Effects of Oligosaccharide Binding on Glycogen Debranching Enzyme Activity and Conformation†

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Received December 21, 1994; Revised Manuscript Received March 9, 1995⊗

ABSTRACT: Glycogen debranching enzyme contains two catalytic activities (4-α-glucanotransferase and amylo-1,6-glucosidase) on its single polypeptide chain, and they are affected differently by the binding of oligosaccharides. Glucose, maltose, and maltotriose are competitive inhibitors of the amylo-1,6-glucosidase activity measured by the hydrolysis of α-glucosyl fluoride, whereas saccharides with four or more glucose units are activators of the same activity, showing apparent "uncompetitive" kinetics. This suggests that they do not bind until the α-glucosyl fluoride is bound. In either case the potency of the effect increases with the length of the oligosaccharide chain. On the other hand, all oligosaccharides tested (maltose to maltohexaose, α-cyclodextrin, and β-cyclodextrin) are competitive inhibitors of the transferase activity and also cause a decrease in the intrinsic fluorescence, both functions again increased by chain length, thus indicating that these saccharides do bind to the free enzyme. These interesting results can be reconciled if the extended main chain resulting from the transferase reaction has to be reoriented into a different binding mode in order to position the α-1,6-linked side-chain glucose into the correct position for the glucosidase reaction. Therefore, activating oligosaccharides behave kinetically as if they had not been previously bound. It is concluded that the main chain of the natural limit dextrin substrate has a different mode of binding for the two catalytic reactions in order to position properly first the maltotetraosyl side chain in the transferase catalytic site and then the glucosyl side chain in the glucosidase catalytic site. All activating saccharides, including glycogen, elicit the same maximal glucosidase velocity, 6-fold the unactivated rate, suggesting that all generate the same enzyme conformation. Circular dichroic spectra yielded estimates of the secondary structure, but these were unaffected by any tested saccharide.

Glycogen debranching enzyme (amylo-1,6-glucosidase/4-α-glucanotransferase, EC 3.2.1.33 and EC 2.4.1.25) removes the 1,6-branch points in the limit dextrin resulting from the action of phosphorylase on glycogen. Its action consists of transferring a maltotriose unit to the "main chain" from the four glucose units attached to this main chain by α-1,6 linkages. This results in an elongated section of α-1,4-linked polymer, leaving a single glucose attached to it via an α-1,6 linkage. The enzyme then cleaves the α-1,6 linkage by which this glucose is attached. The transferase and glucosidase activities are both found on the single large polypeptide chain of this monomeric enzyme (Brown & Brown, 1966; Nelson et al., 1969; Bates et al., 1975), which for the rabbit enzyme has a molecular mass of 177 542 Da as estimated from its cDNA-derived protein sequence (Liu et al., 1993).

Nelson and colleagues used reversible inhibitors and a catalytic site-directed irreversible inhibitor to analyze the two catalytic activities and concluded that the enzyme has overlapping polymer binding sites or interacting sites flanked by a glucosidase site on one side and a transferase site on the other (Gillard & Nelson, 1977; Nelson et al., 1979; Gillard et al., 1980). Liu et al. (1991) showed that the α-1,6-glucosidase activity released the β-anomer of glucose and thus is an "inverting" glucosidase, whereas the transferase retains the α-1,4-configuration. They also showed that the transferase activity can be irreversibly inactivated by a water-soluble carbodiimide in the presence of amines without affecting glucosidase activity measured by the hydrolysis of α-glucosyl fluoride, thus providing further evidence that the two active sites are separate and employ different catalytic mechanisms.

Takrama and Madsen (1988) examined the binding of oligosaccharides, glucose, and glycogen to the debranching enzyme, by comparing the ability of the saccharides to decrease the reaction of sulfhydryl groups with 5,5′-dithiobis-(2-nitrobenzoate), and the results were consistent with the Nelson hypothesis. In addition, Liu et al. (1991) showed that maltohexaose and β-cyclodextrin are acceptors of glucose from β-glucosyl fluoride, suggesting that they must occupy a site near the glucosidase catalytic site. It is worth mentioning that the use of α-glucosyl fluoride as a substrate provides a direct and specific measure of the glucosidase activity, ideally suited for detailed kinetic analysis, whereas the incorporation of [^{14}C]glucose into glycogen, the usual assay, is complicated by the concomitant action of the transferase, which covers up the newly incorporated glucose residue (Brown & Illingworth, 1964). We also observed that

† This research was supported by the Department of Surgery and the Dedicated Health Research Funds of the University of New Mexico School of Medicine.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1995.

