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The design of potential antidiabetic drugs : experimental investigation of a number of β-D-glucose analogue inhibitors of glycogen phosphorylase.

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SUMMARY

α-D-glucose is a weak inhibitor (K_i=1.7 mM) of glycogen phosphorylase (GP) and acts as physiological regulator of hepatic glycogen metabolism; it binds to GP at the catalytic site and stabilizes the inactive T state of the enzyme promoting the action of protein phosphatase 1 and stimulating glycogen synthase. The three- dimensional structures of T state rabbit muscle GPb and the GPb-α-D-glucose complex have been exploited in the design of better regulators of GP that could shift the balance between glycogen synthesis and glycogen degradation in favour of the former. Close examination of the catalytic site with α-D-glucose bound shows that there is an empty pocket adjacent to the β-1-C position. β-D-glucose is a poorer inhibitor (K_i=7.4 mM) than α-D-glucose, but mutarotation has prevented the binding of β-D-glucose in T state GP crystals. A series of β-D-glucose analogues has been designed and tested in kinetic and crystallographic experiments. Several compounds have been discovered that have an increased affinity for GP than the parent compound.

INTRODUCTION

Hyperglycaemia in the non-insulin-dependent form of the desease diabetes mellitus (NIDDM or Type II diabetes) patients results from diminished insulin release and/or insulin resistance that leads to impaired glucose uptake and impaired suppression of hepatic glucose output [1] where the glucose is produced by gluconeogenesis and glycogen metabolism. Glucose is a physiological regulator of hepatic glycogen metabolism that promotes inactivation of GP and acts synergistically with insulin; it controls the activity of GPa by increasing the cooperativity between substrate binding sizes and stabilizing the T state conformation (inactive) which is a better substrate for the protein phosphatase than the R state (active) thus promoting the conversion of GPa to GPb (evidence in [2]). Diminished concentrations of GPa allow the phosphatase to promote activation of glycogen synthase [3]. Thus more efficient conversion of GPa to GPb results in relief of protein phosphatase 1 inhibition and activation of the phosphatase against glycogen synthase. These observations suggest that more potent glucose analogue inhibitors of GP have the potential to shift the balance between glycogen degradation and glycogen synthesis in favour of the latter and may eventually be of therapeutic benefit in the control of blood glucose levels. The determination of the crystal structure of the inactive form of GP [4] has provided a unique opportunity for the design of new and specific inhibitors that might have improved

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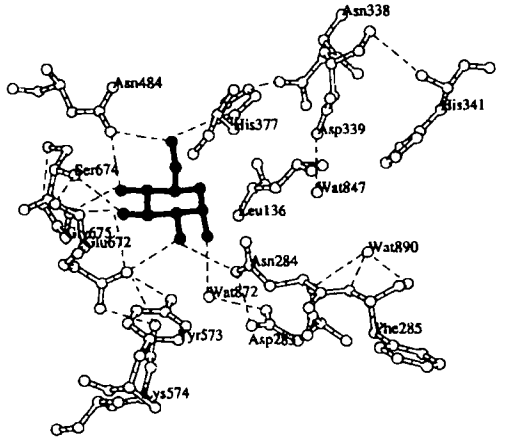


Figure 1. Diagram showing the contacts between GPb and α-D-glucose. Hydrogen bonds less than 3.4 Å are shown (from [2]).

potency, and selectivity for GP. In this work, we report kinetic and crystallographic results on the parent compounds α-D-glucose and β-D-glucose and on a number of β-D-glucose analogue inhibitors of GPb.

