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Conformational Selection of Glycomimetics at Enzyme Catalytic Sites: Experimental Demonstration of the Binding of Distinct High-Energy Distorted Conformations of *C*-, *S*-, and *O*-Glycosides by *E. Coli* β-Galactosidases

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Abstract: We show that the conformational features of the molecular complexes of *E. coli* β-galactosidase and *O*-glycosides may differ from those formed with closely related compounds in their chemical nature, such as *C*- and *S*-glycosyl analogues. In the particular case presented here, NMR and ab initio quantum mechanical results show that the 3D-shapes of the ligand/inhibitor within the enzyme binding site depend on the chemical nature of the compounds. In fact, they depend on the relative size of the stereoelectronic bariers for chair deformation or for rotation around Φ glycosidic linkage.

Introduction

Inhibition of glycosidase enzymes is at the heart of the therapy of numerous diseases, since they are involved in varied and essential biological processes.[1−7] It is generally thought that the best inhibition is obtained when transition-state analogues are considered.[1−5] This is probably due to the gain in energy provided by the binding of a high-energy conformer in the pathway toward the transition state,[1−5] and in the particular case of glycosidases, molecules with a oxocarbenium-like shape would have the best chance of giving the best results.[1−7] In fact, regarding inhibition, divergent results have been shown for different cases, depending on the nature/family of the enzyme and on the chemical nature of the inhibitor used.[8−15] The study and use of nonprocessable substrate analogues is one possible path in the development of new therapeutical agents. On these grounds, the quest for glycosidase inhibitors has led to a group of glycomimetic compounds with the interglycosidic oxygen substituted by other atoms. In particular, *C*- and *S*-glycosyl compounds[16−18] have been shown to be recognized by these enzymes and to behave as moderate to good inhibitors,[19] although controversial results on the conformation of these molecules with respect to the natural compounds have been reported.[20] The existing analogies at the molecular level between mimetics and natural substrates at the catalytic sites, if any, would be of great relevance for the proper design of second-generation molecules with better properties, not only for glycosidase inhibitors, but also for other classes of compounds and enzymes. Obviously, the deduction of the molecular features of an enzyme/substrate complex is a difficult task, due to the fact that the substrate is readily transformed into products. In principle, two ways to deduce the molecular features of an

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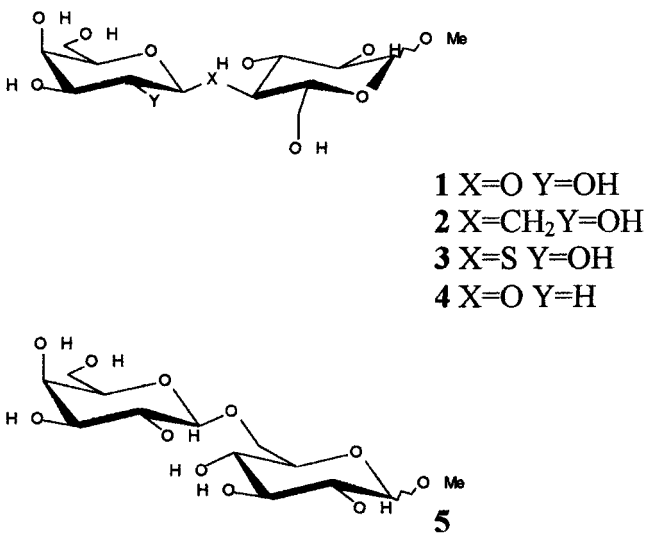
enzyme/substrate complex are useful: studying complexes of either wild type (WT) enzyme/inhibitor[19] or inactive mutated enzyme/substrate.[15,21−34] On this basis, we report herein that the atomic features of the molecular complexes formed between glycosidase enzymes and closely related compounds may differ, thus providing different perspectives of what is actually happening.[35] As a test case, we have chosen *E. coli* β-galactosidase, since it has been widely employed as a model enzyme for studies of glycosidases, which act on disaccharides.[36] *E. coli* β-galactosidase is a retaining glycosidase enzyme with tetrameric structure and one active site per monomer, and its X-ray structure has been recently determined.[37,38] In addition, the nucleophile has been identified as Glu-537, by covalent attachment of an inhibitor to this residue.[39] We recently reported that a high-energy conformer of the *C*-glycosyl analogue (**2**) of lactose (**1**) is bound by WT *E. Coli* β-galactosidase.[19] This result, along with molecular modeling predictions, permitted us to postulate a 3D structure of the complex that, in principle, could be extrapolated to the facts that occur for the catalysis of the natural substrate. Herein, we compare our previous results for β-galactosidase/*C*-lactose (**2**)[19] with those derived for β-galactosidase/*S*-lactose (**3**), and for the complexes of an inactive enzyme (E537Q)[40] and three *O*-glycosides, lactose (**1**), the competitive inhibitor 2′-deoxy lactose (**4**),[41] and allolactose (**5**), the transglycosidation product of lactose hydrolysis (Chart 1). We show that the conformational features of the molecular complexes of enzymes and closely related compounds, in their chemical nature, such as *O*-glycosides and their largely employed *C*- and *S*-glycosyl analogues, may indeed differ. In the particular case presented here, NMR and ab initio quantum mechanical results show that the 3D-shapes of the ligand/inhibitor within the enzyme binding site depend on the chemical nature of the compounds. In fact, they depend on the relative size of the stereoelectronic barriers for chair deformation or for rotation around Φ glycosidic linkage.

