Asymmetric Allosteric Activation of *Escherichia coli* Glucosamine-6-phosphate Deaminase Produced by Replacements of Tyr 121†

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ABSTRACT: Tyrosine 121, a residue located in a α-helical polypeptide segment of glucosamine 6-phosphate deaminase from *Escherichia coli*, has recently been proposed to have a role in the binding of the allosteric activator *N*-acetyl-D-glucosamine 6-phosphate. Accordingly, the site-directed mutants Tyr 121−Thr and Tyr 121−Trp were constructed, to assess experimentally the role of Tyr 121 in the allosteric function of the enzyme. The kinetic study of both mutant forms revealed that the replacements caused striking changes in allosteric activator binding and allosteric properties, when compared to the wild-type enzyme. While the wild-type deaminase behaves as a classical allosteric *K*-system which can be described by the allosteric concerted model, both mutant forms present an asymmetric behavior toward the allosteric activator, which can be described as two distinct half-of-the-sites allosteric activation steps occurring with different affinities for the *N*-acetyl-D-glucosamine 6-phosphate. During the first (high affinity) activation phase, the mutant forms of deaminase behave as mixed *K/V* allosteric enzyme. The biphasic activation curve was also demonstrated by direct binding measurements of the ^{14}C-labeled activator to Tyr 121−Trp and Tyr 121−Thr deaminases. The kinetic analysis of these mutant forms also showed that the threonine replacement produced an important distortion of the enzyme structure reflected in a considerable decrease of its catalytic efficiency. This finding suggests that the Thr replacement at position 121 produces structural perturbations which are absent in the Tyr 121−Trp mutant form of the enzyme, thus emphasizing the structural importance of interactions at this position between the helix and the protein core.

The amino sugars D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) are primarily components of the cell wall and the outer membrane in bacteria like *Escherichia coli*. When amino sugars are absent from the environment, the bacteria must synthesize D-glucosamine 6-phosphate (GlcN6P)[1] from D-fructose 6-phosphate (Fru6P) and L-glutamine using the enzyme glucosamine synthase, encoded by the gene *glmS*. In addition, the amino sugars are useful sources of carbon and nitrogen for the bacteria. The enzymes necessary for the uptake and degradation of amino sugars are coded by the genes of the divergent *nagE* and *nagBACD* operons (Rogers et al., 1988; Plumbridge, 1989; Vogler & Lengeler, 1989). The enzymes involved in these anabolic and catabolic processes need to be carefully regulated, and this regulation is achieved by controls exerted at two levels: first, at the level of enzyme synthesis by transcriptional induction of the degradative genes during growth on the amino sugars (White, 1968; Plumbridge, 1990) and repression of the biosynthetic gene (Plumbridge et al, 1993) and, second, at the level of activity of the degradative enzymes. The key enzyme for this regulation is glucosamine-6-phosphate deaminase (GlcN6P deaminase; EC 5.3.1.10), encoded by the gene *nagB*. This deaminase converts GlcN6P to Fru6P and ammonia (Midelfort & Rose, 1977; Calcagno et al., 1984), a reaction functionally opposite to the metabolic step catalyzed by the biosynthetic enzyme glucosamine synthase.

The deaminase is subject to allosteric activation by *N*-acetyl-D-glucosamine 6-phosphate (GlcNAc6P). This phosphorylated sugar is an extremely important effector for amino sugar metabolism. It is the product of transport of GlcNAc into the bacteria and the substrate of the first enzyme of the GlcNAc catabolism, GlcNAc6P deacetylase, encoded by the *nagA* gene. In addition, GlcNAc6P exerts two regulatory roles: it is the allosteric activator of GlcN6P deaminase and also the allosteric effector of the *nagC* repressor where it plays the role of inducer (Plumbridge, 1991).

The allosteric response of GlcN6P deaminase to intracellular GlcNAc6P results in the increase of enzyme activity at the expense of increasing the affinity for its substrate, GlcN6P. This is a classical *K*-effect, and, indeed, homotropic effects on GlcN6P deaminase can be satisfactorily described by the allosteric concerted model (MWC model; Monod et al., 1965). This is true for both the wild-type enzyme and

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; GlcN6P, D-glucosamine 6-phosphate; GlcNAc6P, *N*-acetyl-D-glucosamine 6-phosphate; CD, circular dichroism.

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some Cys to Ser mutants (Altamirano et al., 1989, 1992).

The spectrophotometric study of the interaction of the enzyme with GlcNAc6P, its allosteric activator, suggested the presence of one tyrosyl residue at or near the allosteric site in each polypeptide chain. This residue can be recognized in spectrophotometric pH-titration experiments because it is particularly acidic (pK of 8.75) and its phenolic hydroxyl was occluded by GlcNAc6P binding. The titration behavior of its phenolic hydroxyl was not modified by the deaminase dead-end inhibitor, 2-deoxy-2-amino-D-glucitol 6-phosphate (D-glucitolamine 6-phosphate, GlcN-ol-6P), but the hydroxyl was entirely protected from deprotonation by a saturating concentration of GlcNAc6P (Altamirano et al., 1994).

A theoretical analysis of the amino acid sequence of the deaminase using a set of indices for the prediction of surface accessibility of amino acid residues strongly suggested that Tyr 121 is the tyrosyl residue involved in this function. This residue is located in a segment predicted as a highly polar helix which gives the highest scores for surface accessibility (Altamirano et al., 1991, 1994). To verify this deduction, the site-directed mutants Tyr 121−Thr and Tyr 121−Trp were constructed to assess experimentally the role of Tyr 121 in the allosteric function of GlcN6P deaminase. The replacement amino acids chosen were threonine, to produce a structurally conservative mutation (Richardson & Richardson, 1989; Serrano & Fersht, 1989), and tryptophan, chosen to maintain an aromatic residue in this position with its ability to establish hydrophobic and aromatic interactions. These two mutant forms of the enzyme were compared to the wild-type enzyme regarding catalytic activity, homotropic cooperativity, GlcNAc6P binding, and allosteric activation. We show here that replacements at position 121 produced striking differences in activator binding and allosteric regulation in the mutant enzymes compared to the wild-type protein. These changes consist of an asymmetric behavior of the mutant enzymes toward the activation by GlcNAc6P, which can be described as two distinct half-of-the-sites allosteric activation steps, occurring with different affinities for GlcNAc6P.

