Mutational Analysis of the Oligosaccharide Recognition Site at the Active Site of *Escherichia coli* Maltodextrin Phosphorylase[†]

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ABSTRACT: A mutagenesis approach was applied to identify specific amino acid residues that are tentatively involved in binding of the oligosaccharide substrate at the active site of *Escherichia coli* maltodextrin phosphorylase. From ten residues located within a proposed channel connecting the enzyme surface with the active site, nine displayed significant effects on the reaction with oligosaccharide substrates when exchanged by mutagenesis. While several mutant enzymes (N258A/D259A/N260A, N307A, E350A, and Y578F) exhibited moderate decreases in apparent binding (about 4−17-fold), two mutations, H536L and E67A, weakenend apparent binding of oligosaccharide substrates by 2 orders of magnitude. Two further mutant enzymes (T346G and H310A) displayed a 10-fold increase in the apparent K_m of the oligosaccharide in the degradation reaction, while binding in the synthesis direction seemed less affected, indicating partially differential binding modes of oligosaccharides in synthesis and degradation. Quite uniquely, the H310A mutant enzyme exhibits a more than 100-fold-lowered K_i for gluconolactone, indicating the existence of an inhibitor binding site similiar to that expected for a carbonium ion-like transition state. This is further confirmed by the finding that glucose, which does not inhibit wild-type enzyme, became an inhibitor of the H310A mutant enzyme ($K_i = 20$ mM). Since mutation of D308 did reduce k_{cat} about 10−100-fold while K_m values remained unchanged, a participation of this residue in transition state binding is probable. The insight into substrate recognition derived from this mutagenesis study corroborates a binding model where maltopentaose fits into the phosphorylase *b* structure in a distorted form.

The majority of natural polysaccharides like starch, glycogen, and maltodextrin are degraded by glycosylhydrolases. Despite large variations in the composition of their substrates, all polysaccharide hydrolases are thought to act by a general acid catalysis mechanism in which two acidic amino acid residues participate in a single or double displacement reaction resulting in inversion or retention of configuration at the anomeric carbon atom of the glycosidic bond (Sinnott, 1990; Henrissat & Bairoch, 1993). As exemplified by glucoamylase, the nature of the polysaccharide binding site is generally well-characterized and consists of several subsites (Hiromi, 1970; Fagerström, 1991). It is assumed that the total affinity of the oligomeric substrate corresponds to the sum of the individual subsite affinities. Cleavage occurs between subsites 1 and 2, where subsite 2 has the highest affinity for the oligomeric substrate. The above model is supported by a wealth of kinetic, mutational, and structural data (Hiromi, 1970; Fagerström, 1990; Sierks et al., 1993).

In contrast to polysaccharide hydrolases, glycogen phosphorylases (EC 2.4.1.1) transfer a glucosyl residue to a phosphate group rather than to a water, resulting in the phosphorolytic cleavage of α-1,4-linked glucose units and formation of α-D-glucose 1-phosphate (Glc-1-P)[1] according to

$$\text{glucose}_n + P_i \leftrightarrow \text{glucose}_{n-1} + \text{glucose 1-phosphate}$$

Another principal difference between the phosphorylase-catalyzed reaction and the reaction of hydrolases is the free reversibility of the phosphorolytic cleavage.

When polysaccharide phosphorylases are compared to other polysaccharide-depolymerizing enzymes like amylases, it becomes immediately evident that there are some fundamental differences in mechanism and structure, despite the assumption that phosphorylases share general acid catalysis [for review, see Madsen and Withers (1986), Palm et al. (1990), and Johnson (1992)]. Accordingly, it may be expected that the substrate recognition site of enzymes acting on the same substrate, but differing in their mechanism, also might be distinguished by the stereochemistry and catalytic interactions within the active site. Such differences would make the comparative study of polysaccharide binding sites highly interesting.

Present knowledge about oligosaccharide binding at the active site of phosphorylases is still scarce. While the binding of either phosphate or Glc-1-P is well-characterized, substantially less is known about productive binding of the polysaccharide for degradation or elongation (Street et al., 1986; Johnson et al., 1990; Becker et al., 1994). In the direction of polysaccharide synthesis, an oligosaccharide with a finite number of glucosyl residues ("primer") is indispensable for elongation by addition of glucose units (French & Wild, 1953). Likewise, in the direction of polysaccharide

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[1] Abbreviations: Glc-1-P, α-D-glucose 1-phosphate; G4, maltotetraose; G5, maltopentaose; G7, maltoheptaose.

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degradation, phosphorolysis is effectively terminated when the residual length of the substrate comes down to a limiting value of glucose residues. The present information on oligosaccharide binding is based mainly on the action pattern derived from kinetic studies with branched and linear oligosaccharides (French & Wild, 1953; Giri & French, 1971). French and Wild suggested a five-glucose unit binding site consisting of four subsites for the primer and one for the glucose moiety of Glc-1-P. For maltodextrin phosphorylase, the minimum length primer, which permits elongation of the oligosaccharide chain, is maltotetraose (G4), while maltopentaose (G5) is the minimum length good substrate for phosphorolysis. Similar to the subsite concept described above, the substrate binding region of the enzyme can be conceived of as an array of tandem subsites with each subsite sterically complementary to and interacting with a single monomer residue of the substrate (Allen, 1980). An intention of this study is to prove whether the subsite concept, proposing that monomer residues remote from the point of catalysis interact with the enzyme, can be applied to glycogen phosphorylases.

Due to the lack of structure information and of suitable substrate analogues, it has not been possible to date to establish a structure−function relationship for oligosaccharide binding sites in phosphorylases. Therefore, being aware that the observed effects may be due to local structural rearrangements induced by the mutation, a site-directed mutagenesis approach appears to be the only practicable way at present to gain further information on how carbohydrates bind at or close to the active site.

