Synthesis and kinetic evaluation of 4-deoxymaltopentaose and 4-deoxymaltohexaose as inhibitors of muscle and potato α -glucan phosphorylases

Renee MOSI and Stephen G. WITHERS¹

Department of Chemistry, University of British Columbia, 2036 Main Hall, Vancouver B.C., Canada VGT 1Z1

α -Glucan phosphorylases degrade linear or branched oligosaccharides via a glycosyl transfer reaction, occurring with retention of configuration, to generate α -glucose-1-phosphate (G1P). We report here the chemoenzymic synthesis of two incompetent oligosaccharide substrate analogues, 4-deoxymaltohexaose (4DG6) and 4-deoxymaltopentaose (4DG5), for use in probing this mechanism. A kinetic analysis of the interactions of 4DG5 and 4DG6 with both muscle and potato phosphorylases was completed to provide insight into the nature of the binding mode of oligosaccharide to phosphorylase. The 4-deoxy-oligosaccharides bind competitively with maltopentaose and non-

competitively with respect to orthophosphate or G1P in each case, indicating binding in the oligosaccharide binding site. Further, 4DG5 and 4DG6 were found to bind to potato and muscle phosphorylases some 10–40-fold tighter than does maltopentaose. Similar increases in affinity as a consequence of 4-deoxygenation were observed previously for the binding of polymeric glycogen analogues to rabbit muscle phosphorylase [Withers (1990) Carbohydr. Res. 196, 61–73].

Key words: glycogen, glycosyl transferase, inhibition, oligosaccharide.

INTRODUCTION

α -Glucan phosphorylases (EC 2.4.1.1) catalyse the phosphorolytic cleavage of polysaccharides such as starch and glycogen to produce α -D-glucose 1-phosphate (G1P), a key metabolic intermediate (for reviews, see [1–3]). Homologues of phosphorylase are present in diverse species ranging from yeast to potato to human. Two of the most extensively studied forms of phosphorylase are the muscle and potato enzymes. Despite their similarities in structural and kinetic properties, strict differences exist in their regulatory mechanisms. Muscle phosphorylase is under both allosteric and covalent control, whereas potato phosphorylase exhibits no such regulation over its activity. In addition, affinities for substrate glucans differ widely between the two enzymes. Muscle phosphorylase prefers highly branched oligosaccharides such as glycogen whereas potato phosphorylase has a higher affinity for linear oligosaccharides. However, previous studies have indicated that, despite their phylogenetic separation, the enzymes operate through a similar mechanism, involving similar transition states. This was demonstrated by the excellent linear free energy relationships ($\rho = 0.998$ and 0.999 , slope = 1.0 and 1.2) obtained when $\log(V_{\max}/K_m)$ values for each of a series of deoxy and deoxyfluoro analogues of G1P with the muscle enzyme were plotted against the same parameters for the potato enzyme [4]. This is supported by the high degree of similarity (> 75%) among the key active-site residues [5,6]. Thus potato phosphorylase represents a model system for studying the mechanism of phosphorolytic cleavage, as the process of catalysis can be separated from complicated regulatory controls.

Although glycogen phosphorylase is a glycosyl transferase, unlike the otherwise analogous sucrose phosphorylase, for which an intermediate was trapped [7], glycogen phosphorylase does not function via a Ping Pong mechanism. It has not, therefore, proved possible to trap a glycosyl-enzyme intermediate simply by incubation with glycogen or oligosaccharides, since a ternary complex is required for the enzyme to be conformationally

activated. In order to form and hopefully trap a glycosyl-enzyme intermediate, some form of incompetent oligo- or polysaccharide is therefore required that is sufficiently close in structure to the natural substrate to bind, but which cannot act as a glycosyl acceptor. Earlier attempts in this direction involved the synthesis of glycogen analogues in which the non-reducing terminal ends of the glucan chains of intact glycogen molecules had been enzymically capped with 4-deoxy- or 4-deoxyfluoro-glucose moieties [8]. Unfortunately, it proved impossible to completely (> 99%) cap all the chain ends and when attempts were made to use this modified material as an incompetent substrate, a low level of transfer (approximately 1%) was still observed, which was sufficient to invalidate the experiment. Interestingly, however, this capped glycogen functioned as a surprisingly tight-binding inhibitor, binding with an apparent affinity 100-fold tighter than that of glycogen itself. In order to probe this further we have completed a chemoenzymic synthesis of two oligosaccharides modified in this manner, 4-deoxymaltopentaose (4DG5) and 4-deoxymaltohexaose (4DG6), and carried out a detailed kinetic analysis of the interaction of these analogues with both the rabbit muscle and potato α -glucan phosphorylases.