MATERIALS AND METHODS

α-D-glucose and β-D-glucose were products of Sigma Chemical Co. The syntheses of the β-D-glucose derivatives will be published elsewhere. The procedures for the preparation of GPb, the determination of initial velocities and the statistical evaluation of the kinetic parameters were described in [2,5]. T state GPb was crystallized from the purified protein as described in [6]. The binding of α- and β-D-glucose in the crystal was carried out by soaking T state GPb crystals for 4 min, at 20 °C, in a freshly prepared (dissolution time=1 min) buffered solution (10 mM BES, 0.1 mM EDTA, 0.02% NaN_3, pH 6.7) containing either 200 mM α-D-glucose or 200 mM β-D-glucose. 2.3 Å resolution were collected on an Arndt-Wonacott oscillation camera at the SERC Synchrotron Radiation Source at Daresbury, UK (Station 7.2). One crystal was used for each experiment and the total time for data collection was approximately 25 min. Data processing and calculations of difference Fourier maps were performed as described previously [7]. The binding of β-D-glucose analogues in the crystal was carried out as detailed in [2,8].

RESULTS AND DISCUSSION

α-D-glucose and β-D-glucose. α-D-glucose on binding at the catalytic site of GPb adopts a well defined $^4C_1$ chair conformation; every potential hydrogen bonding group of glucose makes at least two hydrogen

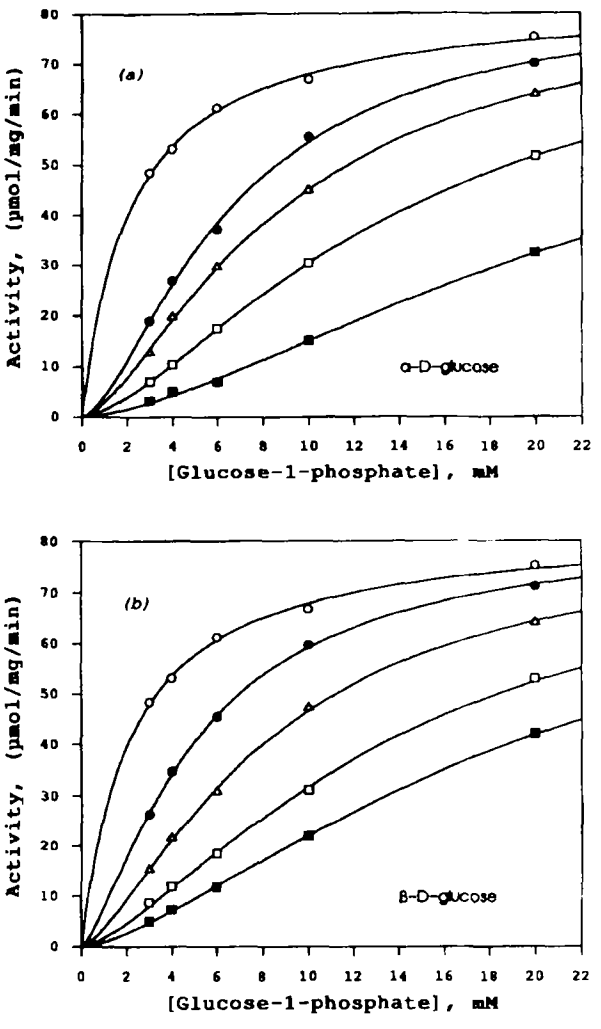


Figure 2. Kinetics of α-D-glucose and β-D-glucose inhibition of GPb with respect to Glc-1-P. (a) The activity of GPb at 30°C and pH 6.8 as a function of substrate concentration in the presence of varying concentrations of α-D-glucose (0 (○), 4 (●), 6 (△), 10 (□), and 20 (■) mM) is shown. Kinetic data were transformed into Hill plots for Glc-1-P which yielded the apparent K_m values and Hill coefficients [12]. The Hill coefficient (n) was 1.5 over a 3-20 mM Glc-1-P concentration range. From the secondary plot of the K_m(app) versus α-D-glucose concentration, a K_i value of 1.7 ± 0.1 mM was determined. (b) As in (a) except that α-D-glucose was replaced by β-D-glucose (0 (○), 10 (●), 20 (□), 40 (△), and 60 (■) mM). Similarly, data analysis provided a K_i value of 7.4 ± 0.7 mM (n=1.5).

