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Binding of Glycogen, Oligosaccharides, and Glucose to Glycogen Debranching Enzyme†

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ABSTRACT: The binding of glucose and a series of oligosaccharides to glycogen debranching enzyme was determined by the ability of the saccharides to decrease the rate of reaction of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoate) (DTNB). At pH 7.2, the strength of binding increases with chain length from glucose to maltotriose to maltopentaose but not to maltohexaose, and the free energies for binding of the oligosaccharides suggest subsites of equivalent affinities for the four glucose units following the initial reducing moiety. The rate of reaction of DTNB with enzyme saturated with saccharide is the same for all compounds, suggesting that all the saccharides, including gluose, induce the same conformational state. The site of binding may be that which binds the α-1,6-linked side chain of the natural limit dextrin substrate. At pH 8.0, this site exhibits similar characteristics, but an additional site, which may bind the four terminal glucose units of the main chain of the natural substrate, is manifested and exhibits different characteristics, including a very low affinity for glucose itself. The binding of glycogen to the debranching enzyme was monitored by centrifugal separation from the protein and exhibits a much lower dissociation constant than that for the oligomers, suggesting that branched polymers have more than one set of subsites.

Amylo-1,6-glucosidase/4-α-glucanotransferase (glycogen debranching enzyme) (EC 3.2.1.33 + EC 2.4.1.25) is a monomeric enzyme of about 165-kilodalton (kDa)[1] molecular mass that encompasses both glucosidase and transferase activities on a single polypeptide chain (Brown & Brown, 1966; Nelson et al., 1979). Following extensive studies with reversible inhibitors and a catalytic site directed irreversible inhibitor, Nelson and colleagues (Nelson et al., 1979; Gillard & Nelson, 1977; Gillard et al., 1980) envisioned this enzyme as possessing a single, overlapping or strongly interacting polymer binding site(s) flanked on one side by a glucosidase site and on the other by the transferase site.

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[1] Abbreviations: α-GSD, α-glucosyl α-Schardinger dextrin; φ-LD, glycogen limit dextrin from the action of phosphorylase on glycogen; DTNB, 5,5′-dithiobis(2-nitrobenzoate); G, glucose; MT, maltotriose; MP, maltopentaose; MH, maltohexaose; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; Nojirimycin, 5-amino-5-deoxy-D-glucopyranose; kDa, kilodalton(s).

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The debranching enzyme shows considerable specificity to α-1,4-glycans which contain α-1,6 branch points. The best substrate for the combined activity of the rabbit skeletal enzyme is glycogen limit dextrin, and the enzyme acts at a much slower rate toward native glycogen and amylopectin (Lee & Whelan, 1971). The glucosidase can remove glucose stubs from 6^3-α-glucosylmaltotetraose (fast B_5) (Brown & Brown, 1966) and from α-1,6-glucosylcyclohexaamylose (α-glucosyl α-Schardinger dextrin) (Taylor & Whelan, 1966). The glucosidase can also catalyze incorporation of glucose into polysaccharides such as glycogen (Hers et al., 1967; Nelson & Larner, 1970). Glucose and the series maltose to maltopentaose were observed to be acceptors of glucosyl segments transferred from glycogen but were not altered when incubated alone with the enzyme. Maltohexaose and higher oligosaccharides were found to be both donors and acceptors of maltosyl and maltotriosyl units (Lee & Whelan, 1971). Amylopectin, glycogen, and limit dextrin are the major substrates for the transferase activity (Nelson et al., 1970).

The transfer reaction of the transferase proceeds with no change in free energy since both the linkage cleaved and the linkage formed are α-1,4; the glucosidase reaction, however, is accompanied by a free energy change of approximately 3–4 kcal (Nelson et al., 1979). Activation energies of 19, 13, and 9 kcal were determined for the combined activity on φ-LD, glucosidase measured by glucose incorporation, and transferase activity on amylopectin, respectively (Nelson & Watts, 1974). These authors suggested that the unusually high activation energy for the combined activity was associated with a conformational change in the enzyme during catalysis.

The debranching enzyme bears a strong resemblance to other glucosidases. Glucosidases are strongly inhibited by glycosylamines and are generally subject to conformational changes on substrate binding (Lai & Axelrod, 1973; Lalêger et al., 1982). For example, Nojirimycin is a potent inhibitor of α-and β-glucosidases (Niwa et al., 1969; Reese et al., 1971) and the debranching enzyme (Gillard & Nelson, 1977). Glucosidase activity, including that of the debranching enzyme, has been found to be susceptible to competitive inhibition by Tris (Gillard & Nelson, 1977; Larner & Gillespie, 1956; Halvorson & Elliw, 1958; Jørgensen & Jørgesen, 1967; Semenza & Von Balthazar, 1974). Studies of the effect of *p*-(chloromercuri)benzoate, DTNB, or a water-soluble alkylcarbodiimide on the debranching enzyme have implicated a thiol group and a carboxylate group at the catalytic site (Gillard et al., 1980; Nelson et al., 1974).

