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# Tyr254 Hydroxyl Group Acts as a Two-Way Switch Mechanism in the Coupling of Heterotropic and Homotropic Effects in *Escherichia coli* Glucosamine-6-phosphate Deaminase<sup>†</sup>

Gabriela M. Montero-Morán,<sup>‡</sup> Eduardo Horjales,<sup>§</sup> Mario L. Calcagno,<sup>\*,‡</sup> and Myriam M. Altamirano<sup>\*,‡,||</sup>

Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México (UNAM), P.O. Box 70-159, Ciudad Universitaria, Mexico City, DF, 04510, Mexico, and Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), P.O. Box 510-3, Cuernavaca, MOR, 62271, Mexico

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**ABSTRACT:** The involvement of tyrosine residues in the allosteric function of the enzyme glucosamine 6-phosphate deaminase from *Escherichia coli* was first proposed on the basis of a theoretical analysis of the sequence and demonstrated by spectrophotometric experiments. Two tyrosine residues, Tyr121 and Tyr254, were indicated as involved in the mechanism of cooperativity and in the allosteric regulation of the enzyme [Altamirano et al. (1994) *Eur. J. Biochem.* 220, 409–413]. Tyr121 replacement by threonine or tryptophan altered the symmetric character of the T → R transition [Altamirano et al. (1995) *Biochemistry* 34, 6074–6082]. From crystallographic data of the R allosteric conformer, Tyr254 has been shown to be part of the allosteric pocket [Oliva et al. (1995) *Structure* 3, 1323–1332]. Although it is not directly involved in binding the allosteric activator, *N*-acetylglucosamine 6-phosphate, Tyr 254 is hydrogen bonded through its phenolic hydroxyl to the backbone carbonyl from residue 161 in the neighboring polypeptide chain. Kinetic and binding experiments with the mutant form Tyr254-Phe of the enzyme reveal that this replacement caused an uncoupling of the homotropic and heterotropic effects. Homotropic cooperativity diminished and the allosteric activation pattern changed from one of the K-type in the wild-type deaminase to a mixed K-V pattern. On the other hand, Tyr254-Trp deaminase is kinetically closer to a K-type enzyme and it has a higher catalytic efficiency than the wild-type protein. These results show that the interactions of Tyr254 are fundamental in coupling binding in the active site to events occurring in the allosteric pocket of *E. coli* glucosamine 6-P deaminase.

Glucosamine 6-phosphate deaminase (GlcN6P deaminase, EC 5.3.1.10)<sup>1</sup> catalyzes the deamination and isomerization of glucosamine 6-phosphate (GlcN6P) producing fructose 6-phosphate (Fru6P) and ammonia. This step is the only allosterically regulated reaction in the pathway of *N*-acetylglucosamine utilization in *Escherichia coli*, and its reaction rate is modulated in response to the *N*-acetylglucosamine 6-phosphate (GlcNAc6P). The concentration of this metabolite, which is the inducer of the *nag* regulon as well as the allosteric activator of the deaminase, increases when the bacteria are cultured in the presence of amino sugars (1–3). The expression of the genes involved in amino

sugar synthesis also depends on GlcNAc6P concentration, so that amino sugar biosynthesis and degradation are regulated in a coordinate fashion (4, 5).

Cooperativity and GlcNAc6P activation of wild-type GlcN6P deaminase can be well explained in the framework of the concerted allosteric model [MWC model, Monod et al., (6)] as shown by Altamirano et al. (7). The allosteric transition from the T to the R conformation, activates the enzyme through an increase of its apparent affinity for GlcN6P or Fru6P which are, respectively, their substrates for the forward and the backward reactions. No change in  $k_{cat}$  occurs in allosteric activation. Accordingly, GlcN6P deaminase can be described as a typical allosteric enzyme of the K-type (7).

We have previously reported spectrophotometric evidence for the participation of a tyrosine residue in the allosteric function of the wild-type GlcN6P deaminase. On the basis of a structure and prediction study, Tyr121 and Tyr254 were the best candidates for this function (8). When Tyr121 was replaced by threonine, tryptophan (7), or serine (Altamirano et al., unpublished material), the three mutant enzymes displayed an altered affinity for the allosteric activator and a biphasic activation behavior. Furthermore, spectral changes that allow monitoring the allosteric transition have been shown by site-directed mutagenesis experiments to be dependent on the presence of Tyr121 hydroxyl group. The

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\* Address correspondence to these authors: Fax: +(525) 6162419. E-mail: myriam@servidor.unam.mx and calcagno@servidor.unam.mx.

