The binding of β - and γ -cyclodextrins to glycogen phosphorylase b: Kinetic and crystallographic studies

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Abstract

A number of regulatory binding sites of glycogen phosphorylase (GP), such as the catalytic, the inhibitor, and the new allosteric sites are currently under investigation as targets for inhibition of hepatic glycogenolysis under high glucose concentrations; in some cases specific inhibitors are under evaluation in human clinical trials for therapeutic intervention in type 2 diabetes. In an attempt to investigate whether the storage site can be exploited as target for modulating hepatic glucose production, α -, β -, and γ -cyclodextrins were identified as moderate mixed-type competitive inhibitors of GPb (with respect to glycogen) with K_i values of 47.1, 14.1, and 7.4 mM, respectively. To elucidate the structural basis of inhibition, we determined the structure of GPb complexed with β - and γ -cyclodextrins at 1.94 Å and 2.3 Å resolution, respectively. The structures of the two complexes reveal that the inhibitors can be accommodated in the glycogen storage site of T-state GPb with very little change of the tertiary structure and provide a basis for understanding their potency and subsite specificity. Structural comparisons of the two complexes with GPb in complex with either maltopentaose (G5) or maltoheptaose (G7) show that β - and γ -cyclodextrins bind in a mode analogous to the G5 and G7 binding with only some differences imposed by their cyclic conformations. It appears that the binding energy for stabilization of enzyme complexes derives from hydrogen bonding and van der Waals contacts to protein residues. The binding of α -cyclodextrin and octakis (2,3,6-tri-*O*-methyl)- γ -cyclodextrin was also investigated, but none of them was bound in the crystal; moreover, the latter did not inhibit the phosphorylase reaction.

Keywords: Glycogen phosphorylase; α -cyclodextrin; β -cyclodextrin; γ -cyclodextrin; oligosaccharide binding; protein-carbohydrate interactions; X-ray crystallography

Glycogen phosphorylase (EC 2.4.1.1; GP) has been a molecular target for structure-assisted design of inhibitors that might prevent unwanted glycogenolysis under high glucose

conditions. Several binding sites in GP, such as the catalytic, the allosteric, the inhibitor, and the new allosteric sites have been explored as specific targets in kinetic, crystallographic, and physiological studies (reviewed in McCormack et al. 2001; Somsak et al. 2001; Treadway et al. 2001; Oikonomakos 2002; Kurukulasuriya et al. 2003). However, the glycogen storage site has not been experimentally validated as a target for modulating hepatic glucose production in type 2 diabetes, and no medicinal chemistry studies have been reported on this target yet. This site was identified from crystallographic binding studies with maltopentaose (G5) and maltoheptaose (G7), that also bind to the catalytic site and act as substrates (Johnson et al. 1988). In GPb, G5

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Abbreviations: GP, glycogen phosphorylase; GPb, muscle glycogen phosphorylase b; GPa, muscle glycogen phosphorylase a; glucose, α -D-glucose; Glc-1-P, α -D-glucose 1-phosphate; α -, β -, γ -CD, α -, β -, γ -cyclodextrin; TM γ CD, octakis (2,3,6-tri-*O*-methyl)- γ -cyclodextrin; G5, maltopentaose; G7, maltoheptaose; RMSD, root-mean-square deviation.

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can bind to the glycogen storage site by filling five major and two minor subsites. The precise role of the glycogen storage site is not fully understood, but it might serve as a region through which the mammalian enzyme is attached to glycogen particles *in vivo*, to produce an effective high concentration of end groups and act as additional regulatory site whereby occupation by glycogen is a prerequisite for catalysis. Also, model building studies (McLaughlin et al. 1984) have shown that the minor site might be consistent with the position of oligosaccharide at an α -1,6 branch position, providing an explanation for the increased affinity of GP for branched polysaccharides like glycogen over linear glucans like maltodextrins (Hu and Gold 1975). Inhibitors, specific for this site, would be therefore of most interest.

Acarbose, a pseudo-maltotetraose with the cyclitol unit of α -1,4-linked to a 4-amino-4,6-dideoxyglucose residue, is a potent inhibitor of many α -glucosidases, α -amylases, and sucrase-isomaltase, and is considered to be an analog of a glucosyl cation like transition state (Truscheit et al. 1981). Binding studies in the crystal of GP α showed that it bound at the major site of glycogen storage site in an analogous way to maltopentaose inhibitor. Kinetic studies have shown that acarbose is a reasonable inhibitor of GP α ($K_i = 26$ mM) in competition experiments with 30 mM G5 (Goldsmith et al. 1987). Apart from acarbose, specific inhibitors of the enzyme that bind at the glycogen storage site have not been previously described.

