbamoylase at acidic pH. The amount of holoenzyme which precipitated in low-ionic-strength buffers in the pH range 5.5–6.1 was determined by Bradford colorimetric assay. Symbols: ●, WT; ■, C109H; and ▲, E119D. (A) MES, 10 mM. (B) Potassium phosphate, 10 mM.

by a linear equation and therefore appeared to be first order. The dissociation rates for C109H were found to be 0.86% holoenzyme min^{-1}, $r^2 = 0.95$, in the phosphate buffer and 0.92% holoenzyme min^{-1}, $r^2 = 0.98$, in the Tris buffer. In contrast, the rates of dissociation for E119D were 0.80% holoenzyme min^{-1}, $r^2 = 0.98$, in the Tris buffer and 0.28% holoenzyme min^{-1}, $r^2 = 0.93$, in the phosphate buffer. No change in stability was observed when 20 mM zinc acetate was included in the assay solution (data not shown). In Figure 4B, the times required for 50% dissociation of the holoenzyme for all enzymes are presented.

All of the proteins were stable at 4 °C in various buffers for months and at 32 °C in the absence of urea throughout the time course of the experiment. Under the conditions reported here, c_3 dissociated much more rapidly than either the mutants or the wild-type enzyme. Therefore, the reported rate of dissociation is dependent on changes to holoenzyme structure induced by the regulatory chain. As the parameter of measure was the dissociation of holoenzyme into smaller subunits, the observed dissociation was dependent on the subunit interactions themselves.

In comparison to wild-type enzyme, the zinc domain-substituted enzymes demonstrated a decreased stability to the urea, and their stability was more dependent on the buffer components. In the Tris buffer, C109H was found to dissociate into its components at a rate ~6 times faster than the wild-type enzyme. E119D was less stable than C109H in the Tris buffer; it dissociated ~10 times faster than the wild-type enzyme. In 40 mM phosphate, the relative stabilities of the altered enzymes were reversed. C109H dissociated ~12 times faster than wild type, while E119D dissociated ~4 times faster than wild type. Thus the order of stability in phosphate was WT > E119D > C109H, and the order of stability in Tris was WT > C109H > E119D. The two mutant enzymes were different from one another in the pattern of their resistance to urea denaturation. C109H showed a difference from one buffer to another in the early phase of the denaturation when it remained holoenzyme for a longer period of time in the Tris buffer. The dissociative rate for C109H was roughly independent of the buffer. E119D had no appreciable lag time in either buffer and showed a buffer-dependent rate of holoenzyme dissolution.

Measurement of Interface Stability in Tripartate-Buffered Urea. The stability of both C109H and E119D in urea buffered with the tripartate components at either pH 7 or pH 8.3 also was determined and compared to the stability of these enzymes in phosphate or Tris buffers (Figure 4A,B). C109H was found to be less stable than E119D. The mutants were denatured much faster in TP buffer than in either phosphate or Tris buffers, while the stability of the wild-type enzyme was not nearly so buffer dependent (Figure 4C). There was little pH dependence on the kinetics of dissociation for either mutant, and so the rapid dissociation of the altered enzymes related instead to the components of the TP buffer itself. In this regard, neither mutant displayed an order of stability which suggested a pH dependence such as was observed in the CTP inhibition of E119D.

DISCUSSION

The mechanism of regulatory modulation by nucleoside triphosphates in aspartate transcarbamoylase is not well understood, and the study of this subject is made more difficult by the scarcity of measurable physical parameters for the enzyme in its regulated state. An early observation on aspartate transcarbamoylase was that the binding site for CTP was located on a regulatory polypeptide chain distinct from the chain to which the substrates bind (Gerhart & Schachman, 1965, 1968). ATP, an activator of the enzyme, was also found to bind to the same site on the regulatory chain, and it competed, albeit poorly, with CTP (Gerhart & Pardee, 1962; Bethell et al., 1968). The two domains of the regulatory chain have unique functions; the N-terminal 100 amino acids constitute the nucleotide binding domain, and the C-terminal 53 amino acids constitute a zinc binding domain which establishes contact with the catalytic subunit (Honzatko et al., 1982). From crystallographic analysis of aspartate transcarbamoylase with either ATP or CTP bound, it has been shown that the two effectors bind to roughly the same residues in the binding pocket (Gouaux et al., 1990; Stevens et al., 1990). Nonetheless, substitutions of a number of binding residues have induced differential effects on the heterotropic regulation (Corder & Wild, 1989; Middleton et al., 1989; Zhang & Kantrowitz, 1988, 1991; Zhang et al., 1988; Wente & Schachman, 1991; Zhang & Kantrowitz, 1992).