0006-2960/95/0434-7056$09.00/0

in the assay of glucosidase activity with α-glucosyl fluoride as substrate the apparent Michaelis constant and maximal velocity of the enzyme increased in the presence of glycogen (Liu et al., 1991). In this paper, the activation, inhibition, and conformational changes of the debranching enzyme caused by oligosaccharide binding are reported.

MATERIALS AND METHODS

Materials. The glycogen debranching enzyme was purified from rabbit muscle by the method described by Takrama and Madsen (1988). The specific activity of the enzyme was 11 μmol min^{-1} mg^{-1}, measured by the coupled activity assay of Gillard and Nelson (1977). α-D-Glucosyl fluoride was kindly provided by Dr. Stephen G. Withers of the University of British Columbia, Vancouver. All other chemicals were commercially available and used without further purification.

Assays. The combined enzyme activity (transferase plus glucosidase) was measured by following changes in the absorbance at 340 nm with limit dextrin as the substrate, in a buffer of 25 mM glycylglycine, 1 mM EDTA, and 1 mM DTT at pH 7.2, according to the coupled enzyme assay method of Gillard and Nelson (1977). The transferase activity was measured spectrophotometrically by following the change in the iodine-limit dextrin spectrum at 620 nm according to the method of Nelson et al. (1970). Glucosidase activity was assayed by following the release of glucose from α-D-glucosyl fluoride as the substrate as described by Liu et al. (1991), with a glucose diagnostic kit (No. 510) provided by Sigma. α-D-Glucosyl fluoride was stored at −20 °C as the solid material, and solutions were freshly prepared for each experiment. The complete hydrolysis of α-D-glucosyl fluoride gave equal amounts of glucose and fluoride, each equivalent to the amount expected from the amount weighed out (Liu et al., 1991). Oligosaccharides were present in the reaction mixture at the designated concentrations. All enzyme assays were carried out at 22 °C. A Shimadzu UV-1201 spectrophotometer with a kinetic module accessory was used for all assays.

Fluorescence spectra were recorded using a Perkin Elmer LS 50 luminescence spectrometer. The excitation wavelength was 280 nm, and the emission spectrum was from 290 to 450 nm with slit 5 for both. The scanning speed was 240 nm/min. The measurements were performed at room temperature in various concentrations of oligosaccharide, 0.1 M sodium phosphate buffer at pH 6.6, and 0.012 μg/mL of debranching enzyme.

Circular dichroic spectra were obtained on a Jasco J-600 spectropolarimeter with a 1-mm path length cell. Spectra were acquired with a 1-nm bandwidth and a step size of 0.2 nm. The scanning speed was 20 nm/min. The buffer conditions were the same as those used in the fluorescence experiments except the enzyme concentration was 0.18 mg/mL.

RESULTS

Effects of Oligosaccharides, Glycogen, and Glucose Binding to the Debranching Enzyme. In this study, it is observed that the binding of oligosaccharides, glycogen, and glucose have different effects on the glucosidase activity. Glucose, maltose, and maltotriose are inhibitors of the glucosidase activity, whereas other oligosaccharides with chain lengths of four glucose units or greater, as well as amylopectin and glycogen, are activators of this activity, as shown in Figure 1. The potency of stimulation increases with the length of the oligosaccharide chain; e.g., the glucosidase activity increased 1.15-, 1.45-, and 1.86-fold in the presence of 20 mM maltotetraose, maltopentaose, and maltohexaose, respectively. Both amylopectin and glycogen are potent activators; in addition, the former is the substrate for the transferase activity, while the latter is a poor substrate for the combined activity (Nelson et al., 1969). It should be noted that the increase in glucosidase activity in the presence of 0.1% glycogen was reduced from 3.65-fold to 1.48-fold by the addition of 56 mM maltotetraose, indicating that glycogen and maltotetraose may compete for the same binding site(s). Another interesting point is that both maltohexaose and α-cyclodextrin contain six glucose units, and at the same concentration of 20 mM, the glucosidase activity was increased 1.86-fold by the former but 2.3-fold by the latter. In contrast to the glucosidase activity, the transferase activity is inhibited by all oligosaccharides tested (Figure 1). The strength of inhibition increases with chain length from maltose to maltohexaose to β-cyclodextrin; e.g., the relative activity was reduced by 8 mM maltose to 0.91, but by 6.8 mM β-cyclodextrin to 0.21.

