The Influence of the Regulatory Chain Amino Acids Glu-62 and IIe-12 on the Heterotropic Properties of *Escherichia coli* Aspartate Transcarbamoylase†

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ABSTRACT: In the structure of *Escherichia coli* aspartate transcarbamoylase with CTP bound [Kosman, R. P., Gouaux, J. E., and Lipscomb, W. N. (1993) *Proteins, Struct. Funct. Genet. 15*, 147−177] Lys-6 and Glu-62 form a salt-link between two regulatory chains. However, recent X-ray structural studies suggest that side chain and backbone interactions existed between Glu-62 and Ile-12. Thus the interaction between Glu-62 and Ile-12 may help to establish the correct conformation of the nucleotide binding site. The present study of two single-site mutant enzymes, Glu-62r→Ala and Ile-12r→Ala, was undertaken to investigate whether the role of Glu-62 is to maintain the stability of the interface between the regulatory chains in the dimer, or interacts with the side chain of Ile-12r to define the nucleotide binding site. For both the mutant enzymes, the maximal velocity, the aspartate saturation at half the maximal velocity, and Hill coefficient were close to wild-type values. The Glu-62r→Ala enzyme showed enhanced regulatory effects with ATP, CTP, and UTP. As a result of this mutation the enzyme losses its ability to discriminate between CTP and UTP. For the Ile-12r→Ala enzyme, the heterotropic properties were reduced or eliminated. The enhanced regulatory effects observed with the Glu-62r→Ala enzyme do not seem to be consistent with the presence of a salt-link between Glu-62r and Lys-6r. However, based upon kinetic data of the unique but completely opposite heterotropic properties of the two mutant enzymes, it is suggested that the side chain interaction between Glu-62r and Ile-12r helps to define the conformation of the effector binding pocket. In this study, we report the properties of both the Glu-62r→Ala and Ile-12r→Ala enzymes and their importance for the heterotropic activation and inhibition of aspartate transcarbamoylase.

Aspartate transcarbamoylase from *Escherichia coli* (EC 2.1.3.2) is an extensively studied allosteric enzyme which catalyzes the committed step in the biosynthesis of the pyrimidine nucleotides: the condensation of L-aspartate and carbamoyl phosphate to form inorganic phosphate and *N*-carbamoyl-L-aspartate (*1*, *2*). The latter continues through the pyrimidine biosynthesis pathway to yield UTP and CTP. The end-product CTP causes heterotropic inhibition of the enzyme, whereas ATP, the end product of the parallel purine biosynthesis pathway causes heterotropic activation of the enzyme (*3*). UTP alone does not exert a significant effect on the activity of the enzyme (*4*), but at pH 7, in combination with CTP, UTP inhibits the enzyme more than CTP alone (*5*). This kind of regulation provides a satisfying logic for ensuring a balance of endogenous purine and pyrimidine pools in the cell.

The aspartate transcarbamoylase holoenzyme is a dodecamer of molecular weight 310 000 comprised of two catalytic trimers (M_r = 100 000) and three regulatory dimers (M_r = 34 000). The binding sites for the substrates are located in the catalytic chains, which are solely responsible for enzyme activity, while the regulatory chains contain binding sites for the nucleotide effectors (*6*, *7*).

Kinetic studies and analysis of the structures of the holoenzyme have established that both ATP and CTP compete for, and bind to the same sites on the six regulatory chains (*8*−*12*). The six sites have been classified into two distinct groups on the basis of their affinity for the nucleotides. Both ATP and CTP bind with high affinity to three of the sites and with low affinity to the remaining three sites (*13*−*17*) such that there is one high-affinity and one low-affinity site per dimer. In the presence of CTP, UTP binds preferentially to the three low-affinity sites, resulting in enhanced affinity for CTP at the three high-affinity sites (*18*).

