NMR studies of the conformation of thiocellobiose bound to a β-glucosidase from *Streptomyces* sp.

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Abstract The conformation of 4-thiocellobiose bound to β-glucosidase from *Streptomyces* sp. has been studied by ^{1}H-NMR transferred nuclear Overhauser effect spectroscopy (TR-NOE). Thiocellobiose behaves as an inhibitor of this glucosidase when cellobiose is used as substrate. NOE measurements and molecular mechanics calculations have also been performed to estimate the probability distribution of conformers of thiocellobiose when free in solution. Experimental data show that, in contrast with the natural O-analogue, thiocellobiose presents three conformational families in the free state, namely *syn*, *anti*-Ψ and *anti*-Φ, whilst only one of them (*syn*) is recognized by the enzyme.

Key words: Transferred NOE spectroscopy; Molecular mechanics calculation; Thiocellobiose; β-glucosidase; *Streptomyces* sp.

1. Introduction

The recognition events among biomolecules are strongly dependent on the three-dimensional shape of the interacting species. The study, at the atomic level, of protein-carbohydrate interactions has become a topic of interest during the last few years [1]. When carbohydrates interact with other biomolecules, it is important to consider that the accessible conformational space around the glycosidic linkages can confer significant flexibility to oligosaccharide structures that can be translated into adaptability within the receptor binding site [2]. Several 3D structures of protein-oligosaccharide complexes have been determined by X-ray crystallography [1–4] and NMR [2,5]. In this context, transferred nuclear Overhauser experiments have been used to obtain information regarding the bound conformation of an oligosaccharide within a protein binding site, which can be compared to that existing in solution [6,7]. This approach is currently employed to study oligosaccharide conformations in several lectin-carbohydrate complexes. Interestingly, these studies have shown that the bound conformation may be either similar to the major one existing in solution or strikingly different to that one [2,8,9].

In the case of oligosaccharide-transforming enzymes, the corresponding interaction between the carbohydrate and the protein is a dynamic process. The substrate is transformed into products and significant structural changes on the substrate molecule are expected to occur along the reaction pathway even at the initial recognition step. In this context, glycosidase enzymes (those that hydrolyze glycosides and oligo- and polysaccharides) have been classified in more than sixty families in base of their sequence homology [10]. The members within a family, along with homology, share similar mechanistic and structural characteristics that are different to those of other glycosidase families [4,11]. At this time there is not much information on how the oligosaccharide substrates are recognized by their appropriate glycosidase. Within this class of enzymes, with the exception of some special cases [12], neither X-ray crystallography nor NMR spectroscopy can be applied directly to analyze substrate-enzyme complexes since the substrate is quickly transformed into products. However, it is possible to extract relevant information from the study of enzyme-inhibitor complexes complemented with some sort of molecular modeling. In fact, several structures of glycosidase-inhibitor or glycosidase-product complexes have been resolved [3,13] and some conformational changes on the saccharide substrate have been predicted to take place upon enzyme binding [14]. This strategy can also be approached using TR-NOE experiments and in fact it has been recently applied in our laboratory to the study of *Escherichia coli* β-galactosidase when complexed with C-lactose [15], a substrate analogue resistant to hydrolysis.

Here we report on the determination of the bound conformation of thiocellobiose, an analogue of cellobiose, within the active site of a β-glucosidase (Bgl3) from *Streptomyces* sp. [16]. The Bgl3 enzyme is a retaining glycosidase showing an exo-like action pattern by releasing glucose units from the non-reducing end of cellooligosaccharides, and it has been classified into the family 1 glycosyl hydrolases [10], upon sequence similarity analysis. TR-NOE experiments have been performed with this aim. Thiosaccharides have been reported to be competitive inhibitors of glycosidases, in accordance with the expected structural resemblance of the corresponding saccharide substrate [17]. In fact, the structure of a thiosaccharide complexed with a glycosidase has been recently solved and used for the study of substrate binding and catalysis [14]. Conformational studies of thiosaccharides are scarce [18–20], and as a first step, it has been necessary to analyse the conformational behaviour of thiocellobiose in solution in order to be compared to that adopted by its O-analogue (cellobiose) [21,22] and to the target glucosidase-bound conformation.

2. Materials and methods

Thiocellobiose was purchased from Toronto Research Chemicals Inc.