Given the structural analogy of cyclic oligosaccharides to linear ones, we investigated the effect of α -, β -, and γ -cyclodextrins (CDs) on the catalytic and structural properties of GP β . The cyclodextrins are torus-like macrorings built up from α -D-glucopyranose units connected with an α -1,4 glycosidic linkage (Fig. 1). The α -CD molecule comprises 6 glucopyranose units, while β - and γ -CD comprise 7 and 8 units, respectively. As a consequence of the 4C_1 conformation of the glucopyranose units, primary and secondary hydroxyl groups are situated on either side of two edges of the ring. The ring is a truncated cone whose cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges, respectively. The secondary O2 hydroxyl group of one glucopyranose unit forms a hydrogen bond with the O3 hydroxyl group of the preceding glucopyranose. As a result, a complete belt of hydrogen bonds is formed in the secondary side that is stronger in β -CD (Makedonopoulou and Mavridis 2000), making it the most rigid and the less water-soluble of all three CDs. In contrast, this hydrogen bond belt is not complete in α -CD (one of the glucose moieties is distorted) and in γ -CD (more distorted conformation), resulting in a higher flexibility and aqueous solubility compared to that of β -CD (Szejtli 1998).

In this work we present a detailed description of the binding of α -, β -, γ -CDs and TM γ CD to GP β through kinetic and X-ray binding studies. The kinetic results show that α -, β -, and γ -CDs are moderate inhibitors of the enzyme, with

Figure 1. Chemical structure of γ -CD and numbering scheme used in a glucose unit (IUPAC-IUB 1983).

γ -CD being the best ($K_i = 7.4$ mM), while TM γ CD did not inhibit GP β . To elucidate the key interactions responsible for inhibition we performed crystallographic binding studies with the above compounds and analyzed the structures of those that showed binding, namely GP β - β -CD and GP β - γ -CD complexes at 1.94 Å and 2.3 Å resolution, respectively. The detailed interactions of β - and γ -CDs with the protein provide a structural explanation for the kinetic properties and show that the cyclic oligosaccharides bind at the glycogen storage site of GP β in a mode analogous to the G5 and G7 binding, with some changes imposed by differences in their conformations. To improve the precision of our understanding of the molecular basis of linear oligosaccharide recognition, we have also determined the crystal structures of the GP β -G5 and GP β -G7 complexes at 2.2 Å resolution.

Results and Discussion

The kinetic parameters (K_i 's) of α -, β -, and γ -CDs are summarized in Table 1. The kinetic experiments with GP β , in the direction of glycogen synthesis, showed that all three

Table 1. Kinetic parameters for maltoheptaose and cyclodextrins

Oligosaccharide	K_i (mM)
Maltoheptaose	1.0 ^a
α -Cyclodextrin	47.1 $\pm$ 5.1 ^b
β -Cyclodextrin	14.1 $\pm$ 3.7 ^b
γ -Cyclodextrin	7.4 $\pm$ 1.3 ^c

^a Data taken from Kasvinsky et al. (1978).

^b Calculated from double-reciprocal plots with varied glycogen concentrations (0.02%, 0.025%, 0.035%, and 0.05% w/v), constant concentrations of Glc-1-P (4.0 mM) and AMP (1 mM) and 9.4 mM oligosaccharide.

^c As above, except that three different concentrations of γ -cyclodextrin (4.7, 9.4, and 14.0 mM) were used. Best fit lines (not shown) were computer generated according to the equation for linear mixed-type inhibition (Segel 1975) by fitting all of the data at once and using the nonlinear regression program *GraFit* (Leatherbarrow 1992). The kinetic parameters determined in this way for GPb inhibition by γ -cyclodextrin were as follows: $V = 147.4 \pm 13.7$ IU/mg, $K_s = 0.023 \pm 0.006\%$ (w/v), $K_i = 7.4 \pm 1.3$ mM.

cyclodextrins exhibited mixed-type inhibition with respect to glycogen substrate at constant concentrations of AMP (1 mM) and Glc-1-P (4 mM). Lineweaver-Burk plots ($1/v$ versus $1/[\text{glycogen}]$) yielded straight lines of which the slopes and the vertical axis intercept increased with increasing concentrations of inhibitor. γ -CD ($K_i = 7.4$ mM) was found to be a better inhibitor than β -CD ($K_i = 14.1$ mM) and α -CD ($K_i = 47.1$ mM). TM γ CD had no effect on the enzymic activity of GPb when added in concentrations varied from

10–50 mM, in the presence of 1 mM AMP, 4 mM Glc-1-P, and 0.035% (w/v) glycogen.