The *Escherichia coli* maltodextrin phosphorylase was employed as a model system for studying oligosaccharide binding in phosphorylases since the bacterial enzyme preferentially binds short linear oligosaccharides.

For the mutagenesis studies, only those residues were selected which met at least two of three criteria.

The residues should be located at or close to a channel with access to the active site which was tentatively proposed to form the oligosaccharide binding site. This channel consists of an about 1.5 nm long region of low electron density connecting the cofactor binding site with the surface of the enzyme in the phosphorylase *b* structure (Barford et al., 1988).

The residues should be conserved in all known phosphorylase primary structures of bacterial, plant, and mammalian origin (Newgard et al., 1989; Hudson et al., 1993).

The residues should be only amino acid residues with charged or hydrophilic amino acid side chains which are commonly involved in carbohydrate binding. In contrast to many other enzymes acting on oligosaccharides, in phosphorylases, no tryptophans and only few aromatic amino acid side chains seem to be involved in carbohydrate binding (Spurlino et al., 1991).

The present study shows that almost all mutations introduced into maltodextrin phosphorylase following the rationale described above affected oligosaccharide binding and catalysis. Effects on binding and catalysis differ significantly, depending on the amino acid residue changed. In most mutant phosphorylases, binding of inorganic phosphate was also affected while binding of Glc-1-P was affected only slightly. Some mutants behaved apparently differently on binding of the oligosaccharide and the second substrate when the effects in the direction of glycogen phosphorolysis or the direction of glycogen synthesis was compared, raising the question of whether different binding modes might exist in both directions. Our results are discussed in relation to a structural model, in which a maltopentaose has been fitted into the active site of phosphorylase *b*.

Table 1: Oligonucleotides Used for Site-Directed Mutagenesis

mutation	restriction site[a]	oligonucleotide
E350A	−*Hae*II	gcgttccagcgcagctggcatcaggg
T346G	+*Nco*I	ggcatcaggccatggtgggtg
E67A	+*Nco*I	caaaaacgccatggagatgtag
N307A	−*Pvu*II	gggtatccgcgagctgaataac
D308A	−*Pvu*II	gggtgggtcgcgttgagctgaataac
N258A/D259A/N260A		ctctatccagccgccgcccatactgc
H537L	+*Xho*I	tgtactcgagcaaacgt
H310A	+*Nar*I	gaacgatacggcgccaactatc
Y578F	−*Bst*NI	gccagataaaagcccggtgc

[a] Restriction site introduced (+) or removed (−) by the oligonucleotide.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strain XL1-Blue [*rec*A1, *end*A1, *gyr*A96, *sup*E44, *lac* (F′ *pro*AB *lac*I^{q}ZΔM15, Tn*10*)] (Stratagene, Heidelberg) was used for recombinant DNA manipulations, and strain CJ236 [*rel*A1, *thi*, *ung*, *dut*, pCJ105] was used for site-directed mutagenesis. Wild-type and mutant phosphorylases were expressed in the maltodextrin phosphorylase deficient strain *E. coli* pop2158 [Δ*mal*-A518, F$^-$, *ara*D139, Δ*lac*U169, *rap*s, *rel*A] (Raibaud et al., 1983). To facilitate mutagenesis, fragments of the *mal*P gene were cloned from the expression vector pMAP101 (Schinzel & Palm, 1990) into the phagemid vector pBluescript II KS+ (Stratagene) giving plasmids pMPHHKS+ and pMPHEKS+ containing a *Hin*dIII/*Hin*cII or a *Hin*dIII/*Eco*RI fragment, respectively.

Site-Directed Mutagenesis. Restriction endonucleases, T4 DNA ligase, and polynucleotide kinase were used as recommended by the manufacturer (Boehringer Mannheim). Oligonucleotides were synthesised on an Applied Biosystems 380A DNA synthesiser and purified by preparative electrophoresis on a denaturing 20% polyacrylamide gel. Alternatively, oligonucleotides were used directly after purification with a S400 spin column (Pharmacia LKB, Freiburg). Site-directed mutagenesis was performed by the method of Kunkel et al. (1987). Uracil-containing DNA was isolated by transforming double-stranded plasmid pMPHHKS+ and pMPHEKS+ into *E. coli* strain CJ236 followed by induction of single-stranded DNA synthesis by infection with helper phage M13KO7. The mutations were introduced into this DNA by mutagenic oligonucleotides. Most mutagenic oligonucleotides contained a second, silent mutation which creates a new restriction site to facilitate screening (Table 1). The mutations were confirmed by restriction analysis and DNA sequencing using the Sequenase Kit (U.S. Biochemicals) or an Applied Biosystems DNA sequencer. DNA fragments containing the mutation were recloned into the expression vector pMAP101.

Enzyme Purification and Characterization. Wild-type and mutant enzymes were obtained from a culture of *E. coli* Δ*mal*A518 harboring the expression plasmid, after induction with maltose as described (Schinzel et al., 1992). Washed cells were disrupted by sonication or by repeated passage

through a French press. The cell-free extracts of mutant enzymes were further purified by ammonium sulfate precipitation, ion exchange chromatography (Q-Sepharose, Pharmacia, Freiburg), and affinity chromatography on a glycogen-modified Sepharose column as described for mutant and wild-type enzymes (Schinzel et al., 1992) Purification of low-activity mutant proteins was followed by SDS−PAGE. Typically, 10 g of bacteria (wet weight) yielded about 60 mg of pure enzyme (>98% as judged by sodium dodecyl sulfate−polyacrylamide gel electrophoresis). Protein concentration was measured by the method of Bradford (1971) or from the absorbance at 280 nm, using $E_{1cm}^{0.1\%} = 1.36$.