Chart 1



Results and Discussion

It is well-known that for ligands, which are not bound too tightly, and as pioneered by Bothner-By and first applied to carbohydrates by Prestegard, the TR-NOE experiment provides an adequate means of determing their bound conformation.[42−45] The conditions for the applicability of this approach are well established, and since the conditions required to monitor TR-NOEs appear to be satisfied frequently by sugar receptors, it has been applied to several studies of lectin- or antibody-bound saccharides.[46,47] Usually, protein−sugar interactions are not extremely strong, there is fast exchange between free and bound states, and the perturbations of the conformational equilibrium of the saccharide (mimetic) upon binding are accessible to observation by NOE.[48] Thus, in our particular case, the conformational analysis of sugars and glycomimetics in the binding site of *E. coli* β-galactosidase was performed on the basis of TR-NOEs that characterize the low-energy regions of the Φ/Ψ energy surface. Different mixing times and protein/ligand molar ratios were systematically used to gain quantitative conclusions. NOE intensities for the ligand were calculated by using a full relaxation matrix in the presence of exchange.[49]

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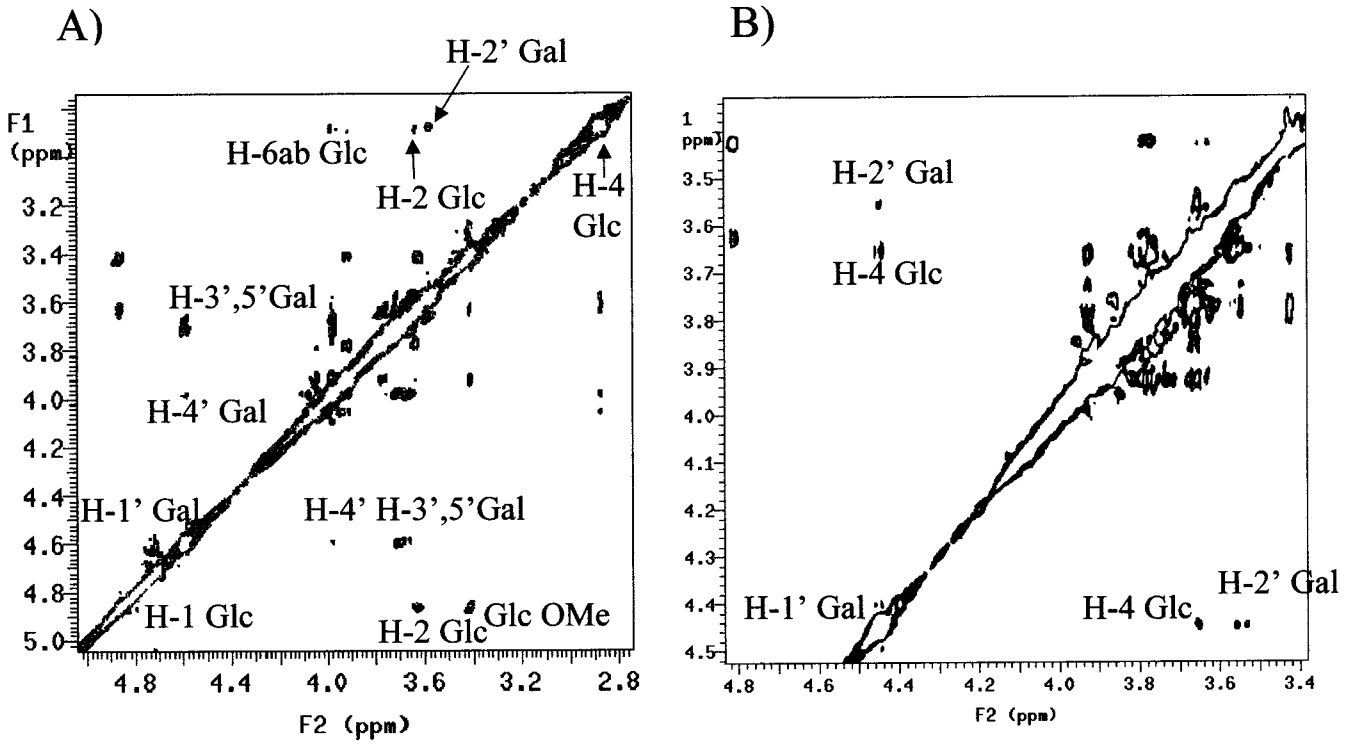

Figure 1. (A) TRNOESY spectrum (mixing time, 150 ms) of methyl *S*-lactoside (**3**) in the presence of WT *E. coli* β-galactosidase (molar ratio, ca. 35:1). Key cross-peaks are indicated. The strongest interresidue peak corresponds to H-2′/H-4. The H-1′/H-3 peak which was the strongest interresidue peak for the free ligand is not observed. Identical results are obtained for the **3**/E537Q complex. (B) TRNOESY spectrum (mixing time, 125 ms) of methyl lactoside (**1**) in the presence of E537Q *E. coli* β-galactosidase (molar ratio, ca. 40:1). Key cross-peaks are indicated. A weak H-1′/H-4 peak is observable, indicating some sort of syn-Φ conformation (see text for discussion). H-1′/H-3′ and H-1′/H-5′ peaks are hardly observed.