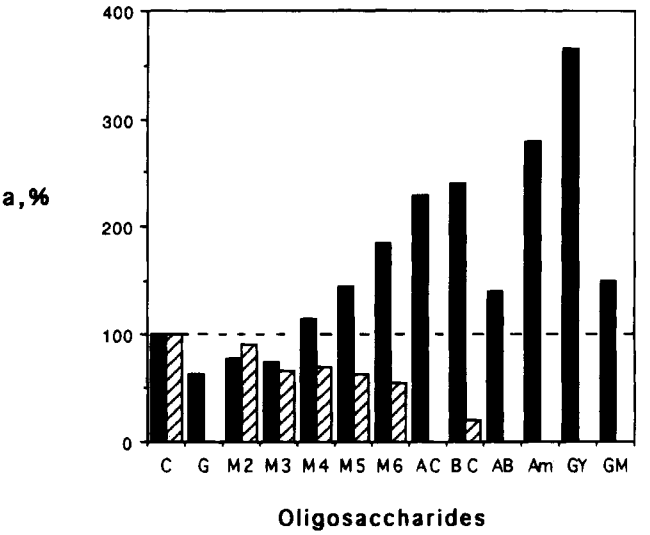

FIGURE 1: Effects of glucose, oligosaccharides, and glycogen on glycogen debranching enzyme activity. The percent enzyme activity is represented by glucosidase in solid bars (Gl) and transferase in hatched bars (Tr): C, native enzyme; G, glucose (65 mM); M2, maltose, 20 mM for Gl and 8.0 mM for Tr; M3, maltotriose, 20 mM for Gl and 7.4 mM for Tr; M4, maltotetraose, 20 mM for Gl and 7.4 mM for Tr; M5, maltopentaose, 20 mM for Gl and 7.5 mM for Tr; M6, maltohexaose, 20 mM for Gl and 7.0 mM for Tr; AC, α-cyclodextrin, 20 mM; BC, β-cyclodextrin, 20 mM for Gl and 6.8 mM for Tr; AB, acarbose, 30 mM; AM, amlopectin, 1%; GY, glycogen, 0.1%; GM, 0.1% glycogen + 56 mM maltotetraose.

Binding Site Relationships between the Substrate of Glucosidase and Oligosaccharides. Since oligosaccharides have different effects on the glucosidase activity depending on their chain length, the relationships of the oligosaccharide binding site(s) with the binding site of the glucosidase substrate, α-glucosyl fluoride, were examined using a Lineweaver−Burk plot. As shown in Figure 2, glucose, maltose, and maltotriose are competitive inhibitors of glucosidase activity. However, the activators, oligosaccharides with a chain length of four glucose units or greater, yield a set of lines that are, within experimental limits, parallel to