UV spectra were recorded on a CARY1 spectrometer. Fluorescence spectra were obtained on a Perkin-Elmer 512 spectrometer at room temperature. Protein concentrations were in the range of 10−50 μM. Cofactor fluorescence was excited at 334 nm, and emission slit widths were set to 2 nm.

Enzyme Assay. Enzyme activity in the direction of oligosaccharide degradation was assayed by measuring Glc-1-P release in the presence of varied concentrations of maltoheptaose and P_i in a coupled assay [50 mM Tris-Ac (pH 6.9), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM NAD^+, 10 μg/mL phosphoglucomutase, and 10 μg of glucose-6-phosphate dehydrogenase] at 30 °C (Schinzel & Palm, 1990). In the direction of oligosaccharide synthesis, the release of P_i from Glc-1-P was determined in 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 50 mM KCl, and 0.1−10 mM Glc-1-P, keeping maltotetraose at saturating concentrations (20 mM) by a modified assay from Saheki et al. (1985). Vice versa, the K_m of the primer molecule maltotetraose was determined in the presence of saturating concentrations of Glc-1-P (20 mM). The phosphate assay was adapted for microtiter plates (Drueckes et al., 1995) which allowed direct evaluation of rates via a computer-controlled microtiter plate reader (Dynatech). Initial velocities were calculated by nonlinear regression analysis (Leatherbarrow, 1992). Heat inactivation was determined by incubating 1−100 μg of mutant phosphorylase in 50 mM 2-(*N*-morpholino)ethanesulfonic acid and 50 mM KCl at pH 6.8 and 60 °C. At the indicated times, aliquots were removed and centrifuged briefly to remove precipitated protein, and the supernatant was assayed for activity as described above.

Inhibitor Studies. To determine inhibition constants, the release of Glc-1-P or P_i, respectively, was measured at different concentrations of each substrate while the concentration of the second substrate was saturating and vice versa in the presence of varying inhibitor concentrations. K_i values were calculated from the initial velocities by nonlinear regression. Because of the rapid hydrolysis of gluconolactone under the experimental conditions ($t_{1/2} \approx 30$ min), the inhibitor was dissolved immediately before use and incubation times in the presence of gluconolactone were restricted to 5 min. No corrections were made for gluconolactone hydrolysis. At higher concentrations (>1 mM), gluconolactone was found to inhibit phosphoglucomutase and glucose-6-phosphate dehydrogenase. For this reason, gluconolactone was used only in concentrations below 0.5 mM. In addition, the concentrations of the auxiliary enzymes of the coupled assay were doubled.

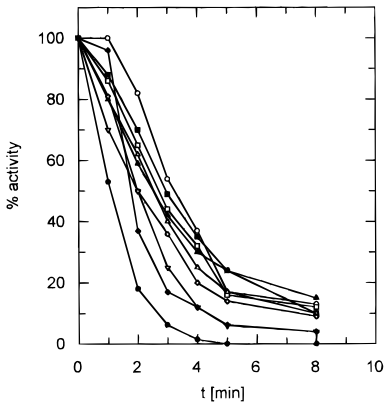

FIGURE 1: Heat inactivation kinetics of wild-type and mutant maltodextrin phosphorylases. The heat inactivation kinetics at 60 °C are shown for wild-type (○) and mutant maltodextrin phosphorylases: E67A, ●; N307A, □; D308A, ■; T346G, △; E350A, ▲; N258A/D259A/N260A, ▽; H310A, ◇; and Y578F, ◆. For details, see Materials and Methods.

RESULTS

Purification and Structural Properties of Native (Wild-Type) and Mutant Maltodextrin Phosphorylases. All phosphorylases, including the wild-type enzyme, were obtained by overexpression of the *mal*P gene carrying the appropriate mutations as described (Schinzel & Palm, 1990). The yields of mutant phosphorylase proteins in the crude cell extract were comparable to that of the wild-type construct. The molecular masses of all mutant phosphorylases were equal to that of the native enzyme, and the absorbance spectra and the OD_{280}/OD_{330} ratio as well as the fluorescence spectra were similar to those of the wild-type enzyme, implying that cofactor content and mode of cofactor binding were not strongly affected by the point mutations.

When tested for the time dependence of heat inactivation at 60 °C, all mutants with the exception of E67A and to a lesser extent D259A exhibited similar kinetics, suggesting that the mutations did not cause significant conformational changes. The E67A mutant enzyme was found to be more susceptible to heat inactivation, indicating that this residue might have a role in maintaining enzyme stability (Figure 1).

Wild-type maltodextrin phosphorylase shows a broad activity optimum centered at pH 6.5 with more than 80% activity remaining over the pH range from 5.5 to 7.0. The pH dependence of phosphorylase reaction appears not to be altered by the mutations despite the fact that, in seven out of ten mutants, protonatable side chains had been replaced by uncharged residues (data not shown).

Kinetic Analysis of Wild-Type and Mutant Maltodextrin Phosphorylases. Tables 2 and 3 summarize the steady state kinetic parameters of native and mutant enzymes in both directions. Among all residues selected for mutation by the criteria discussed in the introductory section, only one mutant enzyme, Y578F, showed no loss in enzymatic activity. In this case, even a small activation of about 10% was observed, although at the cost of a 3-fold-decreased apparent binding.