<sup>‡</sup> Facultad de Medicina, Universidad Nacional Autónoma de México.

<sup>§</sup> Instituto de Biotecnología, Universidad Nacional Autónoma de México.

<sup>||</sup> Present address: Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge, CB2 2QH, U.K. E-mail: mma@mrc-lmb.cam.ac.uk. Fax: + 44 1223 402140.

<sup>1</sup> Abbreviations: GlcN6P, glucosamine 6-phosphate; GlcNAc6P, *N*-acetylglucosamine 6-phosphate.

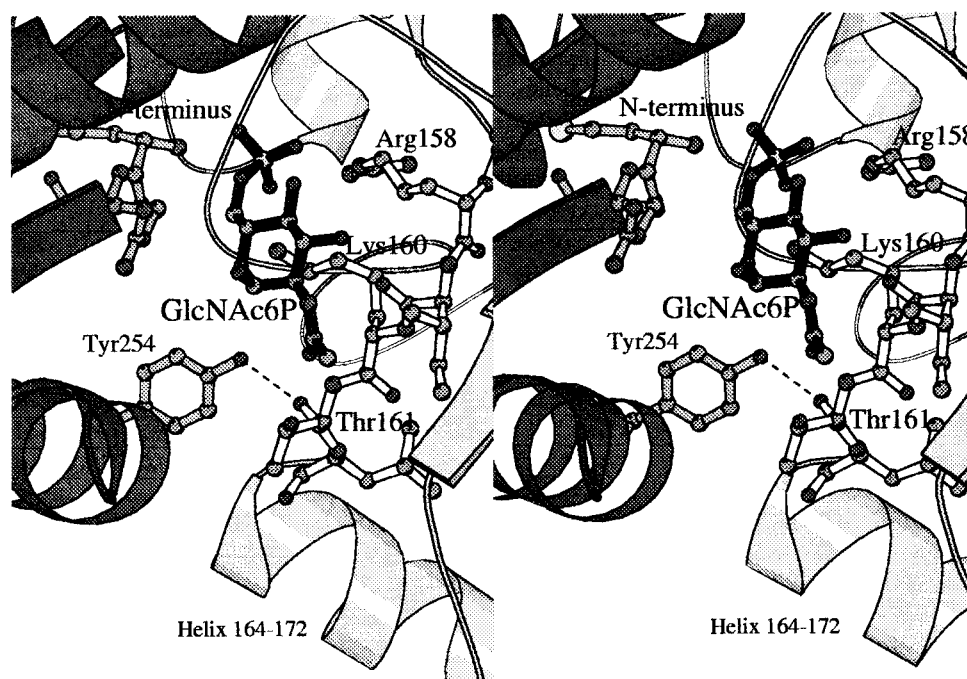


FIGURE 1: Stereo drawing showing the allosteric site of *E. coli* GlcN6P deaminase. A molecule of GlcNAc6P, the allosteric activator of the enzyme, appears bound to the site that is located between two monomers. Each polypeptide chain is represented with different gray shading. The interactions between the bound GlcNAc6P molecule and residues in the protein are described in the text. The dashed line indicates the hydrogen bond between the hydroxyl group of Tyr254 and the main chain carbonyl group at position 161. This figure was generated using the graphics program MolScript (Kraulis, 1991) compiled for Linux.

structural model of the R-conformer of the wild-type enzyme has since become available from crystallographic data at 2.1 Å resolution. The structure of several complexes of *E. coli* deaminase with the active and the allosteric sites occupied by ligands were solved and both sites were unequivocally identified (9). The enzyme is a hexamer of identical subunits arranged as a dimer of trimers and the allosteric sites appear located in the clefts between the subunits forming the trimers. Both neighboring subunits contribute to the structure of the GlcNAc6P binding site (9). The residue Tyr254 is part of a conspicuous intersubunit linkage in the allosteric cleft. Its phenolic hydroxyl group is hydrogen bonded to the backbone carbonyl in position 161 (Thr) of the neighboring subunit. Another important intersubunit contact is established through the phospho group of the bound ligand, which is linked to the N-terminal amino group of one of the subunits and to the side chains of residues Ser151, Arg158, and Lys160 of the facing polypeptide chain. Tyr254, whose hydroxyl group contributes to interchain bonding, is located in the same subunit (i.e., on the same side of the allosteric pocket) as the terminal amino group. The relevant structural details of the allosteric site of *E. coli* GlcN6P deaminase are shown in Figure 1.

