Phosphorylase recognition and phosphorolysis of its oligosaccharide substrate: answers to a long outstanding question

K.A.Watson^{1,2}, C.McClevarty^{1,3}, S.Geremia⁴, S.Cottaz⁵, H.Driguez⁵ and L.N.Johnson^{1,6}

¹Laboratory of Molecular Biophysics, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU,

⁶Oxford Centre for Molecular Sciences, New Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QT, UK,

⁴Department of Chemistry, University of Trieste, Trieste, Italy and

⁵Centre de Recherche sur les Macromolécules Végétales (CERMAV-CNRS), BP 53, F-38041 Grenoble cedex 9, France

³Present address: Department of Biochemistry, University of Leicester, Leicester, UK

²Corresponding author
e-mail: kim@biop.ox.ac.uk

Phosphorylases are key enzymes of carbohydrate metabolism. Structural studies have provided explanations for almost all features of control and substrate recognition of phosphorylase but one question remains unanswered. How does phosphorylase recognize and cleave an oligosaccharide substrate? To answer this question we turned to the *Escherichia coli* maltodextrin phosphorylase (MalP), a non-regulatory phosphorylase that shares similar kinetic and catalytic properties with the mammalian glycogen phosphorylase. The crystal structures of three MalP–oligosaccharide complexes are reported: the binary complex of MalP with the natural substrate, maltopentaose (G5); the binary complex with the thio-oligosaccharide, 4-S- α -D-glucopyranosyl-4-thiomaltotetraose (GSG4), both at 2.9 Å resolution; and the 2.1 Å resolution ternary complex of MalP with thio-oligosaccharide and phosphate (GSG4-P). The results show a pentasaccharide bound across the catalytic site of MalP with sugars occupying sub-sites –1 to +4. Binding of GSG4 is identical to the natural pentasaccharide, indicating that the inactive thio compound is a close mimic of the natural substrate. The ternary MalP–GSG4-P complex shows the phosphate group poised to attack the glycosidic bond and promote phosphorolysis. In all three complexes the pentasaccharide exhibits an altered conformation across sub-sites –1 and +1, the site of catalysis, from the preferred conformation for α (1–4)-linked glucosyl polymers.

Keywords: catalytic mechanism/phosphorylase/oligosaccharide conformation/thio-oligosaccharide recognition/X-ray crystal structure

Introduction

Structural, biochemical and time-resolved studies on rabbit muscle glycogen phosphorylase have contributed to an understanding of the catalytic mechanism whereby the enzyme promotes the phosphorolysis of an α (1–4)

glycosidic bond in glycogen or oligosaccharide substrates. The mechanisms of control by phosphorylation and by allosteric effectors are also understood from crystallographic studies on phosphorylated and unphosphorylated forms of the mammalian phosphorylase (Barford and Johnson, 1989; Barford *et al.*, 1991; Sprang *et al.*, 1991; Johnson, 1992). One question has remained outstanding. This relates to the recognition and conformation of the oligosaccharide component of the substrate when it is bound across the catalytic site. Numerous attempts to observe binding of oligosaccharide substrates with rabbit muscle glycogen phosphorylase (GP) in the active R state were not successful, probably because of the low affinity of the enzyme for linear oligosaccharides. In order to solve this problem we have turned to the *Escherichia coli* maltodextrin phosphorylase (MalP). As suggested by the nomenclature, GP and MalP exhibit distinct differences in their preference for substrates. GP has a high affinity for glycogen and branched polysaccharides and low affinity for small oligosaccharides (Hu and Gold, 1975; Kasvinsky *et al.*, 1978). MalP has low affinity for glycogen but exhibits ~100-fold higher affinity for linear oligosaccharides than GP (Schwartz and Hofnung, 1967; Drueckes *et al.*, 1996). This observation has been exploited to solve the major outstanding problem in phosphorylase catalysis, namely the recognition of the oligosaccharide component of the substrate at the catalytic site.

MalP (EC.2.4.1.1) is a 796 amino acid, non-allosteric, dimeric enzyme that is 46% identical in sequence to GP (Palm *et al.*, 1985, 1987). Unlike GP, MalP is not regulated by phosphorylation or by allosteric effectors but is regulated by control of gene expression (Raibaud and Schwartz, 1984). Phosphorylases are highly conserved from bacterial to mammalian enzymes, but share no homology with other carbohydrate degrading enzymes, with the exception of the noted similarity of the core structure around the cofactor pyridoxal phosphate binding site in GP with a DNA glucosyltransferase modifying enzyme (Artymuik *et al.*, 1995; Holm and Sander, 1995). Phosphorylase isozymes exhibit nearly 100% identity in residues at the catalytic site (Watson *et al.*, 1997) and all utilize the 5'-phosphate of the essential cofactor pyridoxal phosphate (PLP) in catalysis (Palm *et al.*, 1990). The bacterial and mammalian enzymes catalyse the phosphorolytic cleavage from the non-reducing end of α -1–4 linked maltodextrins (Glc)_n to yield α -D-glucose-1-P (Glc-1-P) (Figure 1). The reaction proceeds with retention of configuration. For MalP, the optimal substrate maltodextrin has a minimum length of five glucosyl units for phosphorolysis and four glucosyl units for the reverse reaction of oligosaccharide synthesis (Becker *et al.*, 1994).

Structure determinations of MalP (Watson *et al.*, 1997) and GP have shown that the catalytic site is buried at the centre of the large subunit. It is accessible to the solvent

Fig. 1. The reaction catalysed by MalP and the chemical structure of 4-S- α -D-glucopyranosyl-4-thiomaltotetraose (GSG4).

through a 20 Å long channel that has been proposed as the substrate oligosaccharide binding site. Sub-sites for oligosaccharide recognition are numbered –1 to +4 from the non-reducing end to the reducing end, and the site of phosphorylation is between sub-sites –1 and +1 (Davies *et al.*, 1995). In both the less active T state and the active R state conformations of GP, the catalytic site (sub-site –1) has been defined through binding studies and observations of catalysis in the crystal with monomeric substrates and products such as Glc-1-P and heptulose-2-P, and by binding studies with inhibitors such as glucose, glucose analogues and transition state analogues (Hajdu *et al.*, 1987; Johnson *et al.*, 1989, 1990, 1992; Martin *et al.*, 1990; Duke *et al.*, 1994; Mitchell *et al.*, 1996). The site is situated at the end of the channel, far removed from the bulk solvent and adjacent to the essential cofactor, pyridoxal phosphate. Co-crystallization studies with MalP and maltohexaose (O'Reilly *et al.*, 1997) showed a disaccharide, maltose, bound at adjacent sites to the catalytic site (numbered sub-sites +1 and +2). Analysis of the oligosaccharide mixture from the co-crystallization experiments indicated that both catalysis and hydrolysis of the oligosaccharide had occurred over the period of crystallization.

α (1–4)-O-linked glucosyl oligosaccharides exhibit a preferred conformation in which there is an intramolecular hydrogen bond between the O2 hydroxyl and the O3 hydroxyl of contiguous residues, as observed in maltose (Takusagawa and Jacobson, 1978) and related compounds. Modelling studies from the MalP–maltose complex and from the GP–monosaccharide complexes indicated that an

oligosaccharide bound across sub-sites –1 and +1 could not be accommodated in the preferred conformation if the oligosaccharide were to follow the path of the catalytic channel (O'Reilly *et al.*, 1997). Either there must be a significant conformational change in the enzyme structure, or a significant alteration of the conformation about the α (1,4) glycosidic bond between sub-sites –1 and +1. It was therefore of interest to observe an oligosaccharide substrate bound across the phosphorylase catalytic site and to assess the significance of any conformational change for catalysis.

Acarbose is a pseudo-tetrasaccharide inhibitor of α -glucosidases that can adopt either the preferred conformation or an alternative conformation about the corresponding N-linked glycosidic bond of its acarviosine moiety. Co-crystallization of MalP with acarbose led to oligosaccharide binding in sub-sites +1 to +4 and the identification of the further sub-sites +3 and +4 (O'Reilly *et al.*, 1999). However, in both the MalP–maltose and the MalP–acarbose binding experiments a glycerol molecule, used as a cryoprotectant for the low temperature data collection, blocked the crucial catalytic sub-site –1. Thus the problem of observing an oligosaccharide bound across the phosphorylase catalytic site remained.

In the present work, we developed a method of freezing crystals that avoided the use of glycerol. By carefully excluding phosphate from the purification and crystallization protocols we have been able to co-crystallize the binary MalP complex with the natural substrate maltopentaose (G5). The X-ray crystal structure of the MalP–G5 complex shows for the first time an oligosaccharide bound across the catalytic site in sub-sites –1 to +4 spanning the point of enzymatic cleavage. In order to observe a ternary complex in the presence of phosphate and elucidate the catalytic mechanism, we have exploited the thio-oligosaccharide, 4-S- α -D-glucopyranosyl-4-thiomaltotetraose (GSG4) which is resistant to enzymatic cleavage (Figure 1). Thio-oligosaccharides (Driguez, 1997) have become useful tools for the study of carbohydrate–enzyme interactions (Sulzenbacher *et al.*, 1996). Solution NMR studies (Bock *et al.*, 1994) indicated that the thio analogues showed greater conformational flexibility than the corresponding O-linked and N-linked glucosyl polymers. Comparative studies with the thio and natural pentasaccharide binary complexes showed no significant differences in binding to MalP. Co-crystallization of MalP with the thio-oligosaccharide in the presence of phosphate has allowed us to determine the structure of a ternary enzyme substrate complex. The complex is consistent with previous site-directed mutagenesis data for MalP and provides further understanding of the catalytic mechanism of phosphorylases.