bonds to GPb (Figure 1). In the GPb-α-D-glucose complex, the α-1-OH makes a hydrogen bond to a water molecule (OH8 Wat872). This water in turn is hydrogen bonded to Asp283. Examination of the van der Waals surface of α-D-glucose bound to GPb shows that there is an empty pocket at the β-1-C configuration which represents the pocket recognized in previous binding studies [9]. This β pocket, which is lined by both polar and nonpolar groups, has been the target for most of the additional groups to provide a tighter inhibitor than glucose itself. Modelling studies [2] suggest that β-D-glucose would bind in a mode that places the β-1-OH at a site with few favourable interactions for the β-1-OH and one less hydrogen bond than in the α-D-glucose complex.

The α and β anomers were tested, in kinetic experiments, 1 min after dissolution by using 1-min assays (**Figure 2ab**). Comparison of inhibition constants indicates that β-D-glucose binds with an energy (ΔΔG) of about 0.9 kcal/mol less than α-D-glucose. The time dependent anomerization of α-D-glucose (50 mM) and β-D-glucose (50 mM) is shown in **Figure 3a.** Experimental points fit closely the curves drawn according to a first-order reaction equation. Similar mutarotation constants k (where k is the sum of k_1+k_2, the constants for the two opposing reactions α-D-glucose ↔ β-D-glucose) were found for the two reactions (0.038 min^{-1} and 0.039 min^{-1}, respectively), at 30 °C. Anomerization rates were found to be much lower at 16-25 °C (data not shown), while from the mutarotation data of Pigman & Isbel [10], k values of 0.01455 min^{-1} and 0.01439 min^{-1} can be calculated for α- and β-D-glucose anomerization, respectively, at 20 °C. **Figure 3b** shows a plot of the effects of 10 mM α-D-glucose and 10 mM β-D-glucose on the activity of GPb determined within 2 min and 60 min of sugar dissolution, respectively. Similar inhibition profiles were obtained for the equilibrium mixtures starting from either α or β anomer. The theoretical line drawn in **Figure 3b**, based on the composition of equilibrium solutions of α-D-glucose (36%) and β-D-glucose (64%) and by assuming that the β-anomer is noninhibitory, does not fit the experimental points obtained from the equilibrium solutions. These observations indicate that both α-D-glucose and β-D-glucose are pure, mutually exclusive competitive inhibitors of the enzyme [11].

The crystallographic data processing statistics for the GPb-α-D-glucose and GPb-β-D-glucose complexes were as follows: 147307 and 103489 observations were reduced to 37401 and 38041 independent reflections [91-92% complete to 2.3 Å resolution] with R_m values on intensities of 0.058 and 0.064, respectively. After scaling of the data to the native data set the mean

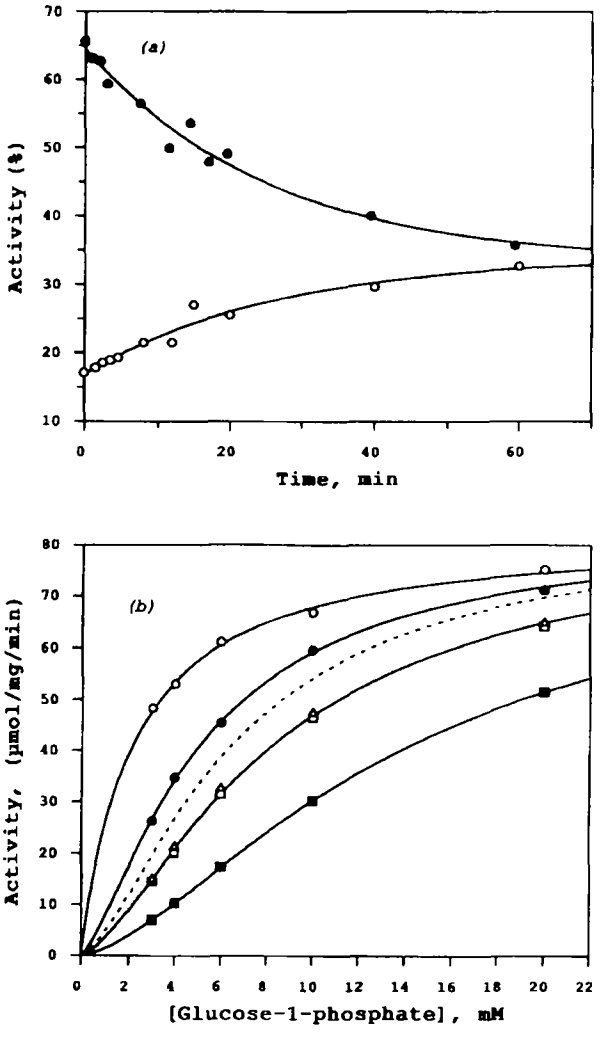
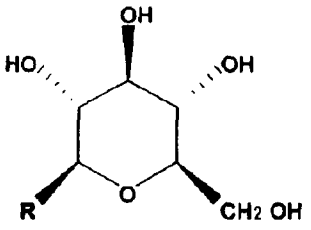
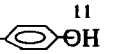