MATERIALS AND METHODS

General

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise noted and were used without further purification. 4-Deoxy- α -maltotriosyl fluoride (4D α G3F) was synthesized as described previously by Lindhorst et al. [9]. ¹H-NMR spectra were recorded on a Bruker WH-400 at 400 MHz and the samples were externally referenced to 2,2-dimethyl-2-silapentane-5-sulphonate ($\delta = 0.015$ p.p.m.). Electrospray mass spectra were recorded on a Sciex AP1-300 mass spectrometer using direct flow injection. The samples were introduced in 100% methanol by a syringe pump driven at

Abbreviations used: 4D α G3F, 4-deoxy- α -maltotriosyl fluoride; 4DG5, 4-deoxymaltopentaose; 4DG6, 4-deoxymaltohexaose; G1P, α -D-glucose 1-phosphate; DTT, dithiothreitol.

¹ To whom correspondence should be addressed (e-mail withers@chem.ubc.ca).

Table 1 Kinetic parameters determined for rabbit muscle and potato phosphorylases with malto-oligosaccharides as substrates

Glycogen was used for muscle phosphorylase and starch for the potato enzyme. G5, maltopentaose; G6, maltohexaose.

(a) Synthesis direction		Phosphorylase			
Substrates		Muscle		Potato	
Variable	Constant	$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	K_m (mM)	$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	K_m (mM)
G1P	α -Glucan	54.9	1.7	32.3	1.9
α -Glucan	G1P	—	0.079*	—	0.046*
G1P	G5	35.0	8.0	30.1	1.6
G1P	G6	40.7	7.1	—	—
G5	G1P	86.1	35	20.7	0.33
G6	G1P	130.5	79.3	—	—

(b) Degradation direction		Phosphorylase			
Substrates		Muscle		Potato	
Variable	Constant	$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	K_m (mM)	$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	K_m (mM)
P_i	α -Glucan	30.3	2.40	7.9	1.9
α -Glucan	P_i	—	—	—	—
P_i	G5	27	6.2	7.2	2.1
P_i	G6	28.3	5.4	—	—
G5	P_i	32.5	17.6	7.2	0.23
G6	P_i	34.4	15.3	—	—

* Values reported previously by Fukui et al. [6]. These values were calculated on the basis of the molar concentration of the non-reducing ends of each glucan.

5 $\mu\text{l}/\text{min}$ in the presence of 5 mM ammonium acetate as an ionization agent.

Rabbit muscle phosphorylase

Rabbit muscle was purchased from Pel-Freeze Biologicals (Rogers, AR, U.S.A.). Rabbit muscle phosphorylase *b* was prepared by the method of Fischer and Krebs using dithiothreitol (DTT) instead of cysteine and recrystallized at least three times before use [10]. Rabbit liver glycogen (type III) was purified with Bio-Rad AG-1X8 (200–400 mesh, Cl^- form) ion-exchange resin (Bio-Rad, Hercules, CA, U.S.A.) to remove nucleotides, and assayed by the method of Dische [11].

Potato phosphorylase

Isolation of potato tuber phosphorylase was performed by a modification of a protocol generously provided by Dr. T. Fukui (Osaka University, Osaka, Japan), itself based on a published procedure [12] (see also [4]). The procedure is identical to that published up to and including the step involving the precipitation of potato phosphorylase using starch in ethanol. The precipitate was redissolved in buffer containing arsenate (5 mM) to enhance starch degradation through phosphorylase-catalysed arsenolysis. The material was loaded on to a DEAE-Sephacel column equilibrated with 10 mM sodium citrate buffer, pH 6.0, at 2 ml/min and washed overnight with the same buffer until the absorbance (280 nm) of the effluent reached baseline levels. A linear gradient of NaCl (4 litres, 0–1 M) was applied to the column (2 ml/min) and 20 ml fractions were collected. Fractions were assayed and those containing phosphorylase were concentrated. This solution was then applied to a Sephacryl S200 gel-filtration column equilibrated with 10 mM citrate at pH 6.0 and

eluted at 1 ml/min with 2 ml fractions. The fractions that absorbed at 280 nm, were pooled on the basis of their purity as assessed by SDS/PAGE.