EXPERIMENTAL PROCEDURES

Biochemicals. Biochemicals and most reagents were from Sigma Chemical Co. (St. Louis, MO). GlcNAc6P was prepared by acetylation of GlcN6P and purified by ion-exchange chromatography, according to Leloir and Cardini (1962). The same procedure was used to synthesize [^{14}C]-GlcNAc6P isotopically labeled in the acetyl group with [1,1′-^{14}C]acetic anhydride (1 mCi/mmol, Amersham) and diluted with nonlabeled acetic anhydride to obtain a specific activity of 1.5 μCi/mmol. The purity of the product was verified by TLC on precoated silica gel plates (Sigma Chemical Co.) using the following solvent systems: (a) isopropanol/15 M ammonia (6:1); (b) ethyl acetate/17 M acetic acid/water/15 M ammonia (6:2:2:1); and (c) ethanol/1 M ammonium acetate/17 M acetic acid/water (5:2:1:1). Spots were visualized by means of reagents for organic phosphates (Hanes−Isherwood reagent) and amino sugars (ninhydrin) as described by Churms (1982). As a general detection procedure, iodine vapors and 5 N H_2SO_4, followed by heating at 110 °C, were used (Churms, 1982). The radiochemical purity of [^{14}C]GlcNAc6P was verified by autoradiographic analysis of TLC plates. To calculate the specific radioactivity of the final product (0.73 μCi/mmol), the GlcNAc6P concentration was determined by a modified Elson−Morgan reaction (Levy & McAllan, 1959), using the pure unlabeled compound as standard.

The GlcN6P deaminase dead-end inhibitor, GlcN-ol-6P, was synthesized and purified as described by Midelfort and Rose (1977); its purity was verified by TLC using cellulose plates and the solvent systems b and c (see above), developed with ninhydrin or general detection procedures.

Mutagenesis and Enzyme Preparation. E. coli wild-type GlcN6P deaminase was prepared from an overproducing strain, as previously described (Altamirano et al., 1991). Amino acid replacements at position 121 were produced by the technique of oligonucleotide-directed mutagenesis using the Kunkel method, as described by Sambrook et al. (1989). The *nagB* gene from pUC(*nagB*) (Altamirano et al., 1991) was cloned into the phagemid vector pTZ18R (Pharmacia) to give pTZ18R(*nagB*). Single-stranded template containing uridine residues for the mutagenesis was prepared as described (Sambrooke et al., 1989). The mutagenic oligonucleotides were 30-mers corresponding to the sense strand of the gene with the Tyr 121 codon near the center. The mutations created were Tyr (TAT) to Thr (TCT) and Tyr to Trp (TGG). Phagemids carrying the mutations were identified by sequencing, and the rest of the *nagB* gene was also sequenced to verify the absence of any secondary mutations. The mutated pTZ18R(*nagB*) single-stranded DNAs were used to transform the Δ*nag* strain IBPC590 (*thi-1*, *argG6*, *argE3*, *his-4*, *mtl-1*, *xyl-5*, *rpsL*, Δ*lacX74*, Δ*nagEBACD*::tc) (Plumbridge et al., 1993). Wild-type and genetically modified forms of GlcN6P deaminase were isolated and assayed using previously reported procedures, essentially allosteric-site affinity chromatography (Calcagno et al., 1984; Altamirano et al., 1991).

The concentration of the wild-type enzyme was calculated from its absorbance at 278 nm at pH 7.7, using its known molar absorptivity [20.0 × 10^4 M^{-1} cm^{-1} (Altamirano et al., 1989)]. The molar absorptivities for the mutant forms of the enzyme were calculated from the spectra of the proteins in 100 mM Tris-HCl buffer, pH 7.7, and the protein concentration in the solution was measured with the method of Bradford (1976) and using the wild-type enzyme as standard. A convenient method to refine the ϵ_{278} values of the mutants involving aromatic amino acid residues is the measurement of the number of the reactive thiol groups under native conditions. We have shown that two cysteine residues, Cys 118 and Cys 239, are reactive in the native deaminase provided that the protein is in the T allosteric conformer. These two thiols per polypeptide chain can be accurately measured with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) (Altamirano et al., 1992). A correction of the molar absorptivities for the Tyr 121 mutant proteins was made taking into consideration the measurement of the thiol groups and the expected stoichiometry of two thiols per polypeptide chain. These values were 21.2 × 10^4 and 17.0 × 10^4 M^{-1} cm^{-1} for Tyr 121−Trp and Tyr 121−Thr GlcN6P deaminase, respectively.

Kinetic Analysis. The reaction of deamination of GlcN6P was measured as previously described (Altamirano et al., 1989). The analyses of the kinetic data were performed using the program ENZFITTER, by R. J. Leatherbarrow (Elsevier Biosoft, Cambridge, U.K.). To test models and simulate

Table 1: Kinetic Properties of Mutant Forms of Glucosamine-6-phosphate Deaminase with Replacements at Tyr 121

	h_{max}	K_m (mM)	k_{cat}[a,b] (s^{-1})	k_{cat}[a,c] (s^{-1})	k_{cat}/K_m ($M^{-1}\ s^{-1} \times 10^5$)	c	L ($\times 10^3$)
wild-type	3.02 ± 0.09	2.01 ± 0.05	295 ± 15	292 ± 17	1.45	0.02	10
Tyr 121−Trp	3.42 ± 0.14	0.67 ± 0.01	41 ± 0.1	97 ± 0.3	1.44	5×10^{-4}	7.7
Tyr 121−Thr	2.93 ± 0.15	2.60 ± 0.02	2.8 ± 0.02	56 ± 0.1	0.215	0.02	3.2

[a] k_{cat} values were calculated taking into account that the hexameric enzyme has six actives sites per molecule. [b] Measured in the absence of the allosteric activator. [c] Measured in the presence of 16 mM GlcNAc6P (mutant forms) or 2.5 mM GlcNAc6P (wild-type).

equations, the program GLE 3.2, by C. Pugmire, Lower Hutt, New Zealand, was used.

Equilibrium Dialysis. The binding of GlcNAc6P to deaminase was measured using standard microcentrifuge polypropylene tubes as dialysis chambers (Reinard & Jacobson, 1989). A dialysis membrane with a cut-off of 14 kDa (Sigma Chemical Co., St. Louis, MO), was used. The tubes with the samples to be equilibrated were shaken for 8 h at 30 °C in an adapted vortex shaker. The concentration of [^{14}C]GlcNAc6P was determined by liquid scintillation counting and taking into account its specific radioactivity. A tube containing the deaminase without the ligand was also prepared to control the enzyme activity after the incubation. Wild-type and Tyr 121 mutant forms of GlcN6P deaminase were shown to be stable during the experiment.

Spectrophotometric Methods. Difference absorption spectra were recorded in a Cary 4 double-beam spectrophotometer at 30 °C. The pH titration of tyrosyl residues in native and mutant forms of the deaminase was performed by recording the spectra of a set of 3.5 μM deaminase solutions, prepared in 50 mM ACES, 25 mM MES, and 25 mM ethanolamine buffer, at various pH values. This buffer system minimizes the change of ionic strength with pH (Ellis & Morrison, 1982). As the fully protonated form of the protein for reference, the enzyme solution at pH 6.0 was employed. The ionization of tyrosyl residues was calculated from the absorbance change at 295 nm, using the molar absorptivity of $2.33 \times 10^3\ M^{-1}\ cm^{-1}$ (Donovan, 1973).