Crystallographic data collection, processing, and refinement statistics for the structure determinations of GPb complexed with G5, G7, β -CD, and γ -CD, respectively, are summarized in Table 2. The overall architecture of the native T-state GPb showing the location of the catalytic, the inhibitor, the allosteric, the new allosteric, and the glycogen storage site is presented in Figure 2. For both the G5 and G7 complexes, electron density maps indicated that five glucose residues (S3 to S7) bound at the major site of the glycogen storage site, and there was no binding at the minor site. For the GPb- γ -CD complex, all glucose residues (S3 to S10) were visible in the electron density map, while for the GPb- β -CD complex the electron density maps suggested partial occupancy of β -CD of the glycogen storage site. No binding was observed at the catalytic site. The electron density maps for GPb crystals soaked with α -CD and TM γ CD indicated no binding at the glycogen storage site. We describe briefly the G5 and G7 interactions at the glycogen storage site and in more detail the γ -CD and β -CD interactions at this site.

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A major and a minor glycogen storage site have been defined from the crystal structures of the GPb-G5 complex at

Table 2. Diffraction data and refinement statistics

GPb complex	G5	G7	β -CD	γ -CD
Resolution ($\text{\AA}$)	30.0–2.2	30.0–2.20	30.0–1.94	30.0–2.3
Outermost shell ($\text{\AA}$)	2.24–2.20	2.24–2.20	1.96–1.94	2.38–2.30
No. of observations	442,007	456,706	714,419	360,103
No. of unique reflections	49,992	49,982	68,504	43,859
R_{symm}^a	0.079	0.096	0.076	0.110
(outermost shell)	(0.426)	(0.467)	(0.499)	(0.483)
Completeness (%)	95.9	95.7	93.4	96.5
(outermost shell) (%)	(85.0)	(84.2)	(85.0)	(84.9)
$\langle I/\sigma(I) \rangle^b$ (outermost shell)	12.7 (2.8)	13.8 (2.2)	13.3 (2.1)	11.0 (2.0)
Wilson B factor ($\text{\AA}^2$)	28.5	32.0	17.9	28.1
R_{cryst}^c	0.182	0.184	0.193	0.189
R_{free}^d	0.215	0.217	0.218	0.232
No. of protein atoms	6575	6571	6597	6571
No. of ligand atoms	56	56	22	88
No. of water molecules	300	304	324	272
RMSD in bond lengths ($\text{\AA}$)	0.006	0.006	0.006	0.006
RMSD in angles ($^\circ$)	1.2	1.2	1.3	1.2
Average B factor ($\text{\AA}^2$)				
Protein atoms	36.2	36.3	28.9	35.3
Water molecules	41.5	41.2	37.3	38.3
Ligand atoms	59.8	61.0	64.3	67.3

^a $R_{\text{symm}} = \sum_i \sum_j |I_i(h) - I_j(h)| / \sum_i \sum_j I_i(h)$ where $I_i(h)$ and $I(h)$ are the i th and the mean measurements of the intensity of reflection h .

^b $\sigma(I)$ is the standard deviation of I .

^c $R_{\text{cryst}} = \sum_h |F_o - F_c| / \sum_h F_o$, where F_o and F_c are the observed and calculated structure factors amplitudes of reflection h , respectively.

^d R_{free} is equal to R_{cryst} for a randomly chosen 5% of the reflections that were not included in the refinement.

Figure 2. A schematic diagram of the muscle GPb dimeric molecule viewed down the twofold for residues 13 to 838. One subunit is colored gray and the other subunit light gray. The positions are shown for the catalytic, the allosteric, the inhibitor, the new allosteric inhibitor site, and the glycogen storage site. The catalytic site, which includes the essential cofactor pyridoxal 5'-phosphate (not shown), is buried at the center of the subunit accessible to the bulk solvent through a 15 Å long channel. Glucose (shown in ball-and-stick representation), a competitive inhibitor of the enzyme that promotes the less active T state through stabilization of the closed position of the 280s loop, binds at this site. The allosteric site, which binds the activator AMP, is situated at the subunit-subunit interface some 30 Å from the catalytic site. The inhibitor site, which binds purine compounds, nucleosides, or nucleotides at high concentrations, and flavopiridol (shown bound; Oikonomakos et al. 2000) is located on the surface of the enzyme some 12 Å from the catalytic site and, in the T state, obstructs the entrance to the catalytic site tunnel. The new allosteric inhibitor site, located inside the central cavity, formed on association of the two subunits, binds N-Benzoyl-N'- β -D-glucopyranosyl urea molecule (shown; Oikonomakos et al. 2002). The glycogen storage site (with γ -CD bound) is on the surface of the molecule some 30 Å from the catalytic site, 40 Å from the allosteric site, and 50 Å from the new allosteric site.