Previously reported kinetic results revealed that the activator exclusively binds to the R conformation, so the T conformer does not contain a functional allosteric site. On the other hand, the substrate GlcN6P binds to both T and R conformers, with higher affinity for the latter (7). Preliminary X-ray diffraction data for the ligand-free enzyme crystallized in the T form, confirmed the disruption of the allosteric site in this conformation. The R → T transition modifies the quaternary structure of the deaminase, producing a rotation of each monomer by 11° around an axis parallel to the 3-fold axis of symmetry of the molecule (9). The analysis of the coordinates of the T-conformer shows that

the intersubunit hydrogen bond between Tyr254 hydroxyl and the facing polypeptide backbone at position 161 is absent in this conformation. In contrast, the T structure presents this tyrosine hydroxyl group hydrogen bonded to the backbone carbonyl oxygen of Pro149. (Horjales et al., unpublished results). These observations suggest that Tyr254 might play an important role in the allosteric function of the enzyme. In this paper, we analyzed the role of the side chain of Tyr254 by means of the construction and study of two site-directed mutants, Tyr254-Phe and Tyr254-Trp. The results presented here illustrate the significant role of Tyr254 in intersubunit interactions coupling homotropic and heterotropic conformational changes in *E. coli* GlcN6P deaminase.

## MATERIALS AND METHODS

**Reagents.** Most chemicals and biochemicals were from Sigma-Aldrich S. A. de C.V, Mexico. The affinity gel used for GlcN6P deaminase purification (*N*-aminoalkyl glucosamine-6-P agarose) was prepared as described (10), except that ECH-Sepharose (Pharmacia) was used instead of *N*-6-aminohexanoyl agarose. The allosteric activator, GlcNAc6P, and its <sup>14</sup>C-labeled form (60 kBq nmol<sup>-1</sup>) were synthesized and purified as described (7).

**Bacterial Strains and Mutagenesis.** Site-directed mutations were obtained by oligonucleotide-directed mutagenesis, using the Kunkel method as described by Sambrook et al. (11) with the *nagB* gene inserted in the single-strand vector pTZ18-R. The mutations created were Tyr(TAT) to Trp(TGG) and Tyr to Phe(TTT). Phagemids carrying the mutation were verified by sequencing, and the entire gene was also sequenced. The plasmids carrying the mutations were used to transform the *Δnag* strain IBPC590 (4). This strain is *ΔlacI* and expresses the deaminase constitutively. Details on the strain and the procedure were already reported (7).

Table 1: Kinetic and Equilibrium Properties of Mutant Forms Tyr254-Phe and Tyr254-Trp of *E. coli* GlcN6P Deaminase, Measured in the Absence of the Allosteric Activator at pH 7.7 and 30 °C

	$h_{\max}^{a,b}$	[GlcN6P] $_{0.5}^a$ (mM)	$k_{\text{cat}}, \text{s}^{-1}^a$ (s <sup>-1</sup> )	$L^c$	$K_R^c$ (mM)	$K_T^d$ (mM)	$c^c$	$\Delta\Delta G_i^{\circ e}$ (kJ mol <sup>-1</sup> )
wild-type	3.02 ± 0.9	4.0 ± 0.2	292 ± 17	10 <sup>4</sup>	2.00	100	0.020 ± 0.001	-9.8 ± 0.0
Tyr254-Phe	1.34 ± 0.11	14.8 ± 3.0	11.4 ± 1.5	2 × 10 <sup>3</sup>	4.50	32	0.14 ± 0.04	-4.9 ± 0.7
Tyr254-Trp	1.86 ± 0.19	2.33 ± 0.3	218 ± 13	4.3 × 10 <sup>3</sup>	0.44	6.1	0.072 ± 0.030	-6.7 ± 1.1

<sup>a</sup> From the fit of experimental data to Hill equation. <sup>b</sup>  $h_{\max}$  is defined as the extreme value of  $d \log [v_0/(V_{\max} - v_0)]/d \log [\text{GlcN6P}]$ , when  $[\text{GlcN6P}] = [\text{GlcN6P}]_{0.5}$ . <sup>c</sup> From the fit of experimental data to eq 1. <sup>d</sup> Calculated as  $K_R/c$ , from the fitted values.  $K_R$  is equal to  $K_m$  of the R conformer, and this value is independently obtained from rate measurements of the enzyme saturated with the allosteric activator. <sup>e</sup> Free energies of interaction between binding sites, calculated as  $\Delta\Delta G_i^{\circ} = -RT \ln 1/c$ . A negative value indicates positive cooperativity.