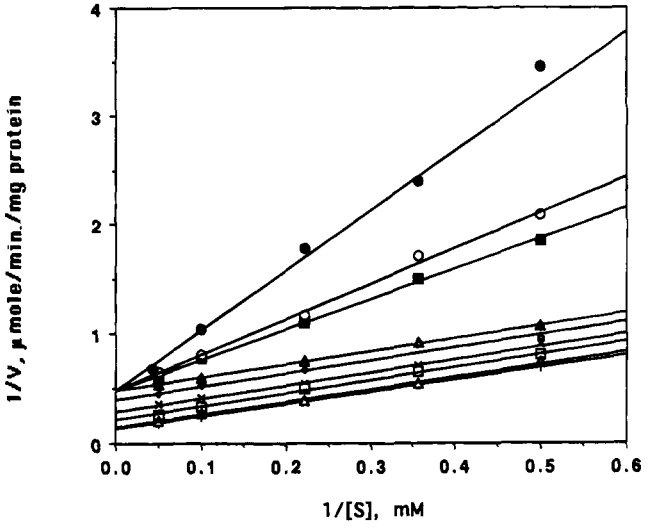

FIGURE 2: Binding relationships between oligosaccharides and the substrate of glucosidase. The incubation mixture contained 0.1 M sodium phosphate at pH 6.6, 2–20 mM α-glucosyl fluoride, and debranching enzyme (0.09 mg/mL): (▲) control; (●) glucose, 65 mM; (◆) maltose, 20 mM; (○) maltotriose, 20 mM; (◇) maltotetraose, 20 mM; (×) maltopentaose, 20 mM; (□) maltohexaose, 20 mM; (△) α-cyclodextrin, 20 mM; (+) β-cyclodextrin, 20 mM.

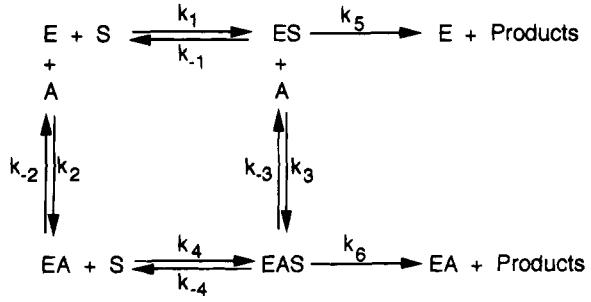

FIGURE 3: General kinetic mechanism, modified from Frieden (1964), for nonessential activation of an enzyme (E) which can bind a substrate (S) or an activator (A) or both. Note that from steady-state treatment the dissociation constant for EA is k_{-2}/k_2, but other equilibria must be designated by Michaelis constants which are complex combinations of rate constants, as discussed in the text.

the control line. This result indicates an "uncompetitive activation", a term adapted from the pattern of uncompetitive inhibition where an inhibitor binds only to an enzyme–substrate complex, but not to the enzyme, although these saccharides do bind the enzyme in the absence of the substrate (Takrama & Madsen, 1988). These activators do not bind at the same site as does α-glucosyl fluoride, so they must bind somewhere else and produce a conformational change which increases both the apparent K_m and the V_{max} value of the glucosidase but not the slope term, which remains at the constant ratio of the K_m to V_{max} values obtained in the absence of any activator.

The effect of a modifier on the activity of an enzyme with only one substrate has been treated by a number of authors, including Segal et al. (1952) and Frieden (1964). Figure 3 is modified slightly from Scheme II of Frieden's paper and is reproduced for clarity. Segal et al. (1952) and Frieden (1964) used both a rapid equilibrium and a steady-state kinetic treatment, but the latter would seem more appropriate in the present case of a hydrolytic enzyme where both substrate and activator appear to cause conformational changes. Taking the steady-state formula for case III of Segal et al. (1952), we may alter it to account for our case, where, in the absence of substrate, there appears to be no binding of activator to the site which increases catalytic activity. Thus $k_2 = 0$, and therefore the dissociation constant for the activator–enzyme complex is ∞, and we arrive at the following equation:

$$\frac{1}{v} = \frac{K_a + [A]}{V_m K_a + V_{ma}[A]} + \frac{K_s}{V_m}\frac{1}{[S]} \quad (1)$$

where V_m = maximal velocity in the absence of activator, V_{ma} = maximal velocity in the presence of saturating substrate and activator, K_s = the Michaelis constant for substrate, and K_a = the Michaelis constant for the activator.

It is important to note that the slope term is not affected by the presence of the activator, as seen experimentally in Figure 2, whereas the intercept term will decrease as activator concentration is increased. The same intercept term is obtained by the rapid equilibrium derivation and by various other manipulations of the equations of Segal et al. (1952) and Frieden (1964). However, for the steady-state treatment of even this minimal mechanism, the Michaelis constants are not simple dissociation constants; rather, $K_s = (k_{-1} + k_5) / k_1$ and $K_a = (k_{-3} + k_6)/k_3$. In reality, these Michaelis constants are likely to be even more complicated combinations of these and additional rate constants.