For **1**−**4**, the key interresidue TR-NOEs that characterize the allowed regions of the Φ/Ψ potential energy map are H1′−H4 and H1′−H6SR for syn-Φ/Ψ, H1′−H3 for syn-Φ/anti-Ψ, and H4−H2′ for anti-Φ/syn-Ψ conformations[50] (Table 1, Supporting Information). The interresidual NOE observed for the competitive inhibitor **2** (Ki, 3.3 mM)[19] in the presence of the WT enzyme is H4−H2′, indicating that the high-energy ($\Delta E = 1.8$ kcal/mol) anti-Φ/syn-Ψ conformer is bound by this enzyme (Figure 1, Supporting Information). Other NOEs involving the interglycosidic CH_2 group are also present. These NOEs are also in agreement with the exclusive recognition of this conformer.[50,51]

Strong intraresidue cross-peaks between H-1/H-3,H-5 pairs were observed for both Glc and Gal rings, indicating $^4C_1$ conformations. Molecular modeling using the X-ray structure of the free enzyme[37,38] demonstrated that this conformer is the only one that can be bound without a significant distortion of the enzyme catalytic site.[19]

This anti-Φ/syn-Ψ conformer cannot be detected for natural lactose.[52] To rule out the possibility that the binding of this unusual conformer could be an artifact, due to the presence of the lipophilic interglycosidic CH_2, similar experiments were performed for the complex between the WT enzyme and the *S*-glycosyl analogue (**3**), with an S atom at the glycosidic linkage. The results (Figure 2) were similar to those described for the CH_2 analogue. The preferred binding of a high-energy anti-Φ/syn-Ψ conformer can be deduced by the strong H-4′/H-2 NOE. Again, no distortion of either the Glc or Gal chairs is observed upon binding by the WT enzyme, since strong intraresidue cross-peaks between all the H-1/H-3 and H-1/H-5 proton pairs were observed. In principle, since the anti-Φ/syn-Ψ conformer is very minor in the free state by NMR (population ca. 10%), a minimum binding energy of $\Delta E = 1.5$ kcal/mol should be provided by the enzyme to bind this competitive inhibitor (**3**, Ki, 7.7 mM) in the high-energy conformation. This value is in the range of that described for **2**.[19] The existence of specific binding to both **2** and **3** was deduced from competitive TR-NOE experiments[19] in which a known potent inhibitor (Ki, 0.1 mM), isopropyl thiogalactose, IPTG, was added to the NMR tube containing the ligand/enzyme solution. It was observed that slightly above an equimolar ratio between IPTG and either ligand, the cross-peaks corresponding to the ligand changed their sign to positive. Those peaks pertaining to IPTG appeared as negative, indicating that both ligands compete for the same binding site, and that the affinity of the enzyme for IPTG is higher than that for the analogues **2** and **3**, as is also deduced from regular Ki determinations.[19]

Obviously, these results for the *C*- and *S*-glycomimetics (**2** and **3**) cannot be directly extrapolated to *O*-lactose **1**. Due to the presence of the exo-anomeric effect in **1**,[53] the energy barriers around Φ and the relative energies of the energy minima will have different values to those of **2** and **3**. On this basis, we

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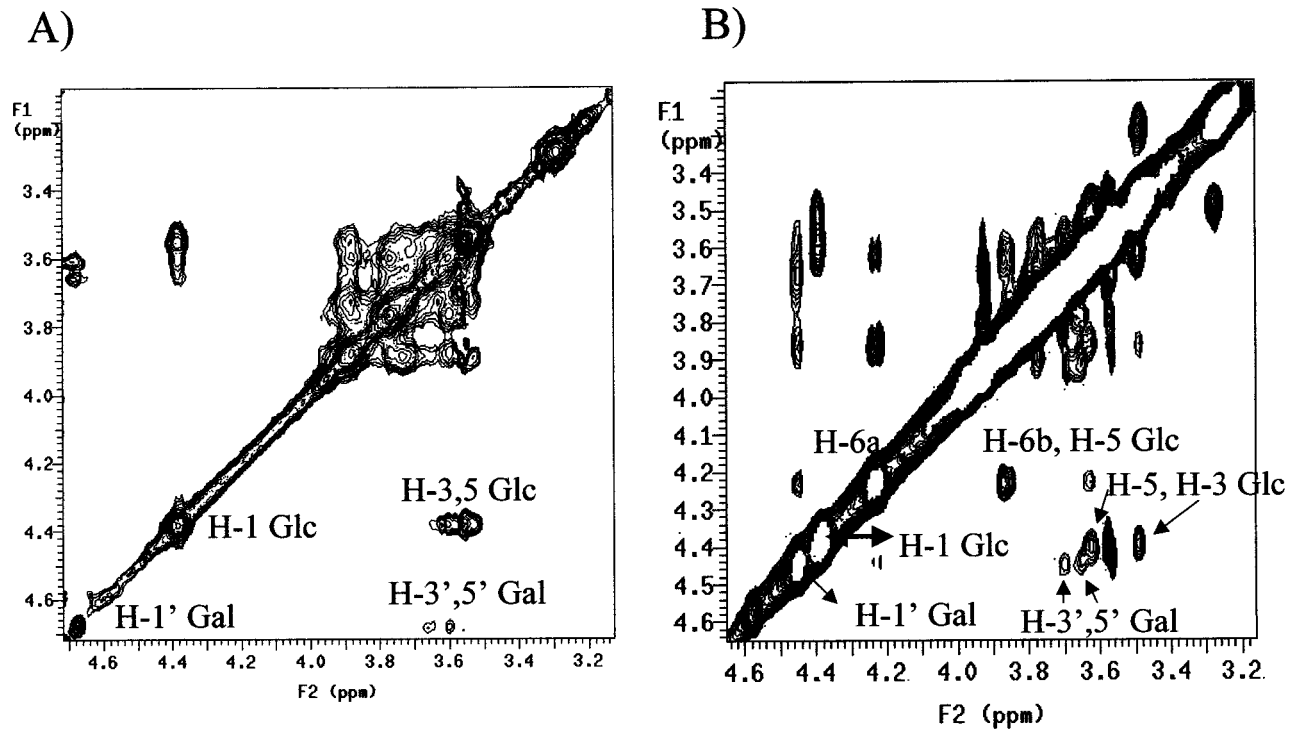