This laboratory announced recently the crystallization of the debranching enzyme in a form suitable for a structure determination by X-ray diffraction methods (Osterlund et al., 1984), and this study is now in progress. The orthorhombic crystals (space group $P2_12_12_1$) were grown from poly(ethylene glycol) solutions containing 10–15 mM oligosaccharides of chain length three to seven glucose units. The enzyme could be crystallized in a cubic form in 0.7 M ammonium sulfate in the absence of saccharide, but in this case, the addition of oligosaccharide caused the crystals to dissolve. These effects of oligosaccharide on crystal growth and morphology are consistent with suggestions that the enzyme may undergo conformational changes when sugars bind.

A later study, designed to improve the limit of diffraction and useful X-ray lifetime of the crystals by altering their growth conditions, found that raising the concentration of maltotriose to 60 mM resulted in improving the limit of diffraction to beyond 3.0 Å (Fitzgerald & Madsen, 1986). Since no information was available on the dissociation constants for the binding of oligosaccharides, it is possible that this finding was simply the result of saturating a site that has a relatively weak affinity. Similarly, although K_m values are available for a number of substrates such as glucose and limit dextrin, only the dissociation constant for glycogen has been determined (in the form of an inhibition constant) (Gillard & Nelson, 1977). Knowledge of the effect of chain length on the binding of oligosaccharides to lysozyme was very useful for mapping the thermodynamic aspects of the binding to each subsite in that enzyme and also contributed to the development of a mechanism of action (Imoto et al., 1972). Such information will be necessary for making proper use of the X-ray diffraction-derived crystal structure of the debranching enzyme when it becomes available and prompted the research which is reported in this paper.

Materials and Methods

The glycogen debranching enzyme was purified from rabbit muscle by extending the method of Taylor et al. (1975) so that an (aminobutyl)agarose column (Sigma) was used first at 21 °C, so that glycogen synthase and phosphorylase kinase both bind, while phosphorylase and the debranching enzyme do not. This was followed by passing the eluate from this step through an (aminobutyl)agarose column at 4 °C so that the phosphorylase binds weakly and the debranching enzyme must be eluted with a salt gradient. The final step was column chromatography on DE-52 (Pharmacia). As discussed here, and by Taylor et al. (1975), these procedures efficiently remove contaminating muscle proteins. As was observed by these authors, the enzyme was homogeneous by the criterion of yielding a single band on SDS–polyacrylamide gel electrophoresis. Electron micrographs also showed a single protein species. The preparations were also used for amino acid sequencing from large protease-derived peptides and gave no indication of inhomogeneity. The specific activity was 11 ± 0.5 μmol min^{-1} mg^{-1} when tested by the combined activity assay of Gillard and Nelson (1977) compared to the values of 8.8 or 8.3 μmol min^{-1} mg^{-1} obtained for preparations using two other methods by Watts and Nelson (1977) or White et al. (1981), respectively. D-Glucose was a Fischer product. DTNB (lot 35F-3653) and maltotriose (95%, lot 115F-0041) were obtained from Sigma. Maltopentaose (91%), lot 10471825-36) and maltohexaose (91%, lots 120210, 10674321-05, and 10719820-06) were products from Boehringer Mannheim. All these chemicals were used without further purification. Protein solutions were dialyzed with Spectrapor membranes (Spectrum Medical Industries). Concentrations of saccharide solutions were calculated on the basis of purities provided by the manufacturers and confirmed by chemical analysis for glucose content.

Absorbances were measured on a Beckman Model DU8 spectrophotometer using a kinetic module assessory which enabled absorbance readings to be recorded continuously at 15-, 30-, or 60-s intervals.

Protein Concentration. Protein concentration was determined at 280 nm using $E^{1\%}_{1cm}$ = 17.5 (Taylor et al., 1975) after exhaustive dialysis for 3 days with several changes of buffer to remove DTT and EDTA from the 25 mM glycylglycine at pH 7.2. The molecular mass of the debranching enzyme was taken to be 165 kDa.

Sulfhydryl Modifications. The following stock solutions were used: 0.1 M K_2HPO_4 buffer, pH 7.2 and 8.0; 37.5 mM EDTA, pH 7.2; and 10 mM DTNB in 0.1 M K_2HPO_4 buffer, pH 6.8. The DTNB solution was prepared freshly for each set of experiments. No increase in absorbance occurred during a set of experiments. Stock solutions of saccharides were

prepared in 0.1 M phosphate buffer at the appropriate pH.