The two site-directed mutations reported here alter only the geometry of the zinc domain of aspartate transcarbamoylase. Each mutation lies at the interface between the catalytic and regulatory chains, and the contact regions within the catalytic chains are in the moving loops which participate in domain closure. Within the interface of the catalytic and regulatory chains, amino acids 109 and 119 are in different positions. Cysteine 109 is part of the interface between the zinc domain and the aspartate binding domain which participates in both C1:R4 and C1:R1 interactions [for nomenclature, see Kantrowitz and Lipscomb (1988)]. While the C1:R4 interface is located solely between the regulatory chain

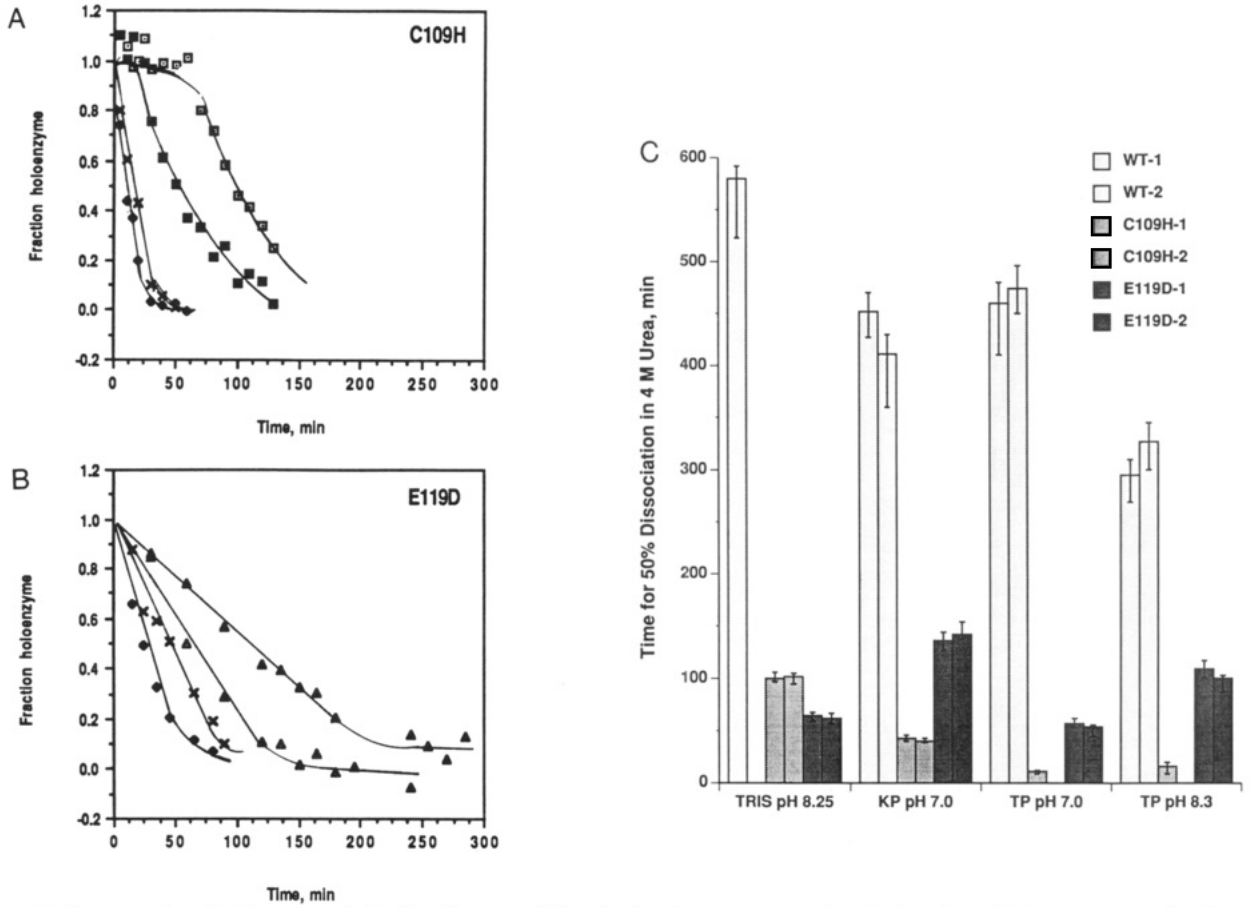

FIGURE 4: Denaturation of holoenzymes in buffered urea and dissociation time course for the C:R interface. Holoenzyme was incubated at 32 °C in 4 M urea, and for each time point the fraction of holoenzyme that remained was determined and plotted vs time of incubation at 32 °C. (A) C109H: ⊡, 0.1 M Tris, pH 8.25; ■, 40 mM potassium phosphate, pH 7; ◆, TP 7; ×: TP 8.3. (B) E119D: △, 0.1 M Tris, pH 8.25; ▲, 40 mM potassium phosphate, pH 7; ◆, TP 7; × TP 8.3. (C) Time for 50% dissociation of holoenzyme into subunits. Each bar represents a single dissociation curve.

and the aspartate binding domain, the C1:R1 interface spans both the aspartate and carbamoyl phosphate domains. Through its involvement in zinc coordination along with cysteines at positions 114, 138, and 141, cysteine at position 109 is in direct linkage to the orientation of the C1:R4 and C1:R1 interfaces. Via the β-sheet of the zinc domain, it is joined to the zinc–allosteric interface as well. Therefore, modification of this residue leaves intact the actual contact residues to both the allosteric domain and the catalytic chain in the mutant, allowing strictly the influence on zinc domain geometry to be assessed. Glutamic acid 119 interacts with H3 of the catalytic chain, which is part of the carbamoyl phosphate binding domain. Replacement of this residue with an aspartic acid leaves the functional group intact but changes the distance from the carboxylic acid function back to the peptide backbone.

