Crystallographic observation of a covalent catalytic intermediate in a β-glycosidase

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The three-dimensional structure of a catalytically competent glycosyl-enzyme intermediate of a retaining β-1,4-glycanase has been determined at a resolution of 1.8 Å by X-ray diffraction. A fluorinated slow substrate forms an α-D-glycopyranosyl linkage to one of the two invariant carboxylates, Glu 233, as supported in solution by ^{19}F-NMR studies. The resulting ester linkage is coplanar with the cyclic oxygen of the proximal saccharide and is inferred to form a strong hydrogen bond with the 2-hydroxyl of that saccharide unit in natural substrates. The active-site architecture of this covalent intermediate gives insights into both the classical double-displacement catalytic mechanism and the basis for the enzyme's specificity.

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The nature of the catalytic intermediate of retaining β-glycosidases has been a matter of controversy for many years. Koshland first suggested a double-displacement mechanism for this class of enzymes wherein a covalent glycosyl-enzyme intermediate is formed upon cleavage of the glycosidic bond[1]. The transition states for the formation and hydrolysis of that intermediate are known to have substantial oxocarbenium ion character[2]. On the basis of pioneering crystallographic studies of hen egg-white lysozyme, Phillips and coworkers suggested a variation of this mechanism in which the intermediate is an ion pair of significant lifetime[3]. Support for this ion-pair intermediate has been obtained from a number of studies, ranging from the early determination of the structure of a tri-(*N*-acetylglucosaminyl lactone)-lysozyme complex[4] to more recent refined high-resolution crystal structures with bound oligosaccharides[5–7]. The structures of these complexes with non-covalently bound glycosides, however, do not explicitly eliminate the possibility of a covalent catalytic intermediate for β-glycosidases.

Evidence from β-glycosidases other than lysozyme has generally favoured a covalent intermediate[2,8]. In particular, isotope effects measured for each of the two catalytic steps, formation and hydrolysis of the intermediate, are inconsistent with an ion pair intermediate. Such α-secondary deuterium kinetic isotope effects provide a measure of the hybridization of the anomeric carbon C-1 of the substrate. Existence of an ion-pair intermediate would require an inverse isotope effect ($k_H/k_D<1$) for the second catalytic step. However, the observed value of $k_H/k_D=1.11$ for this catalytic step is consistent only with a tetrahedral geometry at the C-1 of the intermediate[2,9]. Additional evidence for the nature of the catalytic intermediate has come from mechanism-based inhibitors, such as 2-deoxy-2-fluoroglycosides with relatively reactive leaving groups[10–12], by which a covalent intermediate species accumulates, a process known as trapping. These minimally modified substrates are designed to undergo the first step of catalysis, the formation of a glycosyl-enzyme intermediate, but to hydrolyse very slowly, up to 10^6–10^7-fold slower than the parent sugar[12]. This reduced rate has two sources; one is inductive destabilization of the positively charged oxocarbenium ion-like transition states by the electronegative fluorine, the other is the loss of crucial transition state hydrogen bonding interactions[13,14]. The net result is the accumulation of the covalent intermediate for an extended period of time, as observed both by electrospray ionization mass spectrometry and by ^{19}F-NMR spectroscopy[9–12,15,16]. The covalent intermediate is then turned over at a slower rate, either by hydrolysis or transglycosylation, indicating that this complex is a catalytically competent species[9,16]. Taken together, the above results strongly suggest that trapped fluoroglycosides are representative of the natural glycosyl-enzyme intermediate for retaining β-1,4-glycosidases.