The reaction mixtures contained final concentrations of 1.25 mM EDTA, various concentrations of saccharide, 0.1 M phosphate buffer, and 0.25, 0.5, or 1 μM debranching enzyme in a total volume of 2.95 mL. The reaction was initiated by addition of 50 μL of the DTNB solution after about 5-min preincubation at the controlled temperature of 25 °C. A reagent blank consisted of 2.95 mL of phosphate buffer with 1.25 mM EDTA and 50 μL of DTNB solution. At the concentrations of debrancher used, a sample blank reaction was negligible against a reagent blank. Absorbance readings were made continuously at 412 nm with the reagent blank in the reference cell position. At the concentrations used, 0.045–0.165 mg/mL, the debranching enzyme does not form aggregates which might interfere with the reaction (Gillard et al., 1980).

Total Sulfhydryl Content. Total sulfhydryl content was determined in the presence of 8 M urea or 2% SDS in 0.1 M BES buffer, pH 8.0, after 3 min of reaction. Due to the rapid rate of oxidation of sulfhydryl compounds, especially in the absence of EDTA (Janatova et al., 1968; Gething & Davidson, 1970), EDTA was always included in our reaction mixtures. DTNB is a standard reagent for the specific and sensitive estimation of sulfhydryl groups in native and denatured proteins by absorption measurement at 412 nm (Ellman, 1959; Riddles et al., 1983). The molar absorption coefficient of the released mercaptonitrobenzoate has been determined to range from 11 400 to 14 150 M^{-1} cm^{-1} at pH 8 (Janatova et al., 1968; Riddles et al., 1979). The value of 13 600 M^{-1} cm^{-1} (Ellman, 1959), however, is generally used (Silverstein, 1975; Glazer et al., 1975). Riddles et al. (1979) have also shown that thiol groups can be titrated with DTNB at pH 7.27 where 99.8% mercaptonitrobenzoate ions are present. BES was not employed in experiments with native enzyme because it might bind and interfere (Gillard & Nelson, 1977).

Centrifugal Separation Binding Analysis.[2] Studies of glycogen and glycogen/maltotriose binding to the debranching enzyme were conducted in an RC-5 Superspeed refrigerated centrifuge (Dupont Instruments, Sorval) by the method of Madsen and Cori (1958). The 1-mL mixtures contained purified enzyme and high molecular weight glycogen/maltotriose mixtures. The concentration of debranching enzyme was kept at 1 mg/mL, and the concentration of glycogen varied from 0.25 to 1.0 mg/mL in 25 mM glycylglycine buffer, pH 7.2. For competitive studies, the maltotriose concentration was kept at 25 mg/mL. All linear relationships were fitted by least-squares analysis. The high molecular weight glycogen was prepared from rabbit liver by the method of Bueding and Orrel (1964). Centrifugation was performed at 18 000 rpm at 20 °C for 20 min (maximum of 39000g). Since no protein sedimented in the absence of glycogen, the unbound protein in the supernatant was measured and subtracted from the total protein to yield the bound protein.

Results and Discussion

Total Sulfhydryl Content. Measurements of initial rates indicated that production of mercaptonitrobenzoate was proportional to the concentration of debranching enzyme up to 3 μM. To determine the total thiol content of the enzyme, enzyme concentrations were varied between 0.25 and 1.0 μM. The reaction in either urea or SDS was complete within 5 min (Figure 1). Five independent determinations in each perturbant yielded mean values of 42 ± 2 and 43.8 ± 0.5 –SH

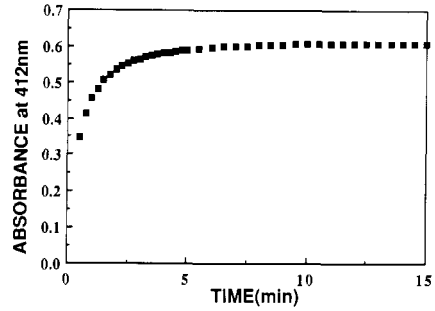

FIGURE 1: Production of mercaptonitrobenzoate from DTNB reaction with the cysteine residues of the glycogen debranching enzyme as a function of time. The reaction was studied in the presence of 2% SDS in 0.1 M BES buffer at pH 8.0 and 25 °C. The concentration of DTNB was 167 μM.