Glycogen debranching enzyme

Glycogen debranching enzyme (E.C. 3.2.1.33, E.C. 2.4.1.25) was purified from rabbit muscle as described previously [13]. In addition, enzyme samples prepared by an identical method were generously donated by Mrs. S. Shechosky and Dr. N. Madsen, University of Alberta, Canada. The combined enzyme activity was measured by following changes in the absorbance at 340 nm with limit dextrin as the substrate, in a buffer of 25 mM glycylglycine/1 mM EDTA/1 mM DTT at pH 7.2 at 30 °C [14]. The glucosidase activity was assayed by measuring the rate of fluoride release using α -glucosyl fluoride as the substrate in 100 mM citrate buffer/1 mM EDTA/1 mM DTT, pH 6.0, at 30 °C using an Orion fluoride-sensitive electrode [15].

Enzymic synthesis and purification of 4DG5 and 4DG6

To 1 equiv. of 4D α G3F (11 mg, 2.24×10^{-5} mol) in 200 mM sodium citrate buffer, pH 6.0, was added maltotriose (1.8 equiv., 20 mg, 4.03×10^{-5} mol) and glycogen debranching enzyme (300 μl , 5 units/ml, 18.3 mg/ml, in 100 mM citrate buffer/1 mM EDTA/1 mM DTT, pH 6.0). The total volume of the reaction mixture was 1.0 ml. The progress of the reaction was checked at 24 h by injecting a small sample of the reaction mixture (10 μl) on to an analytical Waters Dextropak HPLC column (100 $\times$ 8 mm) and eluting with a mobile phase of 100 % double-deionized water at 0.8 ml/min. The products were identified using a refractive-index detector and relative yields were determined by integration of peak areas. The progress of the reaction was monitored

Scheme 1 Enzymic synthesis of 4DG6 from 4DαG3F and maltotriose using glycogen debranching enzyme (Glyx)

Each circle represents one glucose residue linked in an α -1,4 fashion. Each asterisk represents the 4-deoxy moiety found at the non-reducing end of the oligosaccharide. G3, maltotriose; G4, maltotetraose.

Figure 1 HPLC analysis of the products of the reaction of glycogen debranching enzyme with 4DαG3F and maltotriose

Conditions: Dextropak column (10 × 250 mm), 7 ml/min 100% H₂O. The α - and β -anomers of maltohexaose (G6) and maltoheptaose (G7) respectively co-eluted. G3, maltotriose; G4, maltotetraose.

continuously by analytical HPLC as described above until the yield of 4DG6 was optimized. On average, the reaction was stopped after 40–48 h with a typical yield of 45% 4DG5 (8 mg) and 43% 4DG6 (9 mg). Passing the solution through a 100 000 Da molecular-mass cut-off filter (Amicon, Danvers, MA, U.S.A.) to recover the enzyme terminated the reaction. The reaction mixture was purified by loading on to a large Waters Dextropak column (250 × 10 mm) eluted at 7 ml/min using 100% double-deionized water. Fractions containing 4DG5 and 4DG6 as well as any remaining starting material (4DαG3F) were pooled and concentrated by evaporating and then a small sample was injected on to the analytical column to assess purity. Only a single peak was seen in each case. The final products were lyophilized to give white solids. ¹H-NMR (²H₂O, 400 MHz) 4DG5 (selected data only): δ 5.38 (4 H, H-1', H-1'', H-1''', H-1'''), 5.23 (d, $J_{1,2}$ 3.7 Hz, H-1e), 1.95 (ddd, 1 H, $J_{3,4}$ 4.8, $J_{4,5}$ 12.0, $J_{4,5}$ 2.0 Hz, H-4'''), 1.42 (q, 1 H, $J_{3,4}$ 12.0, $J_{4,5}$ 12.0, $J_{4,5}$ 12.0 Hz, H-4a'''). ¹H-NMR (²H₂O, 400 MHz) 4DG6 (selected data only): δ 5.40 (5 H, H-1', H-1'', H-1''', H-1'''), 5.23 (d, $J_{1,2}$ 3.7 Hz, H-1e), 2.00 (ddd, 1 H, $J_{3,4}$ 4.8, $J_{4,5}$ 12.0, $J_{4,5}$ 2.0 Hz, H-4'''), 1.45 (q, 1 H, $J_{3,4}$ 12.0, $J_{4,5}$ 12.0, $J_{4,5}$ 12.0 Hz, H-4a''').