Figure 3. (a) Time-dependent anomerization of α-D-glucose and β-D-glucose at 30°C. Samples were withdrawn at various times from solutions of 50 mM α-D-glucose (O) or 50 mM β-D-glucose (●) incubated at 30°C and tested for GPb inhibition. Assay mixtures contained 4 mM Glc-1-P, 1% glycogen, 1 mM AMP and 10 mM α- or β-D-glucose (pH 6.8, 30°C). Rates were determined at 1 min to minimize mutarotation. As 100% activity was taken the specific activity (51.0 μmol/min/mg) determined in the absence of inhibitor. (b) The activity of GPb as a function of substrate concentration in the absence (O) or presence of 10 mM β-D-glucose (●) or α-D-glucose (■) or the equilibrium mixture of 10 mM α-D-glucose and β-D-glucose (Δ□). The dashed line corresponds to a theoretical line calculated by assuming that only α anomer is inhibitory.

Table I. Kinetic and crystallographic data for GPb-ß-D-glucose analogue complexes[a]

No	Substituent at ß-1-C	K_i (mM)	Polar contacts (distance, Å)		
					
	α-D-glucose	1.7 ± 0.1	O1	OH8	Wat872 (3.0)
1	R = OH	7.4 ± 0.7			
2	R = $O^{9}CH_2CH_2OH$	25.3 ± 5.6	O9	OD1	Asp339 (2.9)
3	R = $CH_2 - {}^{+}N = {}^{3}N = N^{-}$	15.2 ± 0.6	N3	OD1 OH4	Asp339 (2.7) Wat847 (2.6)
4	R = CH_2OH	21.9 ± 3.8		None	
5	R = $CH_2{}^{81}O{}^{82}SO_2CH_3$	4.8 ± 0.4	O81 O82	N OD1	Asn284 (3.1) Asp339 (2.9)
6	R = $CH_2{}^{1}NH_3^{+}$	16.8 ± 5.8	N1	OD1	Asn284 (3.2)
7	R = $CH_2CH_2{}^{1}NH_3^{+}$	4.5 ± 0.9	N1	OD1	Asp339 (3.2)
8	R = $CH_2{}^{8}CN$	9.0 ± 1.1	N8	OH4	Wat847 (3.1)
9	R = *O*(1-6)-D-glucose	16.3 ± 2.9[b]	O2' O3'	NE2 OH2	His341 (3.0) Wat890 (2.7)
10	R = $CO{}^{7}NH_2$	0.44 ± 0.07	O7 N	ND2 O	Asn284 (2.8) His377 (2.9)
11	R = $CO{}^{7}NHCH_3$	0.16 ± 0.03	N	O	His377 (3.1)
12	R = $CO{}^{7}NH$ 	4.4 ± 0.7	O11	NE2 OH2	His341 (2.9) Wat890 (3.1)
13	R = $CO{}^{7}{}^{1}NH{}^{2}NH_2$	0.4 ± 0.1	N1 N2	O OH4	His377 (2.9) Wat847 (3.0)
14	R = $CO{}^{7}{}^{1}NH{}^{2}NHCH_3$	1.8 ± 0.3	N1	O	His377 (3.0)
15	R = $CO{}^{7}NHCH_2CF_3$	8.1 ± 1.8	N	O	His377 (2.8)
16	R = $CO{}^{7}NH$ 	1.3 ± 0.3	O7 N	ND2 O	Asn284 (3.0) His377 (3.3)