In the direction of phosphorolysis, the strongest effects on oligosaccharide binding were seen with E67A and H536L mutant enzymes where the affinities were reduced more than 100-fold for G7 and 5-fold for inorganic phosphate. Enzyme

Table 2: Kinetic Parameters of Wild-Type and Mutant Maltodextrin Phosphorylases in the Direction of Degradation[a]

mutant enzyme	$K_{m\ P_i}$ (mM)	$K_{m\ G7}$ (mM)	k_{cat} (s^{-1})
WT	0.46 ± 0.03	0.39 ± 0.03	24 ± 1
E67A	2.20 ± 0.21	37 ± 2	2.0 ± 0.1
D259A[b]	1.74 ± 0.04	2.68 ± 0.7	8.5 ± 0.7
N307A	1.98 ± 0.03	1.58 ± 0.22	6.7 ± 0.3
D308A	0.80 ± 0.04	0.38 ± 0.02	0.17 ± 0.01
D308S	0.7 ± 0.1	0.5 ± 0.1	2.0 ± 0.1
H310A	2.4 ± 0.2	5.3 ± 0.7	3.50 ± 0.05
T346G	2.0 ± 0.1	3.0 ± 0.1	0.14 ± 0.01
E350A	2.8 ± 0.2	3.9 ± 0.3	0.24 ± 0.01
H536L	2.0 ± 0.3	55 ± 3	0.34 ± 0.01
Y578F	1.60 ± 0.10	1.76 ± 0.26	27 ± 2

[a] Parameters were determined as described in Materials and Methods by using enzyme concentrations from 0.5 (wild-type enzyme) to 50 μg/mL. [b] Triple mutant enzyme N258A/D259A/N260A.

Table 3: Kinetic Parameters of Wild-Type and Mutant Maltodextrin Phosphorylases in the Direction of Synthesis[a]

mutant enzyme	$K_{m\ G1P}$ (mM)	$K_{m\ G4}$ (mM)	k_{cat} (s^{-1})
WT	1.0 ± 0.2	3.6 ± 0.6	46.0 ± 0.1
E67A	0.04 ± 0.01	>100	2.0 ± 0.1
D259A[b]	0.47 ± 0.08	28 ± 3	14.0 ± 0.6
N307A	0.20 ± 0.04	43 ± 4	22.6 ± 0.1
D308A	0.30 ± 0.02	4.2 ± 0.5	0.30 ± 0.01
D308S	0.5 ± 0.1	2.9 ± 0.4	4.1 ± 0.2
H310A	0.21 ± 0.04	5.7 ± 0.8	0.040 ± 0.001
T346G	0.10 ± 0.01	7.5 ± 1.1	0.030 ± 0.002
E350A	0.65 ± 0.05	60	1.7 ± 0.4
H536L	0.9 ± 0.1	>500	1.6 ± 0.1
Y578F	0.64 ± 0.03	12.3 ± 1.5	49 ± 3

[a] See footnote to Table 2. [b] Triple mutant enzyme N258A/D259A/N260A.

activity was also reduced by a factor of 10 or 60, respectively (Table 2). Although determination of exact K_m values was not possible due to the limited solubility of the oligosaccharide substrate, these mutants also showed significantly reduced affinities for maltotetraose in the synthesis reaction. The loss of primer binding seemed comparable to that of the polysaccharide in the degradation reaction. The apparent K_m for Glc-1-P was unaffected in the case of the H536L mutant enzyme but was significantly lower for the E67A mutant enzyme (Table 3).

Oligosaccharide binding was found to be less affected for the N307A mutant enzyme and for the triple mutant enzyme N258A/D259A/N260A, for which approximately 4- and 10-fold-increased K_m values for oligosaccharides were determined in both directions of the reaction (Tables 2 and 3). For the N307A mutant enzyme, apparent binding in the direction of synthesis seemed to be slightly more affected than binding in the phosphorolysis mode (Figure 2). Apparent binding of inorganic phosphate was weakened to the same extent, while binding of Glc-1-P in the synthesis direction was slightly increased. Enzyme activity was only moderately lowered by a factor of 2−4 in both cases.

In the direction of degradation, the H310A mutant enzyme displayed an about 10-fold lower apparent affinity for oligosaccharides. However, in the direction of synthesis, apparent affinity for G4 remained essentially unchanged accompanied by a reciprocal effect on Glc-1-P affinity (Table 3, Figure 2). Furthermore, this mutant enzyme exhibits some unique properties when compared to wild-type enzyme or other mutant enzymes. The first to mention is a significantly

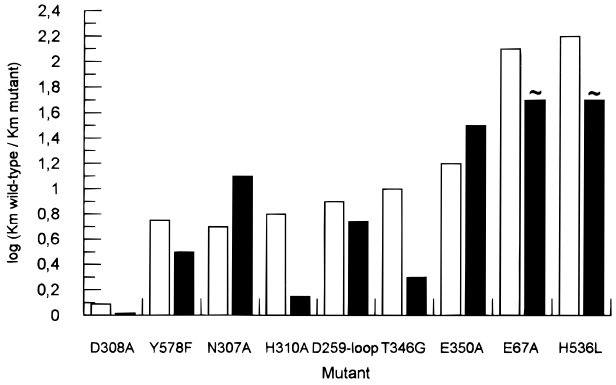

FIGURE 2: Effect of mutations on K_m values of oligosaccharides relative to the K_m of the wild-type maltodextrin phosphorylase. In synthesis, the determination of apparent K_m values of the H536L and E67A mutant enzymes was limited by the solubility of the oligosaccharides; therefore, the values represent lower limits: degradation reaction (G7), open bars; and synthesis reaction (G4), solid bars. G4 = maltotetraose; G7 = maltoheptaose.