In an attempt to characterize the conformation of such a glycosyl-enzyme species, and to provide insights into interactions at the active site that are crucial to substrate specificity and catalysis, we have determined the crystal structure of a trapped fluorocellobiosyl–enzyme complex for the catalytic domain of the xylanase/glucanase Cex (cex-cd) from *Cellulomonas fimi*. Cex-cd hydrolyses either xylan or cellulose with retention of anomeric configuration[11,17,18]. Diffusion of the mechanism-based inhibitor 2",4"-dinitrophenyl-2-deoxy-2-fluoro-β-cellobioside (2F-DNPC) into crystals of cex-cd leads to the formation of an

enzyme–disaccharide complex and the release of dinitrophenol (Fig. 1, 2). The structure of this complex has been determined to an *R*-factor of 0.21 at 1.8 Å resolution, with good stereochemistry (Table 1). The model includes 312 residues, 194 water molecules and 22 ligand atoms. The main chain of the model makes a reasonable fit with the electron density map as shown in Fig. 3, with a mean real-space correlation of 0.93 against the final electron density map. Low values of correlation agree with corresponding high *B*-factors, suggesting larger mobility in these regions. Conversely, high correlation values, such as for the active site residues, correspond to low *B*-values.

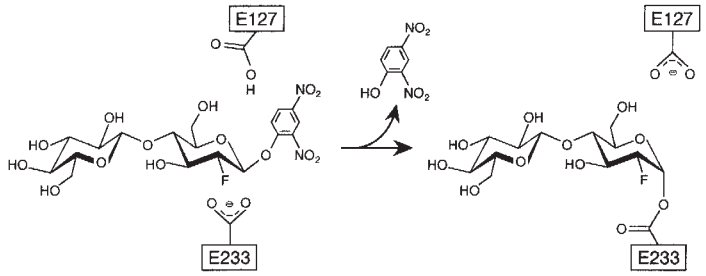

Fig. 1 Cleavage of 2″,4″-dinitrophenyl-2-fluoro-2-deoxy-β-cellobioside (2F-DNPC) by cex-cd releases dinitrophenol and forms a covalent fluorocellobiosyl-enzyme species with Glu 233. In this process, the catalytic nucleophile Glu 233 acts in a concerted manner with the acid/base catalyst Glu 127 (Refs 2,24).

The fluorocellobioside binds covalently by an α-linkage to the *syn* position of the Glu 233 side chain, which in turn is coplanar with the cyclic oxygen of the proximal saccharide. The carbohydrate-binding cleft of homologous xylanases is composed of several subsites[19–21]. In cex-cd, the disaccharide occupies two binding subsites in a deep crevice at the C-terminal end of the $(\beta/\alpha)_8$-barrel motif (Fig.4), without perturbing significantly the conformation of the enzyme. The C-terminal region of the $(\beta/\alpha)_8$-barrel mediates the interactions with the disaccharide by presenting 18 conserved residues into the enzyme's active site, a binding environment that provides a rationale for the substrate specificity of cex-cd.

Table 1 Data collection and refinement statistics

Resolution (Å)	8–1.8	1.95–1.8
Observations	139457	16567
Unique reflections (% completeness)	29464 (99)	5644 (97)
R_{sym} (%)[1]	4.1	28.4
Signal to noise ($<I/\sigma_I>$)	19.7	2.1
Refinement:		
No. reflections ($\|F\|>2\sigma_F$)	24597	4155
R_{factor} (%)[2]	20.8	25.0
No. reflections for free *R*-value	1866	291
free *R*-value (%)	28.3	34.0
Non-hydrogen protein atoms	2396	
main-chains <*B*-factor> (Å^2)	24.2	
side-chains <*B*-factor> (Å^2)	25.9	
Identified water molecules	189	
<*B*-factor> (Å^2)	41.9	
Fluorocellobioside atoms	22	
<*B*-factor> (Å^2)	38.2	
R.m.s. deviation from ideality[3]:		
bond lengths (Å)	0.014	
bond angles (°)	2.0	
improper angles (°)	1.5	
dihedrals (°)	22.4	
Estimated average error in coordinates, Luzzati method (ref. 41) (Å)	0.25	

[1] $R_{sym} = \Sigma|I_i - <I>| / \Sigma I_i$, where I_i is the scaled intensity of the ith measurement and $<I>$ is the mean intensity for that reflection.
[2] $R_{factor} = \Sigma||F_{calc}| - |F_{obs}|| / \Sigma|F_{obs}|$ where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively.
[3] Engh & Huber (ref. 42) stereochemical parameters were used in the refinement.