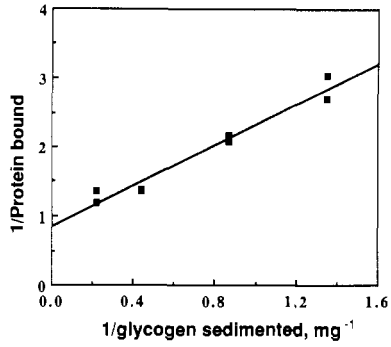

FIGURE 2: Centrifugal separation analysis for the binding of debranching enzyme and glycogen. Data are plotted as a double reciprocal of milligrams of protein bound to glycogen versus milligrams of glycogen sedimented. Studies were conducted in 25 mM glycylglycine buffer containing 1 mM EDTA and 1 mM DTT in 1-mL volumes, and centrifugation was at 18 000 rpm at 20 °C for 20 min.

in urea and SDS, respectively. This agrees with the value of 43 obtained after formic acid oxidation or the 42 obtained in SDS (Taylor et al., 1975).

Glycogen Binding by Centrifugal Separation. The binding studies with glycogen in the presence and absence of maltotriose by sedimentation centrifugation analysis were conducted because attempts made using the DTNB modification method were unsuccessful due to turbidity of reaction mixtures. No sedimentation of debranching enzyme occurred in the absence of glycogen. From a plot of 1/mg of protein bound versus 1/mg of glycogen sedimented (Figure 2), the dissociation constant was determined from the intercept on the abscissa (Madsen & Cori, 1958). The dissociation constant for glycogen and the debranching enzyme was estimated as 1.74 mg/mL. Assuming 6.8% end groups for liver glycogen, this is equivalent to 0.66 mM end groups. Use of a Scatchard plot yielded the same results. Gillard and Nelson (1977), however, found a k_i = 0.56 mg/mL for glycogen inhibition of the debrancher action on ϕ-LD, although an analysis of their data in Figure 22 of their review (Nelson et al., 1979) yields 0.9 mg/mL. The relatively small difference between our results and those of the Nelson group may not be significant since the glycogen preparations were quite different, as were the methods used. Experiments conducted with glycogen in the presence of maltotriose indicate that maltotriose competitively inhibited the binding of glycogen to the debranching enzyme. An inhibition constant of 7.8 ± 0.2 mM for maltotriose was estimated. It should be noted that the K_d determined by the centrifugation method showed good agreement with the dissociation constant obtained from the effect of different concentration maltotriose on the reactivity of the –SH groups, as shown later in Table I.

[2] These studies were conducted by an honors undergraduate student, Heather Harris.

Table I: Dissociation Constants for Saccharide Binding to the Debranching Enzyme at pH 7.2 and 8.0 at 25 °C

saccharide	dissociation constant, K_d (mM)		
		pH 8.0	
	pH 7.2	I	II
glucose	35.0	77.0	432.0
maltotriose	11.7	11.7	30.0
maltopentaose	4.3	2.0	10.0
maltohexaose	5.1	1.3	4.9
glycogen (end groups)[a]	0.66		
maltotriose (competitive)[b]	7.8		

[a] At 20 °C by centrifugal separation. [b] At 20 °C by competing for glycogen binding as determined by centrifugal separation.

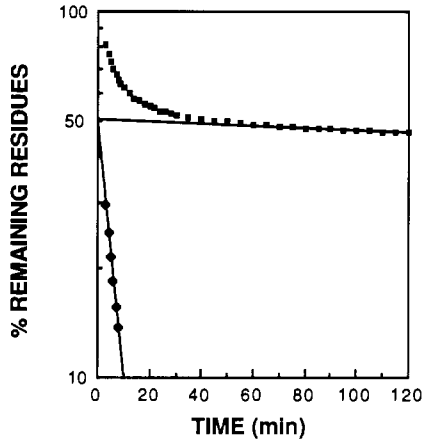

FIGURE 3: Kinetic analysis of the modification of cysteine residues in the debrancher by DTNB as a function of time. The observed rate curve (■) has been resolved into subsets of fast and slow phase reactivities by extrapolating the slow phase back toward time = 0 and subtracting the extrapolated values from the observed values to yield the fast phase (♦). The fraction of remaining cysteine was calculated by taking the total number of such modifiable residues (42) as 100%. The reaction was conducted at 25 °C in 0.1 M potassium phosphate buffer, pH 8.0. The concentration of DTNB in the reaction mixture was 167 μM and that of the debranching enzyme 0.5×10^{-6} M (0.08 mg/mL).

Modification of Cysteine Thiol Groups in the Presence and Absence of Saccharides. The kinetic method of Ray and Koshland (1961, 1962) for modification of amino acid side chains was used to monitor binding of glucose, maltotriose, maltopentaose, and maltohexaose to the glycogen debranching enzyme. Preliminary results indicated that the binding of saccharide can be monitored by measuring the decreased rate of modification of cysteine residues. The rate constants and binding constants were determined from the change in the absorbance of mercaptonitrobenzoate in the presence and absence of ligand under identical conditions.

