Evidence for Two Different Mechanisms Triggering the Change in Quaternary Structure of the Allosteric Enzyme, Glucosamine-6-Phosphate Deaminase†

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ABSTRACT: The generation and propagation of conformational changes associated with ligand binding in the allosteric enzyme glucosamine-6-phosphate deaminase (GlcN6P deaminase, EC 3.5.99.6) from *Escherichia coli* were analyzed by fluorescence measurements. Single-tryptophan mutant forms of the enzyme were constructed on the basis of previous structural and functional evidence and used as structural-change probes. The reporter residues were placed in the active-site lid (position 174) and in the allosteric site (254 and 234); in addition, signals from the natural Trp residues (15 and 224) were also studied as structural probes. The structural changes produced by the occupation of either the allosteric or the active site by site-specific ligands were monitored through changes in the spectral center of mass (SCM) of their steady-state emission fluorescence spectra. Binding of the allosteric activator produces only minimal signals in titration experiments. In contrast, measurable spectral signals were found when the active site was occupied by a dead-end inhibitor. The results reveal that the two binary complexes, enzyme−activator (R_A) and enzyme−inhibitor (R_S) complexes, have structural differences and that they also differ from the ternary complex (R_{AS}). The mobility of the active-site lid motif is shown to be independent of the allosteric transition. The active-site ligand induces cooperative SCM changes even in the enzyme−activator complex, indicating that the propagation pathway of the conformational relaxation triggered from the active site is different from that involved in the heterotropic activation. Analysis of the complete set of mutants shows that the occupation of the active site generates structural perturbations, which are propagated to the whole of the monomer and extend to the other subunits. The accumulative effect of these propagated changes should be responsible for the change in the sign of the $\Delta G^{o\prime}$ of the T to R transition associated with the progression of the active-site occupation, resulting in the predominance of the R over the T forms in the population of deaminase hexamers.

Proteins are dynamic structures in which binding of specific ligands induces conformational changes that are critical for their function in molecular recognition and catalysis. These structural changes are driven by ligand binding energy and often involve different kinds of structural rearrangements of the active site or the whole protein, ranging from local to large-scale conformational changes. Describing these conformational changes is essential to understanding the structural basis of allosteric function (*1*, *2*). Because binding interactions occur at specific sites and involve only a small number of residues, a network of cooperative interactions is necessary for the propagation of binding signals to distal locations within the protein structure (*3*, *4*). Crystallographic data provide us with pictures of the free and ligand-bound structures; the knowledge of these initial and final states helps to characterize the functional conformational changes, but we must rely on physicochemical methods to reveal their mechanisms. Although the interactions at the binding site are mostly mediated by a limited number of residues, the binding Gibbs energy also stabilizes slightly different protein conformers, which involve distant structural motifs (*5*). Many well-known kinetic effects, such as activation by binding of metals or other ligands, and inhibition by ligands structurally dissimilar from the physiological ones, are explained on the basis of the conformational changes at the level of the tertiary folding. However, classic allosteric behavior requires extensive conformational changes involving the quaternary structure of the molecule (*6*, *7*). The crystallographic data usually do not reveal the transitory tertiary relaxations linking the active-site events to the quaternary transition. In many allosteric proteins, allosteric sites are located in the intersubunit interfaces or in free-moving domains, and their occupation by specific ligands stabilizes a particular quaternary arrangement. On the other hand, catalytic sites are usually located inside the subunit fold, and they are functionally linked to interoligomeric interfaces through tertiary relaxation mechanisms. One of the goals of studying allosteric mechanisms is to understand how the initial perturbation associated with ligand binding finds its way toward distant structural components of the subunit. This long-range propagation requires the

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presence in the binding site of residues displaying low structural stability, coexisting with motifs of more rigid geometry essential for efficient catalysis (*3*).

In this research, we have studied the propagation of conformational changes in the allosteric enzyme glucosamine-6-phosphate deaminase (GlcN6P[1] deaminase, EC 3.5.99.6) from *Escherichia coli*, which in many aspects can be considered as a simple experimental model. GlcN6P deaminase catalyzes the deamination and isomerization of glucosamine 6-phosphate (GlcN6P), producing fructose 6-phosphate and ammonium ion (*8*); it is allosterically activated by *N*-acetylglucosamine 6-phosphate (GlcNAc6P). The allosteric kinetics of this enzyme has been well characterized; its regulation by GlcNAc6P results from the change of its apparent affinity for the substrate, while its catalytic constant remains unaffected (*9*, *10*). *E. coli* GlcN6P deaminase contains six identical subunits arranged with 32 symmetry; its crystallographic structure has been determined for the R (PDB entries 1dea, 1hor, 1hot, and 1frz) and T (PDB entries 1cd5 and 1fsf) allosteric states, and the quaternary structural changes produced by the T to R transition have been described as a displacement of two rigid entities within each monomer (*11*, *12*). Its six allosteric sites are located in the intersubunit clefts, while active sites are in pockets partially covered by a motif that isolates them from the bulk solvent. The analysis of the crystallographic *B*-factors suggests that the active-site lid displays a considerable mobility, when the active site is empty either in the T or in the R (enzyme−activator complex) allosteric states of the enzyme (PDB entries 1frz and 1fs5). The mobility of the active-site lid dramatically decreases when the enzyme has both sites, allosteric and catalytic, occupied by the activator and a dead end inhibitor, respectively (*13*). This motif contains some of the phospho group binding residues of the allosteric site (Arg158 and Lys160) and the active site (Arg172). It is a low-stability region that contributes to substrate binding, and which has a key role in the communication between the active and allosteric sites (*14*), while other functional residues, mainly the catalytic ones, are located in areas that are highly structurally stable (*11*−*13*). The contribution of these residues of high conformational stability to the propagation of binding interactions to distant motifs in the protein structure is usually found in allosteric enzymes and is the basis of their function (*3*). It is worth mentioning that attempts to obtain crystals of the enzyme with the active site occupied and the allosteric site free were unsuccessful. However, the Lys160Glu mutant, which lacks one of the phosphate-binding groups in the allosteric site, when cocrystallized with GlcN-ol-6P, yielded crystals with the R structure and only the active site occupied by this dead-end inhibitor. The ligand-free Lys160Glu mutant, as expected, presents the known structure of the T state (*13*, *15*). The superimposition of the α-carbon traces of the monomer of this mutant in the R state with the corresponding wild-type R structures with the active and allosteric sites occupied reveals that both R structures are similar. We can then assume also that the wild-type enzyme produces essentially similar R states upon GlcNAc6P or GlcN-ol-6P binding.