Figure 2. (A) Sections of a TRNOESY spectrum (mixing time, 150 ms) of 2′-deoxy lactose (**4**) in the presence of *E. coli* β-galactosidase E537Q (molar ratio, ca. 30:1). Key cross-peaks are indicated. H-1′/H-3′ and H-1′/H-5′ peaks are hardly observed. Expansions are given in the Supporting Information. (B) Sections of a TRNOESY spectrum (mixing time, 125 ms) of methyl β-allolactose (**5**) in the presence of *E. coli* β-galactosidase E537Q (molar ratio, 38:1). Weak H-1′/H-3′ and H-1′/H-5′ cross-peaks of the Gal ring are observed. Globally, they are eleven times weaker than H-1−H-3, H-1/H-5 for the Glc residue. Thus, indication of chair distortion is provided. A expansion is also given in C.

decided to investigate the conformational behavior of the real enzyme substrates, that is, *O*-glycosides (**1**, **4**, **5**) bound to an inactive enzyme, E537Q. This enzyme was chosen because the key nucleophilic E537 amino acid was mutated, in a conservative way, to the corresponding amide to provide the E537Q mutant enzyme.[40] This approach should permit us to study not only the hydrolytically stable analogues **2** and **3** but also regular glycosides, such as **1**, **4**, and **5**.

As a first case, the study of the TR-NOE recorded for the complexes E537Q/**2** and E537Q/**3** provided identical results to those for the WT enzyme: the anti-Φ/syn-Ψ conformation is the only one recognized in both cases, as deduced from the strong H-4/H-2′ NOE (Figure 2, Supporting Information).

Different results were obtained for the binding of *O*-glycosides **1**, **4**, and **5** to E537Q. In these three cases, and in opposition to the observations for the complexes of **2** and **3** with the WT and E537Q enzymes, the H-1′/H-3′ and H-1′/H-5′ cross-peaks for the all Gal moieties were very weak. Indeed, their intensities are 8−10-fold weaker than the same cross-peaks (H-1/H-3 and H-1/H-5) for the analogous Glc rings. These intensities are contrary to the expectations for a $^4C_1$ chair of the galactose moieties (Figure 2). They also strongly differ from the relative intensities of the H-1′/H-3′ and H-1′/H-5′ (Gal) versus H-1/H-3 and H-1/H-5 (Glc) observed for the competitive glycomimetic inhibitors **2** and **3**.

Specific binding of **1**, **4**, and **5** to the E537Q enzyme was again deduced from competitive TR-NOE experiments, adding IPTG to the NMR tube containing the solution of enzyme/*O*-glycoside. As before, upon addition of a 2-fold excess of IPTG, the cross-peaks of *O*-glycosides vanished, indicating their displacement from the enzyme binding site.

Glycomimetics **2** and **3** (and IPTG) are competitive inhibitors of lactose and, indeed, compete for the same enzyme binding site as the *O*-glycosides (**1**, **4**, and **5**), as demonstrated by the competitive TR-NOE experiments. Therefore, the relaxation environments for a given proton of the galactose and glucose moieties in all the complexes must indeed be similar. In our experimental observations, only the intraresidue H-1′/H-3′ and H-1′/H-5′ cross-peaks of the Gal moieties of **1**, **4**, and **5** showed different absolute and relative intensities to those of **2** and **3**. In all of the compounds **1**−**5**, the corresponding H-1/H-3 and H-1/H-5 cross peaks of the Glc residues remained very similar. Along this reasoning, the observed decrease in the cross-peak intensities of the Gal relative to the Glc protons, observable in the *O*-glycosides, but not in **2**, and **3** or IPTG, which also presents strong H-1′/H-3′ and H-1′/H-5′ cross-peaks in the Gal ring, should indeed be a consequence of the existence of distinct conformational features within the Gal chairs of the *O*-glycosides (**1**, **4**, and **5**) with respect to those of their glycomimetics (**2** and **3**).