Circular dichroism (CD) spectra were obtained in an Aviv CD spectrometer model 62DS using strength-free cells of 5 mm light path. The kinetics of the reaction of the two reactive sulfhydryl groups of GlcN6P deaminase (Cys 118 and Cys 239) with DTNB were measured at pH 7.7 and 30 °C, as described (Altamirano et. al., 1989, 1992), using the double-beam spectrophotometer. From the time course of the reaction, the pseudo-first-order kinetic constants were calculated at different GlcNAc6P concentrations, including zero, and used to calculate the affinity of the protein for this ligand.

RESULTS

Spectrophotometric Titration of Tyr Residues in Tyr 121 Mutant Forms of GlcN6P Deaminase. We have previously demonstrated the presence of a tyrosyl residue with a pK of 8.75, located at or near the allosteric site of the enzyme. This residue was recognized by spectrophotometric pH titration using the red-shift of the main absorption band of the phenolate anion. From theoretical considerations we proposed that this residue is Tyr 121 (Altamirano et al., 1994). To confirm this deduction two mutations were made at this position, Tyr 121−Thr and Tyr 121−Trp. Figure 1 shows the difference near-UV spectra of wild-type enzyme (curve a) and the Tyr 121−Trp mutant form (curve b)

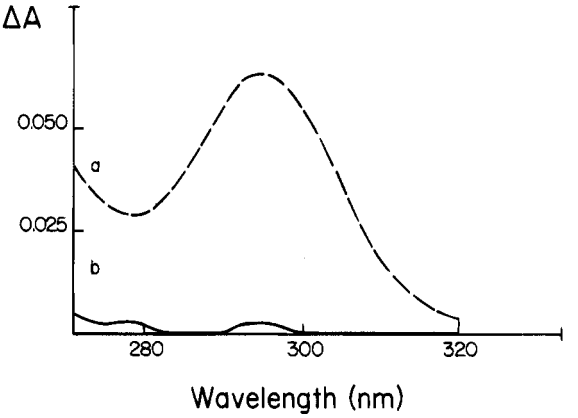

FIGURE 1: Change in difference near-UV spectra of wild-type (curve a) and Tyr 121−Trp GlcN6P deaminase (curve b), produced by the pH increase from 6.0 to 10.5. The spectra were obtained from a 4.5 μM solution of the protein in 50 mM ACES, 25 mM MES, and 25 mM ethanolamine buffer, pH 10.5. The reference cell contained the same enzyme concentration in the same buffer, at pH 6.0. The result obtained with Tyr 121−Thr deaminase was similar to curve b.

obtained as the difference spectra at pH 10.5 and 6.0. In contrast to the wild-type, the difference spectra of the mutant enzyme lacks the peak at 295 nm, which is produced by the red-shift corresponding to the dissociation of the tyrosine hydroxyl. A similar result was obtained with Tyr 121−Thr deaminase (not shown). These results confirm our deduction that the tyrosine residue whose side chain was protected from deprotonation by the allosteric activator is Tyr 121 (Altamirano et al., 1994).

Kinetic Parameters of Mutant Deaminases with Replacements at Position 121. Both GlcN6P deaminases with replacements of Tyr 121 are active and allosteric enzymes as shown in Table 1 (indeed, they were purified by allosteric-site affinity chromatography; see Experimental Procedures). GlcNAc6P, the natural activator of GlcN6P deaminase, is an exclusive-binding allosteric ligand thus inducing hyperbolic kinetics when present at saturating concentrations. The Tyr 121 deaminase mutants require a higher activator concentration, up to 15 mM, to attain hyperbolic kinetics, whereas 0.2 mM is sufficient for the wild-type enzyme. Table 1 gives the fitted values for K_m and k_{cat} and the k_{cat}/K_m ratios for both mutant enzymes; the corresponding values for the wild-type enzyme are included as reference. There is a significant decrease of the k_{cat} values for both mutant forms; Tyr 121−Trp deaminase also has a lower K_m for GlcN6P than the wild-type enzyme. The Tyr 121−Trp mutant enzyme has a k_{cat}/K_m ratio (catalytic efficiency; Fersht, 1985) close to the value for the wild-type enzyme, but the catalytic efficiency of the Tyr 121−Thr enzyme is significantly decreased. It is interesting that, in the absence of the allosteric activator, k_{cat} values are lower for both mutants, a result indicating that they do not behave as allosteric