Stereochemistry of the glycosyl-enzyme attachment

On the basis of a double-displacement catalytic mechanism, it is expected that the covalent intermediate of a retaining β-glycosidase adopts an α-anomeric configuration with a catalytic nucleophile. This catalytic nucleophile for cex-cd has been previously identified as Glu 233 by labelling with the 2-fluorocellobioside and sequencing of the purified glycopeptide[11,22]. In the crystal structure, the C-1 of the proximal saccharide is now confirmed to be tetrahedral and bonded to the conserved residue Glu 233 Oε1 by a covalent linkage of 1.43 Å, in the α-anomeric configuration (Fig. 2). There is no significant element of electron density corresponding to the β configuration. The carbon C-1 of the attached saccharide is found *syn* to the ester group of Glu 233, a preferential location for a nucleophilic attack by a carboxylic acid[23]. In addition, this ester linkage is found to be coplanar with the cyclic O-5 of the attached saccharide (Fig. 5), which may have stereo-electronic implications.

Another conserved acidic residue, Glu 127, which was previously identified as the acid/base catalyst on the basis of kinetic analysis of mutants[24], is located 4.5 Å from C-1 of the substrate and 5.5 Å from the nucleo-phile Oε1. This latter separation agrees with the active sites of other retaining β-1,4-glycosidases, as previously observed[8,25]. The location of the acid/base catalyst is consistent with its role of protonating the glycosidic bond during formation of the glycosyl-enzyme and of deprotonating an incoming water molecule during its hydrolysis.

The nucleophilicity of Glu 233 is likely to be influenced by its immediate environment. In both the complexed (Fig. 5) and the uncomplexed cex-cd, Glu 233 accepts hydrogen bonds from Asn 169 and His 205. The imidazole ring in turn is properly located to hydrogen bond to Asp 235. In the cellobiosyl–enzyme complex, the dyad Asp 235–His 205 is likely to be important in assisting the hydrolysis of the glycosyl–enzyme complex by stabilizing the released carboxylate.

The covalent attachment of the glycosyl adduct to cex-cd in the crystal structure is supported in solution

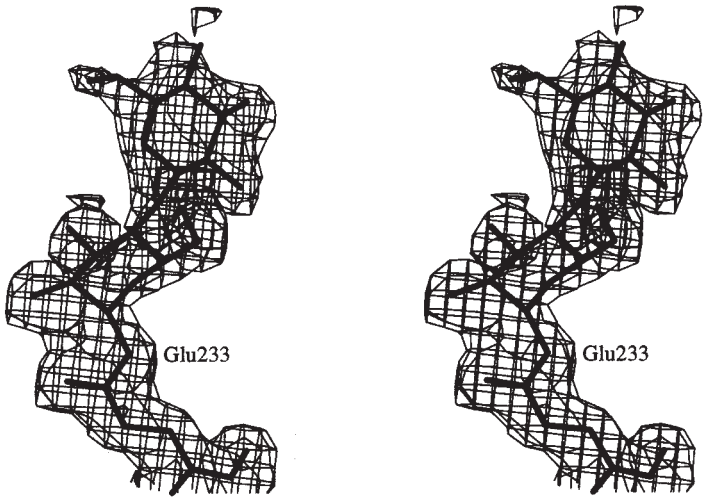

Fig. 2 Electron density map ($3|F_o|-2|F_c|$) shows the fluorocellobioside attached to Glu 233. This map (contour level of 1σ) was generated using phases of the model cex-cd before the incorporation of the disaccharide. The disaccharide was later included with the model and refined. This final model overlays the electron density map shown.