**Enzyme Purification.** *E. coli* wild-type GlcN6P deaminase and the two site-specific mutants, Tyr254-Phe and Tyr254-Trp, were purified by allosteric-site affinity chromatography. The procedure was essentially as previously reported (12), but GlcN6P bound to ECH-Sepharose was used. This activated gel, which has a longer spacer arm than that used in our former preparation, is more efficient and stable. Leakage was practically reduced to zero, and the binding capacity for wild-type deaminase in 100 mM Tris-HCl buffer, pH 7.5, was increased to 30 mg (150 nmol) of protein per mL of wet gel, that is 3–4 times the capacity of the previously reported affinity gel.

The purity of the enzyme preparations was verified by SDS-PAGE and FPLC in a Superdex-200 column (Pharmacia) equilibrated with 75 mM Tris-HCl buffer containing 100 mM potassium acetate (pH 7.5). Both mutant enzymes were hexameric and stable. GlcN6P deaminase was assayed in the direction of Fru6P formation, by the colorimetric measurement of fructose at fixed times, as previously reported (12). The progress of the reaction was always kept below 5% conversion of the initial substrate. Kinetic data were analyzed by nonlinear regression analysis using the program Origin (MicroCal Software, Inc., Northampton, MA). Wild-type enzyme, prepared as already described (12) was used to obtain reference data.

**Molar Absorptivity.** The amino acid substitutions involved aromatic residues, so that the molar absorption coefficients for the mutant deaminases were different from that of the wild-type protein. The new coefficients were initially estimated according to Edelhoch (13) and von Hippel and Gill (14) and refined using sulfhydryl titration as described by Altamirano et al. (7). The molar absorptivities obtained at 278 nm were  $2.1 \times 10^5$  and  $2.4 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  for Tyr254-Phe and Tyr254-Trp mutant forms, respectively.

**Measurement of the Dissociation Constant of the Allosteric Activator.** The dissociation constant of GlcNAc6P was calculated from binding measurements of <sup>14</sup>C-labeled GlcNAc6P using ultracentrifugation. The procedure was essentially the one described by Howlett et al. (15). Samples (200  $\mu\text{L}$ ) containing the enzyme and the ligand were centrifuged for 4 h at 30 °C and 195000g in a Beckman Optima XL-100K ultracentrifuge using the 42.2 Ti fixed-angle rotor ( $r_m = 10.85 \text{ cm}$ ). The rotor was decelerated without braking, taking 3.5 h to stop. Samples contained a final concentration of  $2.5 \text{ mg mL}^{-1}$  of dextran (Sigma D4571, average  $M_r = 81600$ ) to reduce the convective mixing of the sedimented protein as originally proposed by Howlett et al. (15). A 70  $\mu\text{L}$  aliquot was taken for scintillation counting. In our hands, this method gives more accurate results than equilibrium dialysis or flow dialysis.

Table 2: Kinetic Parameters of the Fully Heterotropically Activated Tyr254-Phe and Tyr254-Trp GlcN6P Deaminases from *E. coli*, at pH 7.7, 30 °C<sup>a</sup>

	$K_m$ (mM)	$k_{\text{cat}}$ (s <sup>-1</sup> )	$k_{\text{cat}}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> × 10 <sup>5</sup> )
wild-type	2.1 ± 0.1	302 ± 20	1.44 ± 0.16
Tyr254-Phe	1.0 ± 0.1	75 ± 3	0.75 ± 0.04
Tyr254-Trp	0.9 ± 0.1	248 ± 18	2.75 ± 0.51

<sup>a</sup> For reference, the values for the wild-type enzyme were included.