Reaction between the sulfhydryl groups of the debranching enzyme and DTNB in 0.1 M phosphate buffer at both pH 7.2 and pH 8.0 resulted in a time-dependent modification of cysteine residues as shown on a semilog plot for pH 8.0 in Figure 3. The percent of free thiol groups remaining was calculated by assuming a maximum of 42 modifiable cysteines per mole of enzyme (Taylor et al., 1975). The plot is nonlinear, indicating that not all of the 42 cysteine residues reacted at the same pseudo-first-order rate. This observation suggests the existence of two classes of reactivity to the reagent: a fast phase corresponding to readily available but still shielded –SH groups and a slow phase, indicative of relatively inaccessible residues. Similar biphasic plots were obtained in the presence of all saccharides tested. The data in Figure 3 and those from other similar experiments conducted in the absence of ligand

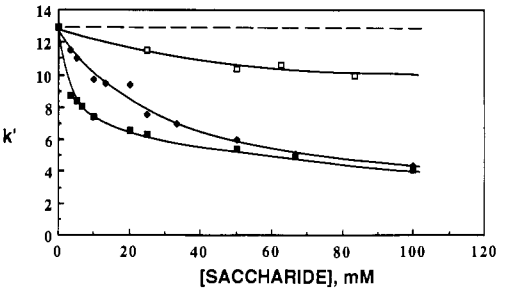

FIGURE 4: Plot of the pseudo-first-order rate constant, k', for the reaction between DTNB and the debranching enzyme against the concentration of various saccharides included in the reaction mixture at pH 8.0. k' was determined as in Figure 3. (□) Glucose; (♦) maltotriose; (■) maltopentaose. Maltohexaose data were omitted for clarity. The horizontal dashed line, $k_1 = (12.9 \pm 1.3) \times 10^{-2}$ min^{-1}, represents the control value to which all values of k' were normalized to allow for minor fluctuations in experiments carried out at different times.

indicate that slightly less than 50% of the cysteine residues form the fast phase, $t_{1/2}$ = 5.4 min. It may be noted that control experiments indicated that the reaction of DTNB with the unprotected –SH groups of glycogen phosphorylase under these conditions has a $t_{1/2}$ of 4 s or less. The remaining thiols, constituting the slow phase, exhibited a $t_{1/2} > 88$ min. Taylor et al. (1975) assigned 17 of 42 –SH groups to the fast phase reaction, a result in reasonable agreement with ours.

At pH 7.2, about 30% of the thiols constitute the fast phase, showing a $t_{1/2}$ = 14.4 min in the absence of ligands, and the half-time for the slow phase was similar to that observed at pH 8.0. Since at either pH the rate of reaction observed is much slower than that of DTNB with model –SH compounds or unprotected –SH groups of proteins, the different rates at the two pHs are a result of the protein's properties and are not due to differences in DTNB or –SH reactivity. It is interesting to note that Cappel et al. (1986) have recently reported a biphasic decrease in activity of the debranching enzyme with the oxidation of sulfhydryl groups by oxidized glutathione. In their work, however, the fast phase was equated to three and the slow phase to seven –SH groups. The different experimental conditions and measurement methods make these results difficult to compare with ours.

Determination of Rate Constants. It is clear from Figure 3 that two first-order rate constants are required to describe the composite rate constant for reactivity of all cysteine residues in the absence of any saccharides. The individual rate constants associated with the fast and slow reactivity groups were determined as illustrated in Figure 3. Subtraction of the values for the contribution of the slow phase, along the extrapolated line of the slow phase, from the observed points, and replotting these differences, gives k_1 (reactivity due to the fast phase alone). The absolute values of these constants are the slopes of the replots of the natural log versus time. Similarly, the apparent first-order rate constant in the presence of saccharide can be determined and is designated k' according to the terminology of O'Sullivan and Cohn (1966). It was noted that the first-order rate constants for the slow phase in the presence and absence of saccharides, regardless of their concentration, were essentially constant and 1 order of magnitude less than the k' values. These results suggest that this latter group of slow-reacting sulfhydryls has very little or no phenomenologically important effect on the actual binding of the saccharides.