Under conditions where the substrate concentration is saturating, the velocity will be solely a function of the intercept term of eq 1 and hence will vary as a function of the concentration of activator. By removing the slope term from eq 1, inverting and subtracting V_m from both sides, and then re-inverting and simplifying, one arrives at

$$\frac{1}{v - V_m} = \frac{1}{V_{ma} - V_m} + \frac{K_a}{V_{ma} - V_m}\frac{1}{[A]} \quad (2)$$

In practice, the glucosidase assays were carried out with 20 mM α-glucosyl fluoride, a concentration 8 times the K_m, and various concentrations of each activator, and the results were plotted according to eq 2, as illustrated in Figure 4 for some representative cases. The resultant kinetic constants are summarized in Table 1 and will be discussed later.

Binding Site Relationships between the Substrate of Transferase and Oligosaccharides. Since it was shown (Figure 1) that all of the oligosaccharides examined in the present study are inhibitors of the transferase, it was necessary to further determine whether these compounds bind at the site where the transferase substrate, amylopectin, binds or at different sites. A Lineweaver–Burk plot of the transferase activity expressed in the presence of various oligosaccharides is shown in Figure 5. The compounds tested, as well as maltose and maltotetraose for which data are not shown, all exhibit competitive inhibition. These results indicate that all of these saccharides tested bind at the substrate site of the transferase.

Summary of Kinetic Constants. Table 1 summarizes the results of the kinetic studies. It is striking that saccharides containing one, two, or three glucose units are all competitive inhibitors of glucosidase activity, whereas all those with four or more units are uncompetitive activators. In each case the potency of the effect increases with increasing chain length. However, the maximal velocity achieved in the presence of activating saccharides is approximately the same, averaging 12.3 ± 0.5 μmol min^{-1} mg^{-1}, or 6 times the rate for the

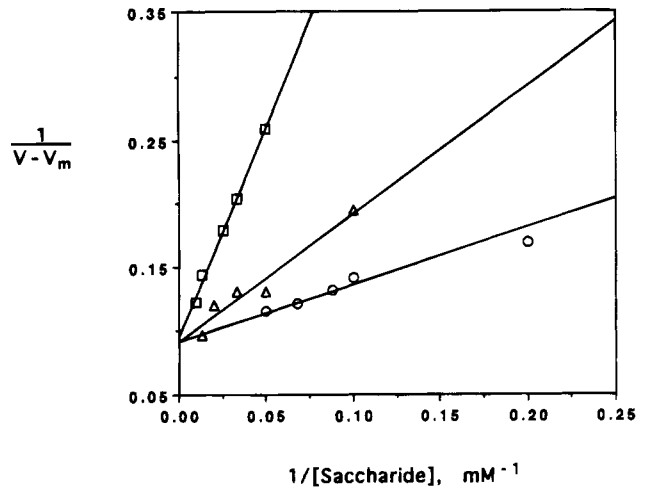

FIGURE 4: Reciprocal plot for the activity of glucosidase acting on 20 mM α-glucosyl fluoride in the presence of various concentrations of the activating saccharide: (□) Maltotetraose, (△) maltohexaose, and (○) β-cyclodextrin. Maltopentaose and α-cyclodextrin were also tested, but for clarity the results are not shown.