Recombinant Bgl3 glucosidase was obtained from cultures of *E. coli* BL21(DE3) carrying the plasmid pET21-HBG3, a pET-21d(+) (Novagen) derivative in which a His-Tag coding sequence was fused to the

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start codon (GTG) of Bgl3 reading frame yielding a protein with an extended N-terminal formed by the sequence MHHHHHHGIH and a deduced molecular mass of 53.6 kDa. For protein purification cell-free extracts of the recombinant strain were obtained and applied onto a 5 ml HiTrap Chelating Sepharose (Pharmacia) column previously charged with Ni^{2+} as a metal ion. Cell extract preparation and chromatography were carried out essentially according to the manufacturer's specifications. Bgl3-containing fractions were pooled, concentrated and desalted by ultrafiltration at 4°C using 50 mM sodium phosphate, pH 7.0, as desalting buffer. Following this procedure an homogeneous enzyme sample was obtained, as judged by SDS-PAGE [23] and Coomassie brilliant blue R-250 staining. Protein concentration was determined by the dye binding method of Bradford [24], using BSA as standard. The pure enzyme was kept at 4°C for short periods or stored at −20°C in the presence of 45% glycerol for prolonged periods.

2.0.1. K_m measurement. Solutions of varying concentration of cellobiose (0–90 mM) were incubated with the β-glucosidase from *Streptomyces* sp. (8 μg/ml), in sodium phosphate buffer 50 mM (50 μl, pH = 6.5) at 37°C for 30 min. The release of glucose from cellobiose was determined using the glucose reagent Trinder (SIGMA): following the manufacturer's protocol, after 10 min incubation with the reagent, the absorbance at 505 nm was measured. The intensity of the color produced is directly proportional to the glucose concentration in the sample. Data were fitted to a Michaelis Menten curve, giving a K_m of 1.4 mM.

2.0.2. Inhibition assay. The enzyme (16 μg/ml) was incubated in sodium phosphate buffer (50 mM, pH = 6.5) at 37°C for 30 min, with cellobiose (1 mM) as substrate, and different concentrations (0–12 mM) of inhibitor (thiocellobiose). The release of glucose from cellobiose was determined as before. Kinetic data were adjusted to a competitive inhibition model, giving a K_i of 6.0 mM.

2.1. Conformational calculations. Molecular mechanics

Glycosidic torsion angles are defined as ϕ H-1′-C-1′-S-C-4 and ψ C-1′-S-C-4-H-4 for thiocellobiose. Relaxed (ϕ, ψ) potential energy maps were calculated using MM2* and AMBER* force fields as integrated in MACROMODEL 4.5 [25], with dielectric constant ε = 80.

All calculations were made for the β anomer of thiocellobiose, assuming that there is not a great conformational difference between α and β reducing end anomers for 1→4 linked disaccharides [21]. Four initial geometries were considered, gggg, gggt, gtgg and gtgt, obtained by combining the positions gg and gt for the primary alcohol group of both glucose moieties. Gauche-gauche and gauche-trans rotamers are defined by the ϖ torsion angle (O5-C5-C6-O6), as −60 or +60°, respectively. The first two characters correspond to the non-reducing glucose moiety, and the other two to the reducing one. The starting positions for the secondary hydroxyl groups were clockwise for both residues. Four relaxed energy maps were obtained following a similar protocol to that described previously [26]. Adiabatic surfaces were built, and the probability distributions calculated for each ϕ, ψ point according to a Boltzmann function.

2.2. NMR experiments

NMR spectra of thiocellobiose were recorded at 299 K in D_2O, using Varian Inova 400 mHz and Varian Unity 500 mHz spectrometers. Proton chemical shifts were referenced to residual HDO at δ 4.76. COSY and TOCSY spectra permitted the assignment of the proton spin systems. 2D NOESY spectra for the free disaccharide were acquired at 700 and 900 ms.

2.3. NOE calculations

NOESY spectra were simulated according to a complete relaxation matrix approach, following the protocol previously described [26], using two different mixing times (700 and 900 ms). The spectra were simulated from the average distances $\langle r^{-6}\rangle_{kl}$ calculated from the relaxed energy maps at 299 K. Isotropic motion and external relaxation of 0.1 s^{-1} were assumed. A τ_c of 55 ps was used to obtained the best match between experimental and calculated NOEs for the H-2/H-4 proton pair.

All the NOE calculations were automatically performed by a home made program, available from the authors upon request [26].