Matrix-assisted laser desorption ionization MS: calculated for C₃₀O₂₅H₅₂ 835.72 (4DG5 + Na⁺), found 835.7; calculated for C₃₆O₃₀H₆₂ 997.86 (4DG6 + Na⁺), found 997.2. Electrospray MS: calculated for C₃₀O₂₅H₅₂ 830.5 (4DG5 + NH₄⁺), found 831; calculated for C₃₆O₃₀H₆₂ 992.9 (4DG6 + NH₄⁺), found 993.

Assay of 4DG5 and 4DG6 solutions

The dinitrosalicylic acid reducing sugar assay according to Burnfeld [16] was used. Stock solutions (10 mg/ml) of both 4DG5 and 4DG6 in deionized water were prepared accurately and assayed along with a series of solutions of maltose of different concentrations, as follows. To samples of sugar (200–1400 μ g) in 1 ml of water was added 1 ml of the assay reagent (1 g of 3,5-dinitrosalicylic acid/100 ml of 400 mM NaOH/30 g of Na/K-tartrate). After the addition of water (8 ml) to each tube, they were boiled for 5 min, then cooled to room temperature and the absorbance was read at 546 nm. The concentrations of the deoxy-oligosaccharide solutions were then determined using a standard curve constructed from the absorbances of the maltose solutions.

Kinetic analysis: synthesis direction

Values of $V_{\max}$ and K_m were determined from the initial rates of saccharide synthesis by monitoring the release of inorganic phosphate from the substrate G1P by the procedure of Fiske and Subbarow as described by Engers et al. [17,18]. All rabbit muscle phosphorylase reactions were conducted in a buffer containing 20 mM glycerophosphate/1.5 mM EDTA/5 mM DTT at pH 6.8. The potato phosphorylase reactions were conducted in a buffer containing 40 mM sodium maleate/250 mM KCl/0.13 mM EDTA, at pH 6.3. Reaction mixtures were 0.2 ml and were incubated at 30 °C for 5 min. Substrate concentrations were varied between 0.2 and 5 times the K_m value ultimately determined wherever possible. Typical enzyme concentrations were 2 μ g/reaction mixture for muscle phosphorylase and 2.5 μ g/reaction mixture for potato phosphorylase. These conditions resulted in less than 1% elongation of the oligosaccharide (as determined from phosphate released).

Kinetic analysis: degradation direction

Values of $V_{\max}$ and K_m were determined from the initial rates of saccharide breakdown by monitoring the rate of G1P production using the phosphoglucomutase/glucose-6-phosphate dehydrogenase-coupled enzyme system as described previously for muscle phosphorylase [19] and by a modification of the procedure used for potato phosphorylase [20]. Buffer conditions were identical to those employed above for the synthesis direction. Final concentrations of coupled enzyme assay components were: for muscle phosphorylase, β NADP⁺ (1 mM), Mg(OAc)₂ (10 mM), glucose 1,6-bisphosphate (1.5 μ M), glycogen (0.5%, w/v), AMP (1 mM), phosphoglucomutase (50 μ g/ml), glucose-6-phosphate dehydrogenase (3 units/ml), imidazole buffer (37.5 mM, pH 6.8) and glycerophosphate buffer (20 mM, pH 6.8); and for potato phosphorylase, β NADP (1 mM), Mg(OAc)₂ (10 mM), phosphoglucomutase (100 μ g/ml), glucose-6-phosphate dehydrogenase (7 units/ml), imidazole buffer (37.5 mM, pH 6.5) and maleate buffer (100 mM, pH 6.5). Reactions were carried out at 30 °C and were generally followed for 5 min. Enzyme concentrations used were 1 μ g/reaction mixture for muscle phosphorylase and 2.5–3 μ g/reaction mixture for potato phosphorylase.

Determination of inhibition constants

The K_i values for 4DG6 with muscle phosphorylase and 4DG5 with potato phosphorylase were determined by measurement of V_{max} and K_m values at a series of inhibitor concentrations (typically five concentrations) which bracket the K_i value to be determined in both directions of analysis. The assay conditions and enzyme concentrations employed were identical to those reported above. The data were plotted in the form of a double reciprocal plot and the value of K_i and the mode of inhibition were determined through a direct fit of the data to expressions for each inhibition mode using the program Gra-Fit [21].