Within the aspartate transcarbamoylase holoenzyme, it is structural determinants of the regulatory chain that entirely account for both its heterotropic and homotropic effects, since the catalytic trimer neither is regulated by NTP nor has any homotropic cooperativity. Indeed, conformational effects at the active site as measured by K_m(aspartic acid) and V_m can be induced by only a C-terminal fragment of the regulatory chain (Markby et al., 1991). Using ATCase enzymes with different regulatory properties, it was shown that the regulatory chain determines both the presence and the type of heterotropic regulation (Shanley et al., 1984; Beck et al., 1989; Wales et al., 1991). These studies were undertaken to understand three aspects of long-distance communication in the heterotropic allostery of this enzyme. First, can mutations in the zinc domain be used to identify residues which contribute to the transmission of the regulatory signals over the 60-Å distance from the allosteric binding site to the active site in the catalytic chain? Second, does the zinc domain interpret or simply relay the signal for the type of heterotropic regulation that originates within the allosteric binding site? Third, what is the nature of the signal for heterotropic regulation?

Residues and Subunit Interfaces. Structural analysis of the regulatory chain of aspartate transcarbamoylase in its unliganded T and PALA-liganded R forms showed no dramatic movement of α-helices or β-sheets; nonetheless, an overlay of the chain trace from each structure revealed differences (Krause et al., 1987). If the allosteric domains were aligned, the zinc domains overlapped, but did not match. If the zinc domains were aligned, the allosteric domain traces drifted apart. Thus, there were changes between the two structures in the geometry and arrangement of the amino acids of this chain. This finding led these authors to consider the effect of regulatory NTP molecules as a kind of torquing action on the much larger conformational changes which were found to be present in the catalytic chains upon binding substrate. To test this model, we designed and synthesized mutants within the zinc domain which would examine the effect of changes to its size, shape, and flexibility on the regulatory behavior of the holoenzyme. A large number of mutants were assayed for their regulatory properties, and only mutants with selectively altered properties were pursued extensively. The screen yielded two such mutants, and the replacements principally reported here are to residues which lie at the C1:R1 interface that is present in both the T and

R crystalline states of the protein (Honzatko et al., 1982; Krause et al., 1987). A significant aspect concerning these residues is that they each indirectly (C109H) or directly (E119D) contact a portion of the catalytic chain which undergoes major movement in the T→R transition.

