The binding of D-gluconohydroximo-1,5-lactone to glycogen phosphorylase

Kinetic, ultracentrifugation and crystallographic studies

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Combined kinetic, ultracentrifugation and X-ray-crystallographic studies have characterized the effect of the β -glucosidase inhibitor gluconohydroximo-1,5-lactone on the catalytic and structural properties of glycogen phosphorylase. In the direction of glycogen synthesis, gluconohydroximo-1,5-lactone was found to competitively inhibit both the *b* (K_i 0.92 mM) and the *a* form of the enzyme (K_i 0.76 mM) with respect to glucose 1-phosphate in synergism with caffeine. In the direction of glycogen breakdown, gluconohydroximo-1,5-lactone was found to inhibit phosphorylase *b* in a non-competitive mode with respect to phosphate, and no synergism with caffeine could be demonstrated. Ultracentrifugation and crystallization experiments demonstrated that gluconohydroximo-1,5-lactone was able to induce dissociation of tetrameric phosphorylase *a* and stabilization of the dimeric T-state conformation. A crystallographic binding study with 100 mM-gluconohydroximo-1,5-lactone at 0.24 nm (2.4 Å) resolution showed a major peak at the catalytic site, and no significant conformational changes were observed. Analysis of the electron-density map indicated that the ligand adopts a chair conformation. The results are discussed with reference to the ability of the catalytic site of the enzyme to distinguish between two or more conformations of the glucopyranose ring.

INTRODUCTION

Glycogen phosphorylase (1,4- α -D-glucan:orthophosphate α -glucosyltransferase, EC 2.4.1.1) catalyses the degradative phosphorolysis of glycogen to form glucose 1-phosphate, the initial stage in the production of metabolic energy in muscle and glucose in liver (Graves & Wang, 1972). The enzyme can exist in at least two structural states in the nomenclature of Monod *et al.* (1965): a T state with low affinity and an R state with high affinity for substrates and activators. The R state can be induced by AMP, substrates and certain substrate analogues such as glucose 1,2-(cyclic)phosphate and UDP-glucose. ATP, glucose 6-phosphate, glucose and caffeine can convert the enzyme from the R state into the T state. A number of studies have demonstrated that glucose causes homotropic co-operativity between substrate sites and exhibits a weakening of binding to the enzyme in the presence of AMP (Helmreich *et al.*, 1967), shows a synergistic inhibition with the strong (K_i 0.2 mM) T-state inhibitor caffeine (Kasvinsky *et al.*, 1978) and promotes dissociation of the tetrameric enzyme (Wang *et al.*, 1965; Helmreich *et al.*, 1967; Withers *et al.*, 1979). In contrast, in the presence of those inhibitors showing a preference for the R-state conformation, the enzyme exhibits hyperbolic saturation curve for the substrate, increased affinity for the nucleotide and stabilization of the tetrameric species (Withers *et al.*, 1979, 1981; Kasvinsky, 1982; Madsen *et al.*, 1983). The kinetic mechanism of the enzyme is Rapid-Equilibrium Random Bi Bi (Engers *et al.*, 1969, 1970), in which the rate-limiting step involves the interconversion of the enzyme-phosphate-glycogen complex. The reaction results in retention of the α -configuration in C-1 and is assumed to proceed

through a glycosyl oxocarbenium ion intermediate that is stabilized by the substrate phosphate itself (Tu *et al.*, 1971; Gold *et al.*, 1971; Street *et al.*, 1989; Johnson *et al.*, 1989, 1990; Palm *et al.*, 1990). Therefore ligands that resemble the half-chair geometry of such an intermediate should be expected to bind strongly to the enzyme (Kraut, 1988).

An understanding of ligand-binding specificity requires knowledge of the three-dimensional structure of the enzyme-ligand complex. The X-ray-crystallographic structure of phosphorylase *b* for both the T state and the R state has been solved (Weber *et al.*, 1978; Sansom *et al.*, 1985; Barford & Johnson, 1989). The crystal structure of the enzyme in the T state has been refined at 0.19 nm (1.9 Å) resolution (K. R. Acharya, D. I. Stuart, K. M. Varvill, B. M. Ouzman & L. N. Johnson, unpublished work), and ligand-binding experiments in the crystal should therefore provide details in terms of ligand-specificity, catalysis and allosteric control. In the T-state structure there is no direct access to the catalytic site from the solvent: loop 280s blocks the channel leading to this site. The binding of heptulose 2-phosphate (1-deoxy-D-gluco-heptulose 2-phosphate) a dead-end product and a potent inhibitor of the enzyme (Klein *et al.*, 1984, 1986), to the catalytic site induces a movement of the side chain of Arg-569 [approx. 0.7 nm (7 Å)] from a buried position to another that brings the NH1 atom close to O-9 of the heptulose 2-phosphate. This movement, which is critical for the formation of the substrate phosphate recognition site, is accompanied by a displacement of the loop carrying the acidic Asp-283 (Hajdu *et al.*, 1987; Johnson *et al.*, 1990). The binding of UDP-glucose, a substrate analogue that belongs to the class of the R-state inhibitors (Madsen *et al.*, 1983), to the catalytic site