by ^{19}F-NMR spectra (Fig.6). It has been shown in a closely related system[15] that this technique can be used to distinguish α-and β-anomeric configurations of a covalently attached fluorosaccharide. The broad resonance at δ -195.5 p.p.m. in the spectrum of cex-cd with 2F-DNPC (Fig. 6*a*) arises from the covalent fluorocellobiosyl–enzyme complex, the large linewidth indicating the covalent attachment. Formation of the intermediate results in a change of chemical shift of less than 3 p.p.m. relative to 2F-DNPC, which is insufficient to draw conclusions about the stereochemistry of the glycosyl–enzyme linkage. However, ^{19}F-NMR chemical shifts of α- and β-2-fluoromannosides differ by approximately 20 p.p.m., sufficient to reliably identify the anomeric configuration of the covalent species[15]. The corresponding 2-deoxy-2-fluoro-4-O-{β-D-glycopyranosyl}-β-D-mannopyranosyl fluoride (2F-GMF) with an axial fluorine at position C-2 was therefore synthesized and found to be a time-dependent inactivator (k_i= 0.069 min^{-1}, K_i=160 mM, measured as in ref. 9) and thus, to form a covalent glycosyl–enzyme intermediate. The large dissociation constant of this inhibitor necessitated use of a large excess of 2F-GMF in order to achieve saturation. Therefore, the only resonances apparent in this region of the spectrum are those from F-2 of the excess 2F-GMF at δ -224.3 p.p.m. and from F-2 of the product of spontaneous hydrolysis at δ -205.4 p.p.m. (Fig. 6*b*). The chemical shift and coupling constants of this latter species confirm it as the more stable α-anomer, but unfortunately this resonance obscures the one due to the covalently linked glycosyl–enzyme intermediate. However, upon dialysis of the sample the ^{19}F-NMR spectrum reveals a resonance at δ -205.6 p.p.m. due to the covalent glycosyl–enzyme (Fig. 6*c*). This chemical shift is consistent only with an α-anomeric linkage.

Relevance of the substituent at the substrate C-2

The fluorine substituent F-2 of the cellobioside substrate is involved in specific interactions with the enzyme. The fluorine F-2 accepts a hydrogen bond from Asn 126 Nδ2 and is close (3.1 Å) to the nucleophile Glu 233 Oε2 (Fig.5). Studies on cex-cd and related retaining β-glycosidases have shown that interactions with the hydroxyl group OH-2 of the natural substrate contribute 30–40 kJ mol^{-1} to the stabilization of the transition states for glycosyl transfer[13,14,26]. Because deoxygenation inductively stabilizes the oxocarbenium ion-like transition states, thus inherently increasing the reaction rate, the observed decreases in reaction rate must be due to the removal of important transition state binding interactions. The value of 30–40 kJ mol^{-1} therefore represents a minimum estimate of the contribution of these interactions. The source of such strong interactions is therefore of considerable interest. While interactions with Asn 126 likely contribute to this stabilization it seems probable that the strongest interaction would be one with Oε2 of Glu 233. Such a hydrogen bond with a charged acceptor could provide the large interaction energies