Results

The overall architecture of the native maltodextrin phosphorylase has been described, and details of the structure compared with the active and inactive forms of mammalian phosphorylase (Watson *et al.*, 1997). In brief, MalP is an α/β protein consisting of two domains (N-terminal domain 19–482; C-terminal domain 483–829, using the rabbit muscle GP numbering) that form the monomeric subunit. The active site lies between these domains, at the centre

Table I. Summary of the data collection and refinement statistics for the three complexes

Data collection	MalP-G5	MalP-GSG4	MalP-GSG4-P
Resolution range	50.0–2.9	50.0–2.9	50.0–2.0
No. of measurements used	74 406	55 829	152 192
No. of unique reflections	38 437	38 856	72 681
Completeness (%)			
All data	79.1	84.5	80.0
Last resolution shell	(3.1–2.9 Å) 75.1 (3.7–3.4 Å) 81.0	(3.1–2.9 Å) 71.5	(2.1–2.0 Å) 64.5 (2.4–2.2 Å) 81.3
Multiplicity	2.2	1.7	2.1
R_{merge}^a	0.18	0.075	0.102
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁
Cell dimensions (Å, °)	$a = 74.45$ $b = 105.69$ $c = 214.91$	$a = 74.74$ $b = 105.02$ $c = 217.96$	$a = 107.41$ $b = 61.50$ $c = 132.38$ $\beta = 105.03$
Final refinement parameters			
No. of reflections used ^b	29 646	32 407	69 025
No. of protein atoms	6753	6753	6753
No. of solvent atoms	190	299	377
No. of cofactor atoms	15	15	15
No. of ligand atoms	56	56	61
$R_{\text{conv}}/R_{\text{free}}^c$	0.245/0.306	0.214/0.277	0.228/0.261
R.m.s. deviation from ideal geometry for the protein			
Bond lengths (Å)	0.008	0.004	0.004
Bond angles (°)	1.33	0.822	0.911
Planar groups (°)	0.876	0.739	0.492
Average B factors (Å ²)			
Main chain	46.3	19.7	23.7
Side chain	47.9	21.5	25.6
Solvent	65.9	41.5	38.3
Oligosaccharide	58.1	20.4	22.9
Phosphate	—	—	60.1
R.m.s. deviation on B factors			
Bond B restraints	5.1	3.9	2.5
Angle B restraints	7.5	5.7	3.8

^a $R_{\text{merge}} = \frac{\sum_i \sum_j |I_{h,i} - I_{h,j}|}{\sum_i \sum_j I_{h,i}}$, where $I_{h,i}$ and I_h are the i^{th} measurement and mean measurement of reflection h , respectively, and the sum is over all

reflections for which more than one measurement is recorded.

^bThis is the number of reflections used in the calculation of R_{conv} . R_{free} calculated for 5% of the data selected at random at the beginning of the structure refinement.

^c $R_{\text{conv}}, R_{\text{free}} = \frac{\sum_h ||F_{\text{obs}h}| - |F_{\text{calc}h}||}{\sum_h |F_{\text{obs}h}|}$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.

of the molecule, and is accessible to the solvent via a channel ~20 Å in length. There is a non-crystallographic 2-fold axis relating two subunits, giving rise to a dimer in the asymmetric unit. The overall quaternary and tertiary protein structures of the three oligosaccharide complexes reported here are similar to the native MalP structure. However there are significant local conformational changes from the native MalP structure in a loop of residues at the catalytic site upon binding the oligosaccharide.

The structures of the MalP-G5, MalP-GSG4 and MalP-GSG4-P complexes

A summary of the data processing statistics and refinement parameters for each of the three MalP-oligosaccharide complex structures is given in Table I. Views of the final $2F_o - F_c$ weighted electron density maps for the three complexes are shown in Figure 2. In each of the complexes the five sugars are clearly defined in sub-sites –1 to +4. Sub-site –1 is the furthest buried in the catalytic site channel and is adjacent to the cofactor PLP. The oligosaccharide binds the length of the channel with sub-site +4 most exposed to the bulk solvent.

Comparison of the isomorphous crystal structures of the MalP-G5 and MalP-GSG4 complexes at 2.9 Å resolution showed that the thio analogue bound in a similar manner to the natural pentasaccharide. The single crystal structure of thio-maltoside (Perez and Vergelati, 1984) demonstrated that the thio analogue has similar structural parameters to those observed for the O-linked maltose with an increase in the distance C1 to C4' of 0.35 Å. In the MalP-G5 complex, the C1···C4' distance is 2.4 Å, compared with 2.9 Å in the MalP-GSG4 complex showing a similar small increase. The binary structures are shown superimposed in Figure 4. The root mean square deviation (r.m.s.d.) in C_α coordinates of the protein atoms is 0.3 Å and the r.m.s.d. between the G5 and GSG4 oligosaccharide coordinates is 0.55 Å. The thio-oligosaccharide (GSG4) is a good mimic of the natural substrate both in its conformation and its interactions with the enzyme.

Crystals of the ternary complex MalP-GSG4-P were obtained under crystallization conditions similar to those for the binary complex of MalP-GSG4, with the addition of 5 mM phosphate to the protein drop. The crystals belong to the monoclinic space group P2₁, a space group that is different from the orthorhombic P2₁2₁2₁ space

Fig. 2. Electron density maps with weighted $2F_o - F_c$ coefficients (see text for details of weighting) for (A) MalP-G5 complex, (B) MalP-GSG4 complex and (C) MalP-GSG4-P complex. Contour levels are 1σ corresponding to $0.19 \text{ e}/\text{\AA}^3$, $0.24 \text{ \AA}^3$ and $0.24 \text{ e}/\text{\AA}^3$ for the G5, GSG4 and GSG4-P maps, respectively. The fifth sugar at sub-site +4 is just out of view.

group of the MalP-G5 and MalP-GSG4 complexes. The crystals diffracted to $2 \text{ \AA}$ resolution but there was some disorder in the direction parallel to the thin edge of the plate-like crystal. Using MOSFLM we were able to extract data to $2.0 \text{ \AA}$ resolution by using a dynamic resolution cut-off applied to individual frames. All the data were used in the refinement but electron density maps were calculated to $2.2 \text{ \AA}$ resolution, where the data are more complete (Table I). The five sugars were immediately apparent in difference maps obtained after refinement of the protein atoms and each sugar could be fitted unambiguously, since all sugar hydroxyl groups could be clearly located. After refinement of the protein and GSG4 atoms, the position of the phosphate group was apparent as the highest peak in the difference Fourier synthesis. The final refinement gave an overall B factor of $60 \text{ \AA}^2$

for the phosphate group. This is higher than expected for a group that makes several contacts to the protein and to the sugars at sub-sites -1 and $+1$. The position of the phosphate ion is nearly identical (overall shift of $0.8 \text{ \AA}$) to the position observed for phosphate in the native MalP structure (Watson *et al.*, 1997) where the B factors were $34 \text{ \AA}^2$. A water molecule modelled at the phosphate site in the MalP-GSG4-P complex did not refine well. It is concluded that the phosphate is present but less well ordered than the other atoms in the structure, possibly as a result of its proximity to the thio linkage of the oligosaccharide (discussed below). The overall structure of the MalP-GSG4-P complex was very similar to the MalP native structure (r.m.s.d. in C_α coordinates $0.8 \text{ \AA}$), where the major difference in structure results from a movement in the 380s loop (discussed below) and, to the

Fig. 3. The monomeric structure of MalP showing the binding of the oligosaccharide in a cleft between the two subunits of the monomer. α -helices are shown in yellow and β -sheet in blue. The view is normal to the 2-fold axis of the dimer from the top of the molecule.

MalP–GSG4 complex (r.m.s.d. in C_α coordinates 0.3 Å). The only difference between the MalP–GSG4 and MalP–GSG4-P complexes is the presence of a water molecule in the former complex that is replaced by the phosphate in the latter MalP–GSG4-P complex, and an accompanying 4.5 Å shift in the position of Arg569. The binding of GSG4 is identical in the binary and ternary complexes.

A summary of the intermolecular contacts for the MalP–GSG4-P complex is given in Table II and shown in Figure 5. The sugar in sub-site –1, buried close to the cofactor, has the largest number of hydrogen bonds and van der Waals contacts with the enzyme. These contacts are similar to those observed for monomeric glucosyl residues binding to the mammalian GP at sub-site –1. Each of the peripheral hydroxyl groups is involved in at least two hydrogen bonds and there are numerous van der Waals contacts, especially to His377. The other sites involve fewer hydrogen bonds but almost all of these involve hydrogen bonds between the sugars and charged residues (Glu88 and Asp339 at subsite +1; Arg292 and His341 at sub-site +2; Arg292 at sub-site +3; and Glu382 and His571 at sub-site +4). The charged–polar hydrogen bonds might be expected to contribute more binding energy than polar–polar hydrogen bonds (Fersht *et al.*, 1985; Street *et al.*, 1986). The van der Waals contacts at sub-sites +2 and +4 are dominated by glucosyl–tyrosyl interactions (to Tyr280 and Tyr613, respectively) as previously noted (O'Reilly *et al.*, 1997, 1999). The contacts from Tyr280 to the sugar involve the C2, C4, O5 and C6 atoms of sub-site +2. The contacts from Tyr613 to the sugar in sub-site +4 also involve the C2, C4, O5, C6 and O3 atoms of the sugar. Both aromatic groups stack with the A side of the glucopyranosyl ring as expected for $\alpha(1,4)$ -linked glucosyl polymers (Johnson *et al.*, 1988). In addition, there are contacts from Tyr613 to C1, O5 and O6 of the sugar in sub-site +3. Hence the contacts from

Fig. 4. Superposition of the MalP–G5 and MalP–GSG4 complexes. The two structures are closely similar.