Regulation of ATCase Activity and Cooperativity. In any buffer, the maximal velocities for the mutants and the wild-type enzyme are identical within experimental error; therefore, the catalytic capability of the mutant enzymes is not impaired or enhanced. By the following argument, the allosteric binding site also is not altered. The average binding constant for CTP to wild-type enzyme has been determined to be at ~70 μM (Matsumoto & Hammes, 1971). In the titrations here, one obtains a qualitative estimate of CTP binding that is consistent with the results from the quantitative binding studies. The concentration required for one-half of the observed net inhibition of wild-type enzyme approximates the binding constant: that concentration of CTP is the amount required to inhibit the enzyme 75% [100% − (50%/2) = 75%]; that value is between 50 and 100 μM CTP. The mutants showed no inhibition by CTP at concentrations up to 10 mM. If the absence of the effect of CTP is due to altered binding at the allosteric site, it is possible to estimate a minimum binding constant from these data. The altered binding constant would be expected to be greater than 20 mM, since a measurable inhibition would have been seen at 10 mM for any binding constant of smaller number. Therefore, in these mutants, the lack of CTP inhibition could be explained by a change in binding affinity for CTP only if the binding constant is increased by greater than 400-fold. When C109H and E119D are inhibited by CTP+UTP, the maximum inhibition again occurs at levels of NTP below 1 mM in curves that parallel the inhibition curve for wild type; thus the allosteric binding site under these conditions is functional and relatively unchanged. This suggests that the altered allostery is not due to binding perturbations at either end of the pathway and, therefore, the mutants are mutants in the regulatory pathway itself.

Kinetics in Phosphate. C109H and E119D each have an $S_{0.5}$ for aspartic acid in the unliganded state that approximates the value found for the CTP-liganded wild-type enzyme. Whether their functional equivalence to CTP-liganded wild-type enzyme can be validated at the structural level is not known at this time. When CTP+UTP are used to inhibit the mutants, the net change in enzyme velocity is ~50%, and this change again is similar to the difference between the CTP- and CTP+UTP-liganded wild-type enzymes. However, two important distinctions here (noted by one reviewer) are that the mutant enzymes still are not inhibited at all by UTP alone and that neither UTP alone (as one would expect for a strict identity with the CTP-liganded state) nor CTP and UTP in combination drive the inhibition to the 95% that is observed for the wild-type enzyme.

Kinetics in Tripartate. In this buffer, the mutants behaved differently from native enzyme and from one another. While C109H was activated by ATP in phosphate buffer at pH 7, it was not activated by ATP in TP buffer at pH 7. Therefore the activation by ATP was dependent on phosphate in this mutant. At pH 8.3, ATP induced a decrease in the Hill coefficient but no apparent change in the $S_{0.5}$(aspartic acid). One can conclude that the response to ATP at pH 8.3, too, is diminished or absent in this mutant.

E119D was similar to C109H in its inhibition by CTP+UTP and in the dependence on phosphate for its activation by ATP at pH 7. However, in TP buffer, both its inhibition by CTP and its activation by ATP were found to be absent at pH 7 and present at pH 8.3. Thus, the mutant E119D, differing in amino acid sequence from wild type only by one side-chain methylene group at position 119, highlights the importance of both the binding of phosphate (CP) and the amino acid pK_a as aspects of the regulatory process. At pH 7, ATP activation is blocked by the absence of phosphate, but it is reversed by a change in pH or the addition of phosphate; thus activation with ATP is again correlated to CP binding, as well as a pH effect on the enzyme structure. The block in CTP inhibition in this mutant appears to be independent of CP or its analogues, but it is completely reversed by pH.