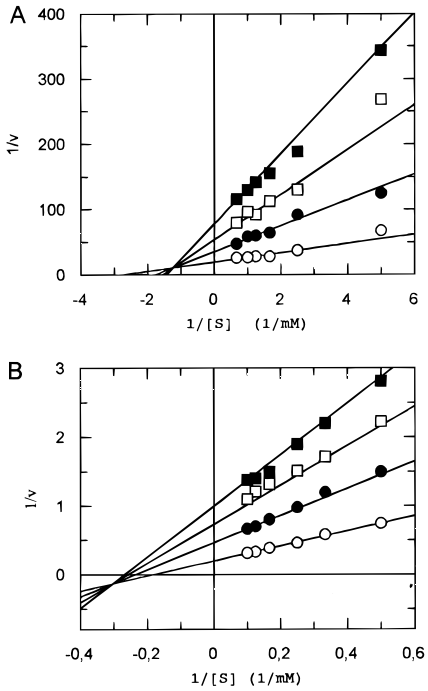

FIGURE 3: Inhibition of H310A mutant maltodextrin phosphorylase by gluconolactone. (A) Inhibition with respect to Glc-1-P at saturating maltotetraose (25 mM) without (○) or in the presence of 15 μM (●), 20 μM (□), and 30 μM (■) gluconolactone. (B) Inhibition with respect to maltoheptaose at saturating P_i (20 mM) without (○) or in the presence of 1 μM (●), 2 μM (□), and 3 μM (■) gluconolactone.

lower enzyme activity in synthesis than in degradation (Tables 2 and 3). Even more interesting are the consequences of this substitution on the inhibitory properties of gluconolactone which is one of most potent inhibitors of the phosphorylase reaction. As with wild-type, maltodextrin phosphorylase and phosphorylase *b* gluconolactone (Gold et al., 1971; Tu et al., 1971) show a noncompetitive inhibition pattern with maltoheptaose and Glc-1-P (Figure 3, Table 4). Due to the relatively low activity in the synthesis reaction and the relatively high concentrations of gluconolactone required, an exact determination of inhibition against maltotetraose as primer was not possible. However, this inhibition seemed to be in the same range as observed for the wild-type enzyme. In contrast, the apparent K_i with

Table 4: K_i Values of Different Inhibitors for Wild-Type Enzyme and the H310A Mutant Maltodextrinphosphorylase

inhibitor	Glc-1-MP[a] K_i (mM)	gluconolactone K_i (mM)	glucose K_i (mM)
wild-type synthesis			
Glc-1-P	0.20 ± 0.04 (c)[d]	0.90 ± 0.17 (nc)	nd[b]
wild-type degradation			
G7	0.26 ± 0.03 (nc)	0.094 ± 0.011 (nc)	nd[b]
P_i	0.36 ± 0.03 (nc)	0.052 ± 0.030 (nc)	nd[b]
H310A synthesis			
Glc-1-P	0.12 ± 0.02 (c)	$(12.2 \pm 0.9) \times 10^{-3}$ (nc)	>50[c]
H310A degradation			
G7	0.12 ± 0.01 (nc)	$(0.92 \pm 0.03) \times 10^{-3}$ (nc)	24.9 ± 0.9 (nc)
P_i	0.22 ± 0.03 (nc)	$(1.32 \pm 0.11) \times 10^{-3}$ (nc)	18.4 ± 0.9 (nc)

[a] α-D-Glucose-1-methylenephosphonate. [b] No inhibition was observed in the presence of concentrations up to 0.5 M glucose. [c] Estimated. [d] (c) competitive inhibition pattern and (nc) noncompetitive inhibition pattern.

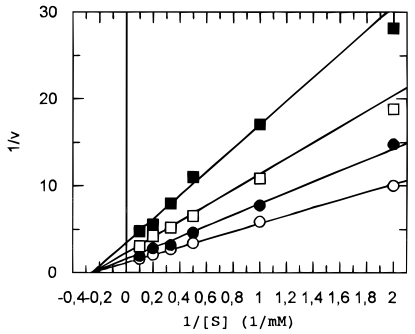

FIGURE 4: Inhibition of H310A mutant maltodextrin phosphorylase by glucose. Inhibition with respect to maltoheptaose at saturating P_i (20 mM) without (○) or in the presence of 15 mM (●), 30 mM (□), and 50 mM (■) glucose.

respect to Glc-1-P and maltoheptaose substrates decreased considerably by a factor of 50−100. The unusual property of the inhibitor gluconolactone to bind differently to the two ternary complexes (Gold et al., 1971) was at least partially retained since a 10-fold lower apparent K_i was observed with respect to maltoheptaose than with respect to Glc-1-P (Table 4). Interestingly, glucose, which seems not to inhibit wild-type maltodextrin phosphorylase at concentrations up to 0.5 M glucose, was found to be an inhibitor of the H310A mutant enzyme with respect to the oligosaccharide substrate with an apparent inhibition constant of 20 mM (Figure 4, Table 4). In this regard and in the noncompetitive inhibition pattern, glucose behaved like gluconolactone. In synthesis (with respect to Glc-1-P), only a weak inhibition was observed, not allowing a precise determination of inhibition constants and pattern. However, an inhibition constant of about 70−150 mM was estimated. In contrast to that of gluconolactone, the inhibition constant of another effective phosphorylase inhibitor, α-D-glucose 1-methylenephosphonate, which is a competitive inhibitor with respect to Glc-1-P, was not changed for the H310A mutant enzyme (Table 4).

As with the H310A mutant enzyme, apparent binding of the oligosaccharide substrate was reduced 10-fold in the direction of degradation for the T346G mutant enzyme while the K_m value for maltotetraose in the synthesis reaction appeared to be unchanged (Figure 2). Furthermore, enzyme activity was also considerably more reduced in the synthesis (about 1500-fold) than in the degradation (200-fold) direction (Tables 2 and 3).