Tyr613 span the two sub-sites +3 and +4. Finally, we note that sub-site +3 appears extraordinarily wet. There are eight water molecules in van der Waals contact with the sugar, although only four of these are hydrogen bonded to the sugar.

Due to the high B factors associated with the phosphate group, we are less certain of the phosphate oxygen positions than for the carbohydrate atoms. Nevertheless, the dynamic refinement has resulted in stereochemically plausible positions that are consistent with the X-ray evidence. The MalP–phosphate contacts are dominated by contacts to an arginine residue, Arg569, where two phosphate oxygens contact the NE and NH2 atoms of the guanidine side chain in a classic binding mode (Johnson and O'Reilly, 1996) (Table II; Figure 5). One of these oxygen atoms also contacts Lys574. A third oxygen is hydrogen bonded to the main chain nitrogen of Gly135, a residue from a glycine-rich loop at the start of an α -helix (Acharya *et al.*, 1991). There is a direct hydrogen bond between one of the oxygens (O2) of the inorganic phosphate and an oxygen (OP1) of the cofactor phosphate and a further phosphate–phosphate contact through water. The cofactor 5'-phosphate is localized by hydrogen bonds from two of its oxygens to two lysine residues (Lys568 and Lys574), while the third oxygen is hydrogen bonded to the main chain nitrogen of a residue at the start of an α -helix. Each of the 5'-phosphate oxygens is also hydrogen bonded to water molecules. This arrangement is very similar to that observed with phosphate bound to the native MalP structure and also to the mammalian enzyme. Finally, in the ternary MalP–GSG4-P complex there are contacts from the inorganic phosphate oxygens (O1 and O4) to the thio-glycosidic linkage [distances 2.9 and 2.8 Å to the S atom, respectively (Table II)]. In the two binary complexes of MalP with G5 and GSG4, where there is no phosphate present, Arg569 adopts an alternative conformation in which it is turned away from the substrate

Table IIA. Intermolecular hydrogen bonds in the MalP–GSG4-P complex

Sub-site	Oligosaccharide atom	Protein atom	Distance (Å)
Sub-site –1 998	O2	Tyr573 OH	2.9
		Pho900 O1	2.8
		Wat1122	2.9
		Wat1277	2.6
	O3	Glu672 OE2	2.6
		Ser674 N	3.3
		Gly675 N	3.2
		Asn484 ND2	3.3
	O4	Gly675 N	3.1
		Wat1085	2.7
Sub-site +1 997	O2	His377 ND1	2.8
		Asn484 ND2	2.7
		Asp339 OD2	2.4
		Asp339 OD2	2.8
	[S4	Pho900 O1	2.9]
		Glu88 OE2	2.8
	O5	Glu88 OE2	2.9
		Gly134 N	3.3
	O6	Leu136 N	2.9
		Arg292 NH2	2.9
Sub-site S+2 996	O2	Wat1162	2.9
		His341 NE2	2.9
		Wat1162	2.8
		Wat1297	2.9
	O3	Asn133 ND2	2.7
		Arg569 NH1	2.7
	O5	Wat1160	2.7
		Arg292 NH2	3.2
	O6	Wat1205	2.7
		Wat1261	2.8
Sub-site S+3 995	O2	Wat1298	2.8
		Arg569 NH1	3.3
		Wat1166	2.7
		Wat1354	3.1
	O5	Glu382 OE1	2.5
		Glu382 OE2	3.3
	O6	His571 NE2	2.9
		Arg569 NH2	2.7
	O1	Tyr573 OH	2.8
		Lys574 NZ	3.2
Phosphate 900	O2	Glc998 O2	2.8
		[Glc997 S4	2.9]
		Lys574 NZ	2.9
		PLP OP1	2.7
	O3	PLP OP2	3.0
		Arg569 NE	2.9
	O4	Lys574 NZ	2.8
		PLP999 OP1	3.0
	O1	Wat1265	2.1
		Gly135 N	2.7

phosphate binding site. The shift in the arginine residue seen on binding phosphate provides additional support for the presence of the phosphate in the ternary complex.

Mutagenesis experiments supported

Extensive site-directed mutagenesis experiments have been carried out with *E.coli* MalP in which the target residues were identified by predicting the likely binding site for an oligosaccharide based on the rabbit muscle GP structure (Drueckes *et al.*, 1996; O'Reilly *et al.*, 1997). The mutagenesis studies have yielded results that are fully consistent with the present direct experimental observations of an oligosaccharide binding to MalP. The mutations Glu88Ala

Table IIB. van der Waals contacts to sugars and phosphate in MalP–GSG4-P complex^a

Sub-site	van der Waals contacts [residue (number contacts)]	Total contacts
Sub-site –1 998	Gly135(3), Leu136(4), His377(15), Thr378(4), Asn484(4), Val455(3), Tyr573(1), Glu672(4), Ala673(2), Ser674(1), Gly675(4), Pho900(9), Wat1085(3), Wat1122(2), Wat1277(2)	61
Sub-site +1 997	Glu88(6), Gly134(6), Gly135(3), Leu136(6), Gly137(1), Tyr280(3), Asp339(4), His341(1), His377(2), Thr378(3), Arg569(6), Pho900(6), Wat1122(2)	49
Sub-site S+2 996	Asn133(6), Tyr280(6), Arg292(3), His341(1), Glu382(4), Arg569(7), Wat1205(1), Wat1130(1), Wat1162(2), Wat1297(2)	33
Sub-site S+3 995	Asp283(1), Glu382(4), His571(2), Arg569(2), Ala610(2), Tyr613(8), Wat1009(2), Wat1037(1), Wat1160(2), Wat1190(1), Wat1205(2), Wat1233(1), Wat1261(2), Wat1298(1)	30
Sub-site S+4 994	Glu382(8), His571(3), Gly612(1), Tyr613(10), Wat1166(4), Wat1209(2), Wat1354(3)	31
Phosphate 900	Gly134(1), Gly135(2), Arg569(12), Tyr573(1), Lys574(4), Glu672(2), Glc997(4), Glc998(9), PLP999(2), Wat1085(2), Wat1122(1), Wat1265(3)	43

^avan der Waals contacts <4.0 Å excluding hydrogen bonds listed in Table IIA.

(sub-site +1), Tyr280Ala (sub-site +1, +2), Asp339Ala (sub-site +1), His341Ala (sub-site +1, +2), Thr378Gly (sub-site –1, +1), Glu382Ala (sub-site +2, +3, +4) and His571Leu (sub-site +3, +4) result in mutant enzymes that exhibit significantly reduced activity. Each of these residues is involved in direct contact with the oligosaccharide (Table II). The results indicate the importance of each of the interactions in locating and directing the oligosaccharide for correct binding for catalysis. The mutant Tyr613Phe showed only slightly changed kinetic parameters, a result that can be rationalized from the structure since phenylalanine at position 613 could perform similar stacking interactions with the sugars in sub-sites +3 and +4. The triple mutant Asn282Ala, Asp283Ala, Asn284Ala also had only slightly changed kinetic properties. The present results show that these residues from the 280s loop play only a minor role in binding oligosaccharide.

Conformational changes

The most significant conformational change in the MalP structure on binding oligosaccharide involves residues 377–384, a region designated as the 380s loop. In the T state (less active) form of GP this loop helps close the catalytic site through hydrophobic and ion pair interactions. In the R state (active) form of GP this loop is displaced from the catalytic site. The movement of the 380s loop in the T–R transition is correlated with the movement of Arg569, the important substrate phosphate binding residue.

Fig. 5. Summary of the intermolecular contacts for the MalP-GSG4-P complex. Contacts to Arg569 are slightly obscured by Tyr573 in this view. Intramolecular hydrogen bonds between adjacent sugars are not shown. Sugars labelled sub-sites +4 to -1 correspond to Glc994–Glc998, respectively. Full details are given in Table II.

Fig. 6. Movement of the 380s loop from its position in the native MalP structure (light lines) to its position in the MalP-GSG4-P structure (darker lines). MalP-G5, -GSG4 and -maltose complexes showed similar conformations of the 380s loop to the MalP-GSG4-P complex while the conformation in the MalP-acarbose was close to that of the native structure. See text for details.

It has been suggested that the changes in the 380s loop in the mammalian GP provide a means of communication between the catalytic site and the glycogen storage site (Barford and Johnson, 1992; O'Reilly *et al.*, 1997).