Abbreviations used: the crystallographic merging *R* factor is defined as $\sum_i |I(h) - I_i(h)| / \sum_i I_i(h)$, where $I_i(h)$ is the *i*th measurement of the intensity of reflexion *h* and $I(h)$ is the mean of these measurements.

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Fig. 1. D-Gluconohydroximo-1,5-lactone *N*-phenylurethane (I) and D-gluconohydroximo-1,5-lactone (II) conformations as deduced from their difference electron-density maps

promotes extensive conformational changes (Oikonomakos *et al.*, 1988). The whole loop 280s is displaced from its initial position and becomes mobile. These structural events leading to the activated R-state conformation have been confirmed with the structural analysis of the R-state phosphorylase *b* (Barford & Johnson, 1989). In contrast, glucose and caffeine have been shown to stabilize the T-state conformation, since they interact with residues of the 280s loop (see Johnson *et al.*, 1989).

In order to obtain information on whether the catalytic site shows a preference for half-chair geometry of the glucopyranoside ring, the binding of D-gluconohydroximo-1,5-lactone *N*-phenylurethane (I) (Fig. 1) in the crystal was studied (Barford *et al.*, 1988). Compound (I) was shown to be a good specific inhibitor of phosphorylase *b*, competitive with glucose 1-phosphate, and it binds to the enzyme almost 10 times more tightly than glucose (Barford *et al.*, 1988; Papageorgiou *et al.*, 1989). X-ray-crystallographic analysis revealed that the sugar moiety of the molecule is located within the glucose 1-phosphate-binding site, with the trigonal geometry at C-1 directing the phenylurethane group into a unique channel and packing it against the 280s loop (Barford *et al.*, 1988). In solution, however, compound (I) was shown to bind to both the R and the T conformations of the enzyme (Papageorgiou *et al.*, 1989). To gain further understanding of the specificity of the catalytic site, the present study reports the effect of the unsubstituted D-gluconohydroximo-1,5-lactone (II) on the kinetic, sedimentation and crystallization properties of phosphorylase. A crystallographic study of the ligand binding to phosphorylase *b* at 0.24 nm (2.4 Å) resolution is also described.

MATERIALS AND METHODS

Materials

The synthesis of compound (II) has been described previously (Beer & Vasella, 1985), and its stability during the solution and crystallographic experiments was checked by t.l.c. (Beer & Vasella, 1985). A 100 mM solution of compound (II) was left for 48 h at 22 °C. Subsequent analysis showed a single band with an R_f value of 0.31, in agreement with Beer & Vasella (1985). Oyster glycogen (Serva) was freed of AMP by the method of Helmreich & Cori (1964). AMP, NADP⁺, glucose 1-phosphate (dipotassium salt) and dithiothreitol were products of Sigma Chemical Co. Yeast glucose-6-phosphate dehydrogenase and rabbit

phosphoglucomutase, purchased from Boehringer Mannheim as suspensions in 3.2 M-(NH₄)₂SO₄, were dialysed against 20 mM-β-glycerophosphate/20 mM-2-mercaptoethanol/1.5 mM-EDTA, pH 6.8, immediately before use. All other reagents were of the highest purity commercially available.

Preparation of phosphorylase

Phosphorylase *b* was isolated from rabbit skeletal muscle by the method of Fischer & Krebs (1962) with the use of 2-mercaptoethanol instead of L-cysteine and recrystallized at least four times. Phosphorylase *a* was prepared from phosphorylase *b* by the action of phosphorylase kinase (Cohen, 1973) and recrystallized three times. Bound nucleotides were removed from both enzymes as described by Melpidou & Oikonomakos (1983).

Determination of phosphorylase activity

Phosphorylase activity in the direction of glycogen synthesis was determined at pH 6.8 and 30 °C by measuring the inorganic phosphate released in the reaction (Fiske & Subbarow, 1925). The enzymes (5–10 µg/ml) were assayed in 47 mM-triethanolamine/HCl buffer, pH 6.8, containing 100 mM-KCl, 1 mM-dithiothreitol and 1 mM-EDTA with 1% (w/v) glycogen and various concentrations of glucose 1-phosphate, AMP and inhibitor as indicated in the Figure legends. Enzyme and glycogen were preincubated for 15 min at 30 °C before the reaction was initiated with glucose 1-phosphate. Final assay volumes were 0.5 ml. Initial velocities were calculated from the pseudo-first-order reaction constants (Engers *et al.*, 1970). Enzyme activity in the direction of phosphorolysis of glycogen, with the auxiliary assay system (Helmreich & Cori, 1964), was measured as described previously (Papageorgiou *et al.*, 1989), except that the final reaction mixtures were 0.75 ml and contained 1.5 µg of phosphorylase *b*, 10 µg of glucose-6-phosphate dehydrogenase and 25 µg of phosphoglucomutase.