2.4. TR-NOESY experiments

β-glucosidase samples were concentrated after repeated cycles of exchange with 50 mM deuterated sodium phosphate containing NaCl (100 mM), in microconcentrators (Centricon p10), and transferred to the NMR tube to give a final pH of 6.5, uncorrected for isotope effects. The final concentration of the enzyme in the NMR tube was 0.1 mM. The regular NOESY sequence was used with mixing times of 250, 350 and 450 ms for a 17:1 molar ratio of thiocellobiose/enzyme at 299 and 303 K. Line broadening of the sugar protons was monitored after the addition of the ligand in order to detect binding.

3. Results and discussion

3.1. Conformational analysis

3.1.1. Molecular mechanics calculations. The structure of 4-thiocellobiose with the atomic numbering is shown in Fig. 1. Torsional angles around the glycosidic linkage are defined as ϕ H-1′-C-1′-S-C-4 and ψ C-1′-S-C-4-H-4. Fig. 2 shows the adiabatic surfaces built from the respective relaxed energy

Table 1
Steric energy values and populations of the low energy regions of thiocellobiose

Min	AMBER			MM2		
	ϕ, ψ	Pop. (%)	E (kJ/mol)	ϕ, ψ	Pop. (%)	E (kJ/mol)
syn	55.1, −11.7	29	40.52	56.8, −7.6	43	94.70
anti-ψ	45.0, 175.5	54	36.73	34.0, 171.3	29	93.52
anti-ϕ	172.8, −2.4	15	38.23	171.6, 1.7	26	93.27

Table 2
Experimental and calculated normalized NOESY intensities (%) for thiocellobiose at 26°C in D_2O solution, at 400 mHz

Proton	Mixing time 700 ms			Mixing time 900 ms		
	Exp	AMBER	MM2	Exp	AMBER	MM2
H-2-H-1	1.3	1.1	1.0	1.6	1.4	1.2
H-2-H-4	3.3	3.8	3.2	4.8	4.8	4.0
H-3-H-1′	2.9	3.8	2.3	3.4	4.8	2.8
H-3-H-5	2.6	3.2	2.6	4.0	4.0	3.2
H-3-H-2	1.9	1.3	1.2	2.0	1.7	1.5
H-3-H-4	1.0	1.2	1.0	1.4	1.6	1.3
H-4-H-1′	2.7	1.4	1.9	3.6	1.7	2.4
H-2′-H-4	n.d.	1.0	1.7	0.7	1.2	2.2

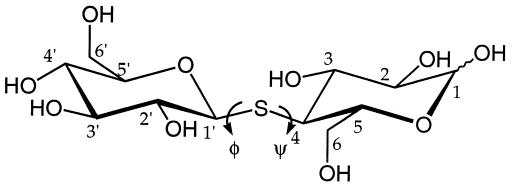

Fig. 1. View of thiocellobiose (1) showing the atomic numbering and the interglycosidic torsional angles (ϕ, ψ).

maps for MM2* and AMBER* force fields, using ε = 80, with the isoenergy contours drawn every 2 kcal/mol. Both force fields predict three low energy regions, similar to that obtained for the natural compound, cellobiose, using the regular MM3 force field at ε = 4 [21]. The geometries of the three minima are very similar for both force fields, with minor differences around the glycosidic linkages (Table 1, Fig. 3), and are in accordance with the exo-anomeric effect [27]. They predict, however, different global minima, and rather distinct population distributions. Using MM2*, the most populated region is that corresponding to the central minimum (*syn*, ϕ, ψ = 56.8, −7.6) although the global minimum is the *anti*-Φ conformer (ϕ, ψ = 171.6, 1.7). AMBER* locates the most populated region around the global minimum, in this case the so-called *anti*-Ψ conformer [28] (ϕ, ψ = 45.0, 175.5).

There are proton-proton short distances which give characteristic NOEs which are exclusive for each one of the three different regions of the conformational map. Consequently, these NOE intensities will be sensitive to their respective populations. For thiocellobiose, these exclusive NOEs are H-4-H-1′, H-3-H-1′ and H-2′-H-4 for the *syn*, *anti*-Ψ and *anti*-Φ conformations, respectively. Therefore, the existence of the different conformational families, in solution or in the bound state, could be detected by the presence of these NOEs. Fig. 4 shows the relevant interresidue proton distances for 1, superimposed on the probability distribution maps.