Mutation of D308 to serine or alanine did not change substrate affinities significantly, but k_{cat} was affected by a factor of 10 and 150, respectively. In fact, D308 was the only amino acid residue where substitution by alanine was without consequences for ground state binding but with distinct effects on k_{cat} (Tables 2 and 3). Although unlikely at the residual activities measured for this mutant enzyme, a wild-type contamination cannot be totally excluded since all K_m values remained unchanged in the mutant enzyme. Therefore, an alternate mutation was introduced at this position to create D308S. This mutant enzyme exhibited an at least 10- or 60-fold higher activity when compared to that of the D308A mutant enzyme, while the K_m values remained unchanged, too (Tables 2 and 3). At the activities measured for this mutant enzyme, a contamination by wild-type enzyme is most unlikely.

By analogy to the phosphorylase *b* structure, the histidine 345 residue was also considered to be involved in carbohydrate binding. Unfortunately, the H345A mutant was not expressed to an extent that any intact protein could be isolated from *E. coli* cells harboring the appropriate vector with the mutagenized gene.

A comparison of the extent to which apparent binding of oligosaccharides was affected in the synthesis and phosphorolysis direction shows that some mutant enzymes were affected predominantly in one direction, while for other mutant enzymes, the effects were shared in both directions. In H310 and T346, the apparent affinities of the oligosaccharide substrate were clearly more affected only in the phosphorolysis reaction, while for the N307A mutant enzyme, the effect on affinity seemed to be 2-fold higher in the synthesis reaction (Figure 2).

Binding at Subsite Five. Glucose residues from the reducing end of oligosaccharide chains longer than five glucose residues contribute little to the binding energy of the oligosaccharide substrate in maltodextrin phosphorylase in the degradation reaction (R. Schinzel et al., in press). Correspondingly, an oligosaccharide chain with four residues together with Glc-1-P saturates the binding requirements in the synthesis mode. Polysugars longer than maltopentaose might be expected to cause different effects on binding kinetics only if additional glucose binding sites would be affected by mutations. To test this, the relative affinities of oligosaccharides longer than four residues in the synthesis reaction or five residues in the degradation reaction were compared for all mutants described here with essentially no effects (data not shown). None of the mutants showed effects with regard to oligosaccharides exceeding four or five residues.

As observed for the wild-type enzyme in the degradation reaction, glucose (with the exception of the H310A mutant

Table 5: Free Energy of Binding Contributed by Subsite 5 in Wild-Type and Mutant Maltodextrin Phosphorylases in the Direction of Oligosaccharide Degradation

	$(k_{cat}/K_m)_{G5}$[a] (mM^{-1} s^{-1})	$(k_{cat}/K_m)_{G4}$[a] (mM^{-1} s^{-1})	$\Delta\Delta G$[b] (kJ/mol)
WT	58 ± 4	0.034 ± 0.004	17.6 ± 1
H536L	0.007 ± 0.002	$(3.8 \pm 0.40) \times 10^{-5}$	10.8 ± 1.6
E67A	0.041 ± 0.004	$(2.45 \pm 0.18) \times 10^{-5}$	16.4 ± 2.0
E350A	0.054 ± 0.005	$(3.5 \pm 0.3) \times 10^{-4}$	11.3 ± 0.5
Y578F	3.7 ± 0.5	0.0053 ± 0.0006	16.2 ± 2.3

[a] Determined directly at $[S] = K_m/10$. [b] Free energy of binding of subsite 5 calculated using $\Delta\Delta G_n = -RT \ln[(k_{cat}/K_m)_{G4}/(k_{cat}/K_m)_{G5}]$ according to Hiromi (1970).

enzyme), maltose, and maltotriose are neither substrates nor inhibitors of the phosphorolysis reaction. In the synthesis mode, the same is true for glucose and maltose which cannot act as primer molecules. However, maltotetraose can be degraded by wild-type enzyme, and maltotriose can act as a primer molecule although at a drastically reduced rate. The low but measurable activities allowed estimation of the free energy of binding contributed by subsite five according to Hiromi (1970). The contribution of subsite five in the E350A and H537A mutant enzymes is reduced significantly (Table 5). The free energy of binding provided by this subsite drops from 18 to about 10 kJ/mol for the H536L mutant enzyme. A similar value was observed for the E350A mutant enzyme, while the contribution of this subsite in the E67A mutant enzyme amounts to about 16 kJ/mol.

DISCUSSION

The existence of distinct glucose binding subsites in the substrate recognition site of phosphorylases was inferred indirectly from the presumed correspondence with the action pattern of small maltooligosaccharides serving as substrates of the reversible phosphorolysis and synthesis of linear polysaccharide chains. Giri and French (1970) suggested that chains of five glucose units (maltopentaose) or chains of four glucose units (maltotetraose) together with Glc-1-P constitute the minimum size of linear oligosaccharides serving as limiting substrates; thus, a model based on the occupancy of five glucose binding subsites was hypothesized.

For lack of appropriate crystallizable enzyme substrate complexes, the location and stereochemistry of glucose subsites could not be solved definitively by X-ray crystallography (Sprang et al., 1991; Johnson, 1992). However, from structures of the phosphorylase *b* complexed with the inhibitor D-gluconohydroximo-1,5-lactone *N*-phenylurethane, a channel could be predicted within which the sugar might bind (Barford et al., 1988). A number of selected amino acid residues located within this channel were functionally probed for their involvement in sugar binding by a site-directed mutagenesis approach.

All mutagenized residues listed in Table 2 and 3 indeed appeared to be involved in polysaccharide binding and reactivity. The effects caused by exchange of individual amino acids differed not only in the extent to which binding or the reactivity was affected but also for their effects on either the degradation or the synthesis reaction.