Table 1: Kinetic Constants for Binding of Saccharides to the Glycogen Debranching Enzyme

	values obtained by measuring glucosidase activity[a]			transferase	chemical
saccharide	K_d (mM)	K_a (mM)	V_{ma} (μmol min^{-1} mg^{-1})	activity,[b] K_d (mM)	reactivity,[c] K_d (mM)[c]
glucose	18				35
maltose	15			28	
maltotriose	11			12	12
maltotetraose		34	13	22	
maltopentaose		25	11	8	4
maltohexaose		11	12	2	5
α-cyclodextrin		5	13	0.8[d]	
β-cyclodextrin		4	12		
glycogen			13[e]		0.7

[a] The dissociation constant, K_d, is measured from the inhibition of glucosidase activity, while K_a and V_{ma} are estimated from the activation of glucosidase activity, as described in the text. V_m for control glucosidase activity was 2.1 μmol min^{-1} mg^{-1}. [b] K_d estimated from the inhibition of the transferase reaction. [c] Data from Takrama and Madsen (1988) determined by the ability of the saccharides to decrease the rate of reaction of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoate). Glycogen binding (end groups) was determined by ultracentrifugal separation. [d] Data of Gillard and Nelson (1977) estimated from the competitive inhibition of α-cyclodextrin with the limit dextrin with a K_i of 0.76 mg/mL, which equals 0.77 mM if one assumes a molecular mass of 984 Da. [e] Measured by Liu et al. (1991) at saturating glycogen (9.6%) and varying α-glucosyl fluoride concentrations.

unactivated enzyme. This suggests that all the saccharides promote the same conformational state of the enzyme, at least as far as can be monitored by the glucosidase activity. The average value of V_{ma} is now similar to the rate for the overall reaction or that measured for the release of glucose from β-glucosyl fluoride in the presence of maltohexaose (Liu et al., 1991) (11.0 and 12.0 μmol min^{-1} mg^{-1}, respectively). Thus the glucosidase reaction, not the transferase reaction, may be rate limiting in the overall reaction.

Fluorescence Spectra of the Debranching Enzyme in the Presence of Oligosaccharides. It has been suggested that oligosaccharide binding may cause a conformational change of the enzyme (Takrama & Madsen, 1988). This study has shown that those saccharides with a chain length of four glucose units or greater do not bind at the glucosidase

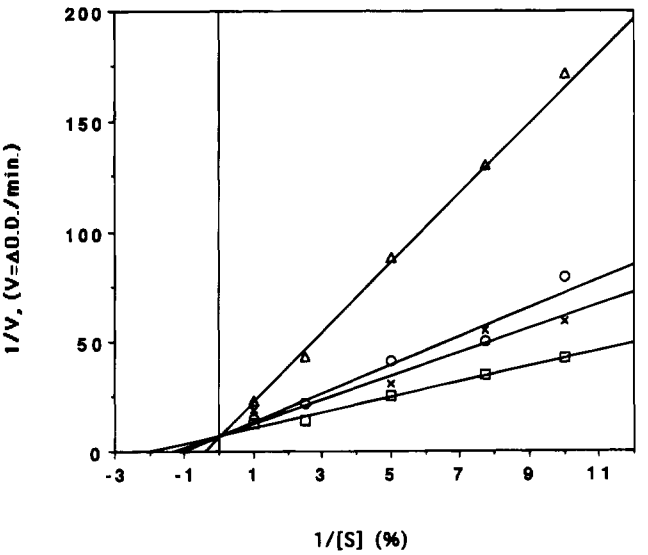

FIGURE 5: The Lineweaver–Burk plot for glycogen debranching enzyme in the presence of oligosaccharides, showing oligosaccharides as competitive inhibitors of the transferase activity. The reaction mixture for the assay contained 0.5% amlopectin, 0.05% gelatin, 0.5 mM EDTA at pH 7.0, 50 mM sodium maleate at pH 5.8, 1 mM 2-mercaptoethanol, and 7.6 μg/mL debranching enzyme: (□) control; (×) maltotriose, 7.35 mM; (○) maltopentaose, 7.51 mM; (△) maltohexaose, 7.04 mM.

substrate binding site but activate the glucosidase activity to change the apparent Michaelis constant and maximal velocity. The hypothesis that these effects may result from conformational changes was tested by observing the fluorescence spectra of the protein in the presence of various oligosaccharides. In the presence of maltohexaose the intensity of the fluorescence emission at 330 nm decreases with increasing concentration of maltohexaose from 0 to 40 mM (data not shown), indicating that the spectral changes are caused by saccharide binding. The binding constant of maltohexaose thus obtained was 15 mM, somewhat higher but in the same range of the values determined by the other methods listed in Table 1. The emission spectra of the enzyme in the presence of various oligosaccharides are shown in Figure 6. The difference in fluorescence increases with the chain length of the oligosaccharides.