2.5 Å resolution (Johnson et al. 1988). The major site is defined by the binding of five glucose units in subsites S3-S4-S5-S6-S7, with the reducing end of the oligosaccharide bound in subsite S3. This site is made up of helices α 12 and α 13 (residues 396 to 418 and 420 to 429, respectively) and the loop connecting the two antiparallel strands, β 15 (residues 430 to 432) and β 16 (residues 437 to 411; Fig. 2). The minor site, defined by the binding of glucosyl residues S8 and S9, lies above the nonreducing end of the major site. It involves GP residues from the top of helix α 12, the loop connecting the antiparallel β -sheets β 8 (residues 198 to 209) and β 9 (residues 212 to 223) and Val354 from helix α 9 (residues 344 to 355). In the complex structures of GPa-G7-caffeine-glucose and GPa-G5-phosphate, seven gluco-

syl moieties (S1 to S7) were found at the major site and four (S8 to S11) at the minor site (Goldsmith et al. 1982, 1989; Goldsmith and Fletterick 1983). In contrast, in the complex GPa-G5-caffeine-glucose, only three glucosyl residues (S4 to S6) and one glucose (S10) were found in the minor site (Goldsmith et al. 1989). In the GPb-heptulose-2-P-G7-AMP complex (Johnson et al. 1990), the minor oligosaccharide site is not occupied.

One molecule of either G5 or G7 bound at the GPb storage site occupying the major site, while no binding was observed at the minor site. The mode of binding and the interactions that G5 and G7 exhibit with GPb are almost identical with those for the GPa-G5-phosphate, GPa-G5-caffeine-glucose, and GPa-G7-caffeine-glucose complexes

(Goldsmith et al. 1982, 1989; Goldsmith and Fletterick 1983) derived from medium-resolution X-ray crystallographic analyses, and that for the GPb–heptulose–2-P–G7–AMP complex (Johnson et al. 1990) determined at a resolution of 2.86 Å resolution. The resolution of the present structures allows us to describe the binding site for G5 and G7 in some detail. Both structures indicated five well-ordered structural waters that form a network of hydrogen bonds that link oligosaccharide to protein residues; these waters were not observed in the previous medium-resolution structures.

The bound G5 has a regular helical structure stabilized by the O2–O3 hydrogen bonds formed between successive sugars with a break between glucose residues S4 and S5 (Fig. 3A). The temperature factors are lower (50–62 Å²) at glucose residues S4, S5, and S6, and increase (65–80 Å²) toward the two ends of the oligosaccharide chain. The O3 hydroxyl group of glucose residue S4 forms a hydrogen bond with Ser429 from helix α 13, and O2 hydroxyl is involved in a water-mediated interaction with Val431 O, Arg426 O, and Gln433 N through another water molecule. The O2 hydroxyl group of glucose residue S5 is hydrogen

Figure 3. (Continued on next page)

Figure 3. (A, C, and E) Diagrams of the 2.2 Å and 2.3 Å sigmaA 2IFol – IFcl electron density map in the vicinity of the glycogen storage site for G5, G7 and γ -CD bound to GPb, respectively. Electron density maps were calculated using the standard protocol as implemented in CNS v1.1 (Brünger et al. 1998) from the GPb model before incorporating the coordinates of G5, G7, and γ -CD, respectively. The maps are contoured at the 1.0σ level. (B, D, and F) Diagrams showing the interactions of G5, G7, and γ -CD with GPb, respectively. GPb residues are drawn as ball-and-stick models, water molecules as cyan spheres, and the oligosaccharide molecules are shown in standard colors. Dashed lines indicate hydrogen bonds.

bonded to N ζ of Lys437 from β -sheet $\beta 16$ and a water molecule; O5 and O6 hydroxyl groups are hydrogen bonded to N $\delta 2$ of Asn407 from helix $\alpha 12$; moreover, O6 hydroxyl groups is hydrogen bonded to Gln401 O through a water molecule (Fig. 3B). Finally, O5 and O6 hydroxyl groups of S3 make a water-mediated hydrogen bond with Phe197 N from a symmetry-related molecule ($\frac{1}{2} + x, \frac{1}{2} - y, \frac{1}{4} - z$).