MalP is constitutively active, and in the native MalP structure, Arg569 is already in its correct position to bind the phosphate substrate, since the crystals were grown from a phosphate-buffered solution (Watson *et al.*, 1997). There is no conserved glycogen storage site in MalP, which provides an explanation for the preference of linear maltodextrin substrates over large branched glycogen substrates. The simplicity of the bacterial enzyme in binding a linear oligosaccharide, allows direct observation of the role of the 380s loop. In MalP the conformational change of this loop results in closure of the catalytic site, which is important both for recognition and correct positioning of the oligosaccharide in the catalytic site (Figure 6). The changes in the pentasaccharide complexes are similar to those observed previously for the maltose complex (O'Reilly *et al.*, 1997), where maltose bound to sub-site +1 and +2. In contrast, in the MalP-acarbose complex where acarbose bound to sub-sites +1 to +4, no closure of the 380s loop is observed. The lack of

movement can be rationalized in terms of the presence of the cyclitol ring, and not a glucopyranosyl ring, in sub-site +1 (O'Reilly *et al.*, 1999). Acarbose, although a potent inhibitor of glucosidases, is a poor inhibitor of MalP with a K_i of 5 mM. The K_i value for GSG4 determined in the direction of glycogen degradation has been found to be 1.5 μ M (R.Schinz, unpublished results). Taken together these observations suggest that, although MalP is able to bind the pseudo-oligosaccharide acarbose with low affinity, high affinity binding of substrates is associated with a conformational change in the 380s loop. The shift in the 380s loop appears to have three consequences for binding oligosaccharide. First, it results in a 2 Å shift in the carbonyl oxygen of His377 that may be significant for catalysis (discussed later). Secondly, it allows Thr378 to contribute van der Waals interactions to the sugar in sub-site +1. Thirdly, it promotes closure of the protein domains through an ionic interaction between Glu382 and His571. Both these residues hydrogen bond to the sugar in sub-site +4. The shift in the position of Glu382 is 13 Å. The mutant enzymes Glu382Ala and His571Leu exhibit nearly 100-fold decrease in k_{cat} and a significant increase in K_m for substrates compared with

Table III. Torsion angles for the $\alpha(1-4)$ glycosidic linkages for the pentasaccharides bound to MalP

	Φ ($^\circ$)	Ψ ($^\circ$)	Distance O2...O3'
MalP-G5			
998-997 (sub-sites -1 to +1)	63	-173	4.4
997-996 (sub-sites +1 to +2)	104	-130	2.8
996-995 (sub-sites +2 to +3)	75	-148	3.8
995-994 (sub-sites +3 to +4)	82	-143	3.6
MalP-GSG4			
998-997 (sub-sites -1 to +1)	80	-203	4.6
997-996 (sub-sites +1 to +2)	91	-124	3.2
996-995 (sub-sites +2 to +3)	81	-137	3.5
995-994 (sub-sites +3 to +4)	86	-144	3.4
MalP-GSG4-P			
998-997 (sub-sites -1 to +1)	68	-189	4.6
997-996 (sub-sites +1 to +2)	91	-125	3.2
996-995 (sub-sites +2 to +3)	81	-131	3.5
996-994 (sub-sites +3 to +4)	94	-148	3.2

Φ = O5-C1-O4'(S4')-C4', Ψ = C1-O4'(S4')-C4'-C5'.

the wild-type enzyme (Drueckes *et al.*, 1996), although both these residues are >15 Å from the site of catalysis.

Oligosaccharide conformation upon binding to phosphorylase

The torsion angles about the glycosidic bonds for the pentasaccharides bound to MalP are summarized in Table III. The torsion angles between the sugars in sub-sites +1, +2, +3 and +4 are not too different from those observed in the crystal structure of maltose (Takusagawa and Jacobson, 1978), and related compounds, where $\phi = 116^\circ$, $\psi = -118^\circ$ (see Table III for ϕ and ψ definitions). For sub-sites +1 to +4 of the MalP-GSG4-P complex, there are intramolecular hydrogen bonds between the O2 hydroxyl of one sugar and the O3' hydroxyl of the adjacent sugar although the distance for the hydrogen bond between sub-sites +2 and +3 is long (3.5 Å) (Table III). The most striking observation is the significant difference in torsion angles for the link between sub-sites -1 and +1 from the preferred values. The torsion angles of $\phi = 68^\circ$, $\psi = -189^\circ$ result in an $\sim 180^\circ$ flip in the sugar in sub-site +1 relative to that in sub-site -1 (Figure 7).

The change from the preferred values for the torsion angles allows the oligosaccharide to bind along the catalytic site channel. With the first sugar fixed in sub-site -1 via hydrogen bonds to each of the peripheral hydroxyl oxygens, the sugar in sub-site +1 is required to adopt an alternative conformation from the preferred conformation in order to avoid clashes with the enzyme, as first recognized nearly 20 years ago (Johnson *et al.*, 1980; Withers *et al.*, 1982). Either the enzyme or the substrate must alter conformation and we can now see that it is the substrate that alters its conformation with a rotation of about -48° on ϕ (resulting in a change of ϕ from 116° to 68°) and a rotation of about -71° on ψ (resulting in a change of ψ from -118° to -189°).

The conformational preference observed for sub-sites -1 to +1 in the MalP complexes is close to a second minimum energy conformer (Rees and Smith, 1975; Perez and Vergelati, 1984; Bock *et al.*, 1994) (Figure 7). The

Fig. 7. Energy diagram computed for α -thiomaltoside from Perez and Vergelati (1984) in which energy contours are drawn by interpolation of energy computed every 5° in ϕ and ψ including hydrogen bonding terms. The conformations for the preferred torsion angles in (A) maltose, (B) methyl-thio-maltoside and (C) the torsion angles observed for the sugars in sub-sites -1 and +1 in the present work are shown.

conformation lies just outside the allowed contours but the calculations do not take into account interactions of the sugars with the enzyme. The energy required for the altered conformation may be provided by the short and strong hydrogen bonds that are made by the sugar hydroxyl in sub-site +1 to acidic residues (i.e. hydrogen bonds between the O2 and O3 hydroxyls to Asp339 and the O6 hydroxyl to Glu88). Similar (but not identical) oligosaccharide conformations have been reported for other enzymes recognizing $\alpha(1-4)$ -glucosyl polymers, as first noted in the pig pancreatic amylase-acarbose complex (Qian *et al.*, 1994) and other glucosidase-acarbose complexes (summarized in O'Reilly *et al.*, 1999), and also observed with glucosyl polymers bound to inactive glucosidase mutant enzymes (Fujimoto *et al.*, 1998; Uitdehaag *et al.*, 1999). The conformation is one that is available to the $\alpha(1-4)$ -oligosaccharide and one that is preferred by phosphorylase.

Discussion

The catalytic mechanism of phosphorylase

Previous structural and biochemical studies with small molecular weight substrates and inhibitors with the mammalian GP led to proposals for the catalytic mechanism (Helmreich and Klein, 1980; Klein *et al.*, 1984; McLaughlin *et al.*, 1984; Johnson *et al.*, 1989, 1990). This mechanism can now be discussed in terms of an oligosaccharide substrate. The α -retaining mechanism proceeds via a double displacement reaction with two steps (Figure 8). In the first step, the 5'-phosphate of the cofactor pyridoxal phosphate promotes general acid attack by the inorganic phosphate on the glycosidic oxygen. The hydrogen bond between the 5'-phosphate of the cofactor and the inorganic phosphate substrate, observed previously with the dead-end product heptulose-2-phosphate in time-resolved experiments (Duke *et al.*, 1994), allows ready

exchange of protons between the two phosphate groups, as observed in ^{31}P NMR experiments (Klein *et al.*, 1984). Protonation of the glycosidic oxygen, between subsites -1 and $+1$, results in cleavage of the glycosidic bond and formation of a carbonium–oxonium ion transition state that is favoured and stabilized by the now negatively charged phosphate. In the second step, the inorganic phosphate acting as a nucleophile, attacks the carbonium

ion C1 carbon leading to formation of the product α -D-glucose-1-phosphate with retention of configuration.

The involvement of the substrate phosphate group as an obligatory part of the reaction mechanism explains why transfer is made preferentially to the phosphate rather than to water. In the binary MalP–G5 and MalP–GSG4 complexes, a water molecule is observed close to the position of the phosphorus of the substrate phosphate in the MalP–GSG4–P complex. The water is within 3.1 Å of the glycosidic oxygen (or sulfur) between subsites -1 and $+1$ and also contacts another water. It is just too far (3.5 Å) to make a strong hydrogen bond to the cofactor 5'-phosphate. Evidently, it would be difficult for the cofactor phosphate to act as an acid–base in promotion of attack by water because the distance is too large to promote a strong interaction and because of differences in the ionization properties of water and phosphate.

Implications for catalysis from the MalP–oligosaccharide complexes

In the present work we have been able to substantiate and expand the proposals for the mechanism with direct observation of the ternary enzyme–thio-oligosaccharide–phosphate complex in the *E. coli* MalP enzyme. Comparison of the binary complexes with the natural substrate, G5, and that with the inactive thio-analogue, GSG4, shows that the oligosaccharides bind in essentially identical conformations, and provides support for the notion that the thio analogue is a good mimic of the natural substrate. The bound oligosaccharides show alteration in the glycosidic torsion angles between sub-sites -1 and $+1$ from the preferred conformation for $\alpha(1-4)$ -linked glucosyl polymers.

In the ternary MalP–GSG4–P complex, the oligosaccharide position is identical to that seen in the binary complexes. The phosphate position is also closely similar to the position observed for the phosphate moiety in the native MalP structure, indicating that the phosphate recognition site is already available in the native enzyme,

Fig. 8. Proposals for the catalytic mechanism of phosphorylase based on the MalP–GSG4–P ternary enzyme substrate complex.