Ultracentrifugation

Ultracentrifugation experiments were performed on an MSE Centrifuge 75 preparative and analytical ultracentrifuge with 10 mg of phosphorylase *a*/ml in 5 mM-β-glycerophosphate buffer, pH 6.8, containing 5 mM-2-mercaptoethanol and 0.1 mM-EDTA at 50000 rev./min at 20 °C. Sedimentation coefficients calculated by the sedimentation-velocity method from direct measurements

of the scanner traces were corrected for viscosity and density of the buffer to water at 20 °C (Oikonomakos *et al.*, 1985).

Protein concentration

Protein was determined from absorbance measurements at 280 nm by using the specific absorption coefficient $A_{1\text{ cm}}^{1\%} = 13.2$ (Kastenschmidt *et al.*, 1968).

Kinetic analyses

Experimental points shown in the Figs. 2–5 are averages of triplicates. Kinetic data presented in the form of a linear double-reciprocal (Lineweaver–Burk) plot were analysed by a non-linear-regression data-analysis program (Leatherbarrow, 1987) by assuming that each rate is subject to about the same proportion of random error (Cornish-Bowden & Wharton, 1988). The program calculates K_m and V_{max} , and the standard errors for these values. Kinetic data presented in the form of Hill (or Dixon) plots were treated by linear-regression analysis (Leatherbarrow, 1987), also using the same type of error distribution. In this case the program only calculates the standard errors of the slopes and intercepts. Standard errors of the $K_{m(\text{app})}$ were therefore calculated by using known relations between standard errors (Squires, 1968). Determination of K_i values was made by replotting $K_{m(\text{app})}$ (slopes or intercepts) versus inhibitor concentration (Segel, 1975) with the use of explicit-weighting linear-regression analysis (Leatherbarrow, 1987), i.e. by providing an explicit value for the standard deviation of each point. In general, the means of the standard errors for the calculated kinetic parameters in all our kinetic analyses averaged less than 10 %.

X-ray crystallography

Tetragonal crystals of phosphorylase *b* grew by the batch method in the presence of 1 mM-IMP and 1 mM-spermine as described previously (Oikonomakos *et al.*, 1985, 1987). Two crystals of approximate dimensions 0.5 mm × 0.5 mm × 2.0 mm were used, and each crystal was soaked for 35 min at 16 °C in thin-walled glass capillary tubes in 10 mM-Bes/NaOH buffer,

pH 6.7 containing 0.1 mM-EDTA, 0.02 % NaN_3 and 100 mM-compound (II). Data to 0.24 nm (2.4 Å) resolution were collected with a Rigaku RU200 rotating-anode X-ray source and graphite monochromator on a Nicolet IPC multiwire area detector (Howard *et al.*, 1987) with 0.2° oscillation frames of exposure time 200 s. Processing of frames was performed with the XENGEN program package (Howard *et al.*, 1987). The structure-factor amplitudes for the ligand-bound protein (F_{PL}) were scaled to the native structure factors (F_p) (with the CCP4 programs CAD and ANSC, modified by S. Gover), where F_p were from cycle 54 of the 0.19 nm (1.9 Å) refinement of phosphorylase *b*. Difference Fourier synthesis was based on the coefficient $m(F_{\text{PL}} - F_p) \cdot \exp ia$, where m is a weight (Sim, 1959, 1960) and phases a were from the native refinement. The final native structure has a crystallographic R factor of 0.191 for 61 344 reflexions ($F \geq 3\sigma$) to 0.19 nm (1.9 Å) resolution. The model contains 6706 protein atoms and 689 water molecules with root-mean-square deviation from standard bond lengths of 0.002 nm (0.02 Å) (K. R. Acharya, D. I. Stuart, K. M. Varvill, B. M. Ouzman & L. N. Johnson, unpublished work). Electron-density maps were examined on an Evans and Sutherland PS330 colour graphics device, on-line to a VAX 6210 computer, with the program FRODO (Jones, 1978, 1985) modified by P. R. Evans. Van der Waals interactions were noted for non-hydrogen atoms whose separation was ≤ 0.4 nm (4 Å). Hydrogen bonds were assigned by FRODO (P. R. Evans, unpublished work) if the two electronegative atoms were less than 0.33 nm (3.3 Å) apart and if the angle between the two atoms and each of their preceding side chain or main chain atoms was greater than 90°.