Binding of Saccharides. The average value of k_1 is $(12.9 \pm 1.3) \times 10^{-2}$ min^{-1} at pH 8 and $(4.8 \pm 0.0) \times 10^{-2}$ min^{-1} at pH 7.2. A plot of the apparent first-order rate constants, k',

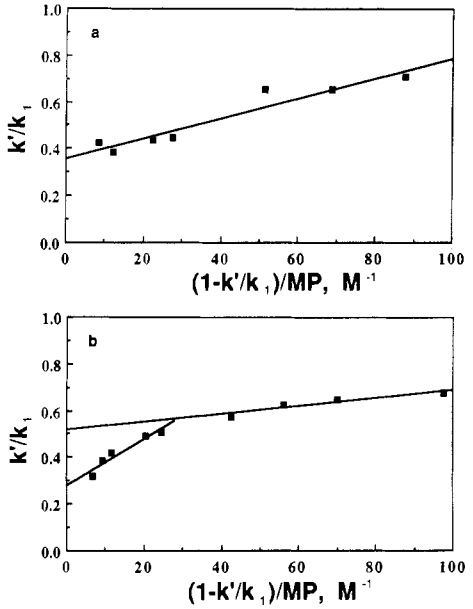

FIGURE 5: Analysis of the data for DTNB modification of the debranching enzyme by the method of O'Sullivan and Cohn (1966). The data were fitted to the equation $k'/k_1 = K_d(1 - k'/k_1)$[saccharide] + k_2/k_1 (see text). k' and k_1 values were determined from plots such as that in Figure 3 for each saccharide at pH 7.2 (a) and pH 8.0 (b). K_d and k_2 were determined from the slopes and y intercepts, respectively. Illustrated is the effect of maltopentaose on the rates of the DTNB reaction with the -SH groups.

for modification of cysteine residues in the presence of the various saccharides versus the concentration of saccharide is shown in Figure 4. The shapes of these curves express saturation phenomena with respect to each saccharide, and the protective effect becomes more pronounced as the substrate increases in size. The simplest explanation for this observation is that with increasing concentration and complexity of the saccharide, the glycogen debrancher becomes less susceptible to attack by the modifying reagent. Possibly, there are subtle changes in the conformation of the enzyme caused only by the binding of saccharide. A plausible implication, consistent with previous observations, is that those global effects caused by saccharide binding may be a direct result of minute or gross changes in enzyme conformation. Conformational changes upon binding of substrate have been suggested for the rabbit muscle debranching enzyme (Brown et al., 1973; Nelson & Watts, 1974). A detailed knowledge of the mechanism involved is not essential for employment of the phenomenon to determine binding constants. Thus, previous binding studies employed inactivation of pyruvate carboxylase with avidin (Scrutton & Utter, 1965), inactivation of creatine kinase by reaction of its -SH groups with iodoacetate (O'Sullivan & Cohn, 1966), and inactivation of glycogen phosphorylase with isocyanate (Avramovic & Madsen, 1968).

Dissociation Constants for Oligosaccharide Binding. The dissociation constant, K_d, and the pseudo-first-order rate constant for the reaction between DTNB and the enzyme–saccharide complex, k_2, were determined by the methods of Scrutton and Utter (1965) and O'Sullivan and Cohn (1966). In a plot of k'/k_1 versus $(1 - k'/k_1)$/[saccharide], the slope gives K_d, and the intercept on the ordinate gives k_2/k_1 from which k_2 is determined. It is relevant to point out that k', the apparent rate constant, is a sum of the weighted rate constants of the free enzyme, k_1, and the complexed enzyme, k_2 (O'Sullivan & Cohn, 1966). Figure 5, panels a and b, illustrates the determination of K_d and k_2 for maltopentaose at pH 7.2 and 8.0, respectively. These plots are representative of those

Table II: Thermodynamic Values for Binding of Saccharides to the Debranching Enzyme[a]

	$-\Delta G$ (kcal mol^{-1})		
		pH 8.0	
saccharide	pH 7.2	site I	site II
glucose	2.26	1.52	0.5
maltotriose	2.63 (0.32)	2.63 (0.56)	2.08 (0.79)
maltopentaose	3.23 (0.30)	3.68 (0.53)	2.73 (0.33)
maltohexaose	3.13	3.93 (0.25)	3.15 (0.42)

[a] $\Delta G = RT \ln K_a$ where $K_a = 1/K_d$ from Table I. Values in parentheses are the $-\Delta\Delta G$ for each additional glucose unit in the oligosaccharide series.

obtained for the other saccharides ranging in size from glucose to maltohexaose.