The interproton distances were obtained from the cross-peak intensities by using the following approach. Two types of intensity references may be taken for the Gal moieties of **1**, **4**, and **5**. Apart from the "external" references (for H-1′/H-3′ and H-1′/H-5′) from the Gal rings of their analogous glycomimetics **2** and **3**, "internal" intensity references provided by their vicinal Glc rings (for H-1/H-3 and H-1/H-5) are also available. Thus, relaxation matrix calculations for the NOEs of the ligand protons in the bound state were carried out. The best fit between observed and expected intensities indicated that the average H-1′/H-3′ and H-1′/H-5′ distances in the Gal rings of enzyme-bound *O*-glycosides **1**, **4**, and **5** is ca. 2.9 Å. This value is far

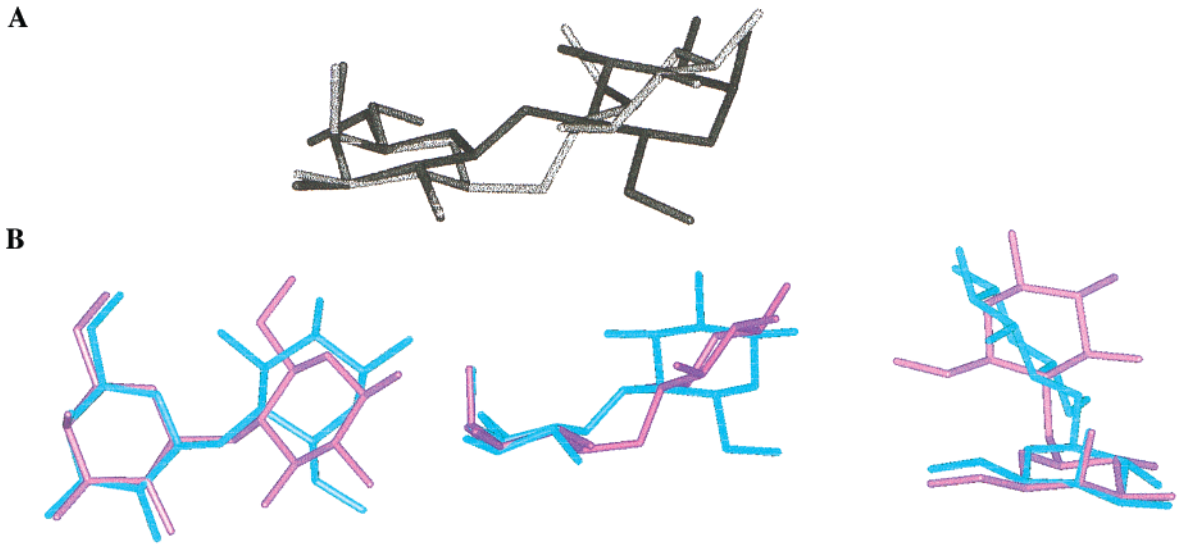


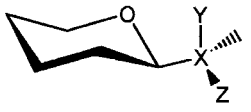
Figure 3. (A) Superimposition of the Gal rings of NMR deduced bound conformations of lactose (**1**), syn-ΦΨ type with distortion of the Gal chair and C-lactose (**2**), and anti-Φ type with no chair distortion. The 3D shape of **2** resembles that of **1** at the binding site. (B) Views from three different sides are shown. The three-dimensional shape of the analogue somehow resembles that of the natural compound, although the glucose ring are twisted by about 90° in both analogues. The galactose ring was used for superimposition since the enzyme is specific for galactose moieties.

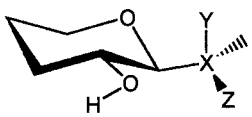
from the 2.4−2.5 Å of the Gal moieties of **2**, **3**, and IPTG, and from the 2.4−2.5 Å for the Glc chairs of **1**−**5**. In principle, the autorelaxation and cross-relaxation behavior of the equivalent sugar protons of **1**−**5** in the enzyme binding site must be similar. Therefore, the 0.4−0.5 Å difference observed between the two cross-peaks that define the $^4C_1$ chair conformation of the Gal moiety (H-1′/H-3′ and H-1′/H-5′) proton pairs of **2** and **3** on one side and of **1**, **4**, and **5** on the other is in agreement with a significant distortion of the galactopyranose chair of the *O*-glycosides. Since normal distances were estimated from the H-3′/H-5′ NOEs, the distortion is focused on the anomeric region of the pyranose. A flattened chair or endo-sofa form with the C5−O5−C1−C2 torsion adopting a small value of ca. −25° probably occurs (Table 2, Supporting Information). Additionally, in the case of E537Q/**1** and E537Q/**4**, the presence of a H-1′/H-4 cross-peak seems to indicate that some sort of syn-Φ conformation exists around the glycosidic linkage.