(A) The oligosaccharide substrate is bound across the catalytic site in subsites -1 and $+1$ and subsequent sites. The O6 hydroxyl of the sugar in sub-site -1 is hydrogen bonded to His377. The O2' and O3' hydroxyls of the sugar in sub-site $+1$ contact Asp339. Other contacts of the sugars are not shown. The phosphate substrate is within hydrogen bonding distance of the glycosidic oxygen and is stabilized in this position by contacts to Arg569 and Lys574. The phosphate is within hydrogen bonding distance of the pyridoxal phosphate 5'-phosphate group whose position is stabilized by contacts to water (shown as asterisks), Lys568, Lys574 and other polar groups, not all of which are shown. The cofactor 5'-phosphate acts as a general acid to promote attack of the inorganic phosphate on the glycosidic oxygen. The oxygen is protonated leading to the cleavage of the C1–O bond and formation of the oxonium–carbonium ion intermediate. (B) The carbonium ion is promoted and stabilized by the inorganic phosphate dianion, the carbonyl oxygen of His377 and the lone pair electrons on the 3' OH of the sugar in sub-site $+1$. The 2' OH group of the sugar in sub-site -1 is polarized by contact with inorganic phosphate. (C) The cleaved oligosaccharide is free to diffuse away. Nucleophilic attack by the inorganic phosphate at the C1 position of the carbonium ion leads to the formation of α -D-glucose-1-phosphate. The position of glucose-1-phosphate has been inferred from the binding of heptulose-2-phosphate in GP. Superimposition of the coordinates shows that the phosphate shifts ~ 1.3 Å in order to make the covalent bond, and Arg569 and Lys574 shift accordingly to retain contact.

and is not dependent on binding an oligosaccharide. The position of the inorganic phosphate is also similar to that observed in time-resolved and inhibitor binding studies with mammalian GP (Duke *et al.*, 1994; Mitchell *et al.*, 1996), supporting the notion that this is the substrate phosphate binding position. The constellation of the cofactor 5'-phosphate, the inorganic phosphate and the glycosidic linkage of the thio-oligosaccharide are fully consistent with the mechanism shown in Figure 8, in which general acid attack on the glycosidic linkage is promoted by the cofactor phosphate via exchange of a proton between the two phosphate groups.

In an alternative mechanism, it has been proposed that the cofactor phosphate acts as an electrophile in order to withdraw electrons from the inorganic phosphate without exchange of protons between them (Withers *et al.*, 1981a,b). This mechanism involves a constrained dianion pentavalent intermediate of the cofactor 5'-phosphate in which one oxygen of the inorganic phosphate approaches the fifth co-ordination position of the cofactor phosphorus atom, as in a transferase reaction. In the present crystal structure there is no evidence for distortion of the 5'-phosphate from tetrahedral geometry, nor are there any additional interactions that could stabilize such geometry. This mechanism is not supported by the present evidence, but it cannot be excluded since the penta-coordinate 5'-phosphate of the cofactor is a transient and highly unstable intermediate that is unlikely to be captured by static X-ray experiments.

Additional factors for catalysis

The present ternary complex suggests three additional factors that are likely to assist catalysis. First, the O2 hydroxyl group of the glucosyl residue in sub-site -1 forms a hydrogen bond to the O1 oxygen of the inorganic phosphate, in addition to the hydrogen bonds with a tyrosyl phenolic group and two water molecules. The interaction with the negatively charged phosphate is likely to result in a reduction of electro-negativity of the O2 hydroxyl which otherwise disfavours the positive charge of the carbonium-oxonium ion transition state intermediate. The hydrogen bond of the O2 hydroxyl with the O⁻ of the phosphate negatively polarizes the O2 atom, and this partial negative charge may be stabilized by the hydrogen bonds to the tyrosyl and the waters. Although donation of a hydrogen bond to the phosphate might reduce the pK_a of the substrate phosphate, the contribution of neighbouring charged groups are likely to influence the pK_a more strongly. Glu672 is just too far (3.5 Å) to participate directly in these interactions. Such electronic effects of the O2 have been shown to be important for glycosidase catalysis (McCarter *et al.*, 1992; Braun *et al.*, 1995). A similar contribution to the stabilization of the transition state has been observed in cyclodextrin glucosyl-transferase (CGTase) (Uitdehaag *et al.*, 1999).

Secondly, as observed previously, the phosphorylase catalytic site contains no negatively charged protein groups in the correct position to promote the carbonium-oxonium ion intermediate, unlike the glycosyl hydrolases where this role is almost universally taken by a carboxylate group. However, we note that the main chain oxygen atom of His377 is directed towards the O5 and C1 atoms of the sugar in sub-site -1 at a distance of 3.2 Å to both

atoms, such that the partial negative charge on this oxygen could provide some stabilization energy. A similar interaction was noted in the complex of GP with nojirimycin tetrazole (Mitchell *et al.*, 1996). Where the attacking group is a negatively charged phosphate group rather than a water molecule, it could be advantageous to have a neutral group acting in a stabilizing role (Wang and Withers, 1995).

Thirdly, the altered conformation about the glycosidic linkage also allows a contact between the O3 hydroxyl of the sugar in sub-site +1 and the O5 ring oxygen of the glucosyl in sub-site -1 (3.1 Å). The O3 atom is also 2.8 Å from the C1 atom of the sugar in sub-site -1. In the MalP-GSG4-P complex, the O3 hydroxyl is involved in a hydrogen bond to Asp339, presumably as a hydrogen donor at pH 6.7, the pH for optimal activity (Schinzel and Drueckes, 1991). This arrangement would allow the lone pair electrons on the O3 atom also to make some contribution to the stabilization of the positive charge on the developing carbonium-oxonium ion. The mutant Asp339Ala exhibits a 100-fold decrease in *k*_{cat} compared with wild type with no significant change in *K*_m values for substrate (Drueckes *et al.*, 1996).

Stereoelectronic effects

In each of the MalP-oligosaccharide complexes, the sugar in sub-site -1 can be accommodated in the usual chair conformation. In particular, the positions of the hydroxyl oxygens of the sugar, located in the 2.0 Å electron density map of the ternary complex, are consistent with a chair conformation and show no indication of a half-chair or sofa conformation, although the possibility of subtle changes cannot be excluded at this resolution. This is in agreement with previous work with GP, which has shown that sub-site -1 can accommodate a range of conformations from half chair to chair, for glucosyl and glucosyl-related residues in sub-site -1, and in contrast to recent work with CGTase where a partial distortion to half-chair was observed (Uitdehaag *et al.*, 1999).

Stereoelectronic theory suggests that in the grouping of atoms O-C1-O there is a possibility of mixing lone pair orbitals on one oxygen with the anti-bonding σ* orbital of the adjacent C-O bond. Such interactions are conformationally dependent and occur when the lone pair orbital on one oxygen is anti-periplanar (180°) to the adjacent C-O bond. The theory, reviewed by Gorenstein (1987), has received experimental and theoretical support but it is not without criticism, mostly concerning the magnitude of the effects and their likely contributions to catalysis. Restricting the discussion to the ⁴C₁ chair conformation of pyranose rings, for α-glycosides the axial C1-O1(O4') bond is anti-periplanar to one of the O5 oxygen lone pair orbitals, resulting in strengthening (and shortening) of the O5-C1 bond [the *endo* anomeric effect (Praly and Lemieux, 1987)]. Such interactions will tend to favour and stabilize a carbonium-oxonium ion. This suggests that α-glycosides with the axial aglycon may be cleaved in the ground state conformation. We note that in the MalP-GSG4-P complex there are no hydrogen bonds to the O5 atom of the glucosyl residue in sub-site -1 that would tend to weaken the *endo* anomeric effect (Leu136 N is 4.2 Å away) and the glucosyl group is in the chair conformation.

The *exo* anomeric effect occurs when one of the lone

pair orbitals on the O1(O4') oxygen is anti-periplanar to the C1-O5 bond, and this will be conformationally dependent on the glycosidic torsion angle ϕ . *Ab initio* calculations suggest that the *exo* anomeric effect will be strongest for a gauche orientation, that is, when $\phi = 60^\circ$ and weakest for an anti orientation, that is, when $\phi = 180^\circ$ (Cramer *et al.*, 1997). Because of the competition for electron deficiency at the C1 atom between the *endo* and *exo* effects in α -glycosides, the *exo* anomeric effect is weaker for α - than for β -glycosides. In β -glycosides, where no *endo* anomeric effect is possible, ring distortion would facilitate glycoside hydrolysis. Such distortions have been observed in lysozyme (Ford *et al.*, 1974; Strynadka and James, 1991; Hadfield *et al.*, 1994), chitinase (Tews *et al.*, 1996) and endoglucanase (Sulzenbacher *et al.*, 1996). For cleavage of the α -C1-O1(O4') bond, catalysis will be assisted if the *exo* anomeric effect is weakened. However, we note that the alternative conformation adopted by the oligosaccharide when bound to phosphorylase ($\phi = 68^\circ$) is such that the *exo* effect is about the same as in the preferred conformation ($\phi = 116^\circ$). This apparent conflict with stereoelectronic theory can be resolved if we compare the conformation of the product, glucose-1-phosphate, at the catalytic site as deduced from studies with heptulose-2-phosphate (Johnson *et al.*, 1990). The torsion angle in heptulose-2-phosphate $\phi = 220^\circ$, is such that would weaken the *exo* anomeric effect. In the reverse reaction the cofactor 5'-phosphate acts as an acid to protonate the phosphate of glucose-1-phosphate and the C1-O1 bond has to be cleaved without direct protonation of the glycosidic oxygen. In such circumstances weakening of the *exo* anomeric effect will be advantageous.

Oligosaccharide conformation and catalysis

What is the significance of the alteration from the preferred geometry about the glycosidic linkage between sub-sites -1 and +1 for catalysis? Several factors are suggested.

(i) The alteration in torsion angles is demanded by the geometry of the catalytic site tunnel. Only in the altered conformation are the catalytic groups correctly aligned with respect to the susceptible bond. In particular, it allows in-plane protonation (Heightman and Vasella, 1999) of the glycosidic oxygen by the phosphate.