The pH-dependent nature of CTP inhibition in E119D implies that the ionization of a key residue(s) within the enzyme is related to its conformation and regulation. In this mutant, the conformation at pH 7 does not allow one of two ionizations to occur. Either residue(s) which are regulated by CTP are not in the ionization state which is regulated or residue(s) which contribute to the CTP regulation itself are not appropriately ionized for the necessary regulatory role that they play.

While E119D is neither activated by ATP nor inhibited by CTP at pH 7 in TP, its activation by ATP is recovered at pH 7 in phosphate buffer, again relating phosphate binding, ATP activation, and amino acid pK_a values, via the noted changes in the isolectric point of the holoenzyme, in a direct relationship to one another.

The amino acid substitutions at positions 109 and 119 each forced a small but distinct change to the overall size and shape of the zinc domain. Mutation to zinc coordinate residue 109 resulted in a mutant with a change in coordinate sphere geometry, but with all of its contact residues still in possession of their side chains. The change of glutamic acid at position 119 to an aspartic acid yielded a mutant enzyme which retained a carboxylic acid at this position, but with a side chain shorter by one methylene group. It is likely, therefore, that these alterations caused a change in the juxtaposition of the zinc domain at one or more of its interfaces with other parts of the holoenzyme.

These amino acid substitutions were not expected to alter the net charge on the protein, but in phosphate buffer, each mutant had an isoelectric point different from that found for the native enzyme. Phosphate binds to the native enzyme as well; nonetheless, it has an isoelectric point which is not altered in phosphate buffer, and so this enzyme must have a way of countering the net increase in negative charge to the phosphate-bound holoenzyme. This mechanism for the counterbalance of charge involves the regulatory chain and is not available in the zinc domain-substituted mutants of altered geometry.

Stability of the Mutant Enzymes in Buffered Urea. While the native enzyme was found to be stable in 4 M urea for several hours, the conservative mutations reported here resulted in enzymes that were dissociated in buffers of phosphate and Tris in a few hours, and they were dissociated in the TP buffer in a matter of minutes. The two mutations induced structurally distinct changes into aspartate transcarbamoylase, as evidenced by the difference in their stabilities. C109H is more stable in 0.1 M Tris, pH 8.25. E119D is more stable in 40 mM potassium phosphate, pH 7. As the exact mechanism of urea denaturation is not known (Creighton, 1984, and references therein), the nature of the changes to aspartate transcarbamoylase by these mutations cannot be described in detail. The overall stability of the region is easily assessed, however, and no assumptions about short- or long-range