a Data from [2] and [8]

b Linear noncompetitive inhibitor

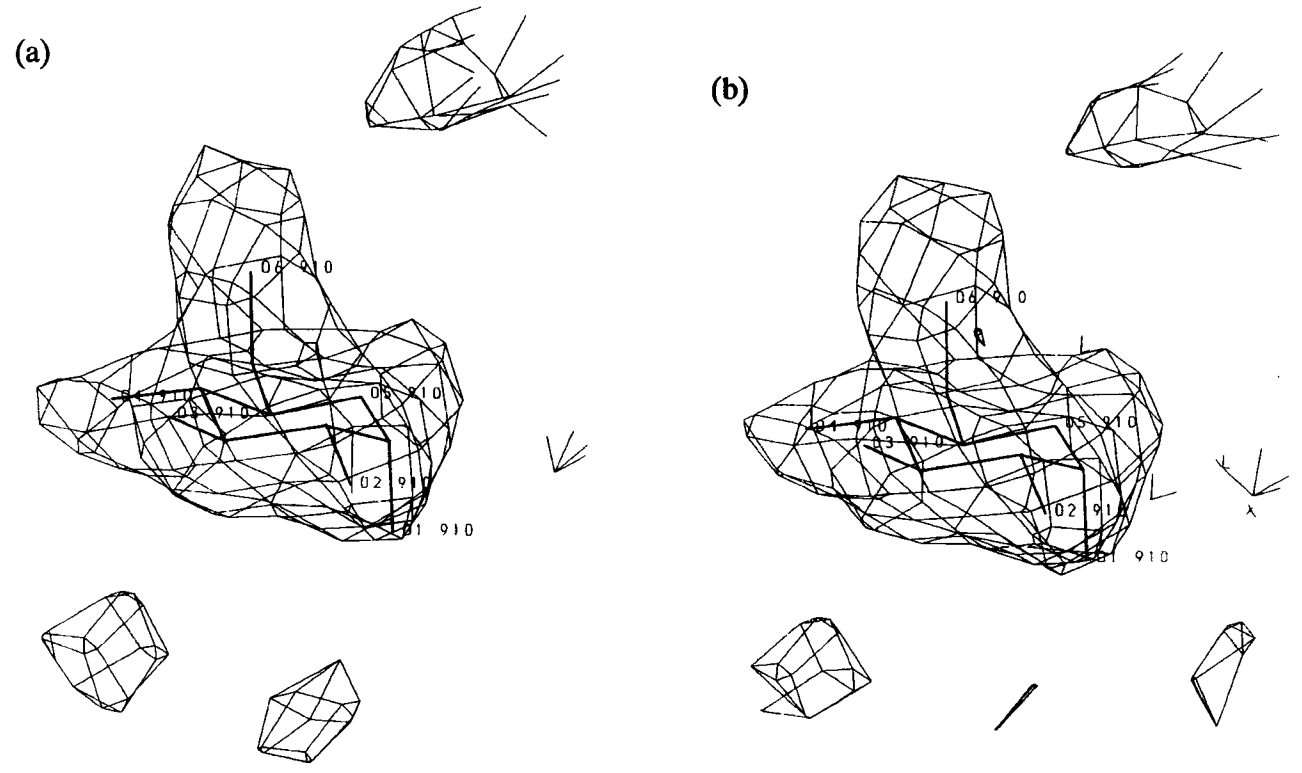


Figure 4. Difference electron density for "α-D-glucose" (a) and "β-D-glucose" (b) at the catalytic site of GPb (positive densities contoured at 3σ). The refined coordinates for the GPb-α-D-glucose complex [2] are shown.