RESULTS

Kinetics

Compound (II) appears to act as a competitive inhibitor with respect to glucose 1-phosphate in both forms of phosphorylase (Fig. 1). The inhibition profile is similar to that obtained with glucose (Helmreich *et al.*, 1967; Papageorgiou *et al.*, 1989),

Fig. 2. Kinetics of compound (II) inhibition of phosphorylase *b* and phosphorylase *a* with respect to glucose 1-phosphate

(a) Double-reciprocal plot for phosphorylase *b* at constant concentrations of AMP (1 mM) and glycogen (1 %) and various concentrations of compound (II). Inhibitor concentrations were: 0 mM (○); 1 mM (●); 2 mM (□); 3 mM (■); 4 mM (Δ). Inset: Hill plots for glucose 1-phosphate. $\log[v/(V-v)]$ was plotted against $\log[\text{glucose 1-phosphate}]$, which yielded the apparent K_m values and Hill coefficients for glucose 1-phosphate (Helmreich *et al.*, 1967); the apparent K_m values and the Hill coefficients (given in parentheses) were 3.2 mM, 6.3 mM (1.27), 9.5 mM (1.37), 13.8 mM (1.31) and 20 mM (1.35) at 0 mM-, 1 mM-, 2 mM-, 3 mM- and 4 mM-compound (II) respectively. (b) As in (a) except that phosphorylase *b* was replaced by phosphorylase *a*. Inset: Hill plots for glucose 1-phosphate; the apparent K_m values and the Hill coefficients (given in parentheses) were 3.2 mM, 7.2 mM (1.43), 11.4 mM (1.28) and 15.9 mM (1.29) at 0 mM-, 1 mM-, 2 mM- and 3 mM-compound (II) respectively.

Fig. 3. Kinetics of compound (II) inhibition of phosphorylase *b* with respect to AMP

Double-reciprocal plot for phosphorylase *b* at constant concentrations of glucose 1-phosphate (2.5 mM) and glycogen (1%) and various concentrations of compound (II). Inhibitor concentrations were: 0 mM (○); 0.25 mM (●); 0.5 mM (□); 1.0 mM (■). Inset: Hill plots for AMP; the apparent K_m values and the Hill coefficients (given in parentheses) were 71 μ M (1.52), 104 μ M (1.50), 124 μ M (1.43) and 161 μ M (1.35) at 0 mM-, 0.25 mM-, 0.5 mM- and 1.0 mM-compound (II) respectively.

where the deviation from linearity in the double-reciprocal plots is interpreted as representing homotropic co-operativity between the substrate-binding sites (Helmreich *et al.*, 1967). Following an approach similar to that of Sprang *et al.* (1982), from a replot of the $K_{m(app.)}$ calculated as described in Fig. 2 versus inhibitor concentration, K_i values of 0.92 mM and 0.76 mM were obtained for phosphorylase *b* and phosphorylase *a* respectively. For phosphorylase *a* with 1 mM-AMP, compound (II) acted as a competitive inhibitor towards glucose 1-phosphate (results not shown), but in this case the inhibitory effect was largely overcome by the presence of nucleotide (K_i 6.1 mM). For phosphorylase *b* the effect of compound (II) on the binding of AMP was also studied at constant concentrations of glucose 1-phosphate (2.5 mM) and glycogen (1%) (Fig. 3). The decrease both in the affinity of AMP for the enzyme and in the Hill coefficient (*h*) is more pronounced in the presence of compound (II) than in the presence of its phenylurethane derivative (Papageorgiou *et al.*, 1989), where no significant effects on the $K_{m(app.)}$ and *h* were observed. When compound (II) was tested in the presence of caffeine, a strong synergistic inhibition of phosphorylase *b* with respect to glucose 1-phosphate was observed (Fig. 4). The interaction constant, which indicates the effect of compound (II) on the binding of caffeine (Segel, 1975), is 0.2, a value similar to that obtained previously for the interaction between glucose and caffeine (Madsen *et al.*, 1983).