These experimental results point out important conclusions: first, that NMR may be used to get insights on the bound conformation on substrates/inhibitors at enzyme catalytic sites, and second, that *E. coli* β-galactosidase (WT or E537Q) always selects a high-energy conformer of its bound sugar or *C*-, *S*-glycomimetic, and that this recognition takes place before the catalytic reaction itself has started. For **2** and **3** (with absent or diminished exo-anomeric effect), the high-energy conformer is produced upon rotation around the glycosidic Φ-angle. In contrast, for *O*-glycosides **1**, **4**, and **5**, the enzyme produces distortion of the chair. Nevertheless, as depicted in Figure 3, the three-dimensional shape of the *C*-glycoside resembles that of **1** in the binding site. In fact, the aglycon orientation is somehow similar with respect to the glycon Gal moiety. For this binding mode, the galactose rings of both compounds were superimposed, since this enzyme is specific for galactosides. The differences are observed in Figure 3B, where it can be seen that there is a twisting of the Glc ring between **1** and **2**. It is noteworthy to consider that the observed bound conformations of **2** and **3** are reminiscent of the deformations in the reaction pathway. Indeed, modelling studies indicate that both types of conformers may be bound without significant distortion of the enzyme binding site (Figures 3 and 4, Supporting Information). Interestingly, despite this 3D similarity, only the Gal rings of the *O*-glycosides (**1**, **4**, and **5**) are bound in a conformation that resembles the postulated oxo-carbenium transition state of glycosidase-mediated hydrolysis.[1−6] Generally speaking, for disaccharides, the distortion from a normal chair conformation at the nonreducing end toward the expected half-chair or sofa conformations for a glycosyl oxo-carbenium ion produces a significant upward shift of the aglycon moiety with respect to the plane defined by C-5, O-5, C-2, and C-3 in the initial $^4C_1$ chair. Several examples of conformational distortion toward sofa, skew-boats, or even boat forms have been recently reported.[22−30] For instance, sofa conformations of the glycon have been observed in crystallographic studies of HEWL mutant lysozyme complexed to oligosaccharide reaction products.[23] Distortion has also been described for a covalent adduct of a mutated T4 lysozyme,[24] and for the complex of a bacterial chitobiase with its substrate.[25] A related binding-induced conformational change has also been observed for a non-hydrolyzable substrate analogue complexed with *Fusarium oxysporum* endoglucanase.[22] It is also necessary to point out that other reported examples have not shown any distortion of substrates or analogues at enzyme binding sites.[31−34]

Why is this behavior different for glycomimetics and glycosides? If deformation is the "natural" expectation, why then are not the chairs of glycomimetics **2** and **3** deformed, rather than the enzyme *just* provoking the rotation around Φ? In principle, we can guess that the origin of the different conformational selection (distortion or rotation) for **1**−**5** is probably in the existence of distinct stereoelectronic barriers for rotation and/or deformation, which in turn depend on the chemical nature of the different compounds. Thus, as a further step, and in order to gain insights on the origin of the different conformational behavior of *O*- versus *C*- and *S*-glycosides, ab initio quantum mechanical calculations (Tables 2−5, Supporting Information) were performed for model compounds (Chart 2) and complemented with other calculations described in the literature.

Chart 2





6	X=O Y=lp Z=lp	**9**	X=O Y=lp Z=lp
6a	X=O Y=H Z=lp	**9a**	X=O Y=H Z=lp
6b	X=O Y=lp Z=H	**9b**	X=O Y=lp Z=H
7	X=C Y=H Z=H	**10**	X=C Y=H Z=H
8	X=S Y=lp Z=lp	**11**	X=S Y=lp Z=lp
8a	X=S Y=H Z=lp	**11a**	X=S Y=H Z=lp
8b	X=S Y=lp Z=H	**11b**	X=S Y=lp Z=H

lp stands for lone pair

It has been reported (HF/6-31G*)[54] that the rotation from syn- to anti-Φ of **6** requires a energy cost (HF/6-31G*) of 2.9 kcal/mol, while that for the 2-ethyl (**7**) and 2-thiomethyl (**8**) analogues is only 1.2−1.3 kcal/mol, respectively. Our B3LYP/6-31G* calculations indicated that the required energy to reach a flattening consistent with the NMR data (C5−O5−C1−C2, −25°) are higher (4.7−5.1 kcal/mol) for the three *O*-, *C*-, and *S*-models, **6**−**8**. At this point, and on this basis, no easy explanation could be given to justify the experimental observations on theoretical grounds, since the cost of the deformation is more than 2.0 kcal/mol higher than that required to rotate around Φ, even considering the exo-anomeric contribution.[55] However, one additional point has to be taken into consideration. For retaining glycosidase enzymes, such as *E. coli* β-galactosidase, and on the way toward the oxocarbonium-like transition state, one of the proposed mechanisms involves protonation of the glycosidic oxygen, prior to the nucleophilic attack.[1−6] Indeed, theoretical studies on model compounds have shown that interglycosidic oxygen protonation facilitates precisely the flattening of the chair.[55] Therefore, ab initio calculations (B3LYP/6-31G*) were carried out, with protonation of either lone pair at the interglycosidic atom in models **6ab** and **8ab**. The data (Supporting Information) showed that the relative energy cost for deformation of the protonated models were significantly smaller than for that of **7**, for which no protonation is possible (i.e., $\Delta E = 3.7$ kcal/mol for **6a** versus **7** (see Table 3, Supporting Information). Moreover, it was 1.5 kcal/mol smaller than the energy required for rotating **6** around Φ. In contrast, for **7** and **8**, the required energies for flattening are higher than those required for Φ-rotation (see Table 3, Supporting Information). Additional calculations were carried out for the corresponding 3-hydroxypyranes **9**−**11**. It was again observed that the energy required for the deformation of the $^4C_1$ of **9** (4.1 kcal/mol) is smaller than that required for the deformation of the $^4C_1$ of the *C*- (**10**, 5.2 kcal/mol) and *S*-analogues (**11**, 4.9 kcal/mol). The reverse situation occurs when rotation around Φ is considered ($\Delta G_{rotation} = 2.1$ kcal/mol for **9** versus 0.9 and 1.0 kcal/mol for **10** and **11**). Protonation of the hydroxylated model glycosides (**9a,b**) additionally favored the $^4C_1$ deformation. (Tables 3−5, Supporting Information).