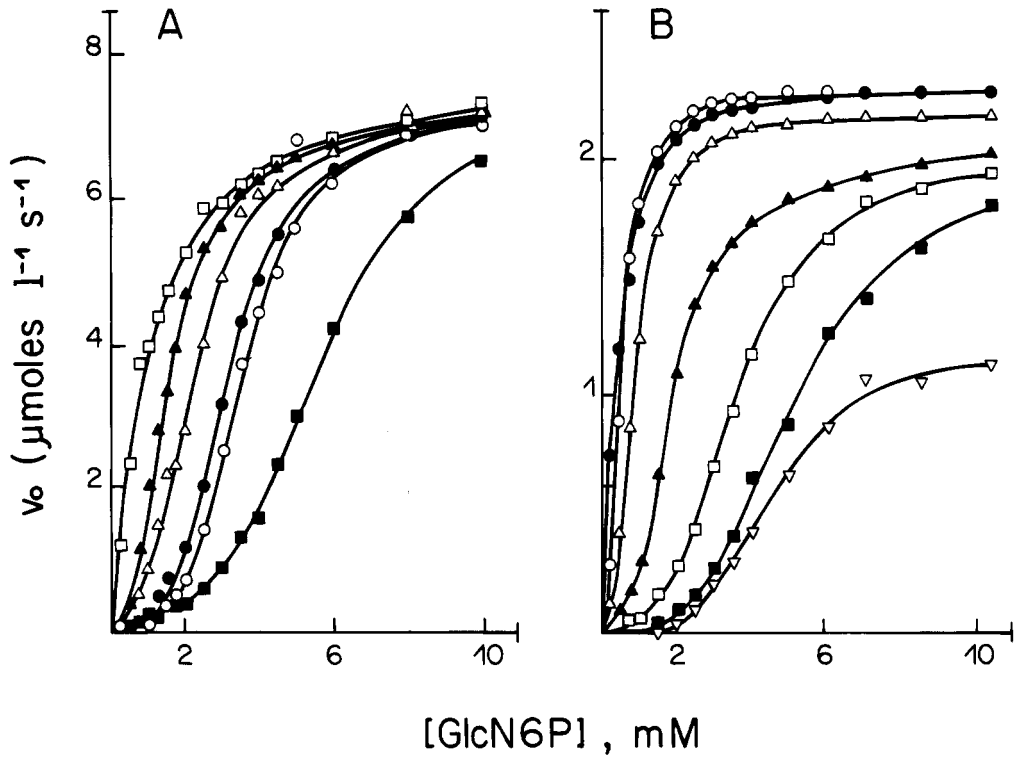

FIGURE 2: (A) Activation of wild-type GlcN6P deaminase by GlcNAc6P. Initial velocities were plotted against substrate concentration at the following activator concentrations: (■), none; (○), 5 μM; (●), 10 μM; (△), 25 μM; (▲), 50 μM; (□) 2.5 mM. Assays were performed using 2 nM deaminase in a final volume of 200 μL, in Tris-HCl buffer, pH 8.0 and 30 °C, as described elsewhere (Altamirano et al., 1989). The curves correspond to the fit of these data to the MWC equation for nonexclusive binding of GlcN6P and exclusive binding for GlcNAc6P. For the curve at zero GlcNAc6P concentration, the number of sites, n, was 5.42 ± 0.22, which can be rounded-off to 6. The fitted value for K_{dis} for GlcNAc6P was 0.033 ± 0.005 mM. The curve obtained at 2.5 mM GlcNAc6P was fitted to the Michaelis–Henri equation. The obtained values for the corresponding kinetic parameters are shown in Table 1. (B) Activation of Tyr 121–Trp GlcN6P deaminase by GlcNAc6P. The plot is similar to that of panel A. The allosteric activator concentrations are as follows: (▽) none; (■) 1 μM; (□) 10 μM; (▲) 25 μM; (△) 50 μM; (○) 10 mM; and (●) 16 mM. The data in the absence of GlcNAc6P can be fitted to MWC equation for the general case (non exclusive binding of substrate). The fitted parameters are shown in Table 1. The kinetics of the activation of this mutant form of GlcN6P deaminase by GlcNAc6P cannot be described using the MWC equation.

K-systems as the wild-type deaminase but display a mixed K and V activation pattern. The Tyr 121–Thr GlcN6P deaminase has a pronounced V-effect, and in the absence of the allosteric activator it is nearly 100 times less active than the wild-type enzyme (Table 1).

Allosteric Activation of Wild-Type GlcN6P Deaminase by GlcNAc6P. We have previously shown that homotropic effects in wild-type *E. coli* GlcN6P deaminase can be accounted for by the MWC model (Monod et al., 1965), assuming nonexclusive binding of the substrate (Altamirano et al., 1989, 1992). The activation of the wild-type GlcN6P deaminase by GlcNAc6P can also be accurately described by this model, considering GlcNAc6P as an exclusive binding ligand; a set of initial velocity versus substrate concentration curves obtained at different concentrations of the allosteric activator is shown in Figure 2A. These data were fitted to the MWC equation for nonexclusive binding of GlcN6P and exclusive binding of GlcNAc6P; from this experiment, a value of 0.033 ± 0.002 mM was obtained for GlcNAc6P dissociation constant.

Allosteric Activation of Mutant Deaminases with Replacements at Position 121. Replacement of tyrosine at position 121 with either threonine or tryptophan produced marked modifications in the kinetics of the allosteric activation of GlcN6P deaminase by GlcNAc6P, which can no longer be described on the basis of a two-state model (Figure 2B and Table 1). This unusual kinetics can be better recognized in the plots of h_{max} (the maximal values of the Hill coefficient)

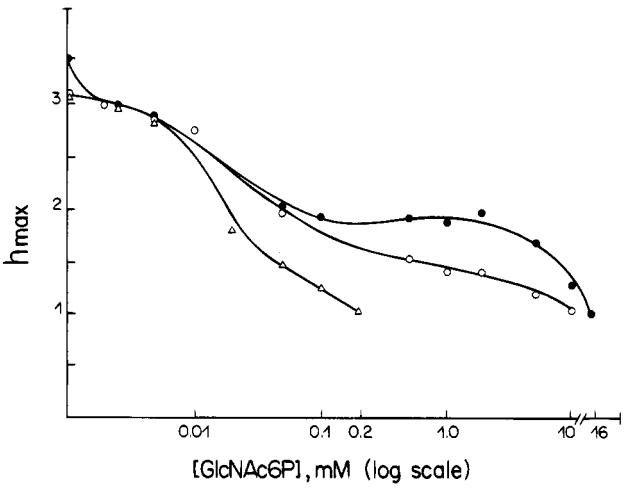

FIGURE 3: Maximal values of the Hill coefficient (h_{max}) plotted versus GlcNAc6P concentration. (●) Tyr 121–Trp deaminase, data from the experiment shown in Figure 2. (○) Tyr 121–Thr deaminase, from an experiment similar to that for Tyr 121–Trp (direct plots not shown). (△) Wild-type enzyme; data from the experiment shown in Figure 2.

versus the log of the activator concentration (Figure 3), where a biphasic activation curve with two distinct activation steps is apparent. This result suggests the existence of a stable conformer of the protein with an intermediate structure; in the case of Tyr 121–Trp mutant enzyme, this species predominates in the GlcNAc6P concentration range from 0.1

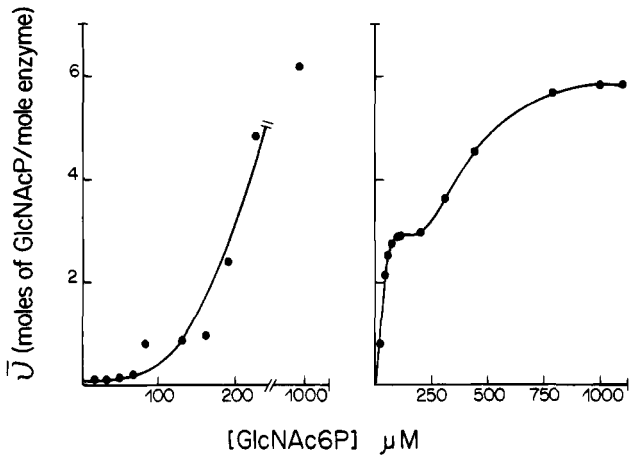

FIGURE 4: (A, left) Binding of GlcNAc6P to GlcN6P deaminase measured by equilibrium dialysis. Microcentrifuge tubes were used as dialysis chambers as described in the text. The enzyme chamber (200 μL) contained a 5 μM GlcN6P deaminase in 0.1 M Tris-HCl buffer, pH 8.0 (30 °C), and 2.5 mM EDTA. The outer chamber (400 μL) contained the same buffer and different amounts of [^{14}C]-GlcNAc6P to yield at equilibrium the desired concentrations of free allosteric activator. From the fit to Hill equation of curve, the following parameters were estimated: $[GlcNAc6P]_{0.5}$, 180 μM; v_{max}, 6.09 ± 0.84 mM; Hill coefficient, 2.51. (B, right) Equilibrium dialysis experiment, performed under the same conditions using the mutant form Tyr 121−Trp.

to 1 mM. From these experiments it appears that, in the first phase of GlcNAc6P activation, the 121 mutant forms of GlcN6P deaminase behave as mixed *K/V* systems, but, during its second activation phase, their allosteric activation is of the *K*-type.