## RESULTS

**Kinetic Properties of Deaminases with Replacements of Tyr254.** The enzyme with Tyr254-Phe replacement showed marked changes in its activity and allosteric properties. Tyr254-Phe deaminase was less active than the wild-type protein; its  $k_{\text{cat}}$  is nearly 25 times lower than the value of the wild-type enzyme, when measured in the absence of GlcNAc6P (Table 1). The  $k_{\text{cat}}$  of the fully heterotropically activated enzyme was remarkably higher than the catalytic constant of the homotropically activated deaminase, i.e., its  $k_{\text{cat}}$  at infinite substrate concentration and without allosteric activator. The  $k_{\text{cat}}$  for Tyr254-Phe deaminase at saturating GlcNAc6P concentration was still four times lower than the corresponding value for the wild-type protein (Table 2). In addition, this mutant enzyme was less cooperative than the wild-type protein and its activation by GlcNAc6P produced a decrease in the  $[\text{GlcN6P}]_{0.5}$  and an increase in the apparent  $k_{\text{cat}}$  values (Figure 2 and Table 1). Therefore, this mutant deaminase is a mixed K and V allosteric enzyme, in contrast to the wild-type deaminase, which behaves as a pure K system. The experimental data obtained in the absence of GlcNAc6P were fitted to the following MWC equation for the case of exclusive binding of GlcNAc6P and nonexclusive binding of GlcN6P (6).

$$v_o = \frac{k_{\text{cat}}n[E_t][Lc\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}]}{L(1 + \gamma)^n(1 + c\alpha)^n + \alpha(1 + \alpha)^n} \quad (1)$$

where  $\alpha = [\text{GlcN6P}]/K_R$ , taking  $K_R = K_m$  (7),  $\gamma = [\text{GlcNAc6P}]/K_d$ ,  $L$  is Monod's (6) allosteric constant, and  $c$  is the ratio  $K_R/K_T$ . The results show an increase in the  $c$  coefficient and a decrease in the allosteric constant  $L$  for the mutant protein when compared to the wild-type enzyme (Table 1). The data in Figure 2A allow us to calculate the apparent affinity of the fully homotropically activated Tyr254-Phe deaminase for GlcNAc6P. The apparent  $V_{\max}$  ( $\Delta V_{\max}^{\text{app}}$ ) obtained from these data, increases hyperbolically as a function of GlcNAc6P concentration, with a fitted  $K_d$

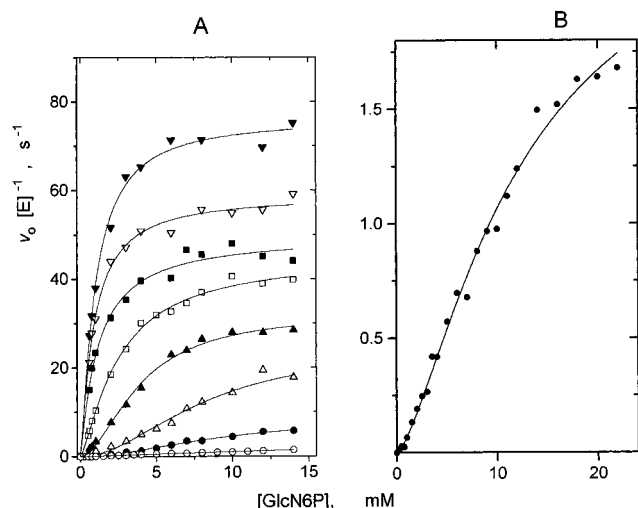


FIGURE 2: (A) Activation of Tyr254-Phe GlcN6P deaminase by its allosteric effector, GlcNAc6P. Initial velocities were plotted versus substrate concentrations at the following GlcNAc6P concentrations: (○) none; (●) 0.2 mM; (△) 0.4 mM; (▲) 1 mM; (■) 2 mM; (▽) 5 mM; (▼) 10 mM. Assays were performed using 11 nM GlcN6P deaminase in a final volume of 200  $\mu$ L, in 40 mM Tris-HCl buffer (pH 7.7) at 30  $^{\circ}$ C, as described elsewhere (12). Data were fitted to the Hill equation except at 10 mM GlcNAc6P, for which the Michaelis equation was used. The fitted kinetics parameters are shown in Tables 1 and 2. (B) The velocity-substrate curve was obtained in the absence of allosteric activator. Data were fitted to eq 1; the fitted parameters are shown in Table 1.