Because of weak electron density, the exact position of the N-terminus of the regulatory chains was not known in the early X-ray structures of the enzyme (*7*). However, higher resolution X-ray data allowed Kosman et al. (*19*) to locate the N-terminus of the R1[1] regulatory chain close to the allosteric effector site. This study also identified a salt-bridge interaction between Lys-6r1[2] and Glu-62r6 across the regulatory dimer interface. A subsequent mutagenesis study showed that alteration of Lys-6r led to diminished response of the heterotropic effectors (*20*). Recent, X-ray structural

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[1] The two regulatory chains of the dimer in the asymmetric unit of the crystal are designated R1 and R6.

[2] An "r" is appended to the residue number to indicate the regulatory chain of the enzyme. A number following the "r" indicates the regulatory chain of the dimer in the asymmetric unit.

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studies of several mutant aspartate transcarbamoylases in our laboratory (manuscript in preparation) suggest that instead of a salt-link with Lys-6r, Glu-62r may have side chain and backbone interactions with Ile-12r, a residue known to have main chain interactions with both effectors (*21*). In these structures, Glu-62r and Lys-6r are more than 10 Å apart. The present study of two mutant enzymes, Glu-62r→Ala and Ile-12r→Ala, was undertaken to investigate whether the role of Glu-62r is to maintain the R1−R6 dimer interface as previously suggested (*20*), or whether the Glu-62r interaction with Ile-12r is important for nucleotide binding and for the heterotropic response of the enzyme by helping to establish the proper structure of the nucleotide binding site.

MATERIALS AND METHODS

Materials. Agar, ampicillin, ATP, CTP, UTP, carbamoyl phosphate, *N*-carbamoyl-L-aspartate, L-aspartate, potassium dihydrogen phosphate, and imidazole were purchased from Sigma Chemical Co. The carbamoyl phosphate was purified by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C. Enzyme-grade ammonium sulfate, electrophoresis-grade acrylamide, agarose, urea and Tris were purchased from ICN Biomedicals and the oligonucleotides from Operon Technologies.

Site-Specific Mutagenesis. The mutant versions of aspartate transcarbamoylase were constructed by introducing specific base changes in the *pyrI* gene, using the Kunkel method (*22*, *23*). The uracil-containing single-stranded DNA required was obtained by infection of *E. coli* strain CJ236 (*22*, *23*) containing the phagemid pEK152 (*24*), with the helper phage M13K07 (*25*). Selection of the mutations were performed directly by dideoxy sequencing (*26*).

Recloning and Confirmation of the Mutations. After each mutation was verified, a DNA fragment containing the mutations was removed with two restriction enzymes and inserted into the plasmid pEK54 (*27*), which had the corresponding section of the wild-type gene removed. Following construction of the plasmids (pEK297 for Glu-62r→Ala and pEK316 for Ile-12r→Ala), each mutation was verified a second time by dideoxy sequencing.

Wild-Type and Mutant Enzyme Purification. The wild-type and the two mutant versions of the aspartate transcarbamoylase were isolated as described by Nowlan and Kantrowitz (*28*) from *E. coli* strain EK1104, containing the plasmid pEK2 (*29*) for the wild-type, pEK297 for the Glu-62r→Ala, and pEK316 for the Ile-12r→Ala enzymes.

Determination of Protein Concentration. The concentration of pure wild-type enzyme was determined by absorbance measurements at 280 nm with an extinction coefficient of 0.59 cm^2/mg. The protein concentration of the mutant holoenzymes was determined by the BioRad version of Bradford's dye-binding assay using the wild-type holoenzyme as a standard (*30*).

Aspartate Transcarbamoylase Assay. The transcarbamoylase activity was measured at 25 °C by either the colorimetric (*31*) or the pH-stat method (*32*). pH stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburet. The saturation curves for aspartate were performed in 0.05 M Tris-acetate buffer, pH 8.3, and in 0.1 M imidazole-acetate buffer, pH 7.0. Assays with the effectors ATP, CTP, and UTP in the presence of CTP were performed at pH 7. All the colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