fractional isomorphous differences (R_{iso}) in structure factor amplitudes from native T state GPb were 0.097 and 0.101, respectively. The two difference Fourier electron density maps showed one major positive peak at the catalytic site. The α-D-glucose molecule fits neatly into this density. There is a weak positive density corresponding to β-1-OH. The glucosyl portion of the β-D-glucose molecule can be fitted to the positive density without any difficulty and each of the hydroxyl groups, except β-1-OH, which is not completely covered by density. Both maps showed the same features, and a double difference Fourier map, calculated by using as coefficients $F_{GPb\text{-}\alpha\text{-}D\text{-}glucose}$-$F_{GPb\text{-}\beta\text{-}D\text{-}glucose}$, was clean at the catalytic site. (There were no traces of any positive or negative density corresponding to the α-1-OH or β-1-OH positions). Thus, although the kinetic data provide a clear indication that β-D-glucose does bind to GPb in solution, this has, however, not been achieved in the crystal, partly because of the long timescales needed for crystallographic data collection compared with enzyme kinetics timescale. The explanation for the lack of β-D-glucose binding in the crystal appears to lie to mutarotation of β-D-glucose to give a mixture of α-anomer and β-anomer and the poorer affinity of the β-D-glucose for GP. The possibility that there is a mixture of bound α and β anomeric molecules at the catalytic sites of the enzyme in the crystal (with a higher proportion of the α anomer) cannot, however, be excluded.

β-D-glucose analogues. A summary of the kinetic and crystallographic results is presented in **Table I.** All the β-D-glucose analogues (with the exception of gentiobiose) were competitive inhibitors with respect to Glc-1-P and bound at the catalytic site. The hydroxyethyl β-D-glucoside (2), modelled to fill the β pocket with a group that would tighten the binding, bound so that the four substituent atoms partially filled the β pocket and allowed a hydrogen bond from O9 to OD1 Asp339 at the end of the pocket. A water molecule (OH4 Wat847), which in the native and α-D-glucose-complexed GPb is hydrogen-bonded to Asp339, is displaced. The compound exhibited a poorer inhibition constant (K_i=25.3 mM) compared to α-D-glucose (K_i=1.7 mM). It was argued that the observed reduction in binding energy (1.6 kcal/mol) is due to the loss of entropy on binding of the flexible β substituent [2] and that replacement of this substituent with a more conformationally restrained functional group should increase binding. This was observed in the β-azidomethyl-1-deoxyglucose (3), even though the potential for 3 to form hydrogen bonds is probably lower than for 2. However, 3 (K_i=15.2 mM) is still a poorer inhibitor

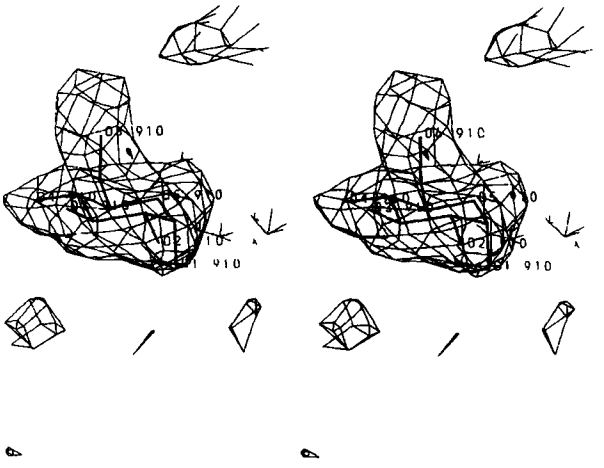


Figure 5. Stereo diagram showing difference electron density for "β-D-glucose" at the catalytic site of GPb (positive densities contoured at 3σ). The refined coordinates for the GPb-α-D-glucose complex [2] are shown.