Knowledge of the mechanism of the local structural changes, which are responsible for the communication between the allosteric and catalytic sites, is fundamental for understanding allosteric proteins. It is reasonable to expect that the initial perturbation associated with ligand binding, which leads to the quaternary allosteric transition, be transmitted through different pathways when starting from the allosteric site or from the active site. To investigate these aspects of the propagation of the allosteric transition from both the active and the allosteric site, we constructed mutant forms of GlcN6P deaminase containing single Trp residues located in rationally chosen positions. With these constructions, we explored the structural changes associated with ligand binding at either the active or allosteric sites, as they were sensed by the changes in the spectral center of mass (ΔSCM) of their fluorescence emission spectra.

MATERIALS AND METHODS

Reagents. Most chemicals and biochemicals were from Sigma-Aldrich S.A. de C.V. The affinity gel used for GlcN6P deaminase purification (*N*-6-aminohexanoylglucosamine-6P agarose) was prepared as described previously (*16*). The dead-end inhibitor, GlcN-ol-6P, was synthesized according to Midelfort and Rose (*8*) and purified as described elsewhere (*17*). ^{3}H-labeled GlcNAc6P (960 kBq μmol^{-1}) and GlcN-ol-6P (250 kBq μmol^{-1}) were prepared by the same procedures, using [^{3}H]acetic anhydride and sodium [^{3}H]-borohydride, respectively, according to previously described procedures (*14*, *17*).

Bacterial Strains and Mutagenesis. Site-directed mutations were carried out by oligonucleotide-directed mutagenesis, using the QuickChange commercial kit from Stratagene. Mutagenesis was carried out on the phagemide pTZ18R*nagB* containing the wild-type gene for *E. coli* GlcN6P deaminase, or a suitable mutant form of the *nagB* gene. The PCR was performed according to the manufacturer's instructions, using *Pfu* DNA polymerase. The mutations were verified by DNA sequencing, and the corresponding plasmids were used to transform the Δ*nag* strain IBPC590. This is a Δ*lacI* strain and expresses the protein constitutively (*18*).

Purification and Assay of Wild-Type and Mutant Forms of GlcN6P Deaminase. E. coli wild-type GlcN6P deaminase and the site-specific mutants were purified by allosteric-site affinity chromatography as previously reported (*9*, *16*). Molar absorptivities were calculated from spectral measurement and thiol group titration, as described by Montero-Morán et al. (*17*). The purity of the enzyme preparations was verified by SDS−PAGE. GlcN6P deaminase was assayed in the direction of Fru6P formation, by the measurement of the fructose 6-phosphate concentration at fixed times, as described elsewhere (*9*). The progress of the reaction was always kept below 5% conversion of the initial amount of substrate. Kinetic data were analyzed by nonlinear regression analysis using Origin 7.0 (MicroCal Software, Inc., Northampton, MA) by fitting them to the following form of the MWC equation:

$$v_0 = \frac{nk_{cat}[E_t][Lc\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}]}{L(1 + c\alpha)^n + (1 + \alpha)^n} \quad (1)$$

[1] Abbreviations: GlcN6P, glucosamine 6-phosphate; GlcNAc6P, *N*-acetylglucosamine 6-phosphate; GlcN-ol-6P, 2-amino-2-deoxyglucitol 6-phosphate; SCM, spectral center of mass.

where L is the MWC allosteric constant ($L = [T]/[R]$), α is the normalized substrate concentration ($[GlcN6P]/K_m^R$), k_{cat} is the catalytic constant, n (the number of binding sites) = 6, $c = K_m^R/K_m^T$, and $[E_t]$ is the concentration of the hexameric enzyme. The parameters k_{cat} and K_m^R were taken from the hyperbolic curves obtained in the presence of 1 mM GlcNAc6P ($K_d^{GlcNAc6P} = 30\ \mu M$).

Fluorescence Spectroscopy. Fluorescence measurements were taken on an ISS (Champaign, IL) PC-1 spectrofluorimeter. The temperature of the cells was kept at 25 ± 0.1 °C. The enzyme concentration was 1 μM in 50 mM Tris-HCl buffer (pH 7.5) and 1 mM EDTA. The saturating concentration of GlcN-ol-6P was 50 μM (25-fold greater than its K_d), whereas for GlcNAc6P, it was 1 mM (33-fold greater than its K_d). Fluorescence measurements were carried out using excitation at 290 nm, and emission spectra were collected at 1 nm steps from 300 to 400 nm. The fluorescence spectral centers of mass (SCM) were calculated from the intensity data (IF_λ) collected at different wavelengths (λ), using the following expression:

$$SCM = \frac{\sum_i^j (\lambda IF_\lambda)}{\sum_i^j (IF_\lambda)} \quad (2)$$

The changes in the SCM (ΔSCM) were fitted to the following form of the Hill equation:

$$\Delta SCM = \frac{\Delta SCM_{lim}[S]^h}{S_{0.5}{}^h + [S]^h} \quad (3)$$

where [S] is the ligand concentration.