The kinetics with respect to phosphate in the direction of glycogen phosphorylase in the presence of either compound (II) or glucose is shown in Fig. 5. Compound (II) appears to act as a linear non-competitive inhibitor with respect to phosphate, in contrast with glucose, which shows non-linear competitive kinetics. Using the general equation for a non-competitive kinetic model (Kasvinsky, 1982), from the secondary plot of the intercepts (Fig. 5a), we calculated an apparent dissociation constant for binding of compound (II) to the enzyme-glycogen-phosphate complex of 1.52 mM, a value that is higher than that for the binding of compound (I) to the enzyme-glycogen-phosphate complex (0.20 mM) (Papageorgiou *et al.*, 1989). In the

Fig. 4. Synergistic inhibition of phosphorylase *b* activity by compound (II) and caffeine

Dixon plot of reciprocal velocity versus compound (II) concentration at various caffeine concentrations and constant concentrations of glucose 1-phosphate (10 mM), AMP (1 mM) and glycogen (1%). Caffeine concentrations were: 0 mM (○); 0.25 mM (●); 0.5 mM (□); 1 mM (■). The intersection point above the horizontal axis, corresponding to the dissociation constant of compound (II) in the presence of caffeine (Segel, 1975), αK_i , equals 0.18 mM. Assuming a K_i value of 0.92 mM, the interaction constant between compound (II) and caffeine, α , is equal to 0.2. The replot of the slopes versus caffeine concentration shown in the inset yields αK_i , the dissociation constant for caffeine in the presence of compound (II), αK_c , is equal to 0.04 mM, and allows the calculation of the dissociation constant for caffeine, K_c , as 0.2 mM.

reverse reaction compound (II) was not synergistic (results not shown) and showed slightly less than additive inhibition in the presence of caffeine.

Ultracentrifugation

Sedimentation-velocity experiments performed at 20 °C with phosphorylase *a* gave the following results. In the presence of 50 mM-glucose the enzyme sedimented approx. 80% as a dimer ($s_{20,w}$ = 8.9 S) and 20% as a tetramer ($s_{20,w}$ = 13.4 S), in agreement with Wang *et al.* (1965), who employed the same buffer in their experiments. Similarly, in the presence of 10 mM- and 20 mM-compound (II), the percentages of dimer and tetramer were approx. 70 and 80% and 30 and 20% respectively. In contrast, compound (I) could not induce any dissociation of the enzyme (Papageorgiou *et al.*, 1989).

Crystallization

Since glucose is known to induce crystallization of phosphorylase *a* in the tetragonal form (Fletterick *et al.*, 1976), the effect of compound (II) on the crystallization of the enzyme was studied. The inclusion of compound (II) rather than glucose in the crystallization medium resulted in large tetragonal crystals within a few days and 22 °C. The final crystallization medium (pH 6.7) contained 20 mg of phosphorylase *a*/ml, 10 mM-Bes/NaOH buffer, 10 mM-magnesium acetate, 0.1 mM-EDTA, 3 mM-dithiothreitol, 10–20 mM-compound (II) and high dilution of seed crystals (10^6) obtained by grinding tetragonal crystals of phosphorylase *b*.

X-ray crystallography

The data-processing statistics were as follows. The final data set consisted of 70630 observations, which were reduced to a

Fig. 5. Kinetics of compound (II) and glucose inhibitions of phosphorylase *b* with respect to phosphate

(a) Double-reciprocal plot for phosphorylase *b* at constant concentrations of AMP (0.5 mM) and glycogen (0.5%) and various concentrations of compound (II). Inhibitor concentrations were: 0 mM (○); 0.5 mM (●); 1.0 mM (□); 2.0 mM (■). Inset: secondary plot of the slopes (▲) and intercepts (▼). (b) As in (a) except that compound (II) was replaced by glucose at the following concentrations: 0 mM (○); 5 mM (●); 10 mM (□); 20 mM (■). Inset: Hill plot for phosphate. The apparent K_m values and the Hill coefficients (given in parentheses) were 1.3 mM, 6.7 mM (1.30), 10.9 mM (1.38) and 20.0 mM (1.33) at 0 mM-, 5 mM-, 10 mM- and 20 mM-glucose respectively. From the secondary plot of $K_{m(app.)}$ versus glucose concentration a K_i value of 2.2 mM can be calculated.

unique data set of 31 627 independent reflexions [83% of the unique set at 0.24 nm (2.4 Å) resolution] with R_m on intensities of 6.2%. After scaling of the data to the native data set the mean fractional isomorphous change was 0.135. The gluconohydroximo-1,5-lactone difference Fourier map showed one major positive peak and a few negative peaks at the catalytic site. The ligand does not appear to induce any conformational changes at the active site, except that His-377 moves slightly and at least one water molecule is displaced. With regard to the other binding sites of the enzyme molecule (allosteric, glycogen-storage and inhibitor sites), the difference map showed no significant peaks.