than α-D-glucose. Compound 4 with a shorter hydroxylated substituent at β-1-C is a poor inhibitor (K_i=21.9 mM) and the crystallographic evidence showed that the O7 makes no hydrogen bonds. The β-mesylate 5 is a reasonable inhibitor (K_i=4.8 mM) and better than β-D-glucose (K_i=7.4 mM); the mesylate group was accommodated in the β pocket with satisfactory van der Waals interactions, one hydrogen bond from O81 to N Asn284 and one potential hydrogen bond from O82 to OD1 Asp339, while OH4 Wat847 was displaced in the complex with β-mesylate. In an attempt to exploit the potential ionic contacts to the Asp339 the compounds β-aminomethyl-1- deoxyglucose (6) and β-aminoethyl-1-deoxyglucose (7) were studied. Compound 6 exhibited a poor inhibition constant (K_i=16.8 mM) and the structural results showed that the distance from the amino group (N1) to Asp339 was too long for an ionic contact. Compound 7, with an additional -CH_2- group, exhibited a favourable interaction between the amino group and the aspartate and a better affinity (K_i=4.5 mM). The water OH4 Wat847 is displaced in the β-aminoethyl-1-deoxyglucose complex. However, the relatively small increase in binding energy (0.8 kcal/mol) between compounds 6 and 7 did not indicate improved inhibition constant that might have been expected from a direct interaction between the amino group and OD1 Asp339 in compound 7. The cyanomethyl compound 8, β-methylcyano-1-deoxy-glucose, was explored to determine the contribution of a three-atom substituent which has a rigid geometry. Compound 8 is a better inhibitor (K_i=9.0 mM) than 2 despite the fact that it has one less atom in the chain for interaction with the enzyme. Gentiobiose (9) bound with the second sugar well located in the β pocket (41 van der Waals contacts) and there were two additional hydrogen bonds. However, the torsion angle O1-C6'-C5'-O5'(48°) differed significantly from the single crystal and theoretical values (-62° and - 60°, respectively). The β-heptonic acid amide (10) proved to be a good inhibitor with K_i=0.44 mM. The X-ray analysis showed that there is one good hydrogen bond from the NH_2 group to the