In cases where mutagenesis caused only slight, less than 10-fold, increases of the K_m values, loss of an uncharged hydrogen bond might account for the change (Fersht et al., 1985; Street et al., 1986). Actually, mutant enzymes which displayed just small changes in affinity are mutations of amino acids with hydrophilic but uncharged side chains like N307A and Y578F. Likewise, small changes observed for a triple mutant containing the corresponding amino acids of the "D283 loop" in phosphorylase *b*, N258A, D259A, and N260A, appear to testify that one of the uncharged residues located in that loop is involved in binding.

Loss as in apparent binding of the size considered equivalent to the loss of a charged hydrogen bond was found with the E67A and the H536L mutant enzymes. The residue corresponding to E67 in rabbit muscle phosphorylase *b*, E88, is located in the core of the enzyme near the active site (Archarya et al., 1991). In the case of the H536L mutant enzyme, the strong and simultaneous effects on the relevant kinetic parameters imply a role of H536 in catalysis through binding of the oligosaccharide substrate both in the ground state and in the transition state. In rabbit muscle phosphorylase *b*, the corresponding H571 is located too far from the active site to become directly involved in oligosaccharide cleavage. Since in the H536L mutant enzyme subsite five contributes only about 10 kJ/mol to binding energy when compared to about 18 kJ/mol for the wild-type enzyme, a participation of H536 in binding of glucose residues at subsite five is most likely.

Besides mutations of amino acid residues preferentially affecting binding in the ground state, one amino acid, D308, has been identified which seems to be primarily involved in transition state binding. Mutagenesis of D308 to either alanine or serine did not influence wild-type apparent binding of the oligosaccharide while enzyme activity was lowered considerably. The more than 10-fold higher activity of D308S mutant enzyme when compared with that of the D308A mutant enzyme implies that the hydroxyl group of the serine side chain can partially substitute the carboxyl group of the aspartate residue in transition state binding.

In three other mutations, H310A, T346G, and E350A, losses in enzyme activities were accompanied by significant changes in apparent affinities (K_m values). The difference in free energy of binding of about 15 kJ/mol is consistent with contacts of E350 to one of the sugar residues by a charged hydrogen bond. Addition of acetate ions caused a partial restoration of the enzyme activity. This suggests that a negative charge at this position is indispensable for a fully active enzyme (P. Drueckes and R. Schinzel, manuscript in preparation). In rabbit muscle phosphorylase *b*, the corresponding residue, E382, is located on the surface of the enzyme at the edge of the active site channel. As indicated by lowered free energy of binding (Table 5), E350 most likely makes contacts to subsite five and the oligosaccharide may bend around the edge of the active site. Amino acid residues near E350 may be crucial for discrimination between binding of short linear oligosaccharides and branched ones like glycogen. This will be studied by introduction of charged amino acids in the vicinity of E350.

For the T346G mutant enzyme, the differences in free energy are even higher than one would expect from the loss of an uncharged hydrogen bond. One explanation could be that substitution of Thr by Gly changes the geometry of other residues relative to the sugar substrate. Since in this mutant enzyme apparent binding of the primer in the synthesis reaction was affected while binding of the oligosaccharide in the degradation reaction was not, this residue appeared to

interact predominantly with the primer molecule and not the oligosaccharide awaiting degradation.

Moderate changes of k_{cat} and a 10-fold loss in apparent binding of the oligosaccharide to the H310A mutant enzyme suggest that H310 is involved in ground state binding of the oligosaccharide substrate in the degradation reaction. The effect on catalytic performance in both directions of the reaction further indicates that binding of the transition state complexes might be affected as well. One of the most remarkable properties of this mutant enzyme, however, is the considerably lowered inhibition constant of gluconolactone. Although not showing an inhibition constant expected for transition state analogues, gluconolactone was considered to fulfill at least partially the requirements for a transition state analogue of the phosphorylase reaction (Tu et al., 1971; Gold et al., 1971). In rabbit muscle phosphorylase as well as in maltodextrin phosphorylase, gluconolactone binds 70−140-fold better to the enzyme/oligosaccharide/phosphate complex ($K_i = 0.025$ mM) than to the enzyme/oligosaccharide/Glc-1-P central complex ($K_i = 3.4$ mM) (Tu et al., 1971; Gold et al., 1971; R. Schinzel, unpublished results). As discussed by these authors, gluconolactone may bind to the area normally occupied by the glucosyl residue that is transferred between oligosaccharide and phosphate. The differences in binding of the central complex were discussed in terms of a steric hindrance of binding of gluconolactone to the enzyme/oligosaccharide/Glc-1-P central complex. The enhanced binding of the inhibitor by the mutant enzyme can be explained by the assumption that steric constraints are relieved, resulting in a more stable enzyme/substrate/inhibitor complex. The conception that substrate and the inhibitor binding site might overlap partially is further confirmed by the finding that glucose, which does not inhibit the wild-type enzyme, was found to function as a noncompetitive inhibitor of the H310A mutant enzyme with respect to Glc-1-P and G7. Therefore, the enlarged gluconolactone binding site of the H310A mutant enzyme appears to be capable of accommodating a glucose molecule.

Mutations that weakened carbohydrate binding also affected binding of inorganic phosphate. This corroborates earlier kinetic studies of rabbit muscle phosphorylase showing that binding of inorganic phosphate, polysaccharide, or Glc-1-P might be stronger with the enzyme binary complex than with the free enzyme (Engers et al., 1970; Graves & Wang, 1972). The observation that phosphate affinity was decreased when oligosaccharide binding was weakend by a mutation supports the assumption that both substrates do not bind independently in the phosphorolysis mode. Since in phosphorylase *b* none of the amino acid side chains corresponding to the mutated residues is within hydrogen bond distance of the phosphate binding site, the effects are most likely exerted by the oligosaccharide itself. Interestingly, none of the mutations weaken apparent binding of Glc-1-P. Thus, in the synthesis mode, primer binding appears not to influence affinity of the second substrate, indicating that in this case the two substrates appear to bind independently.