GlcN-ol-6P Binding Curves from Direct Binding Measurements. Binding curves of the active-site ligand GlcN-ol-6P were obtained from binding measurements of [^{3}H]GlcN-ol-6P using the preparative ultracentrifuge, as previously described (*15*). The enzyme concentration was 0.1 μM in 50 mM Tris-HCl buffer (pH 7.5) and 1 mM EDTA. The saturating concentration of GlcN-ol-6P was 50 μM (25-fold greater than its K_d), whereas for GlcNAc6P, it was 1 mM (33-fold greater than its K_d).

RESULTS AND DISCUSSION

Selection and Validation of Target Positions for the Single-Tryptophan Mutants. This research is based on the study of a set of mutant GlcN6P deaminases containing single Trp residues in selected positions, while Tyr residues replaced natural tryptophans. For simplicity, these mutant forms will be further referenced according to the position of the unique Trp residue in the polypeptide chain. Therefore, 174TRP represents the Trp15Tyr/Phe174Trp/Trp224Tyr mutant form, where both natural Trp residues were replaced with Tyr residues and the Phe174 residue was replaced with Trp. We also studied the single-Trp forms of the enzyme, 15TRP and 224TRP, which retain one of the natural Trp residues and the Trp-depleted (TRP-less) double mutant in which both Trp residues were replaced with tyrosines. Both Trp15 and Trp224 residues are far away from the subunit interfaces or the ligand-binding sites. Other mutants were rationally designed on the basis of the known crystallographic structure of the enzyme. These mutants are 174TRP, which has the probe residue in the active-site lid, 234TRP, where a tryptophan residue is placed in the subunit interface close to the GlcNAc6P binding site, and 254TRP, where the tryptophan residue replaces the tyrosine involved in a key intersubunit contact in the allosteric site. Other mutations introducing Trp residues, such as Tyr121Trp (*16*) and Val249Trp (unpublished result), strongly perturb the allosteric function of the enzyme, and are not useful for the purpose of this research. Figure 1 shows the positions of the natural tryptophans and that of the residues replaced with tryptophans.

All single-Trp mutant forms of GlcN6P deaminase constructed in this research, as well the TRP-less enzyme, are catalytically and allosterically active. All of them conserved almost intact their ability to bind GlcNAc6P, allowing their purification by allosteric-site affinity chromatography. TRP-less, 15TRP, 224TRP, and 234TRP mutants behave like the wild-type enzyme (Table 1). The mutant forms of the enzyme containing the Trp probe in positions 254 and 174 present more noticeable allosteric changes. Tyr254 and Phe174 are important residues for the allosteric behavior of the GlcN6P deaminase, as previously reported (*14*, *17*). In the wild-type deaminase, Phe174 plays an important role in the structure of the active site by anchoring the mobile active-site lid to the remainder of the enzyme structure. The Phe174Ala mutation produces a drastic collapse of the structural stability of the active-site lid and a marked impairment of the enzyme catalytic activity, which is almost entirely recovered by the occupation of the allosteric site with GlcNAc6P (*14*). In contrast, the 174TRP mutant behaves in a manner similar to that of the wild-type enzyme, although its kinetic parameters appear to be somewhat modified. This observation indicates that Trp in position 174 is able to build equivalent hydrophobic interactions like those created by Phe in the wild-type enzyme. In position 254, the wild-type enzyme has a tyrosine residue, which forms an intersubunit hydrogen bond. This interaction switches between two alternative positions in the neighboring subunit, accompanying the R to T allosteric conformerization. The Tyr254Phe mutation removes this interaction and consequently alters the allosteric properties of the enzyme (*17*). Again, a tryptophan residue in this position restores the allosteric properties of the deaminase, which approaches the wild-type kinetics (Table 1). These data show that the chosen single-Trp mutations provide a suitable tool for the study of the allosteric transition through the ligand-induced fluorescence changes of the enzyme.

Binding of GlcN-ol-6P to the Active Site. GlcN-ol-6P, a dead-end inhibitor of *E. coli* GlcN6P deaminase (*8*), is considered an analogue of the putative reaction intermediate 2-amino-2-hydroxyglucosamine 6-phosphate (*11*, *18*). This ligand inhibits the enzyme, displaying linear competitive kinetics with a K_i of 2−3 μM, a value that is nearly 3 orders of magnitude lower than the K_m for GlcN6P. As expected for an enzyme following the symmetry model (*19*), at low substrate concentrations, the inhibitory effect of GlcN-ol-6P produces an activation of the GlcN6P deaminase (Figure 2A). As GlcN6P cannot be used in fluorescence titration experiments because it is the substrate in the unireactant