Homotropic Effects on Tyr 121−Trp and Tyr 121−Thr GlcN6P Deaminases. Although the allosteric activation by GlcNAc6P cannot be described by a two-state allosteric model, the curve of velocity versus substrate concentration obtained without activator (Figure 2B) can be fitted to the MWC equation, as in the case of the wild-type enzyme (Figure 2A and Table 1). The MWC fitted parameters for Tyr 121−Thr deaminase are quite similar to those of the wild-type enzyme, but the Tyr 121−Trp mutant presents an increased homotropic cooperativity mainly produced by a decrease of the *c* parameter (the K_R/K_T ratio), i.e., the nonexclusive binding constant for GlcN6P. In contrast, the velocity versus substrate curves obtained in the presence of several fixed GlcNAc6P concentrations (Figure 2B) cannot be properly fitted to the MWC equation.

GlcNAc6P Binding Measured by Equilibrium Dialysis. The saturation curve of wild-type GlcN6P deaminase by GlcNAc6P, determined by equilibrium dialysis, is shown in Figure 4A. The shape of the curve is sigmoidal with a Hill coefficient of 2.5 and a $[GlcNAc6P]_{0.5}$ of 0.18 mM. When the experiment was performed in the presence of a high, fixed concentration of the dead-end inhibitor, GlcN-ol-6P, to shift the allosteric equilibrium in the direction to the R-conformer, the curve obtained was hyperbolic, with a K_{dis} for GlcNAc6P of 0.035 mM (not shown). The fitted value for the maximal saturation ratio (i.e., the number of allosteric sites per enzyme molecule) was found to be close to the integer six for both experiments.

The binding curve for Tyr 121−Trp obtained under similar experimental conditions is shown in Figure 4B, where a biphasic saturation curve is apparent. This result is consistent with the kinetic data and suggests that GlcNAc6P binding occurs in two successive steps with a stoichiometry of three molecules of activator bound per deaminase hexamer in each step.

Allosteric Activator Binding to Deaminase, Analyzed by CD Spectrometry. The interaction of the activator with the wild-type enzyme was previously analyzed from measurements of the differential CD spectra produced by GlcNAc6P binding. Figure 5A shows a set of CD difference spectra (bound minus free) of wild-type deaminase in the range of 260−310 nm, obtained at different GlcNAc6P concentrations. They show several peaks spanning the aromatic absorption zone (Figure 5A). As discussed elsewhere (Altamirano et al., 1994), the strongest bands in the range 270−290 nm, which can be assigned to tryptophan residues, tyrosine residues, or both, have a regular decrease of their ellipticity when GlcNAc6P concentration is increased. From these spectral changes of the wild-type enzyme, it was possible to obtain a saturation curve of the enzyme by the allosteric activator (Altamirano et al., 1994)

Spectra from a similar experiment using Tyr 121−Trp GlcN6P deaminase are shown in Figure 5B. The results reveal a different and very complex behavior of the mutant enzymes as compared with the wild-type GlcN6P deaminase. In spite of the complexity of the changes observed, some conclusions can be drawn. A positive band at 270 nm is apparent at very low GlcNAc6P concentrations (Figure 5B, spectrum a). This is within the same concentration range of GlcNAc6P which produced a pronounced increase of the k_{cat}, i.e., an allosteric *V*-effect. This peak becomes negative in the micromolar range of GlcNAc6P concentration and decreases steadily during the first phase of the allosteric activation, where the Hill coefficient changes from nearly 3.0 to 2.0 (curves b and c). The comparison of spectra d and e indicate that, in the millimolar GlcNAc6P concentration range, the structural changes associated with activator binding and the allosteric transition are qualitatively different in this range. This almost corresponds to the second activation phase, from Hill coefficient 2 to 1. The negative peak at 267 nm in curve d increases while the peak at 276−278 nm sharpens and decreases, producing an isodichroic point at 272 nm. These changes in the 1−10 mM GlcNAc6P concentration range suggest that binding of this ligand to the apparent low affinity sites probably involves different conformational modifications. This observation is consistent with the changes in sulfhydryl reactivity described below. The far-UV CD spectra of Tyr 121−Trp and Tyr 121−Thr GlcN6P deaminases are similar to the corresponding spectrum of the wild-type enzyme (not shown).

GlcNAc6P−Enzyme Interaction Measured through Its Effect on Sulfhydryl Reactivity. Sulfhydryl groups from Cys 118 and Cys 239 react with several thiol reagents when the enzyme is in the T conformation, but they become completely protected when the T to R transition is induced by saturation with either homotropic or heterotropic ligands (Altamirano et al., 1989, 1992). The kinetics of the chemical reaction of the sulfydryl groups in the presence of GlcNAc6P was analyzed according to the following equation, derived from these observations and the MWC model (see also the Appendix):