On forming the complex with GPb, G5 becomes buried. The solvent accessibilities of the free and bound G5 molecules are 937 Å² and 532 Å², indicating that a surface area of 405 Å² becomes inaccessible to water or that G5 becomes 57% buried in the enzyme complex. Both polar and nonpolar groups are buried; nonpolar groups contribute 175 Å² (43%), and polar groups contribute 230 Å² (57%) of the surface that becomes inaccessible. On the protein surface, a total of 311 Å² solvent-accessible surface area becomes inaccessible on binding G5, of which 180 Å² (58%) is contributed by nonpolar groups. The total buried surface area (protein + ligand) is 716 Å².

GP displays a high affinity for G7 at the glycogen storage site, with a dissociation constant of 1 mM (Kasvinsky et al. 1978), a value similar to that for glycogen. Only five (S3 to S7) out of the seven glucose residues of G7 were defined in the electron density map of the GPb–G7 complex (Fig. 3C). The first two glucose residues could not be observed in the electron density map; similarly, there was no density for these residues in the GPb–heptulose–2-P–G7–AMP complex (Johnson et al. 1990). The conformation of the oligo-

saccharide and its interactions within the glycogen storage site are almost identical to those of G5 (Fig. 3D).

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γ -CD on binding to GPb occupies the major site of the glycogen storage site (Fig. 4). For reasons of comparison, the numbering scheme used for the glucose residues of γ -CD corresponds to that for the linear oligosaccharides G5 and G7. Examination of the electron density in this region indicates that there is poor density for O2 and O6 of S3 and S8, O6 of S7 and S9, O2, O6, and C6 of S10, and there is also a break in the electron density for glycosidic bond between S9 and S10. Glucose residues S4, S5, S6, and S7 of γ -CD are well defined within the electron density map (Fig. 3E).

The interactions between the bound γ -CD and GPb are very similar to those made by G5 and G7 to GPb. There are four direct hydrogen bonds and one water-mediated hydrogen bond between γ -CD and the protein (Fig. 3F). These include a hydrogen bond of the O6 hydroxyl group of S4 to the O γ of Ser429, and three hydrogen bonds of residue S5 (two of the O2 and O6 hydroxyl groups and one of the O5 oxygen) to the side chain atoms of Lys 437 (N ζ) and Asn 407 (N $\delta 2$). In addition, O6 of glucose residue S6 makes an indirect contact to Gln401 (O) via a water molecule. The

Figure 4. The molecular surface of the GPb dimer calculated using the program GRASP (Nicholls and Honig 1991). One subunit is colored green and the other subunit is cyan. The γ -CD molecule is shown as a cpk model and GPb residues interacting with γ -CD are in yellow.

most characteristic feature for γ -CD binding to GPb is the parallel stacking interactions of the glucose plane S5 with the aromatic residue Tyr404 so that the H atom of carbon

atom C4 interacts with the aromatic ring. In the GPb- γ -CD complex, the buried area of γ -CD is $467 \text{ \AA}^2$ and the buried surface in GPb is $391 \text{ \AA}^2$, making a total decrease in solvent-accessible area of $858 \text{ \AA}^2$ compared with the total buried surface area in the GPb-G5 complex of $716 \text{ \AA}^2$.

The geometry of the bound γ -CD is exhibited in Table 3 along with that of the uncomplexed hydrated γ -CD (Harata 1987). Glucose numbering has been changed in the latter to be consistent with the present structure (initial numbering of glucose residues in parentheses). The pyranose rings in both γ -CDs have the usual 4C_1 conformations and the distance and angle values between the glucosidic oxygen atoms O4 are similar. However, there are significant differences both in the deviations of the O4 atoms from their mean plane and in the tilt angles of γ -CD bound to GPb, compared to those of the free γ -CD (Fig. 5). The tilt angles correspond to the dihedral angles formed between each glucose residue and the mean O4 plane, tilt angle close to zero corresponding to a glucose residue perpendicular to the O4 plane, whereas high tilt angle indicates tilting of the primary side of the glucose residues towards the cavity. Table 3 shows that the residues interacting with the protein via hydrogen bonds and their immediate neighbors exhibit maximum tilt angles. The large deviations from the mean O4 plane mentioned above are probably the results of excessive tilting. To the same reason we must attribute the deviations in the intramolecular hydrogen bonds between glucose residues, O3n...O2(n+1), that stabilise the cyclodextrin ring, also observed for resi-