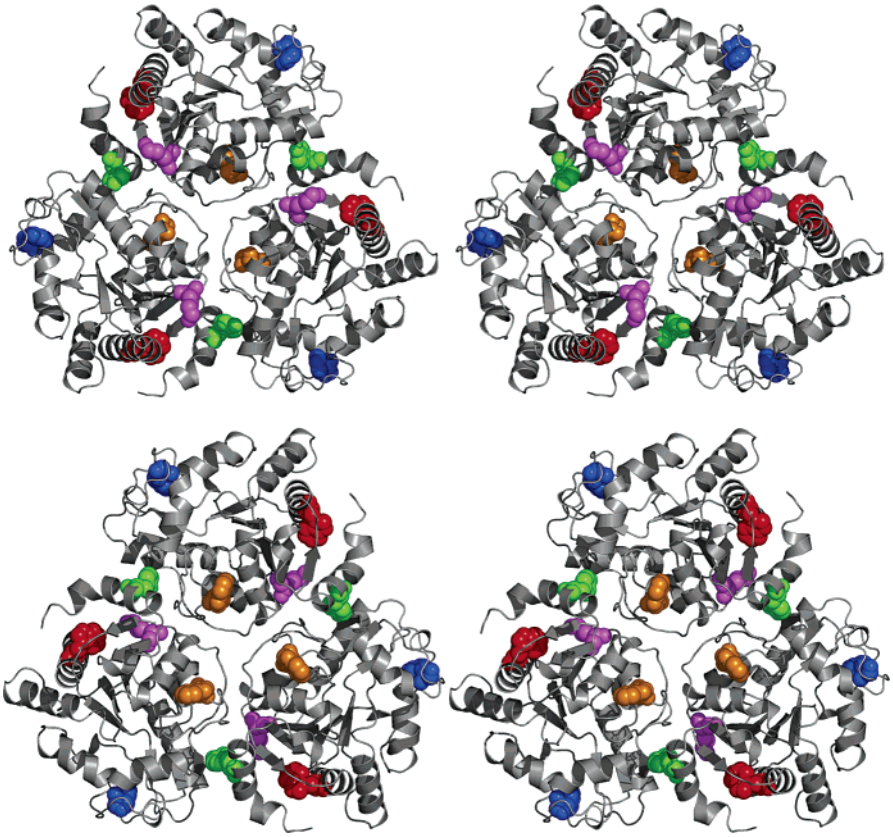

FIGURE 1: Stereoview of a trimer of GlcN6P deaminase showing the position of the probing tryptophans, viewed along the 3-fold axis of symmetry of the molecule: (top) external view of the trimer and (bottom) same trimer viewed from the trimer−trimer contacting face. Trp15 is colored red, Phe174 blue, Trp224 orange, Lys234 green, and Tyr254 magenta.

Table 1: Kinetic Properties of Mutants of *E. coli* Glucosamine-6-phospate Deaminase Containing a Single Tryptophan Residue at the Indicated Positions[a]

enzyme	K_m^R (mM)[b]	$S_{0.5}$ (mM)[c]	k_{cat} (s^{-1})[b]	k_{cat} (s^{-1})[c]	h[c]	K_m^T (mM)[d]	L ($\times 10^{-3}$)[d]	c[d]
wild-type	0.55 ± 0.05	5.5 ± 0.2	158 ± 8	157 ± 6	2.9 ± 0.1	22.0 ± 2	1000 ± 19	0.025 ± 0.0002
TRP-less	0.65 ± 0.04	5.0 ± 0.3	144 ± 9	149 ± 10	2.8 ± 0.1	20.1 ± 0.4	89 ± 22	0.032 ± 0.0007
15TRP	0.50 ± 0.07	4.8 ± 0.3	138 ± 10	135 ± 5	2.4 ± 0.2	16.0 ± 2	80 ± 21	0.031 ± 0.0038
224TRP	0.62 ± 0.09	5.8 ± 0.3	134 ± 10	130 ± 8	2.9 ± 0.2	22.9 ± 3	90 ± 21	0.027 ± 0.0026
234TRP	0.49 ± 0.06	2.4 ± 0.1	159 ± 7	109 ± 6	3.3 ± 0.3	19.9 ± 3	200 ± 1	0.026 ± 0.0002
254TRP	0.26 ± 0.01	0.5 ± 0.1	26.7 ± 1	25.7 ± 2	2.6 ± 0.2	3.9 ± 0.6	0.28 ± 0.029	0.066 ± 0.0154
174TRP	0.49 ± 0.05	3.4 ± 0.2	80 ± 3	74 ± 4	2.9 ± 0.2	17.1 ± 2	80 ± 12	0.028 ± 0.0051

[a] Data for the wild-type and TRP-less enzymes are given for reference. [b] Data obtained in the presence of a saturating concentration of GlcNAc6P and fitted to the Michaelis−Henri equation. [c] Data obtained in the absence of GlcNAc6P and fitted to the Hill equation. [d] Data obtained in the absence of GlcNAc6P and fitted to the Monod−Wyman−Changeux equation (*6*).

direction of the reaction, we used GlcN-ol-6P as the active-site ligand. This inhibitor entirely fills the active site (*11*), and its binding curve displays homotropic cooperativity similar to that of the substrate, as shown in Figure 2B. Saturation with the allosteric activator converts all the deaminase molecules to the R state, yielding a hyperbolic binding curve, because GlcNAc6P binds exclusively to this allosteric state. The fitted MWC parameters from [^{3}H]GlcN-ol-6P binding (Figure 2B) and the kinetics of the wild-type enzyme show that both the substrate and inhibitor display nonexclusive binding behavior, with nearly similar nonexclusive binding coefficients ($c = K_m^R/K_m^T = 0.025$; $d = K_i^R/K_i^T = 0.070$). The allosteric constant L calculated from kinetic or inhibitor binding data has the same value, within experimental error.

Binding of GlcNAc6P to the Allosteric Site. The binding curve of [^{3}H]GlcNAc6P is strongly cooperative, reaching a saturation stoichiometry of 6 mol of activator per mole of hexameric enzyme (Figure 2C). These results indicate that despite the chemical similitude between GlcN6P and GlcNAc6P, the latter is not significantly bound to the active site (Figure 2C) and GlcN-ol-6P does not display affinity for the allosteric site, at least in the explored concentration range (Figure 2B). GlcNAc6P also binds to the allosteric site when active sites are empty, producing the same quaternary R structure, but with some tertiary changes, mainly on the active-site lid (*13*).