$$k_{app} = \frac{k'_1 L}{L + (1 + \gamma)^n} \quad (1)$$

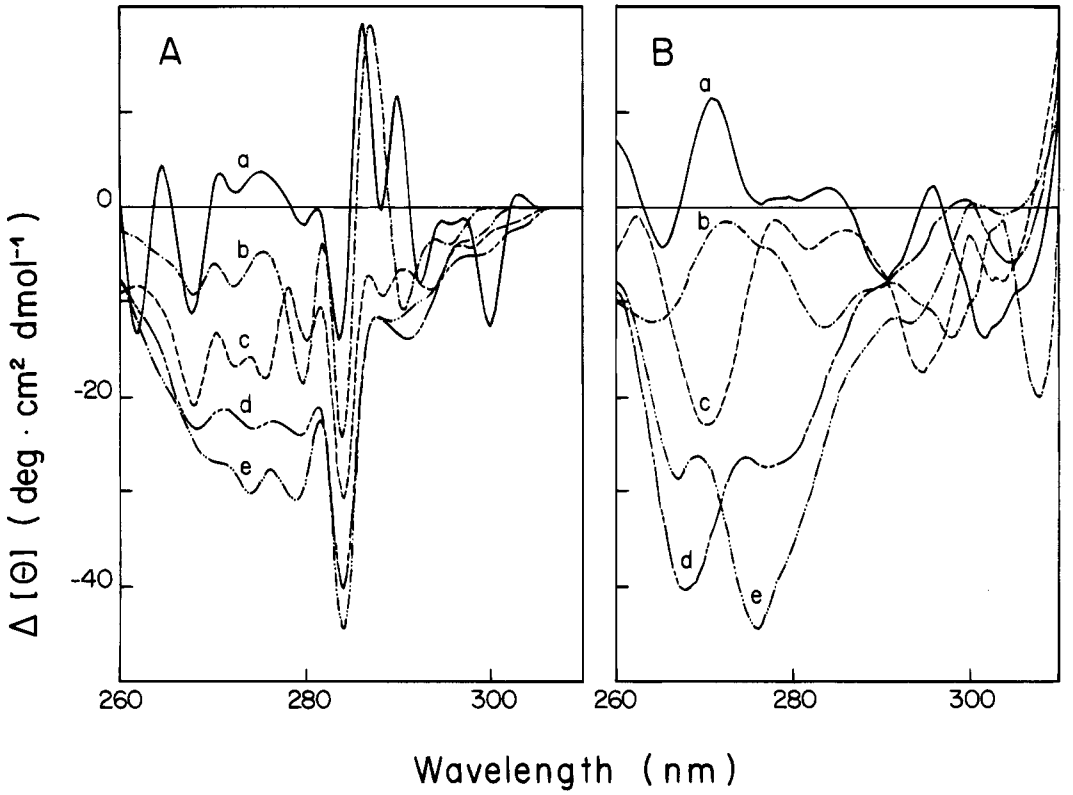

FIGURE 5: CD difference spectra of GlcN6P deaminase produced by the addition of different GlcNAc6P concentrations (bound minus free). (A) Wild-type enzyme (2.5 μM) dissolved in 50 mM Tris-HCl buffer, pH 7.7. Each curve was obtained by subtracting the data from the spectrum obtained in the absence of GlcNAc6P. Activator concentration: a, 25 μM; b, 40 μM; c, 65 μM; d, 90 μM; e, 1 mM. (B) A similar experiment, using 2.5 μM Tyr 121−Trp deaminase, and the following activator concentrations: a, 10 nM; b, 0.1 μM; c, 10 μM; d, 1 mM; e, 10 mM.

Table 2: Protective Effect of GlcNAc6P upon Sulfhydryl Reactivity[a]

	K_{dis}[b] (mM)	$[GlnNAc6P]_{0.5}$[b] (mM)	Hill coefficient[c]
wild-type	0.026 ± 0.006	0.22 ± 0.02	2.95 ± 0.25
Tyr 121−Trp	0.53 ± 0.065	1.20 ± 0.22	3.04 ± 0.31
Tyr 121−Thr	0.85 ± 0.172	1.80 ± 0.26	2.85 ± 0.29

[a] Parameters of curves in Figure 6 fitted to eq 1 and to the Hill equation. [b] Values from the fit of the data to eq 1. [c] Values from the fit of the data to the following form of the Hill equation: $k_{app} = k'_1 - ([A]^h/(K^h - [A]^h))$.

This expression relates the apparent pseudo-first-order rate constant for the reaction of cysteines with DTNB, k_{app}, with the specific concentration of the allosteric activator, γ, which is defined as [GlcNAc6P]/K_{dis}. The other parameters are n, which is the number of active and allosteric sites, and L, the MWC allosteric constant. The experimental curve of k_{app} versus GlcNAc6P concentration fitted to this equation is shown in Figure 6 (panel A for the wild-type and panel B for the Tyr121−Trp mutant form of the enzyme). Essentially the same results were obtained with the Tyr 121−Thr mutant form of the enzyme (data not shown). In both plots it is manifest that the protective effect of GlcNAc6P is cooperative. The fitted values for the dissociation constant of GlcNAc6P at its binding site for the wild-type and both Tyr 121 mutant deaminases are presented in Table 2. The same experimental data were fitted to the Hill equation, and the values for the corresponding Hill coefficients and $[GlcNAc6P]_{0.5}$ are also given in this Table.

The comparison of the chemical reactivities of Cys 118 and Cys 239 thiols toward DTNB at pH 8.0 shows that their reactivity was not affected by the amino acid replacements in position 121, because the second-order rate constants are in all cases 2.5 M^{-1} s^{-1}.

DISCUSSION

Tyrosine 121 Plays a Critical Role in the Structure and Function of GlcN6P Deaminase. The results reported in this paper confirm our previous prediction, based on spectrophotometric data and theoretical considerations, that Tyr 121 is the tyrosine residue located close to or at the GlcNAc6P binding site (Altamirano et al., 1994). Mutations at this position altered the affinity of the enzyme for its allosteric activator. Moreover, these mutations do not just result in an increase of K_{dis} for GlcNAc6P but produce complex modifications involving several aspects of deaminase function.

Kinetics of the Allosteric Activation of GlcNAc6P Deaminase with Replacements of Tyr 121. The kinetic experiments, CD difference spectra, and the direct binding measurements by equilibrium dialysis strongly support the hypothesis that GlcNAc6P binding to the two mutant forms of the enzyme occurs in two successive steps. Although the allosteric transition in Tyr 121 mutant deaminases induced by the homotropic effect of the substrate can be accurately described by the concerted MWC model, the heterotropic effect of GlcNAc6P displays an unusual asymmetric behavior in both mutant enzymes. The present work includes a detailed analysis of the allosteric activation in the wild-type and mutant enzymes. Data on the wild-type enzyme have confirmed the validity of the MWC allosteric model to describe the homotropic effects on the wild-type enzyme as

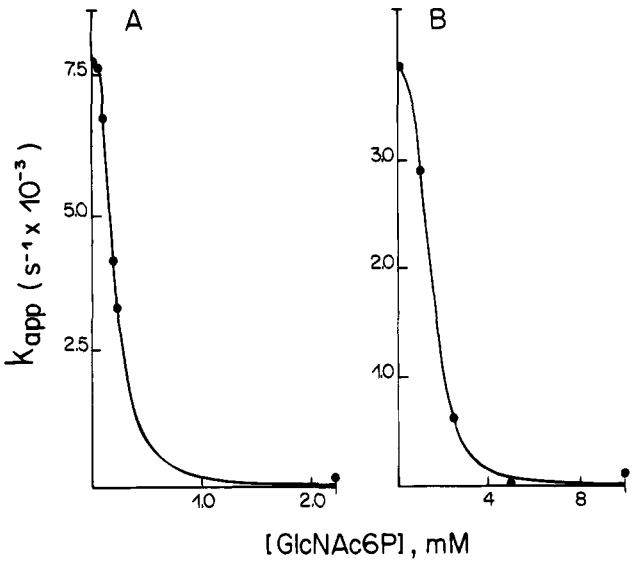

FIGURE 6: GlcNAc6P binding to GlcN6P deaminase, analyzed by means of the protective effect of this ligand on the reaction of Cys 118 and 239 with DTNB. (A, left) Effect of GlcNAc6P on the apparent rate constant of the reaction of GlcN6P deaminase. Samples containing 0.5 μM deaminase in 0.1 M Tris-HCl buffer and 2.5 mM EDTA (pH 7.7), and variable concentrations of GlcNAc6P were treated with 2 mM DTNB. The progress of the reaction was measured with a double-beam spectrophotometer using a blank cell without enzyme. Experimental data were fitted to eq 1, using the following parameters: $k'_1 = 7.8 \times 10^{-4}$ s^{-1} (the observed reaction rate at zero GlcNAc6P concentration) and $n = 6$ (the number of allosteric and active sites). (A) Wild-type enzyme. (B) Tyr 121−Trp GlcN6P deaminase. The fitted values for the variables are shown in Table 2.