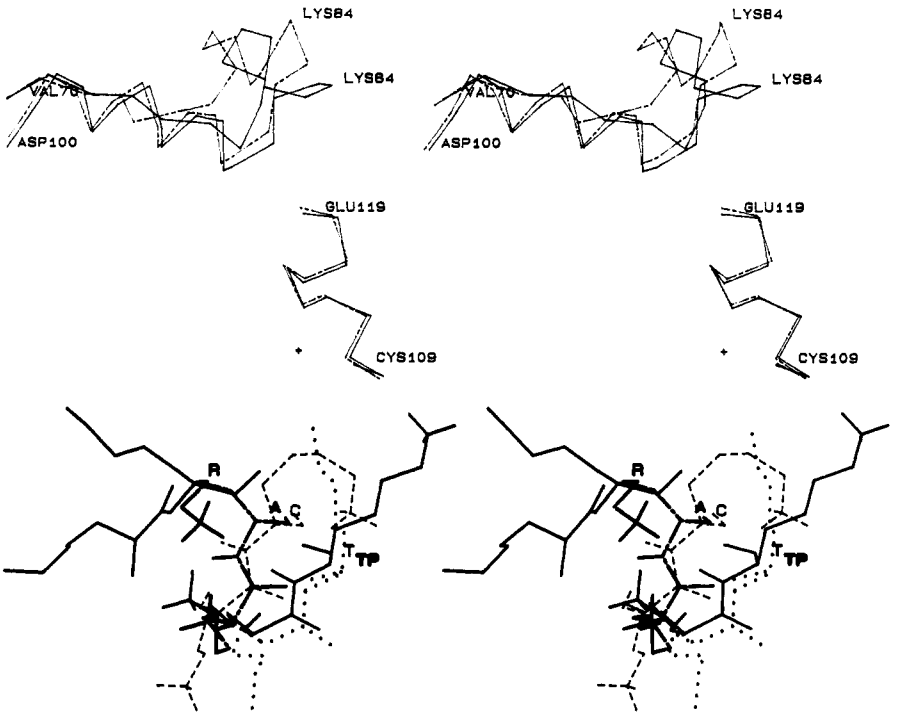

FIGURE 5: Molecular graphics representations of the C1:R1 interface between the regulatory and catalytic chains in and around residue 119 of the regulatory chain and the 80's loop of the catalytic chain. For this study, we used the R structure that has the substrate analogues phosphonoacetamide and malonic acid bound, as it is the most highly refined R structure and the structure that was used as a basis for the NTP-liganded R structures, and it allowed direct comparison to the T-PAM structure as well. (A, top) Cα trace of residues 70–100 of the catalytic chain and regulatory amino acids 109–119 in the T and R forms. Residues 70–76 (background, starting in upper left of figure) are in strand 3 of a β-sheet, and residues 87–100 (foreground, right to left in figure) are in helix 3 of the catalytic chain; the 80's loop consists of the joiner segment of residues 77–86. Regulatory chain amino acids begin with residue 109 in the lower right-hand corner and proceed to residue 119. T, solid line; R-PAM, malonate, dash–dot line. Selected amino acids in each chain are labeled. (B, bottom) Atoms of 80's loop residues lysine 83 and lysine 84 in the R conformation and the liganded T conformers. T, solid line at right side of complex; "T" label is at the αC for lysine 84. TP, dotted line, C, CTP-liganded T, dashed line, A, ATP-liganded T, dash–dot line, R, R-PAM, malonate, solid line, left side of complex.

changes to structure as a result of a site-directed change need be made.

In the absence of denaturants, C109H and E119D are stable enzymes; nonetheless, the effect of urea on their stability is highly pronounced in these mutants of geometry in which the zinc domain structure is compromised. The reduced stability is not due to an unfolding of the regulatory chain due to a simple change in the chelation affinity for zinc, as there was no change to the denaturation kinetics in the presence of exogenous zinc ions (data not shown).

Work from the Allewell laboratory [reviewed in Allewell (1989)] has shown that (1) the solvent accessibility of the catalytic subunits is increased upon NTP binding (Lennick & Allewell, 1981), (2) proton exchange within the zinc domain was greatly increased upon binding to NTP effectors (Burz & Allewell, 1982; Mallikarachchi et al., 1989), and (3) absorption of protons occurs upon substrate binding (Allewell et al., 1979). This phenomenon is not due to an opening or weakening of the C:R interface, as it has been shown that the association constant for this interface was not affected by the binding of NTP effector molecules (Chan, 1975a,b). The observed increase in proton exchange to the zinc domain when aspartate transcarbamoylase is ligated to NTP may be due to a rearrangement of the regulatory chain. Indeed, the solvent accessibility of the zinc domain also may be altered as a result of changes in the interaction between the allosteric and zinc domains that lead to an opening of the structure at this interface (Allewell, 1989). Thus, in the mutants reported here, there may be a direct correlation between the dramatic increase in urea denaturability, an altered interface within the holoenzyme (the C1:R1 or the zinc:allosteric interface or both), and the altered regulatory status.

Relationship of the C1:R1 Interface to Residues of the Active Site. Position 119 interacts with the catalytic chain, through contact with a region of structure known as the 80's loop, at the main-chain atoms of residues 87–89 (Honzatko et al., 1982; Krause et al., 1987; Kim et al., 1987). This loop includes lysine 84, which resides within the active site. Using the program FRODO and the crystallographic coordinates of Lipscomb and co-workers, we demonstrate that the 80's loop and, in particular, the side chain of this residue undergo a large change in conformation in both the T→R transition and in the binding of nucleoside triphosphates (Figure 5).