value of 1.4 mM (not shown). In contrast to the Tyr254-Phe deaminase, the Tyr254-Trp mutant enzyme displayed  $k_{\text{cat}}$  values similar to the wild-type enzyme (Tables 1 and 2). The mutation Tyr254-Trp also produced a decrease in the homotropic cooperativity, but the change was not so marked as in Tyr254-Phe deaminase. The loss of cooperativity in the tryptophan mutant was also due to changes in  $c$  and  $L$ , as in the case of Tyr254-Phe mutant enzyme (Table 1). The activation by GlcNAc6P of the Tyr254-Trp deaminase was essentially of the K-type, as for the wild-type enzyme. Both mutant enzymes, when fully heterotropically activated, exhibit  $K_m$  values which are close to that of the wild-type enzyme.

The Tyr254-Trp mutant enzyme displayed substrate inhibition at high GlcN6P concentrations; neither GlcNAc6P nor the products of the reaction (ammonia and Fru6P) modified the inhibition pattern (not shown).

**Binding of GlcNAc6P by Tyr254-Phe and Tyr254-Trp Deaminases.** The binding of the allosteric activator was measured by ultracentrifugation, using [ $^{14}$ C]GlcNAc6P. The curves for both mutant deaminases displayed positive cooperativity. The corresponding dissociation constants (Figure 3) indicated that the replacement of Tyr254 by phenylalanine or tryptophan reduced the affinity for GlcNAc6P at the allosteric site, by 2 orders of magnitude.

## DISCUSSION

*The Replacements in Position 254 Do Not Affect the Quaternary Structure of the Enzyme.* Deaminase is a dimer of trimers, and the interactions between the subunits in each trimer depend on hydrogen bonds and van der Waals contacts between extensive segments of the polypeptide chains. The available structural data show that the phenolic hydroxyl from

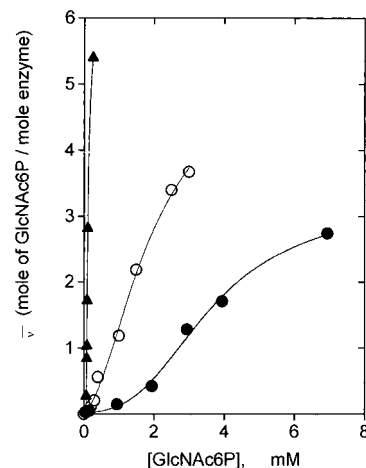


FIGURE 3: GlcNAc6P binding to the mutant deaminases with replacements in position 254. Data were obtained using the ultracentrifugation technique described in the text. Each set of data was fitted to the Monod et al. (6) equation for exclusive binding of the ligand:  $v = \gamma(1 + \gamma)^{n-1}/L + \gamma(1 + \gamma)^n$  (where  $\gamma$  is the specific concentration of the activator,  $[\text{GlcNAc6P}]/K_d$  and  $L$  in this case is the corresponding exclusive-binding allosteric constant). (●) Data for Tyr254-Phe mutant deaminase ( $K_d$ ,  $3.6 \pm 0.4$  mM;  $L$ ,  $7 \pm 4$ ). (○) Binding curve corresponding to the Tyr254-Trp mutant enzyme ( $K_d$ ,  $2.98 \pm 0.96$  mM;  $L$ ,  $48 \pm 40$ ). (▲) Data for the wild-type enzyme, included for reference ( $K_d$ ,  $0.045 \pm 0.002$  mM;  $L$ ,  $54 \pm 10$ ).

Tyr254 contributes to intersubunit contacts in the allosteric site cleft, both in T and R allosteric conformations. However, the binding energy contributed by Tyr254 to the hexamer should be negligible. Accordingly, the replacement of Tyr254 by phenylalanine or tryptophan did not modify the hexameric character of either mutant enzyme.