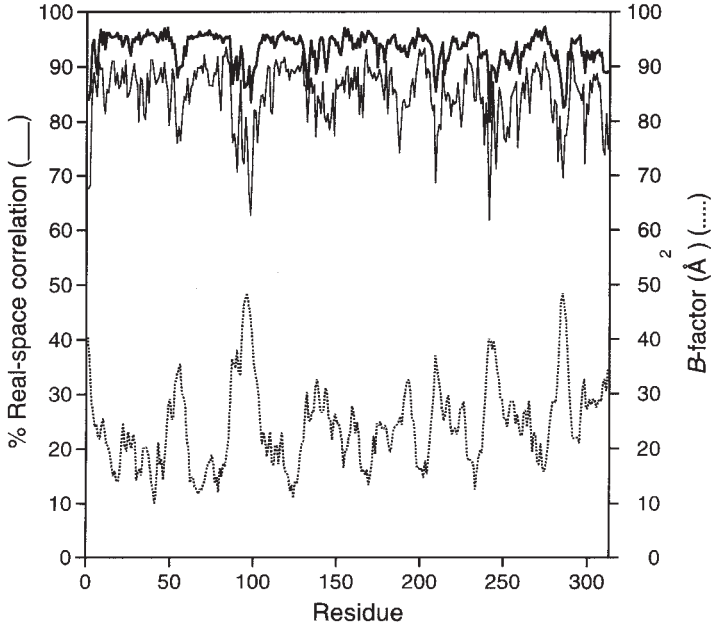

Fig. 3 Main-chain real-space correlation and temperature factor (*B*) profiles. The real-space correlation[36] for the main chain of each residue of the complexed cex-cd model was evaluated against $2|F_o|-|F_c|$ electron density maps, in which phases were derived either from the final model (thick lines) or from simulated-annealing omit maps (thin lines).

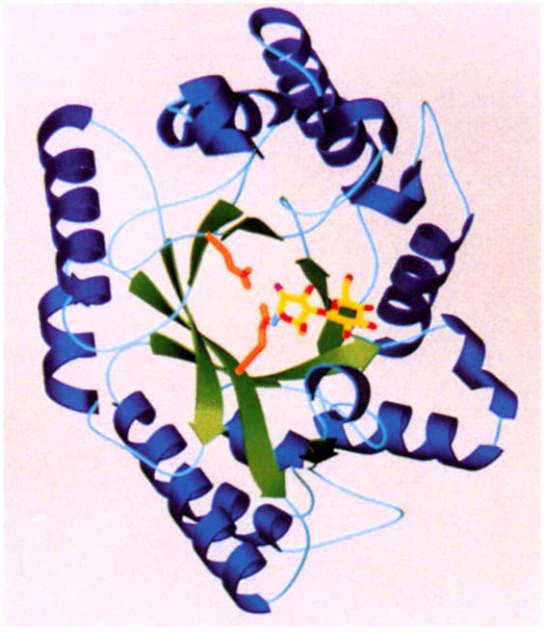

Fig. 4 Fluorocellobioside-binding region in cex-cd. The ligand (red for oxygens, yellow for carbons and magenta for fluorine atom) binds to the nucleophile Glu 233 (orange) in the active site of cex-cd (α-helices in blue and β-strands in green) by a covalent linkage (light blue). The acid/base catalyst Glu 127 (orange) is located 5.5 Å from the nucleophile in this retaining enzyme. This figure was prepared with SETOR[39].

observed, approaching those of the low-barrier hydrogen bonds observed in other systems[27–29]. However, the structure of a good transition state analogue complex will be needed to provide further insights.

Structural basis of the specificity by cex-cd

Cex-cd hydrolyses xylan 40-fold more efficiently than cellulose[9]. Xylan is a β-1,4 linked polymer of D-xylose, a saccharide unit similar to glucose but lacking the hydroxymethyl group on the carbon C-5. To accommodate the C-5-hydroxymethyl group of the distal and proximal saccharides respectively, Gln 87 is now disordered and Trp 281 is rotated by 31° around its CβCγ axis as compared to the uncomplexed structure. These rearrangements would be unnecessary in a xylobiosyl complex, which is consistent with cex-cd's preference for xylobiose over cellobiose at this site.