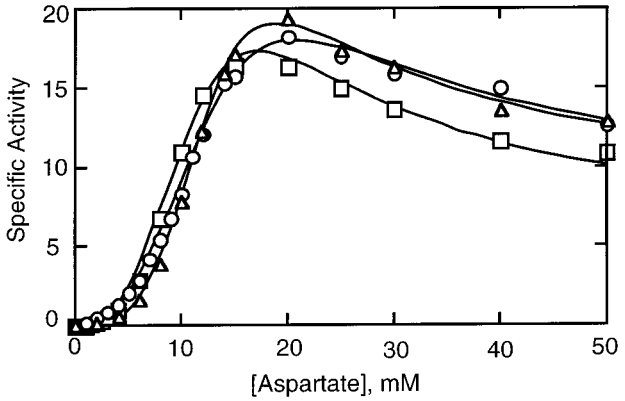

FIGURE 1: Aspartate saturation curves of the wild-type (○), the Glu-62r→Ala (□) and the Ile-12r→Ala (△) aspartate transcarbamoylases at pH 8.3. Colorimetric assays were performed at 25 °C in 0.05 M Tris-acetate buffer at saturating concentrations of carbamoyl phosphate (4.8 mM). Specific activity is reported in millimoles of *N*-carbamoyl-L-aspartate formed per hour per milligram of protein.

Table 1: Kinetic Parameters of the Wild-Type and Mutant Holoenzymes at pH 8.3[a]

enzyme	V_{max}[b] (mmol hr^{-1} mg^{-1})	$[Asp]_{0.5}$ (mM)	n_H
wild-type	17.7 ± 0.8	11.3 ± 0.9	2.7 ± 0.2
Glu-62r→ Ala	17.2 ± 2.5	9.0 ± 0.1	2.8 ± 0.1
Ile-12r→Ala	18.7 ± 0.5	10.7 ± 0.7	3.3 ± 0.4

[a] These data were determined from aspartate saturation curves. Colorimetric assays were performed at 25 °C in 0.05 M Tris-acetate buffer, pH 8.3, at saturating levels of carbamoyl phosphate (4.8 mM). The errors indicated are standard deviations from three or more trials. [b] The maximal velocity represents the maximal observed specific activity from the aspartate saturation curve.

RESULTS

Kinetic Comparison of the Mutant and Wild-Type Holoenzymes. The kinetic parameters derived from the aspartate saturation curves, including maximal observed velocity and $[Asp]_{0.5}$[3] for both the Glu-62r→Ala and Ile-12r→Ala enzymes, were comparable to the corresponding values for the wild-type enzyme (Figure 1). The Hill coefficient for the Glu-62r→Ala enzyme was similar to the value for the wild-type enzyme. However, the Hill coefficient for the Ile-12r→Ala enzyme was slightly larger than the corresponding value for the wild-type enzyme (Table 1).

Enhanced Activation by ATP and Enhanced Inhibition by CTP is Observed for the Glu-62r→Ala Enzyme. The replacement of Glu-62 by alanine at position 62 in the regulatory chain resulted in an enzyme in which the heterotropic effects were significantly enhanced (Figure 2). The maximal inhibition of the Glu-62r→Ala enzyme by CTP was approximately 94% as compared to 75% for the wild-type enzyme. Moreover, the apparent affinity of CTP for the Glu-62r→Ala enzyme was approximately 10-fold higher than for the wild-type enzyme. ATP was also able to activate the Glu-62r→Ala enzyme 330% compared to 230% for the

[3] Abbreviations: $[Asp]_{0.5}$, aspartate concentration required for one-half of the maximal observed specific activity.