(ii) The altered geometry allows the two substrates (either oligosaccharide and phosphate or oligosaccharide and glucose-1-phosphate) to be present at the same time. The reaction mechanism demands that the attacking group is present before the leaving group has left and it also demands that the reaction proceeds with retention of configuration.

(iii) The altered conformation breaks the intramolecular hydrogen bond between the O2 of one sugar and the O3' of the adjacent sugar and the polymer is opened up for attack, whereas in the preferred conformation the O2...O3' hydrogen bond partially prevents access for protonation of the glycosidic bond. The presentation of the glycosidic oxygen to the attacking phosphate is optimized and the lone pair electron on the O3' oxygen may assist in stabilization of the oxonium-carbonium ion.

These considerations provide some rationalization for the alternative conformation adopted by the oligosaccharide when bound across the catalytic site in MalP. It

is striking that in each of the α -glucosidases (amylases and cyclodextrin glucosyl transferases), where the reaction is known to proceed via a two-step mechanism with retention of configuration, a similar but not identical alteration of the conformation from the preferred conformation is observed (summarized in O'Reilly *et al.*, 1999). In contrast, in the single structural example of an α -glucosidase, glucoamylase, in which the reaction proceeds via a single step mechanism with inversion of configuration, the oligosaccharide binds in the preferred conformation for $\alpha(1\text{--}4)$ -linked glucosyl polymers (Aleshin *et al.*, 1996). The significance of the bound oligosaccharide conformation for the presentation of the susceptible glycosidic bond to the catalytic residues merits further investigation.

Materials and methods

Expression and purification of *E. coli* maltodextrin phosphorylase

Maltodextrin phosphorylase was purified with minor modifications as described previously (Schinzel and Palm, 1990; Schinzel *et al.*, 1992). Briefly, four 2 l flasks each containing 700 ml of Luria-Bertani broth were inoculated from an overnight culture of the expression strain (*E. coli* Δ malA518 harbouring the plasmid pMAP101). Maltodextrin phosphorylase was induced with 2 g/l maltose, after ~4 h at 37°C (OD₅₉₅ = 0.8–1.0). A further 16 h of growth was allowed, after which time the cells were harvested by centrifugation. The pellet was resuspended in buffer A (10 mM Tris pH 7.3, 1 mM EDTA), and disrupted by 10 mM MgCl₂, 1:1000 dilutions of 10 mg/ml RNase A, 10 mg/ml DNase I and 50 mg/ml lysozyme for 30 min at 0°C, followed by sonication (5 × 30 s), and then centrifugation to remove the cell debris. The supernatant was fractionated by ammonium sulphate precipitation (35% then 75%) stirring for 30 min at 0°C, and centrifuged. The pellet was resuspended in 5 ml buffer A and dialysed overnight against buffer A. The filtered dialysate was applied to a Q-Sepharose anion exchange column (Pharmacia), equilibrated with buffer A, and eluted using a gradient of 0–0.5 M NaCl in buffer A. MalP elutes as the second peak (~0.2–0.25 M NaCl). These fractions were dialysed against buffer B (20 mM Tris pH 6.9, 2 mM EDTA). The resulting dialysate was loaded onto a glycogen-glycin-Sepharose affinity column (prepared by coupling glycogen to Sepharose through CNBr), equilibrated with buffer B, and eluted with a gradient of 0–0.5 M NaCl in buffer B. The resulting dialysate was applied to a Superdex 200 gel filtration column (Pharmacia) equilibrated against 0.15 M NaCl in buffer B. Maltodextrin phosphorylase eluted as the second peak. The purified enzyme was concentrated to ~10 mg/ml in buffer B and used immediately for crystallization experiments. The final protein concentration was determined from the absorbance at 280 nm, using $E_{1\text{cm}}^{0.1\%} = 1.36$.

Crystallizations

The stock solutions used for the crystallization experiments were 1 M Tris pH 8.5; 50% (w/v) 4K PEG; 2 M LiCl; 50 mM Na₂HPO₄ (P_i). A maltopentaose (G5) solution (100 mM) was prepared in 1 M Tris pH 8.5. GSG4 was made up as a saturated solution (~20 mM) in water. The synthesis of GSG4 will be described elsewhere (N. Payre, S. Cottaz and H. Driguez, manuscript in preparation).

All the crystallization experiments were performed using the hanging drop method and micro-seeding (1:100 dilution) in order to obtain large diffraction quality crystals (typically 100 × 50 × 10 μm^3). For all three complexes, crystals were obtained from a screen of 26–32% 4K PEG, 0.1–0.6 M LiCl and 1 M Tris pH 8.5. The drops for the co-crystallization experiments were set up as follows. For the MalP–G5 and MalP–GSG4 binary complexes, a 4 μl drop consisted of 2 μl of concentrated protein solution, 0.4 μl of G5 or GSG4 stock solution and 1.6 μl of well solution. For the MalP–GSG4–P ternary complex, a 4 μl drop consisted of 2 μl of a 10 mg/ml protein solution, 0.4 μl of GSG4 solution, 0.4 μl of 50 mM Na₂HPO₄ and 1.2 μl of well solution. Two different crystal forms were obtained under these conditions. The GSG4–P ternary complex crystals had a square, plate-like morphology that correspond to space group P2₁ with cell dimensions $a = 107.4$ Å, $b = 61.7$ Å, $c = 126.7$ Å, $\beta = 104.3^\circ$, one dimer/asymmetric unit. The MalP–G5 and

MalP-GSG4 complexes both crystallized in the orthorhombic space group $P2_12_12_1$ exhibiting a rectangular plate-like morphology, one dimer/asymmetric unit, and cell dimensions $a = 74.45 \text{ \AA}$, $b = 105.69 \text{ \AA}$, $c = 214.91 \text{ \AA}$ and $a = 74.74 \text{ \AA}$, $b = 105.02 \text{ \AA}$, $c = 217.96 \text{ \AA}$, respectively. This crystal form is similar to that obtained for the MalP-acarbose complex crystals grown under similar conditions (O'Reilly *et al.*, 1999). A further crystal form could be obtained for the binary complexes in the presence of high concentrations of LiCl and 4K PEG (typically 0.6–0.8 M LiCl and 30–32% 4K PEG). This second crystal form had a prismatic morphology and was found to correspond to a C2 cell with dimensions $a = 170.1 \text{ \AA}$, $b = 111.7 \text{ \AA}$, $c = 120.8 \text{ \AA}$, $\beta = 119.3^\circ$. This is similar to the previously reported MalP-maltose complex (O'Reilly *et al.*, 1997).

Data collection and processing

The crystals were quickly passed through a solution of mother liquor and 20% MPD for flash freezing to 100 K prior to data collection. Glycerol as cryoprotectant was avoided, since previous experiments with MalP had shown that glycerol bound at sub-site -1 and blocked oligosaccharide binding at this site (O'Reilly *et al.*, 1997, 1999). Data to 2.9 Å resolution for the orthorhombic forms of the binary complexes MalP-G5 and MalP-GSG4 were collected using a 300 mm Mar Imaging Plate at the Daresbury Synchrotron Radiation Source station PX7.2 ($\lambda = 1.488 \text{ \AA}$). For the MalP-GSG4-P ternary complex, data to 2.0 Å resolution were collected on PX9.6 ($\lambda = 0.87 \text{ \AA}$) using the ADSC CCD detector.

All three data sets were processed using MOSFLM (CCP4, 1994). Although data to 2.9 Å resolution were collected for the G5 complex, the R_{merge} and the refinement suggested that all the data could be used for the refinement, but for the map calculation only data to 3.4 Å were used (81.0% complete, $R_{\text{merge}} = 0.15$, for outer shell 3.7–3.4 Å). The GSG4-P data were anisotropic resulting in diffraction to 2.0 Å resolution, when the X-rays were normal to the plate-like form of the crystal, but for some images, only to 2.5 Å resolution when the X-rays were parallel to the plate of the crystal. For this reason, a dynamic high resolution cut-off limit in MOSFLM that depends on mean $I > \sigma(I)$ was applied to single frames. Consequently, all the data to 2.0 Å resolution for the MalP-GSG4-P complex were used in the refinement but the map calculation used only data to 2.2 Å (81.3% complete, $R_{\text{merge}} = 0.101$, for outer shell 2.4–2.2 Å).

Phasing and refinement

Both binary structures were solved by molecular replacement in AMoRe (Navaza, 1994) using the 2.4 Å resolution native MalP structure as the starting model. For the MalP-G5 structure, the rigid body refinement in AMoRe gave an R factor of 0.411 and a correlation coefficient (Cc) of 0.643. For the MalP-GSG4 structure, the R factor was 0.390 and the Cc was 0.615. The oligosaccharides (G5 and GSG4) were initially modelled using the biopolymer module of the program SYBYL (Tripos, 1992) and were easily located in the initial difference electron density maps using O (Jones *et al.*, 1991).

The MalP-GSG4-P ternary complex structure was also solved by molecular replacement in AMoRe, using the protein atoms only of the MalP-GSG4 binary complex as the starting model since there were significant differences observed in the region of the catalytic site between the native enzyme and the binary complex structures. The rigid body refinement between 8 and 4 Å resolution of the ternary complex gave an R factor of 0.316 and a Cc of 0.702. The GSG4 substrate was then easily located in an initial difference Fourier electron density map. Once the position of the oligosaccharide had been partially refined, the substrate phosphate anion in the ternary complex was located first by positioning the phosphorous atom from the location of the highest peak in a second difference Fourier calculation, followed by positional and B factor refinement. A subsequent difference Fourier map clearly revealed the position of two of the oxygen atoms. Positional and B factor refinement with these additional atoms, and a final difference Fourier map revealed the location of the last two oxygen atoms. The final ternary complex model was then subjected to simulated annealing, as described below.