The single-crystal structure of compound (II) itself is not known. The structure of the gluconohydroximolactone moiety of compound (I) (Barford *et al.*, 1988) was taken as a basis for the structure of compound (II), and several models were constructed. AM1 energy minimization calculations (made by using the AMPAC option of the QCPE program 506 package) were performed for different conformations of compound (II), and the energies are given in Table 1 to compare the stability of the structures. Calculation indicates several energy minima for several easily interconvertible conformers, and thus a great conformational flexibility. As can be seen from Table 1, the calculated conformations of compound (II) are within 11.3 kJ/mol (2.7 kcal/mol) of their minimum energy conform-

ation. The fitting of each model in the density map was investigated. The best fitting was achieved with compound (II) in the chair 4C_1 and a *gg* conformation about the C-5-C-6 bond (Fig. 6). The half-chair or distorted 4H_3 conformation [almost identical with that observed for compound (I) (Barford *et al.*, 1988) with torsion angles O-5-C-1-N-1-O-7 and C-2-C-1-N-1-O-7 of -2° and -180° respectively] does not account well for the observed density, although there are no steric conflicts with the enzyme. In contrast, no permissible fits could be obtained with the boat $B_{2,5}$, the skewed boat 1S_5 , the boat $B_{3,0}$, and the distorted 1S_3 structures.

The contacts between the atoms of compound (II) and the protein are summarized in Table 2. With the compound (II) molecule in the chair conformation there are 74 van der Waals interactions and nine hydrogen bonds between the ligand and the protein. All the hydroxy groups of the glucose moiety and the oxygen atom of the hydroximo group are involved in hydrogen bonds with protein atoms. Hydrogen-bond interactions formed between the oxygen atoms of the analogue and the atoms of the protein are illustrated in Fig. 7. The hydrogen-bond pattern of the gluconohydroximo-1,5-lactone-phosphorylase *b* complex is similar to that displayed by other glucose and glucosyl derivatives (Johnson *et al.*, 1980; Jenkins *et al.*, 1981; McLaughlin *et al.*, 1984; Hajdu *et al.*, 1987; Oikonomakos *et al.*, 1987, 1988; Barford *et al.*, 1988). In general, since the ligand adopts a chair conformation, there are more similarities with those compounds that have a chair geometry (e.g. glucose) than the compounds with a half-chair geometry [e.g. heptenitol (2,6-anhydro-1-deoxy-D-glucio-hept-1-enitol) and compound (I)] at the glucosyl moiety (Fig. 8). Thus comparison of the binding of compound (II) with that of its phenylurethane derivative compound (I) (Barford *et al.*, 1988) revealed a difference in the direction of the substituents, due to the different geometry of their respective rings (Fig. 8). It is noteworthy that all the glucosyl compounds studied so far bind to phosphorylase *b* with their sugar rings in a *gg* conformation about the C-5-C-6 bond [torsion angles O-6-C-6-C-5-O-5 and O-6-C-6-C-5-C-4 in compound (II) are -77° and 42° respectively]. AM1 calculations, performed for the 4C_1 conformer of compound (II), show that the *gg* rotamer is the

Table 1. AM1 calculations for compound (II)

Conformation	Heat of formation [kJ/mol (kcal/mol)]
$B_{2,5}$	-972.4 (-232.4)
1S_5	-970.7 (-232.0)
$B_{3,0}$	-966.1 (-230.9)
4C_1	-964.0 (-230.4)
4H_3	-963.2 (-230.2)
4H_3 distorted	-961.5 (-229.8)
1S_3 distorted	-961.1 (-229.7)
$B_{3,0}$ flattened	-961.1 (-229.7)

Fig. 6. Stereo diagrams showing best fitting of the chair conformation (a) and half-chair conformation (b) of compound (II) in the difference electron density at the catalytic site

Positive contours at 300 arbitrary units (3 times the root mean square density over the map) only are shown.

Table 2. Hydrogen bonds and van der Waals contacts between compound (II) and phosphorylase *b*

Hydrogen bonds [≤ 0.33 nm (3.3 Å)]	
O-2	OE1 Glu-672 OH Tyr-573 ND2 Asn-284
O-3	OE1 Glu-672
O-4	N Gly-675 OH ₂ Water-897
O-5	ND1 His-377
O-6	ND1 His-377
O-7	N Leu-136
van der Waals contacts [≤ 0.4 nm (4 Å)]	
C-1	Leu-136
C-2	Asn-284, His-377, Glu-672
O-2	Asn-284, Tyr-573, OH ₂ -908, Glu-672
C-3	Glu-672, Gly-675, OH ₂ -897
O-3	Glu-672, Ala-673, Ser-674, Gly-675
C-4	Asn-484, Gly-675, OH ₂ -897
O-4	Asn-484, Ser-674, Gly-675, OH ₂ -897
C-5	Leu-136, His-377, Asn-484, OH ₂ -897
C-6	Gly-135, His-377, Asn-484, OH ₂ -897
O-6	His-377, Val-455, Asn-484
O-5	Gly-135, Leu-136, His-377
O-7	Gly-135, Leu-136, OH ₂ -887, OH ₂ -910
N-1	Leu-136, Asn-284, OH ₂ -910