3.1.2. NMR results. Measurements of nuclear Overhauser enhancements were made and subsequently compared to the previous calculations. The important problem of overlapping among H-3, H-4 and H-5 nuclei, normally present in cellobiose and lactose related disaccharides, is partly solved here due to the nuclear shielding of H-4 produced by the presence of the sulfur atom. Experimental NOEs, compared to those calculated are collected in Table 2. MM2* and AMBER* force fields give a H-4-H-1′ NOE smaller than the experimental one, which probably means that the actual population around minimum *syn* is larger than that calculated, 43% (MM2*) and 29% (AMBER*). The H-3-H-1′ intensity is

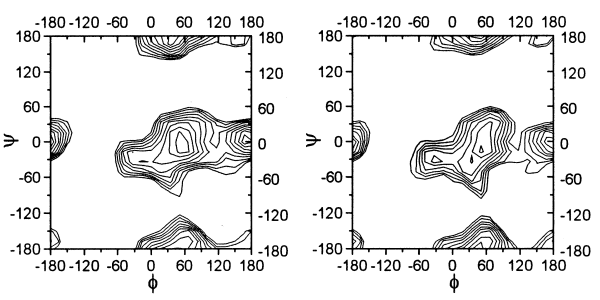

Fig. 2. Comparison of the adiabatic maps calculated by using MM2* (left) and AMBER* (right) force fields.

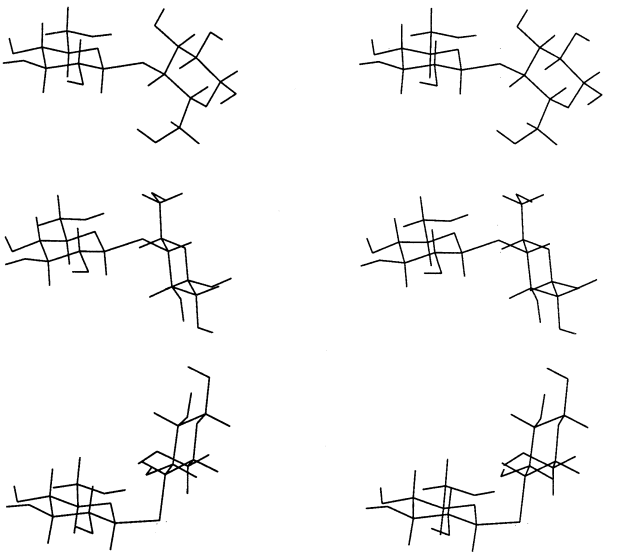

Fig. 3. Stereo view of the major minima of 1 calculated by AMBER*. From top to bottom, conformers *syn*, *anti*-ψ and *anti*-ϕ.

well reproduced by MM2* whereas AMBER* predicts a larger value for it. On the other hand, AMBER* predicts correctly the H-2′-H-4 NOE and MM2* overestimates it. Taking into account all these data, the experimental NOEs could be explained by a population distribution of about 55%, 30% and 15% of *syn*, *anti*-Ψ and *anti*-Φ conformers, respectively. On the other hand, the conformation of the natural compound in solution, cellobiose, may be described in more than 95% by one minimum energy region around the *syn* conformation that is also found in the crystal structures of β-cellobiose, methyl β-cellobioside and cellulose I and II [21,22].

3.2. TR-NOESY experiments

Transferred nuclear Overhauser enhancement (TR-NOE) experiments have been proved to be a useful tool to determine the conformation of the protein-bound ligands. It is being largely used for the study of oligosaccharides bound to lectins and antibodies. In general, it can be applied to those ligands which exchange at relative fast rate between the free and the bound state. The conditions are $K_{-1} \gg \sigma^B$ and $p_b\sigma^B > p_f\sigma^F$, where K_{-1} is the off-rate constant, σ^B and σ^F are the cross relaxation rates for the bound and free ligand, and p_b and p_f are the fractions of bound and free ligand, respectively. NOEs between bound oligosaccharide protons appear strong and negative, as expected for molecules in the slow motion regime.