All three complexes were refined using X-PLOR (Brünger, 1992) following an iterative process of manual model building using O, positional refinement, isotropic B factor refinement, and simulated annealing (starting temperature 2500 K, 5 fs steps for 50 cycles with harmonic restraints applied to the water molecules) and energy minimization. Throughout the initial refinements during model building, 5% of each data set was flagged for calculation of R_{free} . Final X-PLOR refinements for all three complexes were performed by simulated

annealing, followed by restrained anisotropic B factor refinement using a free R flag of between 2 and 5% (equivalent to ~1200 reflections in each complex). For all three complexes, strict NCS constraints were applied to the dimer molecule. The final refinements gave $R/R_{\text{free}} = 0.245/0.306$, $R/R_{\text{free}} = 0.214/0.277$ and $R/R_{\text{free}} = 0.228/0.261$, for MalP-G5, MalP-GSG4 and MalP-GSG4-P, respectively. In each case, electron density maps were calculated using shell-scaling in which the amplitudes were matched and scaled in resolution shells using Rayment weighting (Rayment, 1983; D.I. Stuart, unpublished programs), and 2-fold averaging using RAVE (Kleywegt and Jones, 1994). The additional phasing information to 2.0 Å for the ternary MalP-GSG4-P complex, allowed a different assignment of eight amino acids in the primary sequence that could be clearly traced in the 2.2 Å resolution electron density map. These sequence changes were not observed in the previous 2.4 Å native structure, but are in agreement with the primary sequence reported by F.R. Blattner in the DDBJ/EMBL/GenBank data bank. The assignments using the *E. coli* numbering scheme (with the previous assignment in brackets) are Lys294 (Glu), Glu488(Val), Phe490(Leu), Gln499(Leu), Glu502(Val), Glu522(Asp), His548(Arg), Glu682(Lys) (Palm *et al.*, 1987; Blattner *et al.*, 1997).

The program PROCHECK was used to assess the geometry of the final structures. The co-ordinates for all three complexes have been submitted to the Brookhaven Protein Data Bank.

Acknowledgements

The authors are grateful to Professor Dieter Palm and Dr Reinhard Schinzel for providing us with the MalP expression strain. We thank Dr Stephen Withers for discussions. We wish to thank the staff at SRS Daresbury on Stations PX7.2 and PX9.6 for providing excellent facilities for data collection. Dr Richard Bryan and Dr Yuguang Huang are acknowledged for maintaining excellent computing facilities within the laboratory. This work is supported by the British Diabetic Association, the Medical Research Council and by the European Union Biotechnology Programme (contract BIO4-CT98-0022).

References

- Acharya, K.R., Stuart, D.I., Varvill, K.M. and Johnson, J.N. (1991) *Glycogen Phosphorylase: Description of the Protein Structure*. World Scientific, London, UK.
- Aleshin, A.E., Stoffer, B., Firsov, L.M., Svensson, B. and Honzatko, R.B. (1996) Crystallographic complexes of glucoamylase with malto-oligosaccharide analogs: relationship of stereochemical distortions at the non-reducing end to the catalytic mechanism. *Biochemistry*, **35**, 8319–8328.
- Artymuk, P.J., Rice, D.W., Poirrette, A.R. and Willett, P. (1995) β -glucosyltransferase and phosphorylase reveal their common theme. *Nature Struct. Biol.*, **2**, 117–120.
- Barford, D. and Johnson, L.N. (1989) The allosteric transition of glycogen phosphorylase. *Nature*, **340**, 609–614.
- Barford, D. and Johnson, L.N. (1992) The molecular mechanism for the tetrameric association of glycogen phosphorylase promoted by protein phosphorylation. *Protein Sci.*, **1**, 472–493.
- Barford, D., Hu, S.-H. and Johnson, L.N. (1991) The structural mechanism for glycogen phosphorylase control by phosphorylation and by AMP. *J. Mol. Biol.*, **218**, 233–260.
- Becker, S., Palm, D. and Schinzel, R. (1994) Dissecting differential binding in the forward and reverse reaction of *Escherichia coli* maltodextrin phosphorylase using 2-deoxyglucosyl substrates. *J. Biol. Chem.*, **269**, 2485–2490.
- Blattner, F.R., Plunkett, G., III, Mayhew, G.F., Perna, N.T., Glasner, F.D. (Jan. 1997) submitted to DDBJ/EMBL/GenBank data banks.
- Bock, K., Duus, J.O. and Refn, S. (1994) Conformational equilibria of 4-thiomaltose and nitrogen analogues of maltose in aqueous solutions. *Carbohydr. Res.*, **253**, 51–67.
- Braun, C., Brayer, G.D. and Withers, S.G. (1995) Mechanism-based inhibition of yeast α -glucosidase and human pancreatic α -amylase by a new class of inhibitors. *J. Biol. Chem.*, **270**, 26778–26781.
- Brünger, A.T. (1992) *X-PLOR: Version 3.8: A System for Protein Crystallography and NMR*. Yale University Press, New Haven, CT.
- Collaborative Computational Project Number 4 (1994) The CCP4 suite: programmes for protein crystallography. *Acta Crystallogr.*, **D50**, 760–763.