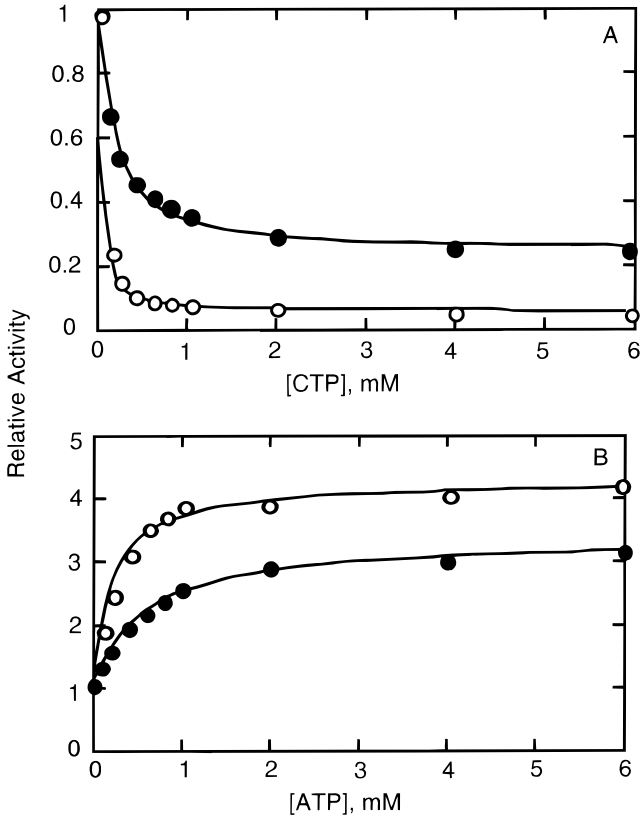

FIGURE 2: Influence of allosteric effectors on the activity of the wild-type (●) and Glu-62r→Ala (○) enzymes. Colorimetric assays were performed at 25 °C in 0.1 M imidazole-acetate buffer at pH 7 in the presence of increasing concentrations of CTP (A) and ATP (B). The aspartate concentration was held constant at one-half the $[Asp]_{0.5}$ of the respective enzyme at a saturating carbamoyl phosphate concentration (4.8 mM).

Table 2: Heterotropic Effects of ATP and CTP on the Wild-Type and Mutant Holoenzymes[a]

	ATP		CTP	
enzyme	activation (%)	K (mM)	residual Activity (%)	K (mM)
wild-type	213	0.57	25	0.16
Glu-62r→Ala	332	0.21	94	0.02
Ile-12r→Ala			35	0.43

[a] Assays were performed at 25 °C in 0.1 M imidazole-acetate buffer, pH 7. In all cases, the carbamoyl phosphate concentration was held constant at 4.8 mM. The aspartate concentrations used were half the $[Asp]_{0.5}$ for each enzyme.

wild-type enzyme; however, the ATP affinity for the Glu-62r→Ala enzyme was only approximately 2-fold higher than for the wild-type enzyme (Table 2 and Figure 2).

The Regulatory Nucleotides Have Significantly Reduced Effect on the Ile-12r→Ala Enzyme. As opposed to the Glu-62r→Ala enzyme, CTP was found to inhibit the Ile-12→Ala enzyme less than the wild-type enzyme (Figure 3). Furthermore, the apparent affinity of the mutant enzyme for CTP was reduced approximately 2-fold (Figure 3A).

ATP did not activate the Ile-12r→Ala enzyme, in fact at low concentrations of ATP, the Ile-12r→Ala enzyme was inhibited slightly. At ATP concentrations greater than 0.5 mM, the extent of the inhibition was reduced, and by 1 mM ATP the inhibitory effect was completely reversed. At 4 mM ATP the activity of the Ile-12r→Ala enzyme was