RESULTS AND DISCUSSION

Malto-oligosaccharides as substrates for phosphorylases

Although kinetic parameters for oligosaccharide substrates of phosphorylase have already been characterized to some extent, they often vary with the assay method and the enzyme source [6,22]. Therefore, it was first necessary to obtain an independent

set of kinetic parameters for maltopentaose and maltohexaose under our operating conditions, and these results are shown in Table 1. Kinetic parameters determined conform completely to the Haldane relationship for a two-substrate random-order equilibrium mechanism [23], a value of $K = 0.79$ being calculated from these parameters, in excellent agreement with the generally accepted value of 0.78. As observed previously [24], the K_m values for G1P with muscle phosphorylase in the presence of maltopentaose and maltohexaose were 4–5-fold higher than those determined in the presence of glycogen. Similarly, K_m values for phosphate were 2–3-fold lower in the presence of glycogen than of oligosaccharides. The K_m values were 100-fold lower for the linear oligosaccharides with potato phosphorylase than with the muscle enzyme, entirely consistent with the preference of the potato enzyme for linear oligosaccharides, as noted previously [6].

Synthesis and purification of 4DG5 and 4DG6

Given the low affinity of muscle glycogen phosphorylase for short oligosaccharides, a minimum of five sugar residues was

Figure 2 Lineweaver-Burk plots for the inhibition of muscle phosphorylase by 4DG6 (I)

(A) [G1P], 72.6 mM; [enzyme], 0.103 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.0125 mM; $\square$, 0.025 mM; $\blacksquare$, 0.06 mM; $\triangle$, 0.125 mM. (B) [Maltopentaose (G5)], 33.5 mM; [enzyme], 0.039 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.0125 mM; $\square$, 0.025 mM; $\blacksquare$, 0.06 mM; $\triangle$, 0.125 mM. (C) [Orthophosphate (Pi)], 23.8 mM; [enzyme], 0.040 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.046 mM; $\square$, 0.093 mM; $\blacksquare$, 0.232 mM; $\triangle$, 0.465 mM. (D) [Maltopentaose], 20 mM; [enzyme], 0.040 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.046 mM; $\square$, 0.093 mM; $\blacksquare$, 0.232 mM; $\triangle$, 0.465 mM.

Figure 3 Lineweaver-Burk plots for the inhibition of potato phosphorylase by 4DG5 (I)

(A) [G1P], 10.0 mM; [enzyme], 0.145 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.685 mM; $\square$, 1.37 mM; $\blacksquare$, 3.43 mM; $\triangle$, 6.85 mM. (B) [Maltopentaose (G5)], 0.9 mM; [enzyme], 0.145 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.650 mM; $\square$, 1.21 mM; $\blacksquare$, 3.03 mM; $\triangle$, 6.05 mM. (C) [Orthophosphate (Pi)], 11.6 mM; [enzyme], 0.049 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.535 mM; $\square$, 2.14 mM; $\blacksquare$, 3.75 mM; $\triangle$, 5.36 mM. (D) [Maltopentaose], 2.79 mM; [enzyme], 0.049 μ M, [I]: $\circ$, 0 mM; $\bullet$, 0.598 mM; $\square$, 1.20 mM; $\blacksquare$, 2.99 mM; $\triangle$, 5.98 mM.

deemed necessary for the incompetent substrate analogue [22]. However, as maltopentaose and longer oligosaccharides are expensive and difficult to modify synthetically, it seemed preferable to carry out the deoxygenation chemistry on the inexpensive oligosaccharide, maltotriose, then to couple this, as its glycosyl fluoride, to another maltotriose using the transferase activity of glycogen debranching enzyme [13,25]. The requisite 4D α G3F was chemically synthesized as described previously [9], and when incubated with glycogen debranching enzyme and the acceptor sugar, maltotriose, both 4DG6 and 4DG5 were produced (Scheme 1). The accumulation of 4DG5 presumably results from the disproportionation reaction also shown in Scheme 1 [26]. Both 4DG5 and 4DG6 were easily purified from the reaction mixture by preparative HPLC using a Dextropak column, as shown in Figure 1. The yields varied depending on the length of reaction and the activity of the enzyme. The reaction was stopped when the yield of 4DG6 had been maximized. Typical yields were 43% 4DG6 and 45% 4DG5. Product purity was assessed by analytical HPLC, with only one peak eluting, and