Table 3. Conformations of γ -CD

Residue	D ^a (Å)	φ ^b (°)	d ^c (Å)	Tilt angles ^d (°)	Torsion Angle (°) O5n-C5n-C6n-O6n	
(a) Bound to the glycogen storage site of GPb					D ₃ ^e (Å)	
S3	4.51	127	0.35	30.5	3.72	−56
S4	4.40	133	−0.60	32.0	2.44	−64
S5	4.47	133	0.07	14.4	2.89	48
S6	4.45	130	0.74	45.3	3.37	−46
S7	4.43	129	−0.77	50.8	2.33	130
S8	4.57	127	0.22	27.9	3.43	53
S9	4.47	136	0.01	4.0	2.63	124
S10	4.43	138	−0.02	8.3	2.93	140
(b) Uncomplexed hydrated γ-CD (Harata 1987)					D ₃ (Å)	
S3 (G4)	4.48	136.1	0.192	4.8	2.76	−62.4
S4 (G3)	4.46	134.6	−0.005	23.8	2.77	−60.6
S5 (G2)	4.46	134.3	−0.173	−2.5	2.91	−60.6
S6 (G1)	4.59	135.8	0.054	16.6	2.88	73.7
S7 (G8)	4.54	134.3	0.126	23.6	2.77	71.9
S8 (G7)	4.43	133.5	−0.056	12.7	2.84	−63.9
S9 (G6)	4.58	137.0	−0.046	13.4	2.81	−57.9
S10 (G5)	4.48	133.5	−0.077	18.4	2.83	72.8

^a O4n...O4(n+1) distances.

^b O4(n-1)...O4n...O4(n+1) angles.

^c Deviations (Å) from the least-squares optimum plane of the eight O4n atoms.

^d Tilt angles between the optimum O4n plane and the mean planes through atoms O4(n-1), C1n, C4n, O4n.

^e Intramolecular hydrogen-bond distances between atoms O3n...O2(n+1) when bound and between atoms O3n...O2(n-1) when free.

Figure 5. Stereoview of the superimposed structures of the free (white) and GPb bound γ -CD (gray).

dues S3–S7. While in the free γ -CD the distance between O3 and O2 hydroxyls range between 2.76 and 2.91 Å (although less symmetrical than in β -CD), in the GPb- γ -CD complex the O3–O2 distances range between 2.33 and 3.72 Å, indicating that some of the intramolecular hydrogen bonds have been broken. Therefore, it seems that binding of glucose residues to GPb is stabilized in conformations that distort the overall shape of the macrocycle. Table 3 also shows the conformations of the primary hydroxyl groups O6, which differ also from those of free γ -CD. The torsion angles O5–C5–C6–O6 indicate that residues S3, S4, and S6 exhibit *gauche–gauche* conformation and the hydroxyl groups point away from the cavity. The torsion angles in residues S5 and S8 are *gauche–trans*, and point towards the interior of the cavity, whereas in residues S7, S9, and S10 exhibit the rarely observed *trans–gauche* conformation. γ -CD presents some additional close contacts in the crystal: C1 and C2 of glucose S3, and C6 of glucose S10 make van der Waals contacts with Asn595, while O2 hydroxyl of S3 contacts Lys596 from a symmetry-related molecule ($\frac{1}{2} + x$, $\frac{1}{2} - y$, $\frac{1}{4} - z$). Because these contacts are not expected to provide much energy for stabilization, we do not think that the deformations described above for γ -CD can be attributed to crystal packing forces.

There are no significant conformational changes of GPb residues upon binding of γ -CD. The comparison of the native GPb structure with the GPb- γ -CD complex (residues 18–249, 262–312, and 326–837) gave an RMSD value of 0.22 Å for C α atoms, indicating that the two structures are similar in their overall conformation within the limits of the resolution of the study. Some minor conformational changes in the protein involve side chains of residues Glu433 and Lys437, which move to allow optimal contacts with the cyclic oligosaccharide in subsites S4 and S5. A small movement is also observed for the side chain of residue Gln408, which is displaced by glucose residue S6. These conformational changes are almost identical to those induced by the binding of G5 and G7. After superimposing the protein C α atoms of the GPb- γ -CD structure onto the GPb-G5 structure, RMSDs values of 4.79, 3.25, 0.67, 0.42, and 1.16 Å were calculated for all atoms of residues S3, S4, S5, S6, and S7, respectively. The corresponding values for

the pair GPb- γ -CD/GPb-G7 were 4.73, 3.33, 0.62, 0.40, and 1.50 Å, respectively. Residues S5 and S6 exhibited the best superimposition whereas residues S3 and S4 show the largest RMSDs between γ -CD and G5 and G7 (Fig. 6). These values are consistent with the differences in the conformation between γ -CD and the linear oligosaccharides because, due to the cyclic conformation of γ -CD, S3 and S4 cannot reach the positions of the corresponding residues in G5 or G7. This is also evident from the differences between the glycosidic angle parameters of γ -CD and G5 (Tables 4). Kinetic experiments showed that γ -CD is a mixed-type competitive inhibitor of GPb with respect to glycogen with a K_i value of 7.4 ± 1.3 mM (Table 1). These data suggest that γ -CD is a less potent inhibitor of GPb than G7, and are also consistent with the crystallographic data.