Therefore, under these theoretical calculations, rotation around Φ is favored for *C*- and *S*-glycosides (**7**, **8**, **10**, and **11**), while deformation of the chair is favored for *O*-glycosides **6** and **9**. These results now agree with the NMR experimental observations for **1**−**5**, and permit us to explain, on the basis of the stereoelectronic features which depend on the chemical nature of the ligand, the distinct experimentally observed conformational selection of *O*-, *C*-, and *S*-glycosyl compounds by *E. coli* β-galactosidase.

It is noteworthy that a similar 3D shape is obtained by the two distinct conformational variations, either chair distortion or rotation around Φ (Figure 3). Thus, the answer to the questions posed above may be as follows: *E. coli* binding site architecture seems to be unable to accommodate ground-state syn-Φ/syn-Ψ disaccharide conformations. Thus, for both glycosides (such as **1**, **4**, and **5**) and glycomimetics (such as **2** and **3**), distortion always has to take place. For *C*- and *S*-glycosides, with absent or reduced exo-anomeric effect, the distortion is easily accomplished by rotation around Φ. However, for *O*-glycosides, with a strong exo-anomeric effect, and due to the higher stereoelectronic barrier for rotation around Φ, the energy required for such rotation is much higher than that necessary for **2** and **3**. Therefore, the enzyme distorts the chair. Although the enzyme binding site has been designed to accomplish such a distortion, there is no need for that in the case of glycomimetics **2** and **3**. In these cases, the less energy demanding rotation around Φ for these compounds gives rise to a similar 3D shape of the glycomimetics in the enzyme binding site (Figure 3).

The case presented herein represents a key example of the different chemical behavior of glycosides and their *C*- and *S*-glycosyl analogues, now at the molecular recognition level. Nevertheless, the use of the *C*- and *S*-glycomimetic compounds has pointed out that binding of high-energy conformers does occur. Although the observed distortion has a different nature, it is evident that these or related glycosyl analogues serve as key probes to explore molecular features of carbohydrate-processing enzymes.

We have focused herein on the conformational, chemical, and stereoelectronic implications of the substitution of the glycosides by glycomimetics to inhibit a model enzyme. The derivation of the precise catalytic mechanism of this enzyme is still far from being unequivocally deduced. The use of the information gathered here, together with that derived from additional biochemical experiments, is currently underway, to clarify the mechanism of this enzyme.

Methods

Source of the Enzyme. The engineered enzyme[19] was obtained by overproduction of a fusion protein of galactosidase and the *C*-terminal choline binding domain (C-LYTA) of the amidase LYTA from *Streptococcus pneumoniae* coded in plasmid pEG40. The protein was purified as described. The concentration of the chimeric protein was estimated by measuring the optical density at 280 nm (0.4 mg/mL). The obtained enzyme was over 95% pure, its activity assayed using ONPG as substrate, and gave a specific activity of 300 mmol min^{-1} of *o*-nitrophenol per milligram of protein at 301 K and pH 7. The obtained K_m for ONPG (0.1 mM) and K_i for IPTG (0.1 mM) were the same as those obtained when the wild-type enzyme was used**.** The E537Q[30] enzyme was handled and purified in a similar manner.

Inhibition Assays. The engineered enzyme (0.05 mg/mL) was incubated in sodium phosphate buffer (50 mM, pH) 7.2) with the substrate ONPG (0.5 mM) in the presence of different concentrations (0, 4, 8, 16, 32 mM) of inhibitor **3**. The reaction was monitored by HPLC. After 5 min of incubation at 310 K, the samples (20 mL) were

(54) Tvaroska, I.; Carver, J. P. *J. Phys. Chem.* **1996**, *100*, 11305.
(55) Andrews, C. W.; Fraser-Reid, B.; Bowen, J. P. *J. Am. Chem. Soc.* **1991**, *113*, 8293.

diluted with 200 mL of cold HPLC elution buffer (acetonitrile 34%, TFA 0.1% in water). The diluted samples were injected in the HPLC system (reverse-phase C18 column, UV detection). The *o*-nitrophenol produced was quantified from the UV absorbance at 320 nm. Kinetic data were adjusted to a competitive inhibition model to obtain the corresponding K_i. Similar inhibition experiments were performed with IPTG.

NMR Experiments. NMR spectra were recorded at 30 °C in D_2O, on a Varian Unity 500 spectrometer. Enzyme samples in sodium phosphate buffer (50 mM, pH 7.2) were exposed to repeated cycles of freeze−drying with D_2O, and transferred to the NMR tube to give a final concentration of 0.037 mM.