most stable one ($\Delta E_{ig} - \Delta E_{gg} = 0.55$ kJ/mol and $\Delta E_{gt} - \Delta E_{gg} = 4.1$ kJ/mol). In contrast, glucose or glucose 1,2-(cyclic)phosphate on binding to phosphorylase *a* in the crystal adopts a *gt* conformation (Sprang *et al.*, 1982; Withers *et al.*, 1982) about the C-5–C-6 bond. In these complexes His-377 moves toward the sugar whereas in the phosphorylase *b* complexes it moves away from the sugar position. This observation indicates that small conformational differences exist between the crystal structures of phosphorylase *b* and phosphorylase *a* at the catalytic site.

DISCUSSION

Compounds (I) and (II) have been shown to be potent inhibitors of the β -glucosidase emulsin (Beer & Vasella, 1986), with K_i values of 2.3 μ M and 98 μ M respectively. Since it was proposed that these compounds might act as transition-state

analogues for the β -glucosidase catalysis and mimic the trigonal geometry required at C-1 for the formation of the carbonium ion intermediate, their interactions with phosphorylase were investigated by solution and X-ray-diffraction techniques. The results reported previously (Barford *et al.*, 1988; Papageorgiou *et al.*, 1989) and here show that, in spite of their structural similarities and the trigonal geometry at C-1, the two compounds act differently. Thus compound (I) is a linear competitive inhibitor for glucose 1-phosphate and exhibits a weak synergistic effect with caffeine, whereas compound (II) is a non-linear competitive inhibitor with respect to glucose 1-phosphate and exhibits a strong synergistic effect with caffeine. In addition, the first inhibitor does not promote the dissociation of phosphorylase *a* to dimers, whereas the second does. Also, the crystallization experiments indicate that compound (II) can induce in phosphorylase *a* the formation of tetragonal crystals, a result consistent with previous results obtained with the T-state inhibitor glucose (Fletterick *et al.*, 1976). A similarity between the two compounds exists only in their inhibitory pattern in the reverse reaction. Both compounds are linear non-competitive inhibitors with respect to phosphate (Papageorgiou *et al.*, 1989; Fig. 4).

Binding studies of the crystals of the T-state phosphorylase *b* at 0.24 nm (2.4 Å) resolution show that both ligands bind at the catalytic site, but compound (II) adopts a chair conformation (Fig. 5) whereas its phenylurethane derivative adopts a half-chair conformation (Barford *et al.*, 1988). Their glucosyl rings therefore occupy a similar but not identical position [C-1–C-1 separation is approx. 0.1 nm (1 Å)] with the hydroximo group occupying a different position [N-1–N-1 separation is approx. 0.2 nm (2 Å) and O-7–O-7 separation is approx. 0.25 nm (2.5 Å) and the *N*-phenylurethane moiety extended to a cavity next to the catalytic site. In the enzyme–compound (I) complex the phenylurethane group is directed to the channel 2 (Barford *et al.*, 1988), but in the enzyme–compound (II) complex the hydroximo group occupies an intermediate position between channel 1 and channel 2. In the binding study by Barford *et al.* (1988) it was found that the phenylurethane group forms hydrogen bonds with the residues of the loop 282–285. In the present study there is very little change in the structure of phosphorylase *b* upon binding of compound (II). It was found that only Asn-284 makes a hydrogen bond with O-2 [the distance between O-2 and ND2 Asn-284 is 0.3 nm (3 Å)] and a van der Waals contact with C-2 of compound (II) [the distance between C-2 and OD1 Asn-284 is 0.4 nm (4 Å)]. The structures of compound (I), compound (II), glucose and caffeine bound at their respective sites therefore allow a rationale for their related inhibitory effects. All four ligands

Fig. 7. Stereo diagram showing the contacts between phosphorylase *b* and compound (II)

Fig. 8. Orthogonal views of the positions of various glucosyl compounds bound at the catalytic site of phosphorylase *b* as compared with the binding of compound (II)

The following compounds are illustrated: (a) compound (I); (b) glucose 1-phosphate (G1P); (c) glucose; (d) heptenitol (H); (e) heptulose 2-phosphate (H2P); (f) (UDP-Glc).