The crystal structures of several bacterial cyclodextrin glucosyltransferases (CGTases; EC 2.4.1.19), the CD producing enzymes, in complex with either linear maltosaccharides or with CD derivatives, have revealed the specific interactions of CDs in the active site of these enzymes (Knegtel et al. 1995; Strokopytov et al. 1995, 1996; Schmidt et al. 1998). In the crystal structures of different CGTases complexed with either α - or β -CD derivatives, the glucose planes of the cyclic oligosaccharide show parallel stacking with the aromatic residues of the active site. The polar contacts between the protein and the cyclic oligosaccharide involve only four glucose units that form direct or water-mediated hydrogen bonds with residues of the active site of the enzyme. Similar mode of binding has also been observed for β -CD bound to the maltodextrin binding protein (Sharff et al. 1993), and α -CD complexed with pig pancre-

Figure 6. Superimposed structures of GPb-G7 (white) and GPb- γ -CD (gray) complexes in the glycogen storage site.

Table 4. Glycosidic angle parameters of maltopentaose and γ -cyclodextrin when bound to the glycogen storage site of GPb

Glucan residues	Maltopentaose			γ -Cyclodextrin		
	φ (°)	ψ (°)	Glycosidic linkage angle (°)	φ (°)	ψ (°)	Glycosidic linkage angle (°)
S3–S4	107	119	117	78	118	114
S4–S5	72	81	116	135	129	117
S5–S6	104	112	114	103	122	114
S6–S7	82	108	116	88	115	116
S7–S8				162	124	116
S8–S9				89	131	115
S9–S10				117	126	115
S10–S3				118	145	113

Torison angles are defined as φ , O5(*n*)-C1(*n*)-O4(*n*-1)-C4(*n*-1); ψ , C1(*n*)-O4(*n*-1)-C4(*n*-1)-C3(*n*-1); glycosidic linkage angle, C1(*n*)-O4(*n*-1)-C4(*n*-1) (IUPAC-IUB 1983).

atic α -amylase (Larson et al. 1994) and soybean β -amylase (Mikami et al. 1993). This mode of binding is analogous to that observed in the present study for γ -CD in GP as expected because the macrocycles are bound on the surface of the proteins mentioned and the round shape does not allow for tight contacts of all glucose units with the proteins.

The binding of β -cyclodextrin to glycogen phosphorylase b

In the electron density maps calculated from the coordinates of the free GPb for the GPb- β -CD complex structure there was enough density in the glycogen storage site to allow fitting of glucose residues S5 and S6 according to the G5/G7 nomenclature. A model of the β -CD molecule was then fitted in the glycogen storage site following the electron density map in these positions, and the complex structure was subjected to one round of refinement (positional and B-factor). Successive refinement cycles significantly improved the quality of the sigmaA 2lFol - lFcl electron density map for glucose residues S5 and S6, but the rest of the map around the β -CD molecule was very poor, and most of the atoms had high temperature factors, indicating partial binding of β -CD to GPb. These crystallographic results are consistent with (1) kinetic data showing that β -CD is a rather poor inhibitor of GPb with a K_i value of 14.1 mM, and (2) the relatively low concentration of β -CD (15 mM) used in the soaking experiment due to limitations in the solubility of β -CD in aqueous solutions. Despite the partial binding of β -CD to GPb observed in the crystal its binding mode is similar to that of γ -CD. The RMSDs between the C $_{\alpha}$ atoms of the β -CD and γ -CD complexes is 0.22 Å. Furthermore, after superimposition of the GPb- γ -CD and GPb- β -CD complex structures, glucose residues S5 and S6 of γ -CD superimpose to their counterparts in β -CD with RMSD values of 0.54 and 0.73 Å, respectively.