Cex-cd has been reported to preferentially cleave a cellobiose unit rather than glucose from the non-reducing end of cellulose. This preference is also reflected in the greater activity with aryl cellobioside substrates than arylglucosides[9,11]. In the crystal structure, on binding of fluorocellobioside, cex-cd does not undergo significant conformational changes, as shown by a r.m.s. deviation of 0.2 Å for the Cαs between the complexed and free enzyme. A closer analysis reveals that the proximal saccharide makes hydrogen bonds with Lys 47, His 80 and Asn 126 whereas the distal saccharide significantly contributes through hydrogen bonding with residues Glu 43, Asn 44, Lys 47 and Trp 273 (Fig. 5). One of these residues, the side chain of Lys 47, shows a significant decrease in its temperature factor, from 23.6–15.8 Å^2, which may be explained by its interactions with the ligand. Of the residues 43–47, four are conserved and this sequence forms a loop which joins the β-strand β2 to the α-helix α2a (ref. 25). This loop may play a dominant role in the preference of the enzyme for the release of disaccharides rather than monosaccharides.

Among the water molecules located in the structure, one forms a hydrogen bond to C-4-OH of the distal saccharide, whereas another is found within hydrogen-

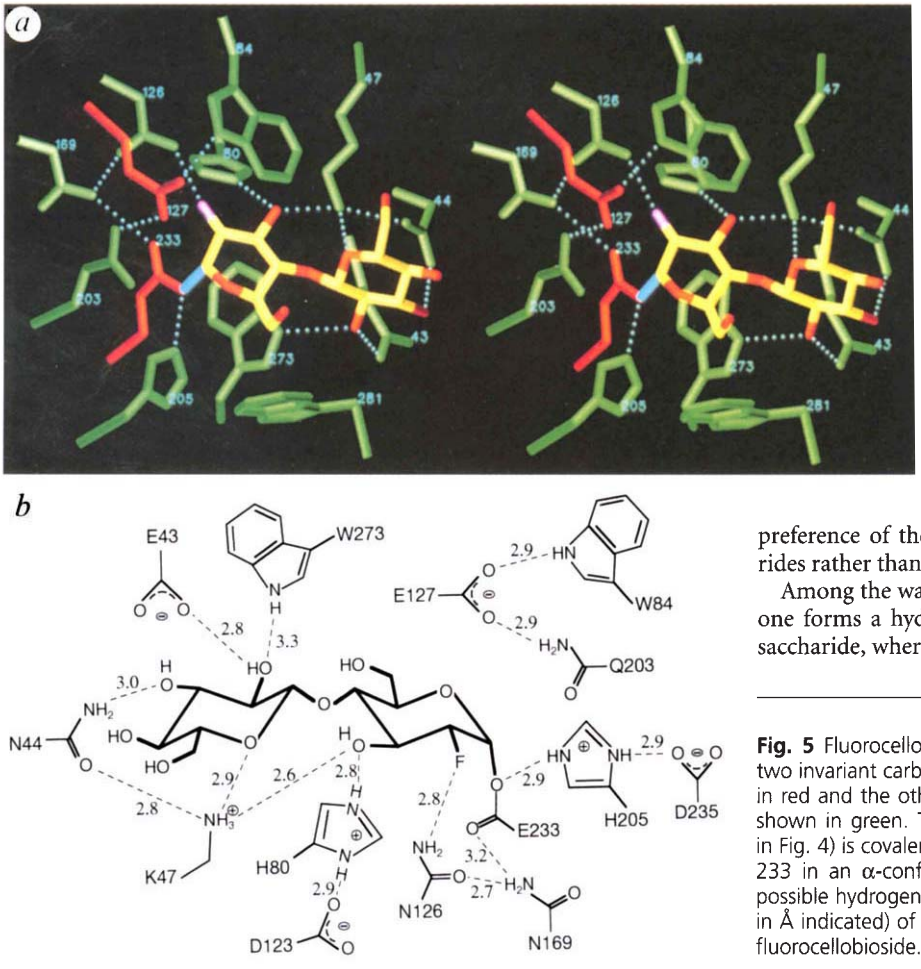

Fig. 5 Fluorocellobioside interactions with cex-cd. *a*, The two invariant carboxylates Glu 127 and Glu 233 are shown in red and the other conserved residues of cex-cd[25,40] are shown in green. The fluorocellobioside (colour scheme as in Fig. 4) is covalently bonded (blue) to the nucleophile Glu 233 in an α-configuration. *b*, Schematic diagram of the possible hydrogen bond network (dashed lines with length in Å indicated) of cex-cd involved in the recognition of the fluorocellobioside.