The mutations at position Tyr254 affect the active-site properties. Both GlcN6P deaminases with replacements in position 254 present modified catalytic properties with respect to the wild-type protein. These can be related to changes of Tyr254 hydrogen bonding with the neighboring polypeptide chain in the allosteric site. The structure of the R conformer shows the phenolic hydroxyl of this residue hydrogen bonded to the backbone carbonyl at position 161 (9) (PDB entries 1DEA, 1HOR, and 1HOT). This is one of the few constraints to the flexibility of the motif loop-helix-loop 161–185, which contains the helix 5 (164–174) and forms the lid of the active site. This motif presents a high flexibility in all the referred structures, as shown by the corresponding temperature coefficients reported with the model coordinates. The active-site lid connects the active and the allosteric sites; its N-terminal extremity is rigidly connected to a short  $\beta$ -strand (158–161) with two positively charged residues. These are Arg158 and Lys160, which bind the phosphate moiety of the activator at the allosteric site (Figure 1). Close to the C-terminal end of helix 5 is Arg172, whose side chain is part of the active site and binds the substrate phospho group. Therefore, we can envisage the change of catalytic and allosteric properties of the enzyme brought about by Tyr254-Phe mutation, as a consequence of a flexibility increase of the active-site lid. Nevertheless, the possible role of a conformational change of the lid can also be considered. The  $k_{\text{cat}}$  value measured in the absence of the allosteric activator is 25-fold lower in this mutant. On the other hand, full heterotropic activation increases its  $k_{\text{cat}}$  (the so-called V-effect). This is consistent with the

hypothesis that, in the homotropically activated R conformer, which lacks the hydrogen bond 254-OH-O161, the active-site lid is less rigidly anchored and has a higher degree of disorder. When GlcN6P deaminase is activated by GlcNAc6P binding (heterotropic activation), the phospho group of the activator becomes linked to the charged residues Arg158 and Lys160, thus partially reducing the flexibility of the active-site lid. Thr161 also contributes to this stabilization of the lid in heterotropic activation, because its O $\gamma$  is hydrogen bonded to the carbonyl oxygen of GlcNAc6P acetamido group in the enzyme-activator complex. These observations are consistent with the increase of  $k_{\text{cat}}$  values produced by the allosteric activator in Tyr254-Phe deaminase. An entropic effect produced by the putative increased freedom of the 161–185 segment in this mutant form of the enzyme can also explain the observed increase of  $[\text{GlcN6P}]_{0.5}$  values (Table 1). In contrast, the Tyr254-Trp mutation results in a pure K-system with a similar catalytic activity to that of the wild-type protein. This result suggests that Tyr254-Trp deaminase can build a similar intersubunit contact as the wild-type enzyme. To verify this, the Tyr254-Trp replacement was modeled from the crystallographic coordinates of the R conformer using the program O (16). When a tryptophan side chain was placed in position 254 using the second most common tryptophan rotamer, the Ne1 atom from the tryptophan residue presents hydrogen bond geometry (distance of 3.5 Å) with the CO oxygen at 161 in the neighboring monomer.

**The Role of the Tyr254 in Homotropic Cooperativity of GlcN6P Deaminase.** Tyr254-Phe deaminase has a reduced homotropic effect with respect to the wild-type enzyme, which is consistent with the higher  $K_R/K_T$  ratio ( $c$  in eq 1) and the lower value for the Monod et al. (6) allosteric constant,  $L$  (Table 1). It has been shown that  $c$  and  $L$  are not independent parameters (17, 18), and there are many examples of relaxation of the T conformer by site-directed mutagenesis which cause a decrease of both  $K_T$  and  $L$  and it has not been possible to alter these parameters independently (19, 20). From  $c$  values and using the expression  $\Delta\Delta G_i^\circ = -RT \ln 1/c$  to calculate the free energies of intersubunit interaction (21), we obtained the values shown in Table 1. The free energy of interaction gives a quantitative measure of cooperativity, as has been emphasized by Forsén and Linse (22). The changes in  $c$  or  $\Delta\Delta G_i^\circ$  are more marked in the enzyme with the phenylalanine replacement, suggesting that the constraints produced by the intersubunit interactions involving Tyr254 in the wild-type T conformer are important to keep low the substrate affinity in this allosteric conformation. It should be noted that Tyr254 mutants did not lose all of the homotropic effect and that the interactions discussed here cannot be considered as the sole structural determinant of the cooperative kinetics of GlcN6P deaminase. It is probable that other intersubunit links are present and contribute to the stability of the T form and to the differential affinity for GlcN6P in the T and R conformers.

**Catalytic Constants of Deaminases with Replacements of Tyr254.** It is interesting to compare the catalytic efficiencies,  $k_{\text{cat}}/K_m$  of the wild-type enzyme, and the mutants Tyr254-Phe and Tyr254-Trp (Table 2). In the former enzyme, the mutation causes a marked decrease of the  $k_{\text{cat}}/K_m$  ratio if compared with the wild-type protein. In contrast, the enzyme with the tryptophan replacement has an improved catalytic

efficiency, although this change was not selected by evolution. This suggests that the selective pressure for an efficient allosteric control (high  $K_T$ ) is more important than selection for a higher  $k_{\text{cat}}/K_m$  ratio.