initially proposed (Altamirano et al., 1989, 1992). Additional data in this paper show that the extension of the model is also valid for the activation kinetics. Further verification of the applicability of this model to the wild-type enzyme comes from the kinetics in the presence of the dead-end inhibitor GlcN-ol-6P. This is a ligand of the active site which, under hyperbolic kinetics, behaves as a linear competitive inhibitor ($K_i = 0.002$ mM). Previous experiments (Altamirano et al., 1994) and the results of equilibrim dialysis experiments reported here indicate that GlcN-ol-6P is exclusively bound to the R-form. In addition, the kinetics of wild-type inhibition by GlcN-ol-6P were accurately described by the following equation derived from the MWC model (Segel, 1975):

$$v/V = \frac{Lc\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha + \theta)^{n-1}}{L(1 + c\alpha)^n + (1 + \alpha + \theta)^n} \quad (2)$$

In this expression, α and θ are, respectively, the specific concentrations of GlcN6P and GlcN-ol-6P, and c is the nonexclusive binding constant for GlcN6P. Other symbols have the same meaning as in eq 1. It was derived assuming nonexclusive binding of GlcN6P and exclusive-binding of GlcN-ol-6P. A paradoxical activation of wild-type deaminase at low concentrations of the competitive inhibitor (0.2−0.5 times K_i) is implicit in this equation, and, indeed, it has been experimentally observed (M. M. Altamirano and M. L. Calcagno, unpublished results).

The Allosteric Activation of Tyr 121 Mutant Forms of GlcN6P Deaminase Occurs in Two Consecutive Half-of-the-Sites Steps. The kinetics of GlcNAc6P activation of both GlcN6P deaminases with replacements in position 121 can no longer be described by a concerted allosteric model. Both mutant enzymes behave asymmetrically with half of the allosteric sites displaying a nearly normal affinity for GlcNAc6P, while the other half of the sites present an affinity at least one order of magnitude lower. The halfway conformation, with an intermediate homotropic cooperativity, is particularly evident in the Tyr 121−Trp mutant (Figure 3). This biphasic activation pattern was also observed in equilibrium dialysis experiments, where a first binding phase in the micromolar range was observed for both 121 mutant GlcN6P deaminases (Figure 4B). Data of GlcNAc6P binding to the wild-type enzyme, and presented here as a reference (Figure 4A), show six allosteric sites per hexameric molecule and the kind of binding curve that is expected for an allosteric K-system.

Structural Evidence of a Two-Step Heterotropic Effect in Mutant Forms of GlcN6P Deaminase Containing Substitutions at Tyr 121. We have previously shown that the reactivity of two thiols groups, those of Cys 118 and Cys 239, can be used to monitor the allosteric transition of the enzyme because their reactivity is entirely eliminated by the T to R conformational change (Altamirano et al., 1992). We can use, therefore, the values for the apparent reaction rate constant of the modification of these thiols with DTNB, measured at different GlcNAc6P concentrations, to evaluate the GlcNAc6P binding curve and the corresponding K_{dis}, according to eq 1. Both wild-type and Tyr 121 mutant deaminases give simple curves that can be fitted to this equation, but the mutant enzymes have a higher apparent K_{dis}. The comparison of these results with kinetic and equilibrium dialysis data suggests that the major conformational change detected by the loss of sulfhydryl reactivity is associated with the second phase of the activation curve, i.e., the transition from the intermediate conformer to the fully activated enzyme. This, in turn, suggests that the full R conformation in both Tyr 121 mutants is not attained until the three low affinity binding sites for GlcNAc6P are occupied by this ligand. The curve for GlcNAc6P binding in the presence of a saturating concentration of the dead-end inhibitor GlcN-ol-6P is hyperbolic. These data confirm that the enzyme has six allosteric sites per molecule and suggest that the latter ligand binds exclusively or almost exclusively to the R conformer.

The spectropolarimetric titration experiments with the mutant enzymes with GlcNAc6P, in spite of the complexity of the spectra obtained, support the hypothesis of a biphasic binding of the allosteric activator. The set of curves in Figure 5B suggests that the structural events accompanying GlcNAc6P binding and the allosteric transition produced by the heterotropic effect are more intricate in Tyr 121 mutants than expected for a concerted allosteric model.

It is remarkable that the allosteric transition produced by a homotropic ligand, the substrate, is not affected in Tyr 121 mutants of the enzyme. This could imply that suppression of Tyr 121 interferes with local conformational changes associated with GlcNAc6P binding, which triggers the allosteric transition when the allosteric site is occupied.

Difference Free Energy Profiles of Mutant Deaminases with Replacements of Tyr 121. In the experiments performed in the presence of 16 mM GlcNAc6P, both Tyr 121 mutant forms of GlcN6P deaminase attain hyperbolic kinetics. The corresponding K_m(GlcN6P) and k_{cat} values can be used to calculate the difference in the free energy profiles for the

reaction between the wild-type enzyme and the mutants, as proposed by Fersht (1974). This allows us to analyze the consequence of the site-directed mutations on catalysis (Wilkinson et al., 1983). The validity of this approach to calculate differences in $\Delta G^{\ddagger}$ values has been discussed by Fersht et al. (1992). The comparison of Tyr 121−Trp and Tyr 121−Thr mutant deaminases yields interesting differences. The Trp substitution produces mainly a change in K_m for GlcN6P (Table 1). Assuming K_m as a K_{dis}, we can estimate the change in the $\Delta G°$ for the formation of the enzyme−GlcN6P complex in the ground state, $\Delta\Delta G°_{GlcN6P}$, as 2.9 kJ mol^{-1}. The positive value says that the mutant protein binds the substrate more tightly than the wild-type protein. If the mutation does not affect the interaction between the enzyme and its substrate in the transition state, the catalytic term k_{cat}/K_m should be unchanged, which is the case with this mutant form. The free energy associated with this process will be represented as $\Delta G^{\ddagger}_T$. As the change in k_{cat} produced by the mutation is not independent of the values of $\Delta\Delta G°_{GlcN6P}$ and $\Delta\Delta G^{\ddagger}_T$ (Fersht, 1974), but given by the difference $(\Delta G°_{GlcN6P} + \Delta G^{\ddagger}_T)_{wild\text{-}type} - (\Delta G°_{GlcN6P} + \Delta G^{\ddagger}_T)_{mutant}$, the k_{cat} value must necessarily change. The free energy profile of Tyr 121−Thr GlcN6P deaminase is qualitatively different. From data in Table 1, we know that the $\Delta\Delta G°_{GlcN6P}$ did not appreciably change, but k_{cat}/K_m decreases. The change expressed as $\Delta\Delta G^{\ddagger}_T$ is −4.6 kJ mol^{-1}. The negative sign in this case means that the mutation decreases the stability of the enzyme−substrate complex in the transition state. These results provide evidence that the Thr replacement produces structural perturbations which are absent in the Tyr 121−Trp enzyme and seriously affect the interaction between the enzyme and the substrate in its transition state.