Fluorescence of Residues Distant from Both Ligand-Binding Sites (Trp15 and Trp224). Trp15 is located in the center of the helix 1 in the section of the molecule that moves as a rigid body in the allosteric transition (*12*). The side chain of Trp15 points to the C-terminal α-helix (helix 8), forming an H bond between the indole N and the backbone carbonyl oxygen of Asn262. The corresponding N−O distances are 2.95 Å in the T state and 3.4 Å in the R state (PDB entries 1fsf and 1frz, respectively). The other natural tryptophan,

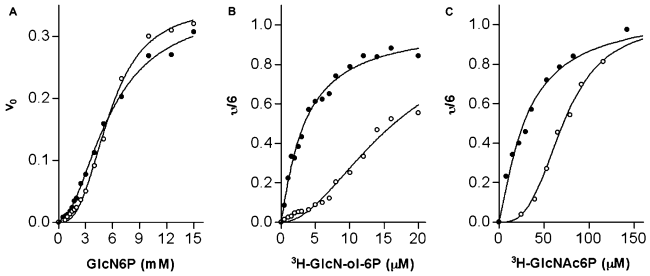

FIGURE 2: (A) Initial rates (in arbitrary units) at 30 °C and pH 7.5 for the wild-type enzyme obtained in either the absence (○) (h = 2.8 ± 0.2 and $S_{0.5}$ = 5.6 ± 0.2) or presence (●) (h = 1.9 ± 0.1 and $S_{0.5}$ = 5.7 ± 0.3) of the competitive inhibitor GlcN-ol-6P at 2 μM ($K_i^{GlcN-ol-6P}$ = 2 μM). (B) Fractional saturation, v/6 (moles of ligand bound per equivalent of active site), as a function of GlcN-ol-6P concentration obtained from direct binding measurements using [^{3}H]GlcN-ol-6P for the wild-type GlcN6P deaminase. Data were obtained in either the absence (○) (h = 1.9 ± 0.1 and $S_{0.5}$ = 16.4 ± 0.5 μM) or presence (●) (h = 1.0 ± 0.0 and $S_{0.5}$ = 3.4 ± 0.1 μM) of the allosteric activator GlcNAc6P at 1 mM ($K_d^{GlcNAc6P}$ = 30 μM). The fitted value for the nonexclusive binding constant for GlcN-ol-6P (d, defined as K_i^R/K_i^T) is 0.070 ± 0.008. (C) Fractional saturation, v/6, as a function of GlcNAc6P concentration obtained from direct binding measurements using [^{3}H]GlcNAc6P for the wild-type GlcN6P deaminase. Data were obtained in either the presence (●) (h = 1.2 ± 0.1 and $S_{0.5}$ = 30.6 ± 2.3 μM) or absence (○) (h = 3.1 ± 0.2 and $S_{0.5}$ = 71.7 ± 3.4 μM) of 50 μM GlcN-ol-6P.

Table 2: SCM Changes Caused by the Addition of the Active-Site Ligand GlcN-ol-6P to the Mutant Enzymes 15TRP and 224TRP

enzyme	ΔSCM_{lim} (nm)	h	$S_{0.5}$ (μM)
wild-type	0.83 ± 0.02	2.40 ± 0.35	1.97 ± 0.13
15TRP	−0.97 ± 0.14	1.06 ± 0.15	28.00 ± 5.78
224TRP	1.21 ± 0.04	1.71 ± 0.17	5.98 ± 0.38

Trp224, is located close to the 3-fold axis of symmetry in the so-called internal module (*12*), which remains almost motionless during the quaternary conformational change.

Fluorescence changes caused by the allosteric transition and ligand binding were studied for the wild-type enzyme and mutant forms 15TRP and 224TRP. The allosteric transition may be triggered either from the active site, upon binding GlcN-ol-6P, or from the allosteric site upon binding GlcNAc6P, the physiological allosteric activator.

The GlcN-ol-6P-induced allosteric transition of the wild-type enzyme causes a bathochromic shift of 0.83 nm in the spectral center of mass (Table 2), reported as the ΔSCM_{lim}, as defined in eqs 3 and 4. By contrast, the T to R transition starting from the allosteric site did not cause significant spectral changes. When the enzyme is titrated with GlcN-ol-6P, the plot (not shown) of ΔSCM versus the competitive inhibitor concentration displays homotropic cooperativity with a Hill coefficient of 2.4 (Table 2). The fluorescence changes of wild-type GlcN6P deaminase result from the combined emission of both tryptophan residues, which are located in different positions in the molecule. It is reasonable to expect that the data obtained would be complex, as interference between their spectral properties can occur. Because of this, the single-Trp mutant enzymes 15TRP and 224TRP were studied in the same way as the wild-type enzyme. The 15TRP mutant shows a behavior contrasting with that of the wild-type enzyme. The titration curve with GlcN-ol-6P for this enzyme shows a blue shift (ΔSCM_{lim} = −0.97 nm) instead of the red shift observed for the wild-type enzyme (not shown). The corresponding titration curve is hyperbolic, whereas the GlcNAc6P binding did not produce any spectral change. The absence of cooperativity in GlcN-ol-6P binding and the lack of spectral changes upon GlcNAc6P binding suggest that the detected conformational perturbation is not related to the allosteric transition; i.e., the Trp15 residue does not sense the quaternary conformational change. The recorded spectral change is probably caused by tertiary structural changes restricted to each subunit. In accordance with this hypothesis, GlcNAc6P binding does not produce any fluorescence signal.

The mutant enzyme 224TRP did not display fluorescence changes upon allosteric activator binding, while the titration with the dead-end inhibitor produces results similar to those obtained with the wild-type enzyme. 224TRP deaminase displays a red-shifted SCM (ΔSCM_{lim} = 1.21 nm), and the corresponding binding curve is sigmoid [h = 1.7 (not shown)]. This cooperativity could result from the opposite effects of ligand binding upon SCM when both Trp residues are present.

This set of data indicates that the allosteric transition that is initiated from the allosteric site does not modify the surroundings of Trp residues located in remote positions 15 and 224. Thus, SCM did not change with GlcNAc6P binding, while GlcN-ol-6P binding is sensed and assessed through a SCM change. This observation suggests that when the allosteric transition is triggered from the active site, the associated conformational relaxation propagates through a different and probably more complex pathway than when the transition starts from the allosteric site.