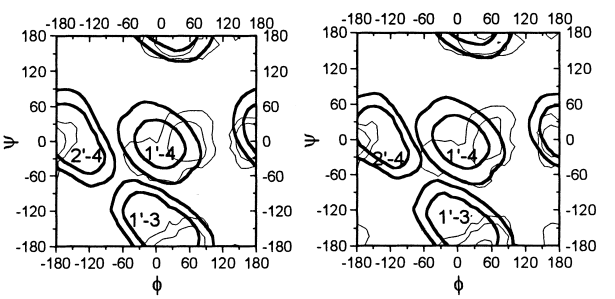

Fig. 4. Superimposition of the relevant proton-proton short distances on the probability maps determined for 1 using MM2* (left) and AMBER* (right) force fields.

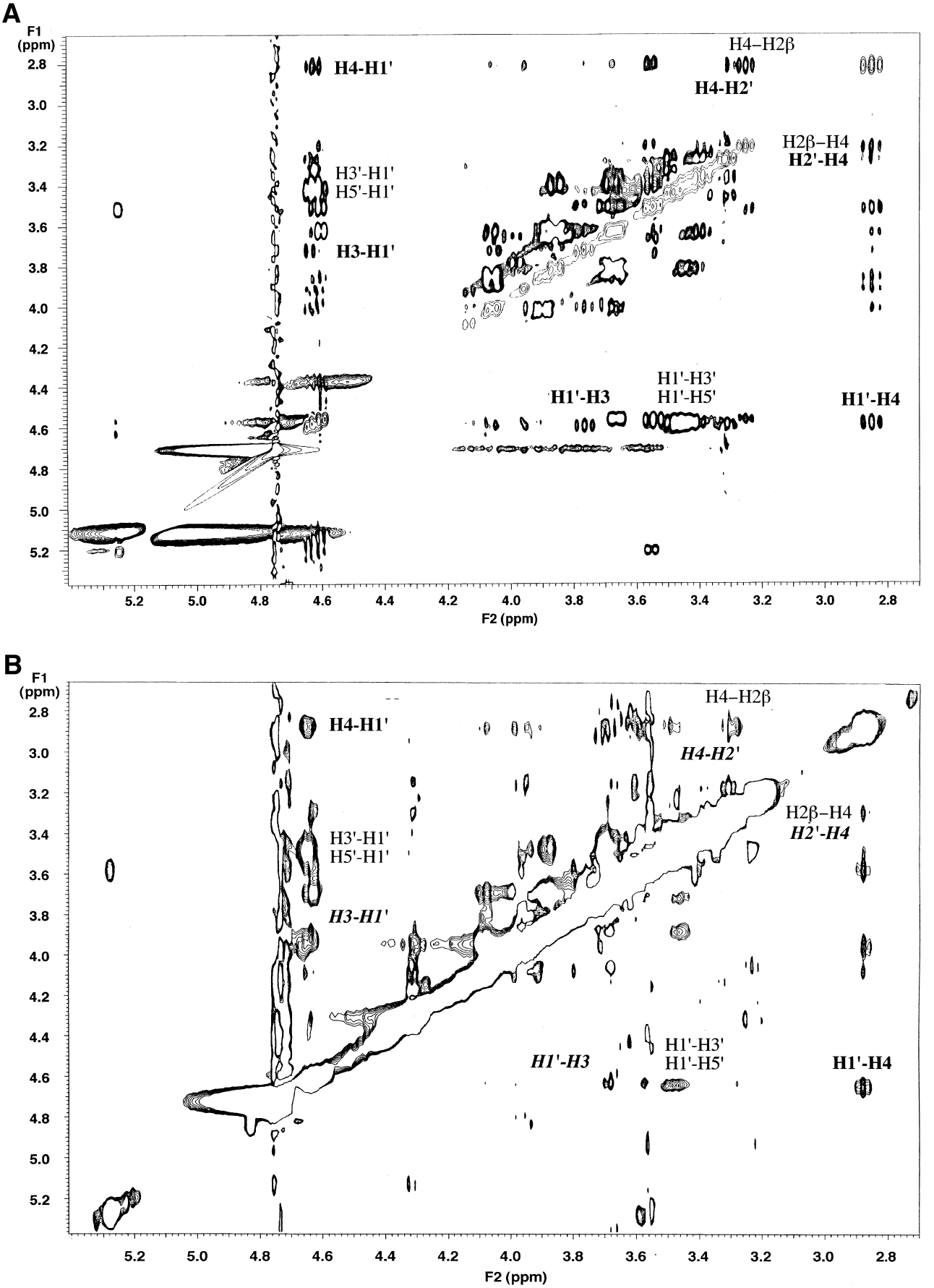

Fig. 5. NOESY ^{1}H-NMR spectra of 1 in the absence (A) and in the presence (B) of β-glucosidase, acquired at 900 and 250 ms respectively. Relevant NOEs are labeled: Exclusive NOEs are indicated in bold. Labels in italics indicate absence of NOE.