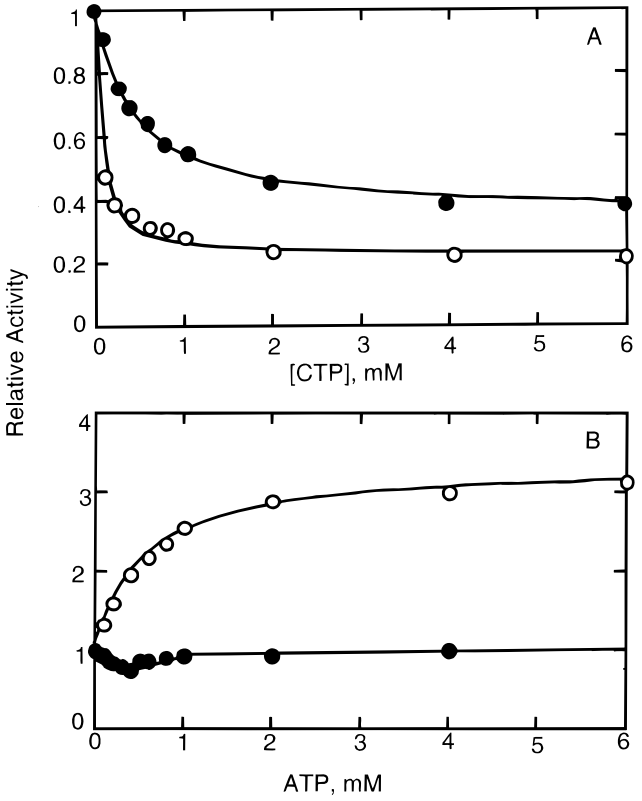

FIGURE 3: Influence of allosteric effectors on the activity of the wild-type (○) and Ile-12r→Ala (●) enzymes. Colorimetric assays were performed at 25 °C in 0.1 M imidazole-acetate buffer at pH 7 in the presence of increasing concentrations of CTP (A) and ATP (B). Experimental conditions are the same as indicated in the legend to Figure 2.

comparable to the activity of the enzyme in the absence of ATP (Figure 3B).

The Effect of UTP Alone on the Glu-62r→Ala and Ile-12r→Ala Enzymes. The wild-type enzyme does not bind UTP as strongly as it binds CTP, and UTP alone only inhibits the enzyme about 20% in the absence of CTP (*5*, *9*). In contrast, the Glu-62r→Ala enzyme is 70% inhibited by UTP alone, which is similar to the extent of inhibition caused by CTP alone on the wild-type enzyme (Figure 4). This is completely opposite to that observed with the Ile-12r→Ala enzyme. As seen in Figure 4, UTP alone has no effect whatsoever on the activity of the Ile-12r→Ala enzyme.

Influence of UTP in the Presence of CTP on the Glu-62r→Ala and Ile-12r→Ala Enzymes. For wild-type enzyme at pH 7, the addition of UTP in the presence of saturating concentrations of CTP causes additional inhibition of the enzyme (*5*). For the Glu-62r→Ala enzyme, when UTP was added in the presence of 4 mM CTP, only a small amount of additional inhibition was observed (Figure 5). It appears that the high levels of CTP inhibition observed for the Glu-62r→Ala enzyme diminish the effect of UTP in the presence of CTP.

UTP alone does not exert a significant effect on the Ile-12r→Ala enzyme (Figure 4), and when UTP is added in the presence of CTP, no additional inhibition is observed (Figure 5). However, low concentrations of UTP, when added to the enzyme saturated with 4 mM CTP, cause a small inactivation; however, as the concentration of UTP is increased this small inhibition is reversed, and at high

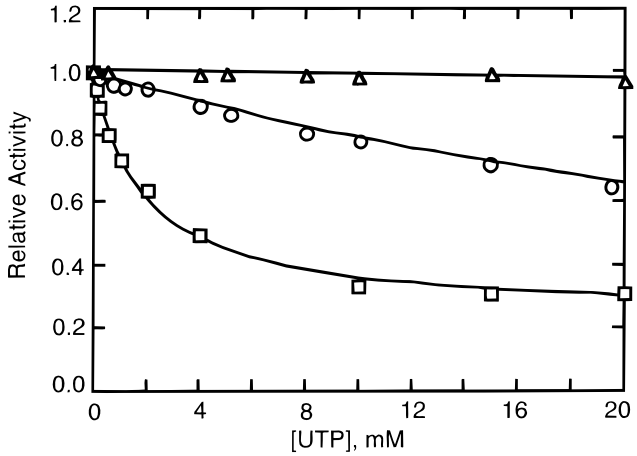

FIGURE 4: Influence of UTP on the activity of the wild-type (○), Glu-62r→Ala (□), and Ile-12r→Ala (△) enzymes. Colorimetric assays were performed at 25 °C in 0.1 M imidazole-acetate buffer at pH 7 in the presence of increasing concentrations of CTP. Experimental conditions are the same as indicated in the legend to Figure 2.