Fluorescence of Residues in the Allosteric Site (254TRP and 234TRP deaminases). The allosteric sites are in clefts formed between the subunits in each trimer. Two positions were selected for placing tryptophan residues as fluorescent probes in the allosteric site: Tyr254 and Lys234. Neither of these residues is directly involved in GlcNAc6P binding; Tyr254 is part of a previously described switching intersubunit contact (*17*), while Lys234 is positioned in the external part of the allosteric-site cleft, exposed to the solvent. Lys234 has not been related to any specific function, either in catalysis or in allosteric regulation.

Mutant 254TRP. The titration of the mutant 254TRP with the active-site ligand GlcN-ol-6P, measured through the SCM change, yields a sigmoidal saturation curve [h = 1.7, ΔSCM_{lim} = −1.8 nm (Table 3)]. A SCM hyperbolic change of the same sign is obtained by titration with GlcNAc6P (Figure 3A,B). In both cases, saturation with the nonvaried ligand abolishes the spectral change. These results suggest that the signal comes from the displacement of the 254 switch which, according to Montero-Morán et al. (*17*), retains its function when a Trp residue replaces Tyr254. When the T to R transition starts from the active site, the response to the dead-end inhibitor is cooperative, as expected for an allosteric ligand. Saturation with GlcNAc6P abolishes the spectral change because the inhibitor binds to an enzyme that already is in the R state. This result strongly suggests that the observed SCM change does not arise from local changes in the active site when GlcN-ol-6P binds. In the same way, saturation with the inhibitor abolishes the spectral change induced by GlcNAc6P binding (Figure 3C). These observations indicate that the signal coming from the allosteric site, where the reporter Trp was placed, is due to local structural

Table 3: SCM Changes Produced by the Addition of either GlcN-ol-6P or GlcNAc6P to Mutant Enzymes Having a Single Trp Residue at the Indicated Position

	titration with GlcN-ol-6P			titration with GlcNAc6P		
enzyme	ΔSCM_{lim} (nm)	h	$S_{0.5}$ (μM)	ΔSCM_{lim} (nm)	h	$S_{0.5}$ (μM)
254TRP	-1.78 ± 0.03	1.7 ± 0.2	0.075 ± 0.004	-1.15 ± 0.09	1.0 ± 0.0	141.7 ± 19.4
254TRP[a]	NE[b]	–	–	NE[b]	–	–
234TRP	-2.69 ± 0.21	2.1 ± 0.2	4.21 ± 0.70	biphasic	–	–
234TRP[a]	-3.38 ± 0.13	1.9 ± 0.2	5.40 ± 0.42	NE[b]	–	–
174TRP	-5.07 ± 0.19	1.1 ± 0.1	22.79 ± 1.97	NE[b]	–	–
174TRP[a]	-1.64 ± 0.07	1.4 ± 0.2	5.41 ± 0.54	-2.12 ± 0.36	1.0 ± 0.0	108.3 ± 21.9

[a] Enzyme saturated with the ligand that binds the site which is not being titrated. [b] No effect.