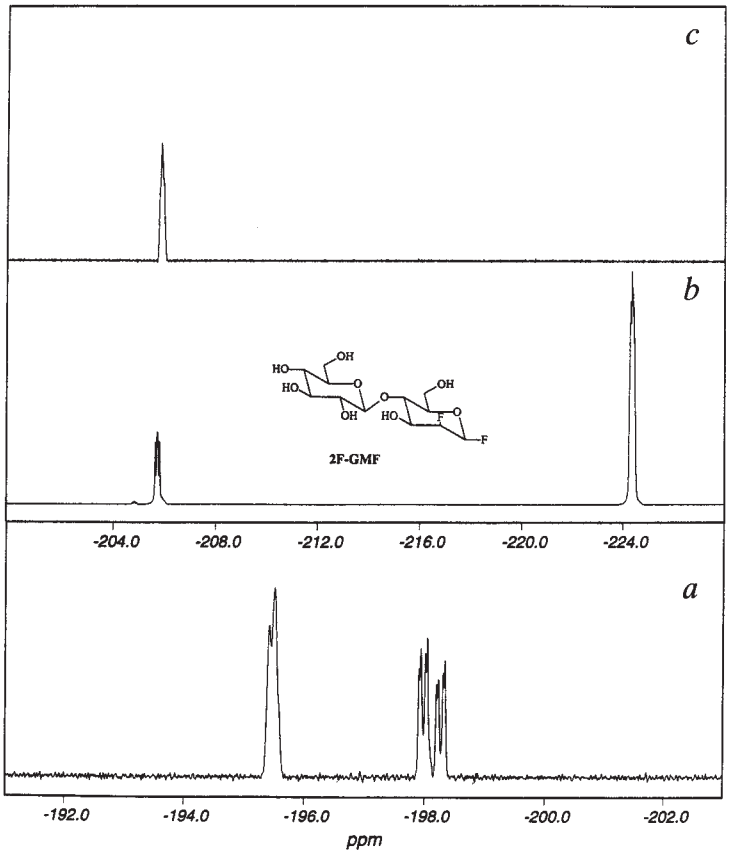

Fig. 6 ^{19}F-NMR spectra of cex-cd with 2-fluoro-disaccharides. *a*, cex-cd (0.2 mM) plus 2F-DNPC (1.0 mM). The peak at δ -195.4 p.p.m. with a broader line width was assigned to the covalent intermediate. The peaks at δ -198.1 p.p.m. and -198.4 p.p.m. are unliganded 2F-DNPC and hydrolysed products respectively. *b*, Cex-cd (0.2 mM) plus 2F-GMF (30 mM). *c*, Same as in (*b*), after dialysis. ^{19}F-NMR spectra were acquired essentially as described previously[15].

bonding distance to both the acid/base catalyst Glu 127 and the active-site residue Gln 203. Although it is 4.0 Å away from the sugar C-1, this latter water molecule is the most likely candidate to be the nucleophile in the second step of catalysis. Whether or not this designated water actually performs the nucleophilic attack on C-1 or forms a hydrogen bond with an incoming nucleophilic water molecule still remains an open question.