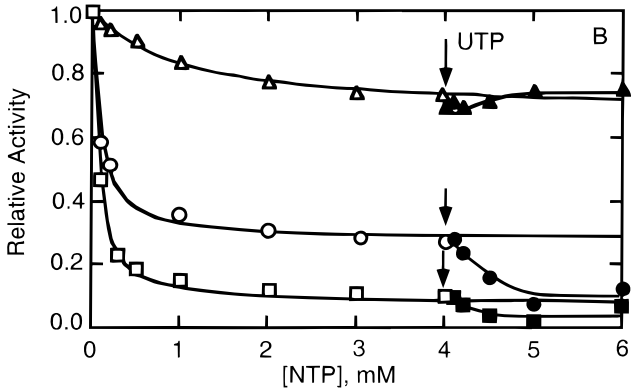

FIGURE 5: Influence of UTP in the presence of CTP on the activity of the wild-type (●), Glu-62r→Ala (■) and Ile-12→Ala (▲) enzymes. At CTP concentrations of less than or equal to 4 mM, no UTP was present for the wild-type (○), Glu-62r→Ala (□), and Ile-12→Ala (△) enzymes. At NTP concentrations greater than 4 mM, the CTP concentration was held constant at 4 mM and UTP was added. The ordinate therefore corresponds to the total nucleotide concentration [NTP] = [CTP] + [UTP]. Assays were performed in 0.1 M imidazole-acetate buffer pH 7.0. The aspartate concentration was held constant at the $[Asp]_{0.5}$ of the respective enzyme, and a saturating concentration of carbamoyl phosphate was used (4.8 mM).

concentrations of UTP little effect is observed (Figure 5). The influence of UTP in the presence of CTP for the Ile-12r→Ala enzyme is similar to that observed for ATP alone (Figure 3B).

DISCUSSION

Early X-ray structures of aspartate transcarbamoylase failed to locate the position of the N-terminus of the regulatory chain due to weak electron density in this region of the structure (*7*). However, Kosman et al. (*19*) reported the position of the R1 regulatory chain N-terminus, close to the allosteric effector binding site, in a structure of the enzyme complexed with CTP. An alanine scanning mutagenesis study established that the elimination of the side chain of Lys-6r led to reduced inhibition by CTP and reduced activation by ATP (*20*). The Kosman et al. (*19*) study showed that Lys-6r formed an interaction with Glu-62r across the interface between the two chains of a regulatory dimer (R1−R6). Thus, the mutagenesis study of Lys-6r (*20*) added support to the proposal of Stevens and Lipscomb (*33*) that the R1−R6 interface plays an important role in the transmission of the allosteric signal from the nucleotide binding site to the active site 60 Å away. The reduction in heterotropic effects observed when Lys-6r was substituted by alanine was believed to be due to perturbation of the R1−R6 dimer interface, caused by disruption of the salt-link, which in turn might hinder the compression or expansion of the regulatory dimers upon nucleotide binding (*20*). However, it is difficult to rationalize the results reported here for the Glu-62r→Ala enzyme on the basis of this hypothesis. The replacement of Glu-62r by alanine should disrupt the R1−R6 interface in a similar fashion as when Lys-6r was replaced by alanine. Therefore, one would expect the efficiency of transmission of the heterotropic signal to be compromised as observed for the Lys6r→Ala enzyme (*20*), not enhanced as observed for the Glu-62r→Ala enzyme.