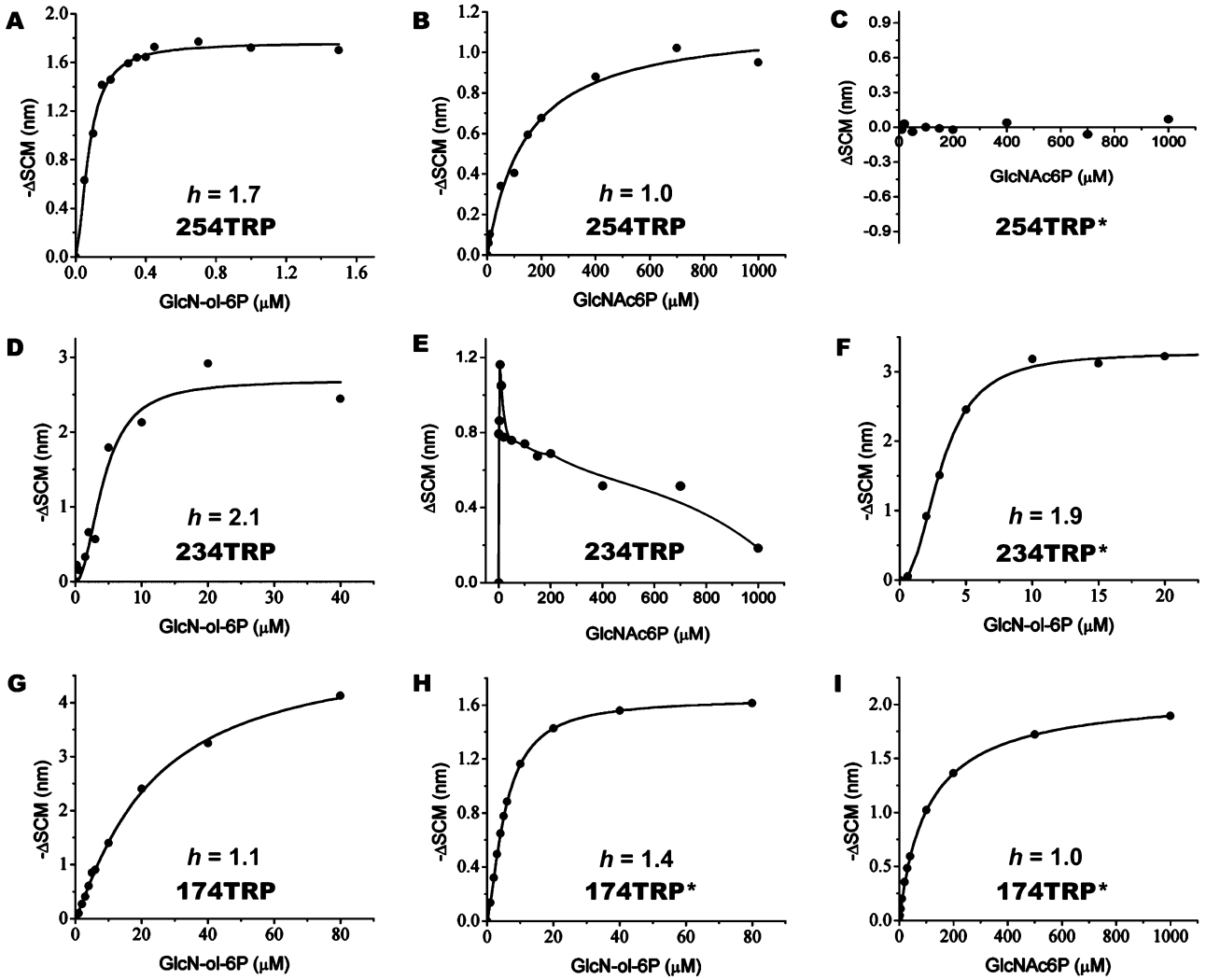

FIGURE 3: Changes in the spectral centers of mass of fluorescence spectra produced by titration of the different enzyme mutants with the active-site (GlcN-ol-6P) or allosteric-site (GlcNAc6P) ligands: (A) 254TRP mutant titrated with GlcN-ol-6P, (B) 254TRP mutant titrated with GlcNAc6PA, (C) 254TRP mutant saturated with GlcN-ol-6P titrated with GlcNAc6P, (D) 234TRP mutant titrated with GlcN-ol-6P, (E) 234TRP mutant titrated with GlcNAc6P, (F) 234TRP mutant saturated with GlcNAc6P and titrated with GlcN-ol-6P, (G) 174TRP mutant titrated with GlcN-ol-6P, (H) 174TRP mutant saturated with GlcNAc6P and titrated with GlcN-ol-6P, and (I) 174TRP mutant saturated with GlcN-ol-6P and titrated with GlcNAc6P. Asterisks indicate the presence of either 50 μM GlcN-ol-6P when titrating with GlcNAc6P or 1 mM GlcNAc6P when titrating with GlcN-ol-6P.

changes associated with GlcNAc6P binding. However, this signal accompanies the allosteric transition when the 254 switch moves from its position in the T state to that of the R state, as a part of the propagated conformational change that eventually produces the allosteric transition. A remarkable property of this mutant is its high affinity for the substrate (Table 1) and for the competitive inhibitor, as reflected in the $S_{0.5}$ value calculated from the SCM changes with GlcN-ol-6P (Table 3).

Mutant 234TRP. The other selected position for placing a reporter tryptophan was position 234 where the wild-type enzyme has a lysine. The saturation curve obtained by GlcNAc6P titration yields a complex response; the ΔSCM values plotted versus GlcNAc6P concentration show a biphasic change (Figure 3E). The fluorescence signal is suppressed by saturation with GlcN-ol-6P, thus proving that it comes from the quaternary change and not just from GlcNAc6P binding. The biphasic nature of the curve reveals

its complex structural origin, involving components of opposite sign that are sensed from this particular position of the reporter Trp. At first sight, this two-step response can be considered as the combination of a positive signal, related to the quaternary change and completed at a low GlcNAc6P concentration, and a negative signal derived from the induced fit at the allosteric site. It must be taken into account that according to previous kinetic and structural evidence (*9*, *11*), binding a single activator molecule is enough to shift the hexamer to the R state. The abolition of both components of the biphasic curve in Figure 3E by GlcN-ol-6P saturation does not necessarily imply that this effect is unrelated to the allosteric-site filling by GlcNAc6P. It may indicate that the closing of the active-site cavity by GlcN-ol-6P drives the enzyme into a different structure; therefore, the reporter Trp no longer detects the allosteric-activator binding. The titration of the mutant 234TRP with the active-site ligand, GlcN-ol-6P, produces a large cooperative blue shift of the SCM [$h = 2.1$, $\Delta SCM_{lim} = -2.7$ (Table 3 and Figure 3D)]. This signal appears to be associated with the allosteric transition starting from the active site. Nevertheless, the titration of the mutant 234TRP, previously saturated with GlcNAc6P, also produces a cooperative blue shift of the SCM [$h = 1.9$, $\Delta SCM_{lim} = -3.4$ nm (Table 3 and Figure 3F)]. This cooperative signal appears to arise from the propagated interactions that trigger the allosteric transition when it is initiated from the active site. In this case, the quaternary change has already occurred because the enzyme was previously saturated with the allosteric activator. The observed spectral changes suggest that the network of interactions that starts from the active site and propagates across the subunit remains operative in the enzyme−GlcNAc6P complex. There are no reasons to assume that the interactions that trigger the allosteric transition from the active site must occur when the transition starts from the allosteric site. The observed cooperativity for GlcN-ol-6P binding may be produced by this kind of "futile triggering" of interactions starting in the active site of the enzyme when it is in the R conformer. This statement, of course, leads us to postulate that the R state in the E−GlcNAc6P complex (say, R_A) and the R state in the ternary complex E−GlcN-ol-6P−GlcNAc6P (say, R_{AS}) are not conformationally identical. It is worth noting that the reporter Trp residues at the allosteric site are not able to detect structural changes in the conversion of R_S into R_{AS}, although these R states are not necessarily identical, as will be shown in the next section.