Table I summarizes the dissociation constants for the various saccharides at pH 7.2 and 8.0. The K_d for glucose at pH 7.2, 35 mM, is in the range of the two values of K_m (32 and 43 mM) which have been reported previously for the incorporation assay of the glucosidase reaction (Nelson & Larner, 1970; Gillard & Nelson, 1977). However, since the kinetic mechanisms for this enzyme are not known in detail, it is not possible to assign a physical meaning to a K_m value or to expect that it should necessarily be equated with a dissociation constant. As pointed out earlier, our determination of the dissociation constant for maltotriose based on the inhibition by this compound of glycogen binding is similar to the value based on the protection of sulfhydryl groups against reaction with DTNB. The strength of binding of saccharides at pH 7.2 increases with chain length to a maximum of five glucose units. Glycogen, however, binds much more tightly, suggesting that branched polysaccharides may take advantage of additional binding sites. The phosphorylase limit dextrin may bind even more tightly since it has a K_m of 0.11 mM whereas the K_m for glycogen was estimated at 0.29 mM end groups (Gillard & Nelson, 1977). These results are analogous to those for α-amylase, where it was found that the α-limit dextrin binds more tightly than maltose (Levitzki et al., 1963).

At pH 8.0, the biphasic nature of the binding curves can be analyzed into two sets of dissociation constants, suggesting two sites which manifest strong and weak binding for the saccharides. The strong binding sites appears to be similar to the single site seen at pH 7.2, with a greater increase in binding as chain length increases. The weak binding site may represent a second site for the binding of branched polymers which is not evident for unbranched oligomers at a more physiological pH. The increased pH may permit an unmasking of this site whereas at pH 7.2 this may require the cooperative action of a branched saccharide.

In Table II, the free energies for binding the oligosaccharide series are computed for the data of Table I. For technical reasons, the most reliable data pertain to the strong binding site at pH 8.0. The increased energy of binding on going from glucose to maltotriose is 0.56 kcal per glucose unit, and from maltotriose to maltopentaose an almost equivalent value of 0.53 kcal. This result suggests strongly that all four glucose units beyond glucose have binding subsites with equivalent affinities. The sixth glucose unit, however, contributes very little to the strength of binding, suggesting that, if glucose itself occupies the first subsite, only five subsites are available in total. A similar situation is seen at pH 7.2 except that the incremental energy for each subsite is smaller than at pH 8.0, and it is clear that the sixth glucose unit appears not to contribute at all. These results are reminiscent of the classical observations made with lysozyme, where increasing chain length of the saccharide resulted in increasing strength of binding, allowing calculation

Table III: Rate Constants for the Reaction between DTNB and the Enzyme–Saccharide Complex at pH 7.2 and 8.0 at 25 °C[a]

	k_2 ($\times 10^2$ min^{-1})		
		pH 8.0	
saccharide	pH 7.2	I	II
glucose	1.8	6.4	
maltotriose	1.0	7.5	2.0
maltopentaose	1.7	6.2	3.3
maltohexaose	1.4	6.6	4.9

[a] Mean value for k_2 at pH 7.2 is $(1.5 \pm 0.3) \times 10^{-2}$ min^{-1}. [b] Mean value for k_2 at pH 8.0 and site I is $(6.7 \pm 0.4) \times 10^{-2}$ min^{-1}.

of the free energy of binding for each subsite (Imoto et al., 1972). For lysozyme, however, the free energies of binding ranged from 1.7 to 4.6 kcal per subsite, except for that involved in catalysis. Presumably, the debranching enzyme involves several more subsites, all with relatively weak binding, to bind glucose residues of the main chain in order to achieve the quite strong binding affinities seen with glycogen and, especially, limit dextrin.

The results for the secondary binding site observed at pH 8.0 are more difficult to interpret, but the single glucose binds with an energy equivalent to that of the other subsites rather than much more tightly, as it did for the stronger binding site. We might speculate that in this case we may be probing the subsites for the main chain whereas the strongly binding site may associate with the side chain.

Table III records the derived values for k_2, which represent the pseudo-first-order rate constants for the reaction between DTNB and the enzyme which is fully saturated with saccharide. At pH 8.0, k_2 is essentially constant at $(6.7 \pm 0.4) \times 10^{-2}$ min^{-1}, representing an average of 52% of the rate with the unliganded enzyme. This suggests that all the saccharides, including glucose, drive the enzyme to a single conformational state and that glucose may therefore adhere to part of the same series of binding subsites. The implication is that the reducing-end glucose of all the saccharides binds to the same subsite as does glucose. Since glucose must bind to the glucosidase site, one may tentatively predict that the transfer site for the oligosaccharide is contiguous with the glucosidase site for the single glucose moiety which undergoes reversible α-1,6 bond formation. At pH 7.2, the average k_2 is $(1.5 \pm 0.3) \times 10^{-2}$ min^{-1}, representing an average of 31% of the rate with the free enzyme. The implications of this result are the same as those on which we speculated for pH 8.0, above. These results are in contrast to the situation seen for the weak binding site at pH 8.0, where k_2 shows a continual increase with the chain length of the saccharide. The meaning of this result is not readily apparent but emphasizes again the different character of this site.