An Altered Orientation of the N-Terminus Allows an Interaction between Glu-62r and Ile-12r. Although the electron density maps are weak in the area of the N-terminal of the regulatory chain, the analysis of a series of mutant aspartate transcarbamoylases in our laboratory (manuscript in preparation) suggests that the N-terminus is in a slightly different conformation than that previously reported (*19*). In these mutant structures there is no interaction between Lys-6r1 and Glu-62r6; instead Glu-62r forms van der Waals interactions with both the side chain and backbone of Ile-12r. A possible link between Glu-62r and Ile-12r may be important for effector response since Ile-12r is located in the effector binding pocket and has main chain carbonyl and amide interactions with both ATP and CTP (*12*, *34*). Since the allosteric domain of the regulatory chain is composed of a relatively rigid β-sheet, the side chain interaction between Glu-62r and Ile-12r may help to maintain the overall shape of the effector binding pocket.

The Replacement of Glu-62r by Alanine May Expand the Nucleotide Binding Site. Although Glu-62r does not directly interact with any of the regulatory nucleotides and is not directly in the allosteric binding pocket, the marked alteration in heterotropic response, when it is replaced by alanine, clearly indicates that it has a strong influence on the nucleotide binding site. The kinetic data presented here suggest strongly that side-chain interactions between Glu-62r and Ile-12r help to maintain the structure of the nucleotide binding pocket. When Glu-62r is replaced by alanine, the side chain interactions with Ile-12r are lost which may allow the backbone in the vicinity of Ile-12r to shift position toward Glu-62r opening the regulatory binding site (Figure 6). This alteration in the conformation of the binding site is reflected in the enhanced heterotropic effects observed for the Glu-62r→Ala enzyme.

Further support for this theory is provided by the effect of UTP on the Glu-62r→Ala enzyme. For the wild-type enzyme, UTP binds weakly in the absence of CTP and causes a 20% reduction in activity when present alone (*9*). In contrast to the wild-type enzyme, UTP alone has almost the same effect on the Glu-62r→Ala enzyme as CTP does on the wild-type enzyme. The kinetic data obtained in the presence of CTP alone show that CTP has the same inhibitory effect on the Glu-62r→Ala enzyme as the com-

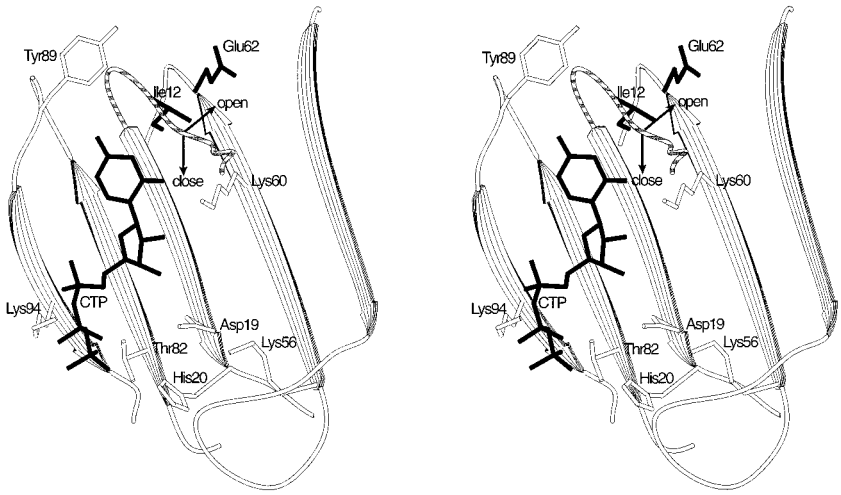

FIGURE 6: Stereoview of the allosteric domain of the regulatory chain of aspartate trancarbamoylase complexed with CTP. Shown are the five strands of β-sheet that make up the domain along with key residues shown by mutagenesis to be important for the heterotropic properties of the enzyme. The N-terminal loop comprising residues 7−14 is indicated by the striping. When the bulky Ile-12 is replaced by alanine, the N-terminal loop is capable of repositioning, resulting in a closing of the nucleotide site. When Glu-62r is replaced by alanine, the N-terminal loop is capable of repositioning, resulting in a opening of the nucleotide site. This figure was drawn with SETOR (*36*).

bination of CTP and UTP has on the wild-type enzyme. Thus, as a result of the mutation the enzyme loses its ability to discriminate between CTP and UTP. The more open nucleotide binding site proposed above may relax the constraints that allowed the wild-type enzyme to distinguish between CTP and UTP. Since the X-ray data indicate that there are no side chains directly involved in the discrimination between CTP and UTP, these data suggest that the exact conformation of the binding site is critical for distinguishing between the two nucleotides.