by reducing sugar analysis of accurately prepared solutions, which confirmed the expected concentrations, within 5%. MS analysis confirmed the constitution of the products and ^1H -NMR was used to confirm the linkage of the sugars. Peaks were observed at δ 5.4 p.p.m. in both 4DG5 and 4DG6, as expected for the anomeric protons of sugar residues involved in α -(1-4) linkages, with no resonances due to α -(1-6) branchpoints at δ 4.96–5.00 p.p.m. [4]. Further, the methylene protons of the 4-deoxy moiety are clearly seen at δ 1.45 and 2.00 p.p.m.

4DG5 and 4DG6 as incompetent substrates for phosphorylase

Due to the limited supply of the deoxygenated oligosaccharides, 4DG6 was studied as an inhibitor of muscle phosphorylase only and 4DG5 was only studied with potato phosphorylase. In order to ensure that these deoxygenated analogues did not function significantly as substrates when tested as inhibitors in the degradation direction, the substrate activities of both 4DG5 and 4DG6 were first studied. No significant degradation was observed

Table 2 Inhibition parameters determined for rabbit muscle (4DG6) and potato (4DG5) phosphorylase

G5, maltopentaose.

Phosphorylase	Variable substrate	K_i (mM)	Inhibition mode
(a) Synthesis direction			
Muscle	G1P	6.4	Non-competitive
	G5	2.1	Competitive
Potato	G1P	0.1	Non-competitive
	G5	0.04	Competitive
(b) Degradation direction			
Muscle	P _i	7.4	Competitive
	G5	2.5	Non-competitive
Potato	P _i	1.0	Non-competitive
	G5	0.06	Competitive

under the conditions used for normal assays, consistent with the approximately 400-fold lower k_{cat}/K_m measured previously for 4-deoxyglucose 1-phosphate compared with G1P [4,27]. The binding modes of 4DG6 and 4DG5 to muscle and potato phosphorylase respectively were determined by completing a full inhibition analysis in both the synthetic and degradation directions. As seen in Figures 2(A), 2(C), 3(A) and 3(C), 4DG6 and 4DG5 competitively inhibit binding of maltopentaose in both the synthetic and degradation directions for muscle and potato phosphorylases, but bind non-competitively with respect to either G1P or orthophosphate (Figures 2B, 2D, 3B and 3D). These results indicate that the 4-deoxy-oligosaccharides and G1P or orthophosphate bind simultaneously to the enzyme at independent sites and suggest that the 4-deoxy-oligosaccharides are binding in the substrate acceptor sites.

In keeping with the previously observed high affinities of 4-deoxy-glycogen derivatives for phosphorylase [8] the K_i values measured for 4DG6 and muscle phosphorylase were approximately 40-fold lower than the K_m value measured for maltohexaose (Tables 1 and 2). A similar but somewhat smaller difference in binding affinity was observed when 4DG5 was tested as an oligosaccharide analogue for potato phosphorylase in either reaction direction. In that case the K_i values were about 10-fold lower compared with the K_m values for maltopentaose. These higher affinities are not, therefore, some artifact of the polymeric substrate, glycogen, but represent real differences in affinity [9.2 kJ/mol (2.2 kcal/mol) and 5.4 kJ/mol (1.3 kcal/mol) respectively] caused by the substitution of the hydroxyl by hydrogen. The source of this increased affinity is unclear, but it may indicate that the hydroxyl group is involved in a destabilizing ground-state interaction that is not present for the deoxy analogue. Such ground-state destabilization, if not present at the transition state, could be an important element of catalysis for this enzyme [28].

The relatively high binding affinity of muscle phosphorylase for 4DG6 may prove useful in X-ray crystallographic studies of oligosaccharide binding to phosphorylase, since no structures containing productively bound oligosaccharides are currently available. A recent attempt to determine such a structure by co-crystallizing *Escherichia coli* maltodextrin phosphorylase with maltohexaose unfortunately revealed only a disaccharide bound non-productively [29]. The higher affinity of the deoxy-oligosaccharides may well improve occupancy. These analogues may also prove useful in attempts to trap a glycosyl-enzyme intermediate since they could be used, in conjunction with G1P, to

form incompetent ternary complexes with glycogen phosphorylase.

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