Fluorescence of a Reporter Tryptophan in the Active-Site Lid (174TRP deaminase). The Phe174Ala mutation, as previously reported, produces the complete loss of the binding properties of the active site (*14*). A Trp residue placed in this position restores the active-site function (Table 1). The enzyme with a Trp residue in this position produces a large essentially hyperbolic blue shift ($\Delta SCM_{lim} = -5.1$ nm) of SCM upon GlcN-ol-6P binding (Figure 3G). The magnitude of this change suggests that the induced fit at the active site involves a large modification of the Trp174 surroundings, which become more hydrophobic. The titration with the allosteric activator does not produce spectral changes at all, thus indicating that the signal produced is derived from local conformational changes at the active site and is not related to the quaternary allosteric change. This result suggests that the lid motif, where the reporter Trp was placed, moves in a manner that is independent of the T to R transition. This is consistent with the known allosteric kinetics of GlcN6P deaminase, whose T and R states are both able to bind the substrate or dead-end inhibitors, although with different affinities. It is reasonable to expect that the lid motif would retain a considerable mobility in either allosteric state of the enzyme, keeping the catalytic site accessible to its ligands. When the GlcNAc6P-saturated 174TRP deaminase was titrated with GlcN-ol-6P, the allosteric equilibrium was already displaced to the R state. However, a sigmoid saturation curve ($h = 1.4$) resulted from the measured blue shift [$\Delta SCM_{lim} = -1.6$ nm (Figure 3H)]. This change, which is smaller than that for the ligand-free form of the enzyme, confirms that the active-site lid motif conserves an important part of its mobility in the R state. This, in turn, proves the existence of some structural differences between the R_A and R_{AS} complexes. This conclusion is consistent with the reported values for the crystallographic *B*-factors of these complexes (*13*).

The observed cooperativity for the titration with GlcN-ol-6P of the allosteric activator-saturated mutant 174TRP is an unexpected result. The dead-end inhibitor binds hyperbolically to the R state of the wild-type enzyme, as observed in direct binding experiments (Figure 2B). This property can be related to the interactions originating in the active site, as already discussed for the mutant 234TRP. We can postulate again that the propagation of the quaternary change, triggered by active-site ligands, remains operational even when the allosteric transition has occurred. This futile triggering is, in any case, propagated to the whole hexamer, hence the sigmoid character of the curve. The titration of the 174TRP−GlcN-ol-6P complex with the allosteric activator gives fluorescence spectra whose ΔSCM varies hyperbolically with this ligand concentration (Figure 3I). The curve relates to the structural change from the R_S complex to the R_{AS} complex and provides a spectral proof that both conformers are structurally different. In contrast to the R_A and R_{AS} species, whose structural models were obtained by X-ray crystallography, crystals of the wild-type R_S complex (that is, with its allosteric sites empty) were never obtained (E. Horjales, personal communication).

Structural and Thermodynamic Considerations. In the active site, Trp174 produces ΔSCM values that can be directly related to the conformational mobility of the active-site lid. The results presented for the 174TRP mutant reveal the functional link between the lid of the active site and the GlcNAc6P binding site; this is easily explained because the antiparallel A′−C′ β-sheet is a shared structure between the allosteric site and the active-site lid (*11*, *14*). They also show that even in the R state of the enzyme, the lid region keeps its conformational mobility until GlcN-ol-6P is bound.

This set of data shows that active-site occupation produces changes in Trp fluorescence in the five positions that have been studied. Contrastingly, the occupation of the allosteric site produces signals only in the mutant forms carrying the probing Trp residue in the allosteric site. The induced fit produced by the binding of the ligand to the active site propagates a structural change, which probably involves the whole tertiary structure of the subunit. On the other hand, the quaternary change induced by binding at the allosteric site propagates the quaternary transition as a rigid body

concerted motion of the hexamer subunits. The multiple signals produced by the active-site occupation in the mutant deaminases used in this research reveal the existence of a network of interactions that are triggered upon binding to the active site. This network appears to be independent of the triggering mechanism producing the allosteric transition by the allosteric-site occupation, as in the case for the futile triggering proposed for the active-site titration of the R forms of the enzyme.

The existence of two very different mechanisms for triggering the allosteric transition is required for the control of the activity of the enzyme according to the fluctuating concentration of involved metabolites. For an allosteric equilibrium between two conformational states with different affinities for an active-site ligand, the free energy of this allosteric transition is (*20*)

$$\Delta G^{\circ\prime}{}_i = \Delta G^{\circ\prime}{}_0 + i\Delta G^{\circ\prime}{}_W \quad (4)$$

where $\Delta G^{\circ\prime}{}_i$ is the free energy of the allosteric transition from T_i to R_i, i is the number of molecules of substrate (or other active-site ligand) bound to the oligomeric protein, $\Delta G^{\circ\prime}{}_0$ is defined as $RT \ln L_0$, and $\Delta G^{\circ\prime}{}_W$ is defined as $RT \ln c$ and is a measure of the free energy associated with the active-site affinity change caused by the allosteric transition. The value of $\Delta G^{\circ\prime}{}_i$ becomes zero when $[T_i] = [R_i]$; thus, the amount of bound molecules that shifts the allosteric equilibrium to this point (i_{eq}) may be calculated as

$$i_{eq} = -\Delta G^{\circ\prime}{}_0/\Delta G^{\circ\prime}{}_W = -\ln L/\ln c \quad (5)$$

For high L values and when $c < 1$ (nonexclusive substrate binding), as is the case in *E. coli* GlcN6P deaminase and many other allosteric enzymes, i_{eq} is a number close to half of the number of binding sites. This implies a mutual dependence of c and L parameters, as was theoretically demonstrated by Rubin and Changeux (*21*) and Johannes and Hess (*22*). In practice, this means that when half of the active sites are occupied (when $i = n/2$, then $L_i = 1$), the next bound molecule produces a considerable shift in the allosteric equilibrium ($L_{n/2+1} \ll 1$). Experimentally, this appears as the minimal requirement of $n/2$ substrate molecules bound to produce the allosteric transition, as found by Kantrowitz et al. for fructose 1,6-bisphosphatase (*23*). Whereas the allosteric site must detect low concentrations of GlcNAc6P for a sensitive response to the cell's need to convert amino sugars into Fru6P, the active site should be less responsive to keep the metabolic flux at a suitable level in the absence of the allosteric effector. This different response is a direct consequence of the nonexclusive binding of active-site ligands.

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