For TR-NOE spectra, three ligand/enzyme ratios between 24/1 and 75/1 were employed. No T_2-filter or short spin lock pulse SL ($T_1\rho$-filter) was employed to remove the background protein signals. In fact, due to huge size of the enzyme (more than 4000 amino acids for tetramer), the baseline is almost flat, and there is no need to use the filter schemes, which could lead to a modification of the ligand proton intensities. Nevertheless, TR-ROESY experiments were also carried out to detect and to exclude peaks due to spin diffusion effects. A continuous wave spin lock pulse was used during the 250 ms mixing time. Key NOEs were shown to be direct cross-peaks, since they showed different sign to diagonal peaks. In some cases, these experiments allowed the identification of spin-diffusion effects, since the observed Gal H-1/Gal H-4 and Gal H-1/Gal H-6ab cross-peaks in TR-NOESY changed their sign in TR-ROESY spectra. No attempt was made to perform a quantitative analysis of these spectra, due to the fact that for the high ligand/enzyme ratio, the contribution of the signals of the free ligand was fairly important.

TR-NOESYs were also recorded after adding variable amounts of IPTG (isopropyl α-D-thiogalactopyranoside) to verify the existence of specific binding. In these competitive TRNOE experiments the known inhibitor, IPTG, was added to the NMR tube containing the ligand/enzyme solution. It was observed that slightly above an equimolar ratio between IPTG and either ligand, the cross-peaks corresponding to the ligand changed their sign to positive, while those of IPTG appeared as negative. These observations indicate that both ligands compete for the same binding site, and that the affinity for IPTG is higher than that for the analogues.

Since it is impossible to accurately deduce the position of the protons in the enzyme binding site, only the protons of the ligand were considered for the relaxation matrix calculations, following the protocol employed by London[39] as described.[19] Normalized intensity values were used since they allow correction for spin relaxation effects. The overall correlation time τ_c for the free state was always set to 0.15 ns[40] and the τ_c for the bound state was estimated as 500 ns according to the molecular weight of the enzyme ($\tau_c = 10^{-12}\ W_M$). To fit the experimental TRNOE intensities, exchange-rate constants between 100 and 1000 s^{-1} and external relaxation times ρ^* for the bound state of 0.5, 1, and 2 s were tested. The best agreement was achieved by using $k = 300\ s^{-1}$ and $\rho^* = 1$ s.

The approach used to obtain the distances in the bound state was based on the fact that glycomimetics **2** and **3** are competitive inhibitors of lactose and, therefore, compete for the same binding site as the *O*-glycosides (**1**, **4**, and **5**), as is also demonstrated by the competitive TR-NOE experiments with IPTG. Therefore, unless a conformational change had taken place, the relaxation (environment) of H-1 Gal in the complexes of E537Q with **2** and **3** should be similar to the relaxation of the same H-1 Gal in the complexes of E537Q with **1**, **4**, and **5** (obviously, except for the presence of the CH_2 in **2**, which is accounted for the by relaxation matrix calculations). A similar reasoning may be applied to every proton in the ligands. Thus, the significant relative cross-peak intensity changes of the Gal versus the Glc protons, in **1**, **4**, and **5**, but not in **2** and **3**, should indeed be a consequence of the existence of distinct geometrical or conformational features (Gal H-1/H-3 and H-1/H-5 interproton distances) for the *O*-glycosides (**1**, **4**, and **5**) and their *C*- and *S*-glycomimetics (**2** and **3**).

Ab initio quantum mechanical calculations were carried out with GAUSSIAN 98. The full optimizations of the geometries were performed at the HF/6-31G* and B3LYP/6-31G* levels (which should also be valid for *S*-glycosides), using the gradient optimization routines in the program without any constraints, except for ω = C5−O5−C1−C2, which was kept fixed. Details are given in the Supporting Information. For **6**−**8**, five conformations (ω = −65/−40/−25/−15/0°) were minimized. Three geometries were considered for **9**−**11** (ω = 65/−25/0°). Docking of the NMR conformations within the enzyme binding pocket was performed with AMBER, using the X-ray structure of the enzyme.[27,28] The Gal moiety of **1**−**5** was superimposed onto the corresponding geometry of the previously optimized WT/**2** complex.[19] Energy minimizations of the complex were performed by using the seven hydrogen bonds established between Gal and the protein as distance restraints. The obtained geometry of the complex showed that the NMR-derived distorted conformations of **1**, **4**, and **5** perfectly fit within the binding site (Figure 4, Supporting Information).

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Supporting Information Available: Schematic view of the main three low-energy minima of lactose, showing the key interresidue distances that correspond to NOEs, view of the galactopyranose distorted chair indicating the corresponding interproton distances; sections of the TR-NOESY spectra of compounds **1**−**6** in the presence of *E. coli* β-galactosidase E537Q (molar ratios ranging from 24 to 46:1, mixing times ranging from 75 to 150 ms), with relevant cross-peaks indicated; a schematic view of the complex between **1** and *E. coli* β-galactosidase after docking experiments with the NMR-derived conformation of **1** and X-ray structure of the enzyme; and tables and details of the quantum mechanical calculations (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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