The Structural Role of Tyr 121. The changes produced by the mutations studied here affect not only GlcNAc6P binding and the subsequent induction of the allosteric transition but also interactions occurring at the active site and the transmission of local conformational changes that produce the concerted allosteric transition. This strongly suggests that Tyr 121 must play a key structural role in the architecture of the deaminase monomer, and its replacement by Thr or Trp produces a significant conformational alteration. It would appear that some structural roles of Tyr 121 are still played by the Trp residue in the Tyr 121−Trp mutant, as shown in the discussion of the change in the free energy profiles caused by the mutations. Because Tyr 121−Thr mutant, but not Tyr 121−Trp, is affected in its ability to attain the optimal conformation in the ES‡ complex, we can presume that an aromatic residue in this position is structurally important for the stability of the protein in its transition state. Data in Table 1 show also that GlcNAc6P significantly contributes to the stabilization of the active structure of Tyr 121−Thr deaminase. It is interesting to remark that this structural role of Tyr 121 can be mimicked by the Trp 121, but the latter replacement produces the same biphasic GlcNAc6P binding as threonine replacement.

The CD far-UV spectra of Tyr 121−Trp and Tyr 121−Thr mutant enzymes are essentially identical and similar to the wild-type spectrum, thus indicating that the structural changes in Tyr 121−Thr are not major and do not produce an improper folding of a part of the protein. Large nonlocal folding changes has been invoked in the case of some single-site mutations of aspartate transcarbamoylase (ATCase) from *E. coli*. Site-directed mutagenesis studies of this enzyme have shown that single amino acid replacements can produce recognizable alterations in the enzyme structure that can be propagated to positions distant from the site of the mutation (Wente & Schachman, 1991; Peterson & Schachman, 1992). ATCase is an allosteric *K*-enzyme, and its allosteric transition has been shown to be concerted (Perutz, 1990; Lipscomb, 1994), but amino acid replacements for Gln 231 produced a mixed *K/V* behavior. The ATCases with substitutions of Gln 231 also present an apparent decoupling of the homotropic and heterotropic effects, so their kinetics are no longer consistent with the two-state model used to describe the allosteric kinetics of the wild-type enzyme (Peterson et al., 1992). Similarly, the site-directed mutants of GlcN6P deaminase at position 121 produced a diversity of functional changes which indicate that the tyrosine residue at this position plays a key structural role.

Concluding Remarks. The comparison of kinetic and structural data of the well characterized allosteric enzymes shows that evolution has selected structures undergoing symmetric allosteric conformational changes, in which the control of the enzyme activity is exerted through the modification of its affinity for the substrate, without changing the catalytic parameters (Perutz, 1990). These *K*-systems provide a better control of the enzyme activity by its allosteric effectors in the presence of nonsaturating substrate concentrations than *V*-systems. In a pure *V*-system, at a substrate concentration close to the corresponding $[S]_{0.5}$, the curve of initial velocity versus activator concentration is a hyperbola. A more sensitive response of the reaction rate to changes in the activator concentration occurs if the curve is sigmoidal, as is the case in the *K*-systems. This is one of the reasons why these allosteric *K*-systems may have been preferred over *V*-systems by natural selection. It is interesting to observe how easily mixed *K/V* allosteric systems are obtained by constructing mutants with single amino acid replacements, thus strongly suggesting that an important selective pressure exists to eliminate them.

The present results stress how site-directed mutations can alter, in many cases indirectly, the critical balance of interactions responsible for the complex kinetics of an allosteric enzyme. They also emphasize how the predictive techniques for protein structure, combined with chemical and physicochemical techniques, give results that are perfectly complementary and valuable in identifying targets for mutagenesis.

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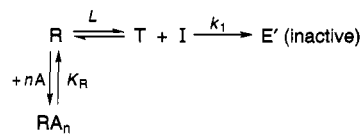
APPENDIX

Kinetics of the Modification Reaction of an Allosteric Protein in the Presence of an Exclusive-Binding Protective Ligand. Scheme 1 shows the reaction sequence for the chemical modification of a two-state allosteric protein in which only the T conformer reacts with the modifying reagent, I, in the presence of the ligand A. This ligand

Scheme 1



protects completely the protein from reaction and binds exclusively to the R form. The chemical modification is irreversible, and the modified enzyme is inactive. This scheme takes into account our observations of the reaction of the sulfhydryl groups of the native GlcN6P deaminase with DTNB (Altamirano et al., 1989, 1992).

The fraction of the enzyme in the R-state ($\bar{R}$) is given by the following expression (Monod et al., 1965):

$$\bar{R} = \frac{(1+\gamma)^n}{L + (1+\gamma)^n}$$

where γ is the specific concentration of the exclusive-binding ligand A, n is the number of binding sites and L is the allosteric constant. The fraction of the enzyme in the T form, $\bar{T}$, is

$$\bar{T} = 1 - \bar{R}$$

or

$$\bar{T} = \frac{L}{L + (1+\gamma)^n} \quad (1)$$

The pseudo-first-order rate equation for the protein modification is

$$v = k''_1 E_t[\bar{T}] \quad (2)$$

where $k''_1 = k_1[\mathrm{I}]$ and E_t is the total concentration of the unmodified enzyme; $E_t[\bar{T}]$ is then the fraction of the enzyme susceptible to chemical modification. From eqs 1 and 2, we have

$$v = E_t \frac{k'_1 L}{L + (1+\gamma)^n}$$

The apparent rate constant for the modification reaction in the presence of the allosteric ligand, is

$$k_{app} = \frac{k'_1 L}{L + (1+\gamma)^n} \quad (3)$$

The plot of k_{app} versus γ gives a downward S-shaped curve, intercepting the vertical axis in $k_{obs} = k'_1$ (see Results, Figure 6). The following expressions are linear transformations of equation 3:

$$\log \frac{k'_1 - k_{app}}{k'_1 k_{app}} = n \log (1+\gamma) + \log L$$

$$\log \frac{k'_1 - k_{app}}{k_{app}} = n \log (1+\gamma) - \log L$$

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