In conclusion, four different cyclodextrins were tested for inhibition of GPb. Of these, γ -CD is the best inhibitor with a K_i value of 7.4 mM, which is in the same order of magnitude as that reported for the linear oligosaccharide G7 (Kasvinsky et al. 1978). The crystallographic binding studies on GPb with γ -CD and β -CD revealed that the cyclic oligosaccharides bind at the glycogen storage site by anchoring two of their glucose residues to subsites S4 and S5 in a manner similar to the binding of the linear oligosaccharides G5 and G7. However, the inhibition constant of γ -CD is almost seven times higher than that of G7, and that of β -CD is even higher (Table 1). The lower potency of γ -CD is explained by the crystallographic results, which show that G5 or G7 are involved in a more extensive network of interactions with GPb than γ -CD. This is mostly due to additional interactions made by glucose residues S3 and S6 that are not observed in the GPb- γ -CD complex. Residues S3 and S6 cannot interact with the protein due to the shape of γ -CD itself. γ -CD binds at the glycogen storage site of T-state GPb at approximately the same position as that of G5 (or G7), and there is very little change in the structure of the enzyme. The structure of the γ -CD complex helps to explain the kinetic properties of the inhibitor. On binding to the glycogen storage site, γ -CD is able to inhibit glycogen binding to this site directly and glycogen binding to the catalytic site indirectly. Further kinetic and structural studies with oligosaccharide analogs are in progress, with the aim to identify the structural requirements of the glycogen storage site for the design of more effective enzyme inhibitors.

Materials and methods

Kinetic experiments

AMP, Glc-1-P (dipotassium salt), glycogen, maltopentaose, maltoheptaose, and other chemicals were obtained from Sigma-Aldrich

Chemie GmbH. Cyclodextrins were obtained from Cyclolab. Rabbit muscle GPb was isolated, purified, recrystallized, and assayed as described (Oikonomakos et al. 2002). Care was taken to ensure that the average increase in glycogen chain length did not exceed two glucose units (Kasvinsky et al. 1978).

Crystallization and data collection

T-state tetragonal ($P4_32_12$) GPb crystals were grown as described previously (Oikonomakos et al. 2000). The γ -CD, G5, and G7 complexes were obtained by soaking native GPb crystals with 150 mM γ -CD, 70 mM maltopentaose, or 70 mM maltoheptaose, respectively, in fresh solutions of mother liquor (10 mM Bes, 0.1 mM EDTA, pH 6.7), for at least 2 h prior to data collection. Data to 2.3 Å resolution for the γ -CD, G5, and G7 complexes were collected at room temperature on an Image Plate RAXIS IV mounted on a Rigaku Ru-H3RHB rotating anode generator with a belt drive rotating anode ($\lambda = 1.5418$ Å). Data for the α -CD and the β -CD complexes, obtained by soaking native GPb crystals with 15 mM β -CD (11 h) or with 120 mM α -CD (1 h) in mother liquor, were collected on Station X11 of the EMBL Outstation, on a MAR Research Image Plate. Data for TM γ CD complex obtained by soaking native GPb crystals with 100 mM TM γ CD in mother liquor, for 1 h, were collected from a single crystal using a rotating anode source ($\lambda = 1.5418$ Å) and an 18-cm small Mar detector. Crystal orientation and integration of reflections, interframe scaling, partial reflection summation, data reduction, and postrefinement were all performed using DENZO and SCALEPACK (Otwinowski and Minor 1997).

Structure refinement

All crystallographic refinements were carried out with CNS version 1.1 (Brünger et al. 1998), using bulk solvent corrections. All data were included with no sigma cutoff. The starting protein structure was the refined model of the room temperature GPb–caffeine complex (code 1GFZ; Oikonomakos et al. 2000) with caffeine removed. Alternating cycles of manual building with the program O (Jones et al. 1991), and torsion angle dynamics, conjugate gradient minimization, and restrained individual B-factor refinement using the maximum likelihood target function as implemented in CNS were performed until the R_{free} value for every model could not be improved any further. A summary of the data processing statistics and refinement parameters are given in Table 2. Analysis of the Ramachandran plots (CCP4 1994) showed that all residues lie in the allowed regions. Geometric parameters were determined by programs SHELXL (Sheldrick and Schneider 1997).

The structures were analyzed with the graphics program O (Jones et al. 1991). Solvent-accessible areas have been calculated for oligosaccharides in isolation and when bound to provide an estimate of the molecular surface area involved in binding to the enzyme, by using the program NACCESS (Hubbard and Thornton 1993). GP structures were superimposed over well-defined residues using LSQKAB (CCP4 1994). Coordinates for the 2.2 Å resolution GPb–maltopentaose, 2.2 Å resolution GPb–maltoheptaose, 1.94 Å resolution GPb– β -cyclodextrin, and 2.3 Å resolution GPb– γ -cyclodextrin complexes have been deposited with the RCSB Protein Data Bank (<http://www.rcsb.org/>; codes 1P29, 1P2B, 1P2D, and 1P2G, respectively). All figures were prepared with the program MOLSCRIPT (Kraulis 1991) and rendered with Raster3D (Merritt and Bacon 1997).

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