Implications

The structures of complexes of other affinity-labelled glycosidases have been reported previously. Examples include adducts with glycosyl epoxide affinity labels of lysozyme[30] and a β-1,3-1,4-glucanase[31]. However, these complexes are not structural analogues of a covalent intermediate since they are not catalytically competent and there is no direct linkage between the enzyme's catalytic nucleophilic side chain and C-1 of the saccharide. In addition, the structure of a T4-lysozyme mutant complexed with a substrate covalently attached at the site of mutation has been determined[32], but that complex is not the natural intermediate in catalysis. The use of fluoroglycosides as mechanism-based slow substrates for structural studies does address directly the stereochemistry of the disaccharide attached to the enzyme's catalytic nucleophile, in addition to allowing a description of a β-1,4-glycosidase in the context of a covalent intermediate. As the glycosyl–enzyme complex is generated in the crystal, the activity of the uncomplexed cex-cd enzyme in the crystalline state is confirmed. Moreover the covalent complex presented here unambiguously identifies two saccharide-binding subsites of the enzyme and their specific carbohydrate-enzyme interactions. In particular, the proximity of the fluorine atom F-2 to Glu 233 Oε2 suggests a rationale for the importance of the hydroxyl group at this position. In addition, this structure provides useful insights into the role of specific interactions in determining the biotechnologically important specificity for xylan *versus* cellulose.

Methods

Formation of the complex. The catalytic domain of recombinant β-glycanase Cex (cex-cd) was crystallized as previously described[33]. A crystal of native cex-cd was soaked for 11 h in 0.5 mM 2″,4″-dinitrophenyl-2-fluoro-2-deoxy-β-cellobioside (2F-DNPC) in a 10 μl drop of the reservoir solution. The drop was suspended over 1ml of 15% polyethyleneglycol 4000 and 100 mM acetate (pH 4.6). An equivalent crystal form was also obtained by crystallization of the complexed enzyme (data not shown). X-ray diffraction data to 1.8 Å were measured on a San Diego Multiwire Systems area detector and then reduced as previously described for the native cex-cd[25]. The data reported here were measured on a single crystal, which belongs to space group $P4_12_12$ with unit cell dimensions a=b=88.17 Å and c=81.10 Å.

Structural analysis and refinement. The position of the cex-cd model[25] was optimized by rigid-body refinement and then refined with X-PLOR (version 3.1)[34]. The simulated annealing protocol was applied to the model, in which the temperature was decreased from 3000–300 K by steps of 25 K, followed by standard positional and individual isotropic B-value refinements. The refinement progress was monitored with the free R value[35]. Model work and analysis was done using the program O[36]. In order to further analyse the model, simulated-annealing omit-maps[37] were computed for the whole polypeptide chain, in which segments of 10–13 residues were omitted prior to rounds of simulated annealing with the temperature decreasing from 1000–300 K. The side chain of residue Gln 87 is disordered and treated as an Ala in the model. After several rounds of model building and positional refinement, water molecules were added. Each peak above 3σ in a $|F_o|-|F_c|$ map was visually examined and assigned as a water molecule if it met the following criteria: the peak centroid was within hydrogenbond distance (2.5–3.5 Å) to at least one non-hydrogen atom of either the protein, the ligand or another water molecule; acceptable hydrogen bond angles; temperature factor B≤70 Å^2; consistent with a peak on $2|F_o|-|F_c|$ and $3|F_o|-2|F_c|$ electron density maps. No bulk solvent correction was used with the model. Following refinement of the resulting model, the disaccharide was incorporated into the model and refined with an occupancy of 0.75. A modified version of the files param3_mod.cho and toph3.cho (X-PLOR) were used for the disaccharide restraints and applied as follows: bond lengths of the ligand were restrained by the weights and distances suggested with the exception of the covalent linkage which was restrained to ideal values of 1.43 Å with the same weight as the rest of the saccharide; restrained weights on angles involving the ligand carbons C-1 and C-2 were reduced to 10% of the weight applied for the remaining of the disaccharide; dihedral angles were released for angles involving the ligand's carbons C-1 and C-2 whereas full

dihedral restraints were applied to the rest of the disaccharide. The resulting refined model contains 2396 non-hydrogen protein atoms, 22 disaccharide atoms and 194 water molecules and has good agreement with geometrical parameters for well determined structures as verified using PROCHECK[38]. The atomic coordinates are being deposited with Protein Data Bank (PDB entry code 1EXP).

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