- Cramer,C.J., Truhlar,D.G. and French,A.D. (1997) Exo-anomeric effects on energies and geometries of different conformations of glucose and related systems in the gas phase and aqueous solution. *Carbohydr. Res.*, **298**, 1–14.
- Davies,G.J., Tolley,S.P., Henrissat,B., Hjort,C. and Schulein,M. (1995) Structures of oligosaccharide-bound forms of the endoglucanase V from *Humicola insolens* at 1.9 Å resolution. *Biochemistry*, **34**, 16210–16220.
- Driguez,H. (1997) Thio-oligosaccharides in glycobiology. *Top. Curr. Chem.*, **187**, 85–116.
- Drueckes,P., Boeck,B., Palm,D. and Schinzel,R. (1996) Mutational analysis of the oligosaccharide recognition site at the active site of *Escherichia coli* maltodextrin phosphorylase. *Biochemistry*, **35**, 6727–6734.
- Duke,E.M.H., Wakatsuki,S., Hadfield,A. and Johnson,L.N. (1994) Laue and monochromatic diffraction studies on catalysis in phosphorylase *b* crystals. *Protein Sci.*, **3**, 1178–1196.
- Fersht,A.R. *et al.* (1985) Hydrogen bonding and biological specificity analysed by protein engineering. *Nature*, **314**, 235–238.
- Ford,L.O., Johnson,L.N., Machin,P.A., Phillips,D.C. and Tjian,R. (1974) Crystal structure of a lysozyme-tetrasaccharide lactone complex. *J. Mol. Biol.*, **88**, 349–371.
- Fujimoto,Z., Takase,K., Doui,N., Momma,M., Matsumoto,T. and Mizuno,H. (1998) Crystal structure of a catalytic-site mutant α -amylase from *Bacillus subtilis* complexed with maltopentaose. *J. Mol. Biol.*, **277**, 393–407.
- Gorenstein,D.B. (1987) Stereo electronic effects in biomolecules. *Chem. Rev.*, **87**, 1047–1077.
- Hadfield,A.T., Harvey,D.J., Archer,D.B., MacKenzie,D.A., Jeenes,D.J., Radford,S.E., Lowe,G., Dobson,C.M. and Johnson,L.N. (1994) Crystal structure of the mutant D52S hen egg white lysozyme with an oligosaccharide product. *J. Mol. Biol.*, **243**, 856–872.
- Hajdu,J., Acharya,K.R., Stuart,D.I., McLaughlin,P.J., Barford,D., Oikonomakos,N.G., Klein,H. and Johnson,L.N. (1987) Catalysis in the crystal: synchrotron radiation studies with glycogen phosphorylase *b*. *EMBO J.*, **6**, 539–546.
- Heightman,T.D. and Vasella,A.T. (1999) Recent insights into inhibition, structure and mechanism of configuration-retaining glycosidases. *Angew. Chem. Int. Ed.*, **38**, 750–770.
- Helmreich,E.J.M. and Klein,H.W. (1980) The role of pyridoxal phosphate in the catalysis of glycogen phosphorylases. *Angew. Chem. Int. Ed.*, **19**, 441–445.
- Holm,L. and Sander,C. (1995) Evolutionary link between glycogen phosphorylase and a DNA modifying enzyme. *EMBO J.*, **14**, 1287–1293.
- Hu,H.-Y. and Gold,A.M. (1975) Kinetics of glycogen phosphorylase a with a series of semisynthetic, branched saccharides: a model for binding of polysaccharide substrates. *Biochemistry*, **14**, 2224–2230.
- Johnson,L.N. (1992) Glycogen phosphorylase: control by phosphorylation and allosteric effectors. *FASEB J.*, **6**, 2274–2282.
- Johnson,L.N. and O'Reilly,M. (1996) Control by phosphorylation. *Curr. Opin. Struct. Biol.*, **6**, 762–769.
- Johnson,L.N., Jenkins,J.A., Wilson,K.S., Stura,E.A. and Zanotti,G. (1980) Proposals for the catalytic mechanism of glycogen phosphorylase prompted by the crystallographic studies on glucose-1-phosphate binding. *J. Mol. Biol.*, **140**, 656–680.
- Johnson,L.N., Cheetham,J., McLaughlin,P.J., Acharya,K.R., Barford,D., Phillips,D.C. (1988) Protein-oligosaccharide interactions: lysozyme, phosphorylase, amylases. *Curr. Top. Microbiol. Immunol.*, **139**, 81–134.
- Johnson,L.N., Hajdu,J., Acharya,K.R., Stuart,D.I., McLaughlin,P.J., Oikonomakos,N.G. and Barford,D. (1989) In Herve,G. (ed.), *Glycogen Phosphorylase b*. CRC Press, Boca Raton, FL, pp. 81–127.
- Johnson,L.N., Acharya,K.R., Jordan,M.D. and McLaughlin,P.J. (1990) The refined crystal structure of the phosphorylase-heptulose 2-phosphate-oligosaccharide-AMP complex. *J. Mol. Biol.*, **211**, 645–661.
- Johnson,L.N., Hu,S.-H. and Barford,D. (1992) Catalytic mechanism of glycogen phosphorylase. *Faraday Disc.*, **93**, 131–142.
- Jones,T.A., Zou,J.-Y., Cowan,S.W. and Kjeldgaard,M. (1991) Improved methods for building protein models in electron density maps and location of errors in these models. *Acta Crystallogr.*, **A47**, 110–119.
- Kasvinsky,P., Madsen,N.B., Fletterick,R.J. and Sygusch,J. (1978) X-ray crystallographic and kinetic studies of oligosaccharide binding to phosphorylase. *J. Biol. Chem.*, **253**, 1290–1296.
- Klein,H.W., Im,M.J., Palm,D. and Helmreich,E.J.M. (1984) Does pyridoxal 5'-phosphate function in glycogen phosphorylase as an electrophilic or a general acid catalyst? *Biochemistry*, **23**, 5853–5861.
- Kleywegt,G.J. and Jones,T.A. (1994) Halloween...masks and bones. In Bailey,S., Hubbard,R. and Waller,D. (eds), *Proceedings of the CCP4 Study Weekend*. SERC Daresbury Laboratory, Warrington, UK, pp. 59–66.
- McCarter,J.D., Adam,M.J. and Withers,S.G. (1992) Binding energy and catalysis. *Biochem. J.*, **286**, 721–727.
- McLaughlin,P.J., Stuart,D.I., Klein,H.W., Oikonomakos,N.G. and Johnson,L.N. (1984) Substrate-cofactor interactions for glycogen phosphorylase *b*. A binding study in the crystal of heptenitol and heptulose-2-phosphate. *Biochemistry*, **23**, 5862–5873.
- Martin,J.L., Withers,S.G. and Johnson,L.N. (1999) Comparison of the binding of glucose and glucose-1-phosphate derivatives to T state glycogen phosphorylase *b*. *Biochemistry*, **29**, 10745–10757.
- Mitchell,E.P., Withers,S.G., Ermert,P., Vasella,A.T., Garman,E.F., Oikonomakos,N.G. and Johnson,L.N. (1996) Ternary complex crystal structures of glycogen phosphorylase with a transition state analogue nojirimycin tetrazole and phosphate in the T and R states. *Biochemistry*, **35**, 7341–7355.
- Navaza,J. (1994) AMoRe: an automated package for molecular replacement. *Acta Crystallogr.*, **A50**, 157–163.
- O'Reilly,M., Watson,K.A., Schinzel,R., Palm,D. and Johnson,L.N. (1997) Oligosaccharide substrate binding to *E. coli* maltodextrin phosphorylase. *Nature Struct. Biol.*, **4**, 405–412.
- O'Reilly,M., Watson,K.A. and Johnson,L.N. (1999) The crystal structure of the *E. coli* maltodextrin phosphorylase-acarbose complex. *Biochemistry*, **38**, 5337–5345.
- Palm,D., Goerl,R. and Burger,K.J. (1985) Evolution of catalytic and regulatory sites in phosphorylase. *Nature*, **313**, 500–502.
- Palm,D., Goerl,R., Weidinger,G., Zeier,R., Fischer,B. and Schinzel,R. (1987) *E. coli* maltodextrin phosphorylase: primary structure and deletion mapping of the C-terminal site. *Z. Naturforsch.*, **42c**, 394–400.
- Palm,D., Klein,H.W., Schinzel,R., Buehner,M. and Helmreich,E.J.M. (1990) The role of pyridoxal-5'-phosphate in glycogen phosphorylase catalysis. *Biochemistry*, **29**, 1099–1107.
- Perez,S. and Vergelati,C. (1984) Structure and conformational analysis of methyl α -thiomaltoside. *Acta Crystallogr.*, **B40**, 294–299.
- Praly,J.-P. and Lemieux,R.U. (1987) Influence of solvent on the magnitude of the anomeric effect. *Can. J. Chem.*, **65**, 213–223.
- Qian,M., Haser,R., Buisson,G., Duée,E. and Payan,F. (1994) The active centre of a mammalian α -amylase. Structure of the complex of a pancreatic α -amylase with a carbohydrate inhibitor refined to 2.2 Å resolution. *Biochemistry*, **33**, 6284–6294.
- Raibaud,O. and Schwartz,M. (1984) Positive control of transcription initiation in bacteria. *Annu. Rev. Genet.*, **18**, 207–231.
- Rayment,I. (1983) Molecular replacement method at low resolution: Optimum strategy and intrinsic limitations as determined by calculations on icosahedral virus models. *Acta Crystallogr.*, **A39**, 102–116.
- Rees,D.A. and Smith,P.J.C. (1975) Polysaccharide conformation. Part IX. Monte Carlo calculation of conformational energies for disaccharide and comparison with experiment. *J. Chem. Soc. Perkin II*, 836–840.
- Schinzel,R. and Drueckes,P. (1991) The phosphate recognition site of *Escherichia coli* maltodextrin phosphorylase. *FEBS Lett.*, **286**, 125–128.
- Schinzel,R. and Palm,D. (1990) *Escherichia coli* maltodextrin phosphorylase: contribution of active site residues Glu637 and Tyr538 to the phosphorolytic cleavage of alpha-glucans. *Biochemistry*, **29**, 9956–9962.
- Schinzel,R., Palm,D. and Schnackerz,K.D. (1992) Pyridoxal 5'-phosphate as a 31P reporter observing functional changes in the active site of *Escherichia coli* maltodextrin phosphorylase after site directed mutagenesis. *Biochemistry*, **31**, 4128–4133.
- Schwartz,M. and Hofnung,M. (1967) La maltodextrine phosphorylase d'*Escherichia coli*. *Eur. J. Biochem.*, **2**, 132–145.
- Sprang,S.R., Withers,S.G., Goldsmith,E.J., Fletterick,R.J. and Madsen,N.B. (1991) The structural basis for the association of phosphorylase *b* with AMP. *Science*, **254**, 1367–1371.
- Street,I.P., Armstrong,C.R. and Withers,S.G. (1986) Hydrogen bonding and specificity. Fluorodeoxy sugars as probes of hydrogen bonding in the glycogen phosphorylase-glucose complex. *Biochemistry*, **25**, 6021–6027.
- Strynadka,N.C.J. and James,M.N.G. (1991) Lysozyme revisited: crystallographic evidence for distortion of an *N*-acetylmuramic acid residue bound in site D. *J. Mol. Biol.*, **220**, 401–424.

- Sulzenbacher,G., Driguez,H., Henrissat,B., Schülein,M. and Davies,G.J. (1996) Structure of the *Fusarium oxysporum* endoglucanase I with a non-hydrolysable substrate analogue: substrate distortion gives rise to the preferred axial orientation of the leaving group. *Biochemistry*, **35**, 15280–15287.
- Takusagawa,F. and Jacobson,R.A. (1978) The crystal structure of maltose. *Acta Crystallogr.*, **D34**, 213–218.
- Tews,I., Perrakis,A., Oppenheim,A., Dauter,Z., Wilson,K.S. and Vorgias,C.E. (1996) Bacterial chitobiase structure provides insight into catalytic mechanism and the basis of Tay–Sachs disease. *Nature Struct. Biol.*, **3**, 638–648.
- Tripos Associates Inc. (1992) Sybyl Molecular Modeling Software, St Louis, MO.
- Uitdehaag,J.C.M., Mosi,R., Kalk,K.H., van der Veen,B.A., Dijkhuizen,L., Withers,S.G. and Dijkstra,B.W. (1999) Catalysis in the α -amylase family—X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase. *Nature Struct. Biol.*, **6**, 432–436.
- Wang,Q. and Withers,S.G. (1995) Substrate-assisted catalysis in glycosidases. *J. Am. Chem. Soc.*, **117**, 10137–10138.
- Watson,K.A., Schinzel,R., Palm,D. and Johnson,L.N. (1997) The crystal structure of *E. coli* maltodextrin phosphorylase provides an explanation for the activity without control in this basic archetype of a phosphorylase. *EMBO J.*, **16**, 1–14.
- Withers,S.G., Madsen,N.B. and Sykes,B.D. (1981a) Active form of pyridoxal phosphate in glycogen phosphorylase. Phosphorus-31 nuclear magnetic resonance investigation. *Biochemistry*, **20**, 1748–1756.
- Withers,S.G., Madsen,N.B., Sykes,B.D., Tagaki,M., Shimomura,S. and Fukui,T. (1981b) Evidence for direct phosphate phosphate interaction between pyridoxal phosphate and substrate in the glycogen phosphorylase mechanism. *J. Biol. Chem.*, **256**, 10759–10762.
- Withers,S.G., Madsen,N.B., Sprang,S.R. and Fletterick,R.J. (1982) Catalytic site of glycogen phosphorylase: Structural changes during activation and mechanistic implications. *Biochemistry*, **21**, 5372–5382.

Received May 25, 1999; revised and accepted July 7, 1999