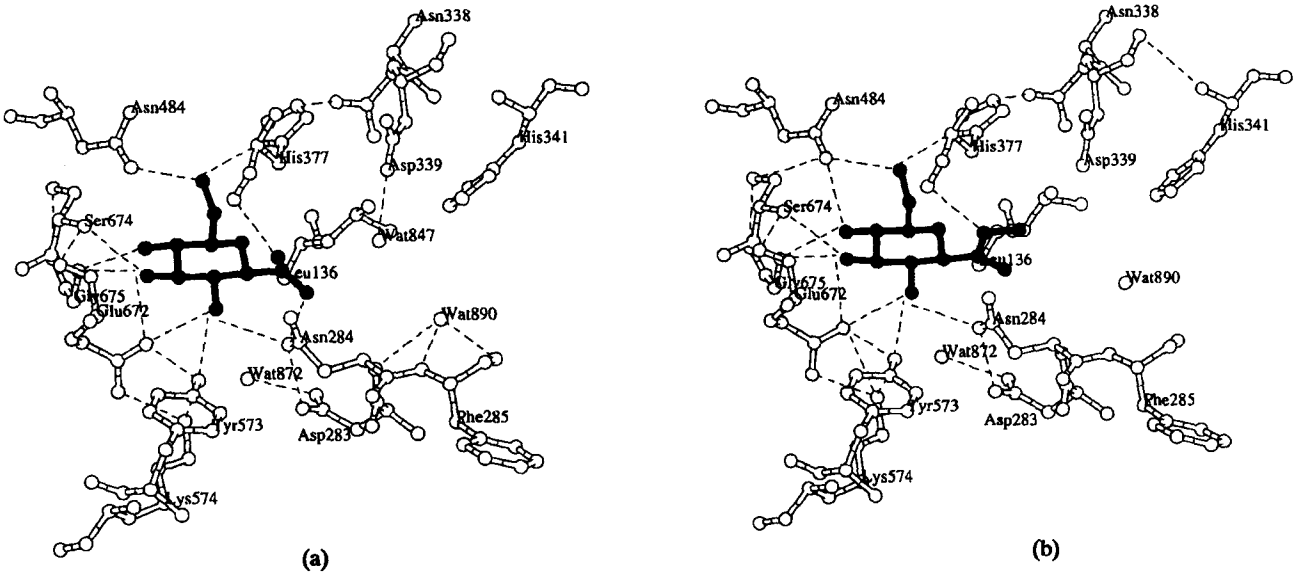


Figure 6. Contacts between β-glucoheptonamide 10 (a) or β-heptonic acid methylamide 11 (b) and GPb (from [8]).

main chain O of His377 and one poor hydrogen bond from the carbonyl oxygen O7 to ND2 Asn284 (**Fig. 6a**). The methyl amide 11 is the best inhibitor with a K_i value of 0.16 mM. The amide nitrogen makes one hydrogen bond directly to O His377. The methyl group makes 3 van der Waals contacts to non-polar groups (His377 and Thr378) and 3 van der Waals contacts to polar groups (OD1 Asp339, O His377 and ND_2 Asn284), while displacing a water molecule (OH4 Wat847) (**Fig. 6b**). The two compounds 10 and 11 are significantly more potent inhibitors than α-D-glucose but attempts to improve potency by making further substitutions to either 10 or 11 have not led to a better inhibitor. In the complex with 12, the aromatic group fits in the β pocket with 23 van der Waals interactions to the ring. However, there were small adjustments in the protein structure and glucosyl moiety [8] needed to accommodate the phenyl ring. The hydrogen bonds to the amide are also less favourable than in 10. The hydroxyl group is hydrogen-bonded to NE2 His341 and to OH2 Wat890 but these hydrogen bonds appear to make little contribution to the binding energy (K_i=4.4 mM). The hydrazide 13 bound in a very similar mode to the methyl amide 11. However, the water OH4 Wat847 had reappeared and the N2 atom is hydrogen-bonded to this water. Thus, the replacement of the $-CH_3$ group of 11 by the polar $-NH_2$ group in 13 was anticipated to produce an even tighter affinity of 13 for the enzyme. Compound 13 did not, however, show improved affinity. Compound 13 is a slightly poorer inhibitor (K_i=0.4 mM) than compound 11 (K_i=0.16 mM) and a possible explanation for the small difference in K_i may lie in the energy required to dehydrate the hydrazide 13 on transferring to the buried environment of the protein. The methyl hydrazide 14 was a poorer inhibitor (K_i=1.8 mM) than the unmethylated hydrazide 13. The additional methyl group displaced the water OH4 Wat847 and there was no hydrogen bond from the N2 atom. Compound 15 was a worse inhibitor (K_i=8.1 mM) than compound 14 and this is because the fluorine atoms are placed close to Asp339 (2.9 Å). The cyclopropane derivative 16 with a rigid non-polar group is a better inhibitor (K_i=1.3 mM) than 15 and comparable to the methyl hydrazide 14 (K_i=1.8 mM). Accommodation of the cyclopropane group in the pocket required some shifts of the glucosyl and amide moieties [8] with respect to their positions in the methyl amide 11.

In conclusion, these studies have led to some generalizations concerning the interactions between β-D-glucose analogues and GPb. (1) The chemical nature of the hydrogen-bonding atoms and the directionality of the hydrogen bond are important. (2) The smaller the loss in conformational entropy, either in the form of flexibility or in the form of unusual conformation, of the ligand on binding to the enzyme, the greater the affinity. (3) Complementarity of van der Waals interactions is an important determinant for the overall affinity; part of the difficulty in fitting additional groups into the β pocket arises from the mixed polar/non-polar nature of the binding pocket. (4) Ligand or protein desolvation effects are also important; the energy required to dehydrate a compound on binding at the catalytic site of GPb is not fully compensated by hydrogen bonds to the protein.

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