In conclusion, the observation that some mutations affect binding of the oligosaccharide in a different way in oligosaccharide degradation and synthesis, the opposite effects of mutations on binding of the second substrates whether P_i or Glc-1-P, and the differential effects of mutation of H310 on activity in either direction of the reaction give further support to the hypothesis that the oligosaccharide might bind in different modes in synthesis and degradation as predicted from the differential effects of 2-deoxyglucosyl analogues of the substrates in the direction of synthesis or degradation (Becker et al., 1994). This would require a conformational transition occurring during catalysis. Such a rearrangement would explain the difficulties in X-ray crystallography of ternary enzyme/substrate complexes to get an electron density of oligosaccharides bound to the active site of phosphorylases. However, since not all mutations cause such differential effects, the binding sites should overlap for the most part for the synthesis and degradation mode. Most likely, only one or two subsites are rearranged in the course of the catalytic reaction.

Table 6: Possible Phosphorylase *b*−Maltopentaose Interactions[a]

glucose residue	hydroxyl group	amino acid residue	*E. coli* numbering
5	O2, O3	Glu 382	350[b]
5	O6	His 571	536[b]
5	O6	Arg 770	*b,d*
4	O2	Glu 382	350
4	O3	Ala 383	351[c]
3	O6	Asp 339	308
3	O3	His 341	310
3	O2	His 341	310
3	O2	Ala 383	351[c]
2	O6	Glu 088	67
2	O3	Asp 339	308
2	O3	His 377	345
2	O3	Thr 378	346
2	O2	Leu 136	114[c]
2	O2	Asp 339	308
2	O2	His 377	345
2	O2	Thr 378	348

[a] This compilation is derived from a model in which a maltopentaose molecule was fitted into the phosphorylase *b* structure (kindly provided by L. N. Johnson, Oxford). Possible side chain contacts in a distance of less than 4 Å to the hydroxyl groups of the sugar are shown. Interactions of glucose residue 1 (G1) are not listed. [b] At a distance of <6 Å, no contacts to other amino acids were found within this range. [c] Contacts to the backbone. [d] The corresponding residue in *E. coli* is not an arginine but a proline; mutagenesis of this residue has no influence on the *E. coli* enzyme (P. Drueckes, unpublished results).

Model of the Oligosaccharide Binding Site. To establish a structure−function relationship of oligosaccharide binding to the active site in phosphorylases, the results from the mutagenesis studies were compared with the proposed stereochemistry of a maltopentaose molecule modeled into the phosphorylase *b* structure by L. N. Johnson (personal communication). A reasonable fitting was obtained if one allows for a distortion of the oligosaccharide molecule between the second and third glucose unit. To this end, the positions of the amino acid residues that correspond to the mutated residues in the bacterial enzyme relative to the maltopentaose molecule were examined. Indeed, with only few exceptions, the individual glucose residues of the model appear to be in a position to make contacts to amino acids that were found to interact with the oligosaccharide by the mutagenesis approach (Table 6, Figure 5). Within the limits of the theoretical model, the satisfactory agreement between the model and the mutagenesis experiments supports the assumption that the polysaccharide might be bound in a distorted form.

In this model, none of the amino acid side chains appears to make direct contact with glucose residue five (Table 6). However, H571 (corresponding to H536 in maltodextrin

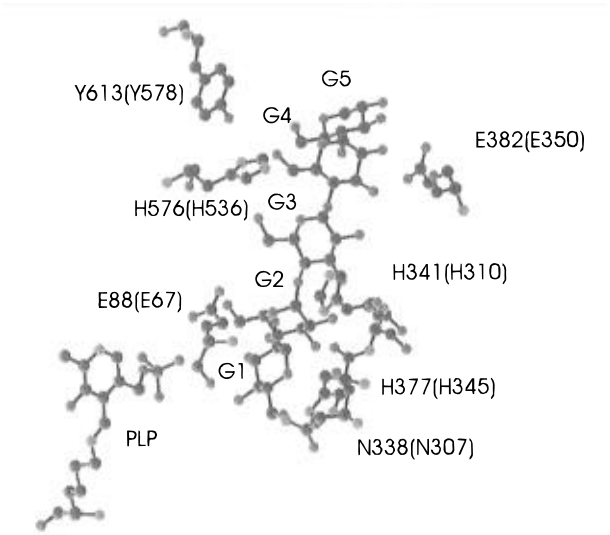

FIGURE 5: Position of amino acid side chains (phosphorylase *b* numbering, *E. coli* numbering in parentheses) found to be involved in oligosaccharide binding in maltodextrin phosphorylase to a maltopentaose molecule fitted into the structure of phosphorylase *b* (L. Johnson, personal communication). See Table 6 for listing of the corresponding maltodextrin phosphorylase residues.

phosphorylase) and E382 (E350) are the residues next to this subsite. Y613 (Y578) seems even more distant from subsite five of the sugar model, but it is within hydrogen bond distance of H571. Hence, the participation of Y613 with substrate binding is most likely indirect or mediated by other side chains, most likely H571. Only the side chain of E382 (E350) seems to be in the vicinity of glucose residue four. Comparable to glucose interactions at subsite four, only two suitable amino acid side chains are located near the glucose residue at subsite three. The positions of D339 and H341 in rabbit muscle phosphorylase are taken up by D308 and H310 in maltodextrin phosphorylase, respectively. However, the glucose residue at subsite two appears to make a multitude of contacts with residues previously considered for their involvement in oligosaccharide binding. This observation, together with the predicted distortion of glucose residue two, suggests that binding of the oligosaccharide molecule at subsite two might be critical for the activity of maltodextrin phosphorylase.

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