Nelson and his colleagues proposed that the debranching enzyme would have a maltotriosyl binding site, associated with the transferase activity, and another polymer binding site which would bind the main chain of the ϕ-LD (Gillard & Nelson, 1977; Nelson et al., 1979). The data in this paper are consistent with this concept and provide some details of the binding sites. In addition, on the basis of differential effects of proteases and inhibitors on the transferase, glucosidase, and combined activities of the enzyme, it was suggested that the catalytic sites for the transferase and glucosidase activities were separate, such that after the transferase action took place the 1,6-linked glucose unit would rotate into the glucosidase catalytic site. The evidence cited could also be interpreted as involving protein conformational changes to account for the expected large spatial shifts expected for the transferase activity, and, less likely, a single catalytic site involving some different functional groups for the two activities. In this speculative interpretation, the binding sites for the maltotriosyl moiety which is transferred and the glucose residue which is cleaved would be contiguous. The data presented in this paper are consistent with this hypothesis, and we look forward to testing it with the X-ray crystallographic structural and binding studies.

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The ATP/AMP Binding Site of Pyruvate,Phosphate Dikinase: Selective Modification with Fluorescein Isothiocyanate†

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ABSTRACT: Pyruvate,phosphate dikinase from *Propionibacterium shermanii* is strongly inhibited by fluorescein 5′-isothiocyanate (FITC). The time course of inactivation is biphasic, but the dependence of the pseudo-first-order rate constants on the inhibitor concentration indicates the formation of a reversible complex with the enzyme prior to covalent modification. The substrate/product nucleotide pairs MgATP and MgAMP protected against inactivation, while in the absence of Mg^{2+}, both the nucleotides were ineffective. Previously, an essential lysine at the ATP/AMP subsite of the enzyme from *Bacteroides symbiosus* had been implicated by use of the 2′,3′-dialdehyde of AMP (oAMP) [Evans, C. T., Goss, N. H., & Wood, H. G. (1980) *Biochemistry 19*, 5809]. The inhibition by FITC was competitive with MgAMP, and a multiple inhibition analysis plot indicated that binding of oAMP and FITC was mutually exclusive. These observations suggest that FITC and oAMP bind at the nucleotide binding site and probably to the same reactive lysine that is modified by oAMP. With peptide mapping by high-performance liquid chromatography, FITC was found to be a suitable probe for isolating the peptide from the ATP/AMP subsite.

The overall reaction catalyzed by pyruvate,phosphate dikinase (EC 2.7.9.1, pyruvate,orthophosphate dikinase) from *Propionibacterium shermanii* and *Bacteroides symbiosus* (now classified as *Clostridium symbiosus*) involves three partial reactions, each of which is catalyzed at a distinct subsite:

$$\text{ENZ-His} + \text{ATP} \rightleftharpoons \text{ENZ-His}\sim{}^{\beta}\text{P}^{\gamma}\text{P} + \text{AMP} \quad (1a)$$

$$\text{ENZ-His}\sim{}^{\beta}\text{P}^{\gamma}\text{P} + \text{P}_i \rightleftharpoons \text{ENZ-His}\sim{}^{\beta}\text{P} + {}^{\gamma}\text{PP}_i \quad (1b)$$

$$\text{ENZ-His}\sim{}^{\beta}\text{P} + \text{pyruvate} \rightleftharpoons \text{ENZ-His} + {}^{\beta}\text{P-enolpyruvate} \quad (1c)$$

The phosphoryl moiety of the phosphoryl-enzyme intermediate (ENZ-His∼P)[1] was shown to be bound to the enzyme through a phosphoramidate linkage to the 3′-nitrogen of a histidine residue (Spronk et al., 1976). The pyrophosphoryl group of the ENZ-His∼PP intermediate has also been shown to be linked to a histidine residue after stabilization of the ENZ-His∼PP by diazomethylation (Phillips & Wood, 1986). The amino acid sequence surrounding the phosphorylated histidine residue has been sequenced (Goss et al., 1980). This essential histidyl residue is considered to be centrally located

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[1] Abbreviations: PPDK (dikinase), pyruvate,phosphate dikinase; ENZ-His∼P, phosphoryl pyruvate,phosphate dikinase; ENZ-His∼PP, pyrophosphoryl derivative of the dikinase; P-enolpyruvate (PEP), phosphoenolpyruvate; oAMP, 2′,3′-dialdehyde of AMP; FITC, fluorescein 5′-isothiocyanate; FTC, fluorescein thiocarbamyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NEM, *N*-ethylmorpholine; SDS, sodium dodecyl sulfate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; NADH, reduced nicotinamide adenine dinucleotide; TEA, triethylamine; AUFS, absorbance units full scale.

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