stabilize the geometry of the 280s loop occurring in the T state since they interact with residues of this loop. These features can explain the behaviour of the analogue compound (II) in the kinetics (in the direction of glycogen synthesis), in the ultracentrifugation and crystallization experiments, but are not

consistent with some of the solution properties observed with analogue compound (I), i.e. the linearity in the double-reciprocal plots with respect to substrate and the stabilization of the tetrameric phosphorylase *a* (Papageorgiou *et al.*, 1989). The major difference between compounds (I) and (II) is the bulky

phenylurethane group. In order to accommodate this in the channel, compound (I) has to adopt a half-chair conformation. The energy difference between chair conformation and half-chair conformation for these compounds is not very great, and hence the enzyme is able to influence ligand conformation. It may be suggested that the ability of the analogue compound (I) to favour T-state or R-state enzyme has to do with ability to interact with 280s loop. ^1H -n.m.r. experiments (B. Bernet, D. Beer & A. Vasella, unpublished work) indicate that an equilibrium exists between the chair, half-chair and/or boat conformers of compounds (I) and (II) in solution with a strong influence of the solvent upon the conformational equilibrium and a relatively strong participation of the chair conformers in the equilibrium. If the chair conformation of compound (I) is the most stable one in solution, it would displace the 280s loop, giving rise to R-state-like structure. It cannot do this in the crystal structure, where the enzyme is locked in the T state and the 280s loop blocks the binding of the chair conformation of this analogue at the catalytic site. In order for the chair conformation of compound (I) to bind, steric constraints have to be overcome. This steric hindrance is relieved during the structural rearrangement of the 280s loop, when UDP-glucose (with a chair geometry for the glucosyl residue and a bulky substituent at C-1) binds to the enzyme (Oikonomakos *et al.*, 1988). Thus, although the T-state catalytic site is able to accommodate compound (I) in half-chair conformation without significant changes in the enzyme structure, it cannot accept the additional bulk of phenylurethane group attached to a chair ring. Compound (II), on the other hand, is free to bind as chair and hold the 280s loop in place.

The kinetic data on inhibition of phosphorylase *b* by compound (II) (Fig. 5) showed that this inhibitor was linear non-competitive with respect to phosphate and failed to show synergism with caffeine. This indicates kinetic behaviour identical with that observed with glucono-1,5-lactone (Gold *et al.*, 1971; Tu *et al.*, 1971; Kasvinsky, 1982) and glucal (Kasvinsky, 1982), which possess a half-chair conformation, but different to that observed with glucose (Helmreich *et al.*, 1967; Fig. 4a), glucose 1-fluoride (Ariki & Fukui, 1977; Kasvinsky, 1982), methyl α -glucoside (Kasvinsky, 1982), UDP-glucose (Engers *et al.*, 1970) and glucose 1,2-(cyclic)phosphate (Hu & Gold, 1978), which possess chair conformations and produce either non-linear or linear competitive kinetics with respect to phosphate. It may be suggested that the chair conformation of compound (II) is not favoured in the direction of glycogen phosphorylation, and likewise for compound (I). Boat conformations are impossible, but other conformations such as half-chair cannot be excluded. Although the half-chair conformation of compound (II) is energetically less stable than the chair conformation (Table 1), the energy difference between the two conformations is, however, small. It is reasonable therefore to assume that the conformation of this inhibitor as bound to the enzyme can be determined from the interactions it makes with the enzyme. ^{19}F -n.m.r. experiments with 6-fluoropyridoxal 5'-phosphate-reconstituted phosphorylase (Chang *et al.*, 1986) have shown that the ternary complexes enzyme-AMP-glycogen-glucose 1-phosphate and enzyme-AMP-glycogen-phosphate exhibit different chemical shifts for the ^{19}F nucleus, indicating that the two complexes have different conformations. The enzyme therefore may impose steric restrictions on the conformational freedom of the ligand or it may stabilize unfavourable conformations by formation of hydrogen bonds. Such enzyme effects are not included in the AM1 calculations (carried out for the state *in vacuo*) and must be inferred from the type of enzyme inhibition and the structure of the enzyme-ligand complex. However, in the tetragonal crystals of phosphorylase *b*, no binding of either oligosaccharide or phosphate substrates at the catalytic site has been observed.

Ligand-binding experiments with R-state crystals (Barford & Johnson, 1989) and X-ray analysis to high resolution may help to resolve this problem.

In conclusion, the most likely explanation for both the solution and structural effects of compounds (I) and (II) on phosphorylase is that an equilibrium exists between two (or more) conformations in solution and the selected bound conformation depends upon the interactions that it makes with the different enzyme states. Both compounds are good inhibitors of the enzyme, but considerably less potent than expected for a transition-state analogue. The results further support the notion (Barford *et al.*, 1988) that the catalytic site of phosphorylase has no great preference for a half-chair or chair conformation of a glucosyl residue, and that, in contrast with the situation for β -glycoside-recognition enzymes, distortion of the glucopyranose ring towards half-chair geometry and promotion of trigonal geometry at C-1 is not an obligatory first step in the catalytic reaction.

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