The 80's loop is situated between two elements of secondary structure; strand 3 of the major β-sheet of the CP domain and helix 3. When α-carbon traces of the unliganded T and the PAM+malonate R structures are compared (Figure 5A), it can be observed that the elements of secondary structure do not change orientation either within themselves or with respect to one another; however, the connecting 80's loop undergoes a rearrangement which involves a change in the puckering pattern and an uplifting of the residues which appear to form a protruding flap in the T state. The influence of the zinc domain directly on the pucker of the 80's loop can be envisioned. The loop would absorb the full force of any push or pull from the zinc domain, since a rocking motion would not be transmitted to the α-helix or the β-strand, as there is no change in orientation there. Along with backbone movement in the 80's loop, the ϵ-NH_2 groups of lysines 83 and 84 undergo a large movement that results in changes to their orientation to

the Cα backbone, to each other, and to the elements of secondary structure. In the R conformers of aspartate transcarbamoylase, there is no difference in either the Cα position or the side-chain position for lysine 84 for the unliganded, ATP-bound, and CTP-bound structures (Gouaux et al., 1990), while in the T conformers, both aspects of the lysine 84 position are altered when either PAM or NTP is bound (Figure 5B). The Cα position for residue 84 is roughly the same for the T and T-PAM structures, but the side-chain location is drastically different. In the T-PAM structure, the side chain of this residue extends up and away from the peptide backbone in a manner quite similar to the configuration of this residue in the R form, while the α-carbon remains pinned into the loop conformation of the T structure. The Cα position of the NTP-ligated structures is slightly removed from the same position in the unliganded structure, but this position is the same for both the ATP- and the CTP-ligated structures. However, again the side-chain conformation of this lysine is distinct for each enzyme form, as the resultant location of the ε-NH2 group is different.

Amino Acid Side-Chain Chemistry and Regulation of ATCase by Nucleoside Triphosphates. In early chemical modification studies by Stark and co-workers, a correlation between phosphate and pK_a similar to that reported here has been noted. Lysine 84 of the catalytic chain is selectively modified at pH 8 by pyridoxal phosphate, but not by pyridoxal (Greenwell et al., 1973; Kempe & Stark, 1975). This reaction requires that the ε-NH_2 group of lysine be unprotonated, and that form typically is not favored near neutral pH. Therefore the pK_a of this residue is likely altered by ~2 pH units in a manner that is dependent on phosphate. It is possible that glutamic acid 119 of the regulatory chain is involved in maintaining the lowered pK_a of this lysine side chain due to its position relative to lysine 84.

Additionally, we see a relationship between phosphate and the net charge with both mutants. In the absence of phosphate C109H, E119D, and the wild-type enzyme have similar isoelectric points, but in the presence of phosphate the mutants have isoelectric points that are elevated by ~0.4 pH unit. Thus it appears that this protein has an isoelectric point that is conformationally dependent, and different conformation(s) are represented by C109H and E119D in phosphate.

Lysine 84 has been implicated in the catalytic reaction directly by chemical modification studies from the Stark laboratory (see above) and by site-directed mutagenesis, where conservative replacements at this position yielded inactive enzymes (Robey et al., 1986). Site-directed replacement of this residue by either glutamine or arginine produced a mutant with no activity, again suggesting that both geometry and charge are critical to the lysine at this position. Spectrophotometric analysis of holoenzyme modified at residues 83 and 84 with a probe that absorbs in the visible range yielded further evidence that NTP binding induces conformational effects at this position. ATP binding induced a hyperchromic shift, and CTP binding, a hypochromic shift, at 430 nm; here again it was shown that CP must be bound to observe the ATP effect (Schachman, 1988; Hensley & Schachman, 1979).

We propose that there is a direct relationship between the conformation of the regulatory chain, the ε-NH_3 position and pK_a of lysine 84, and the regulation of aspartate transcarbamoylase. This hypothesis may be tested kinetically, or using the simple tools of protein chemistry discussed here, one could titrate the pK_a of lysine 84 as well as determine the isoelectric point of the holoenzyme under conditions of its regulation by nucleoside triphosphates.

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