**Concluding Remarks.** The study of the site-directed mutants Tyr254-Phe and Tyr254-Trp of GlcN6P deaminase demonstrate the key role of the intersubunit contacts between neighboring polypeptide chains involving Tyr254 in the mechanism of the allosteric transition of *E. coli* GlcN6P deaminase. The system formed by Tyr254 hydroxyl and the backbone carbonyl groups at positions 161 or 149 functions such as a two-way switch, shifting from one position to another in the T  $\rightarrow$  R transition. When the switch is in T position, the hydroxyl group from Tyr254 is bound to backbone CO at position 149. This intersubunit interaction may be responsible for one of the constraints that determine the properties of the T conformer in the wild-type deaminase. The rearrangement of the quaternary structure as a consequence of the allosteric activation in the wild-type enzyme moves the switch to the second position, releasing this constraint, while Tyr254 hydroxyl establishes a new interaction with the carbonyl backbone at position 161. This interaction involves the N-terminus of helix 5, which is part of the motif forming the lid of the active site and connecting it to the allosteric site. Then, the change of the switch to the R position contributes to the fixation of the lid to the core of the enzyme. The replacement of the crucial Tyr254 residue by phenylalanine increases the mobility of the lid, thus generating the impaired catalytic function of Tyr254-Phe deaminase.

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## REFERENCES

- White, R. J. (1968) *Biochem. J.* 106, 847–858.
- Plumbridge, J. A. (1990) *J. Bacteriol.* 172, 2728–2735.
- Plumbridge, J. A. (1991) *Mol. Microbiol.* 5, 2053–2062.
- Plumbridge, J. A., Cochet, O., Souza, J. M., Altamirano, M. M., Calcagno, M. L., and Badet, B. (1993) *J. Bacteriol.* 175, 4951–4956.
- Plumbridge, J. A. (1995) *EMBO J.* 14, 3958–3965.
- Monod, J., Wyman, J., and Changeux, J. P., (1965) *J. Mol. Biol.* 12, 88–118.
- Altamirano, M. M., Plumbridge, J. A., Horjales, E., and Calcagno, M. L. (1995) *Biochemistry* 34, 6074–6082.
- Calcagno, M. L., Campos, P. J., Mulliert, G., and Suástegui, J. (1984) *Biochim. Biophys. Acta* 787, 165–173.
- Oliva, G., Fontes, M. R. M., Garratt, R. C., Altamirano, M. M., Calcagno, M. L., and Horjales, E. (1995) *Structure* 3, 1323–1332.

10. Altamirano, M. M., Hernández-Arana, A., Tello-Solís, S., and Calcagno, M. L. (1994) *Eur. J. Biochem.* 220, 409–413.
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
12. Altamirano, M. M., Plumbridge, J. A., Hernández-Arana, A., and Calcagno, M. L. (1991) *Biochim. Biophys. Acta* 1076, 266–272.
13. Edelhoch, H. (1967) *Biochemistry* 6, 1948–1954.
14. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem* 182, 318–3260.
15. Howlett, G. J., Yeh, E., and Schachman, H. K. (1978) *Arch. Biochem. Biophys.* 190, 809–819.
16. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr., Sect. A* 47, 110–119.
17. Rubin, M. M., and Changeux, J.-P. (1966) *J. Mol. Biol.* 21, 265–274.
18. Imai, K. (1982) *Allosteric Effects in Haemoglobin*, Cambridge University Press, Cambridge.
19. Fermi, G., and Perutz, M. F. (1981) *Atlas of Molecular Structures in Biology: Haemoglobin and Myoglobin*, Oxford Clarendon Press.
20. Perutz, M. F. (1990) *Mechanisms of Cooperativity and Allosteric Regulation in Proteins*, Cambridge University Press, Cambridge.
21. Wyman, J. (1964) *Adv. Protein Chem.* 19, 223–286.
22. Forsén, S., and Linse, S. (1995) *Trends Biochem. Sci.* 20, 495–497.
23. Kraulis, P. (1991) *J. Appl. Crystallogr.* 24, 946–950.

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