The Glu-62r→Ala enzyme also exhibits enhanced affinity for the nucleotides. In addition to the possible structural alterations to the binding site mentioned above, the enhanced binding of the negatively charged nucleotides may be due to the loss of the negatively charged side chain of Glu-62r thereby making the binding site more electropositive.

Truncation of the Side Chain of Ile-12r May Allow for a Compression of the Allosteric Binding Pocket. As in the case of the Glu-62r→Ala enzyme, it is difficult to rationalize the results obtained for the Ile-12r→Ala enzyme on the basis of published structures, in which Ile-12r has main chain interactions with both ATP and CTP (*12*, *35*). If the role of Ile-12r in the regulatory process is limited only to these main chain interactions, no effects would be expected when Ile-12r is replaced by alanine. The dramatically different heterotropic effects associated with the replacement of Ile-12r suggest that there has been an alteration in the backbone. All of the effectors bind poorly to the Ile-12r→Ala enzyme and exhibit reduced heterotropic effects. We can speculate that in the absence of the isobutyl side chain of Ile-12r the loop encompassing residues 12r-16r collapses, resulting in a more closed binding pocket (Figure 6). This compression may alter the structure of the binding pocket sufficiently to prevent the nucleotides from binding with the same affinity as they do to the wild-type enzyme.

Indirect evidence of a possible backbone shift around Ile-12r comes from an examination of the UTP inhibition data. The only difference between CTP and UTP is at the 4 position of the pyrimidine ring, where CTP has an amino group and UTP has a carbonyl group. However, a more subtle difference exists in the protonation state of the nitrogen at the 3 position of the pyrimidine ring, which is protonated in UTP and deprotonated in CTP. Since the backbone of Ile-12r forms hydrogen bonding interactions with both the N3 nitrogen and the 1-amino group of the pyrimidine ring (*21*), the subtle differences in the mode in which these nucleotides interact with the backbone of Ile-12r must play a significant role in the discrimination between them. Therefore, the altered effects of CTP and UTP for both the mutant enzymes is indirect evidence in support of a backbone shift of Ile-12r due to the amino acid replacements.

The effect of ATP on the Ile-12r→Ala can be explained by two opposing processes. ATP binds poorly and in fact initially causes inhibition; however, a reversal of inhibition is observed at ATP concentrations greater than 1 mM. From these results, it appears that the nucleotide binding pocket has been altered to an extent that the enzyme is unable to recognize ATP. Since there is structural asymmetry between the two binding sites of the regulatory dimer (*7*), the binding of ATP may be causing slight inhibition at one site and slight activation at the other.

Glu-62r and Ile-12r Work Together to Maintain the Geometry of the Nucleotide Binding Site in the Wild-Type Enzyme. Compression of the binding pocket when Ile-12r is replaced by alanine is again consistent with the theory that the side chains of Ile-12r and Glu-62r function to maintain the structural integrity of the binding site. Glu-62r is a residue which is far away from the binding region and does not have side chain interactions with the nucleotides, yet when it is altered, strong effects are observed on the heterotropic properties. This fact in itself suggests that Glu-62 is able to exert an influence on the binding of the regulatory nucleotides. This must necessarily be an indirect effect and therefore must be transmitted via one or more amino acid residues, which in turn have interactions at the allosteric site. The results of this study suggest that Glu-62r and Ile-12r work together to maintain the conformation of the nucleotide binding site and changes in either one of these residues perceptibly alters the binding site to produce dramatically different heterotropic effects. At this point, it

is not possible to establish if there are other residues involved in this process, but a strong case can be made for the fact that interactions between Glu-62r and Ile-12r provide a simple but elegant way for the enzyme to control the structure of its regulatory site and therefore control its own regulatory processes.

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