The ability of thiocellobiose for the inhibition of β-glucosidase was firstly tested through kinetic measurements (see Section 2), in order to have an idea of its association capacity. The measured K_i was 6.0 mM for a competitive inhibition model. TR-NOESY experiments recorded at different mixing times showed important differences compared to those obtained for the free sugar (Fig. 5). The H-3-H-1′ and H-2′-H-4 NOEs, that characterize the *anti*-Ψ and *anti*-Φ conformers of thiocellobiose in solution, are not present in the spectrum of the complex. The only exclusive NOE observed is the H-4-H-1′ NOE, indicating that the *syn* conformation is the only one recognized by the enzyme. Therefore, β-glucosidase from *Streptomyces* sp., selects only one conformation of those present in free solution. In fact, the bound conformation corresponds to the major conformation of thiocellobiose in solution, which also turns to be the global minimum of the natural compound, cellobiose. The interaction between thiocellobiose and this β-glucosidase does not force the inhibitor molecule to make significant conformational changes. In addition, deformations of the pyrenoid rings are not observed either, since the Gal H-1/H-5 and H-1/H-3 NOE cross peaks which define the $^4C_1$ chair conformation are fairly strong. However, it has been reported using X-ray crystallography that *Fusarium Oxysporum* endoglucanase I (a retaining glycosidase from family 7) complexed with a thiooligosaccharide substrate analogue, provokes a conformational variation of the pyrenoid ring at the point of cleavage. In fact, the regular $^4C_1$ chair is distorted towards a twist boat form that positions the leaving group in a quasi-axial orientation, thus facilitating the breaking of the glycosidic bond [14]. A similar conformational change on the substrate molecule upon binding has been proposed for myrosinase, a family 1 enzyme that is able to hydrolyse thioglycosides. In this case, the conclusions were based on the structure solved for a covalent glycosil-enzyme reaction intermediate [29] complemented with molecular modeling. Although the recognition of glycosides in distorted conformations (half chair or skew boat) was early proposed to facilitate the action of glycosidases [30], still it is an open question up to what extent such deformation is a necessary initial step in the reaction pathway [31]. In fact, other different conformational changes of the glycoside have been proposed to occur upon substrate binding to glycosidases. For example, for a family 8 glucanase [13], a conformation of the substrate molecule with Φ close to 180° around the scissile glycosidic bond has been invoked to exist, based upon the structure of the enzyme product. A similar torsional angle has been observed by using TR-NOE experiments for C-lactose, a non-hydrolyzable substrate analogue [15], in its complex with *E. coli* β-galactosidase (from family 2).

In conclusion, *Streptomyces* β-glucosidase (family 1), differs of the other mentioned glycosidases since it selects a conformation of thiocellobiose which is similar to the most populated one of the natural substrate in solution. The selection by a protein of only one conformer of those present in the free state for oligosaccharide molecules has also been reported by us and by others. In fact, we have shown that in some cases the protein binding site provides a perfect arrangement to accommodate only one of these three conformational regions. On the other hand, the entropy penalty for the recognition of only one conformer is probably high and its implications for enzyme inhibitor design should also be considered. The inhibition constant estimated for thiocellobiose against the β-glucosidase (K_i = 6.0 mM) was higher than the K_m obtained for cellobiose (1.4 mM), under the same experimental conditions, thus indicating a lower affinity for the thioanalogue than for the substrate. When the 3D structure of this β-glucosidase becomes available (the X-ray determination of the structure is actually in progress, J.A. Perez-Pons, personal communication) it will be possible to perform molecular modeling studies of the inhibitor within the active site of the enzyme, as it has been done for other enzymes [32,33,15]. This modeling will give some insights whether or not the substrate analogue, in the observed *syn* conformation, can be easily accommodated at the enzyme binding site, thus simulating the binding mode of the natural substrate. Further studies with other glycosidase enzymes and other non-hydrolyzable analogues are in course in order to discriminate whether there is a general pattern of enzyme substrate recognition among the members of a glycosidase family or if that interaction depends mostly on the particular substrate structure or on the enzyme structure details and mechanistic particularities.

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