Circular Dichroic Spectra of the Debranching Enzyme with and without Oligosaccharides. The circular dichroic (CD) spectrum of the debranching enzyme was performed in 0.1 M sodium phosphate, pH 6.6, at room temperature to determine whether the binding of saccharides causes any change in the secondary structure of the enzyme. The CD spectrum of the native enzyme (Figure 7) was analyzed using the method of Saxena and Wetlaufer (1971). In Table 2 the contents of α-helix, β-structure, and random structure estimated for glycogen debranching enzyme are summarized and compared with X-ray crystallographic data for hog pancreas α-amylase (Buisson *et al.*, 1971). Both enzymes catalyze similar reactions and have the four consensus sequences commonly found at the carboxy terminal of β-strains in the 8-fold α/β barrel domains of amylases and glucanotransferases (Liu et al., 1993). It will be interesting to check these structural contents when the X-ray structure of this enzyme becomes available.

The debranching enzyme in the presence of β-cyclodextrin yields the same CD spectrum as the native enzyme (Figure 7). The same results are observed with other oligosac-

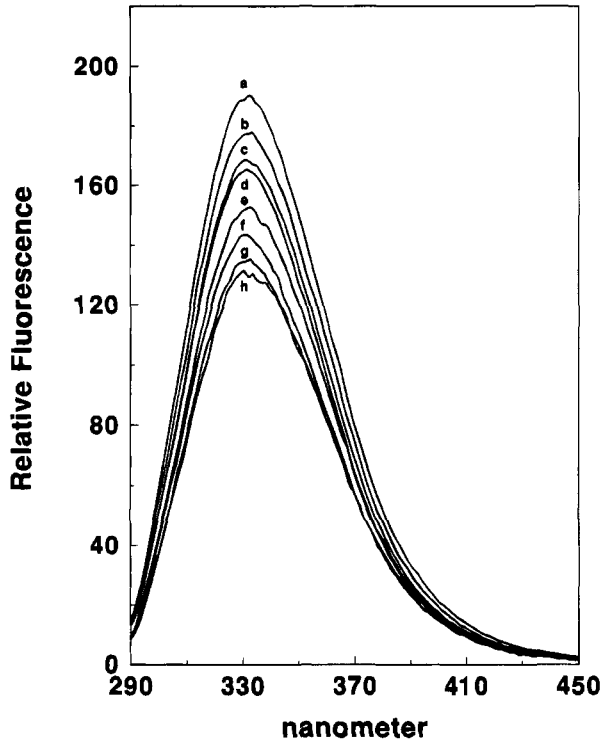

FIGURE 6: Fluorescence spectra of glycogen debranching enzyme in the presence of oligosaccharides. The measurements were carried out in 0.1 M sodium phosphate, pH 6.6, 20 mM oligosaccharides except for the control, and 0.012 mg/mL debranching enzyme, at room temperature: (a) control, (b) maltose, (c) maltotriose, (d) maltotetraose, (e) α-cyclodextrin, (f) maltopentaose, (g) β-cyclodextrin,and (h) maltohexaose.

charides from maltose to maltohexaose and α-cyclodextrin (data not shown), indicating that the secondary structure of the enzyme is not affected significantly by the binding of oligosaccharides.

DISCUSSION

The effects of oligosaccharides on the debranching enzyme's glucosidase activity support the hypothesis that there are two binding sites: glucose, maltose, and maltotriose bind to one site where the glucosidase substrate, α-glucosyl fluoride, binds, while those oligosaccharides with four or more glucose units bind at the other site and activate the glucosidase activity. All oligosaccharides tested are competitive inhibitors of the transferase, suggesting that the substrate binding site for the transferase contains both of the binding sites; therefore, on first consideration, the substrate binding site for glucosidase should be a part of the substrate binding site for the transferase. However, considering the three competitive inhibitors of glucosidase, the glucose moiety must occupy the same site as does α-glucosyl fluoride, both because it is a competitive inhibitor and because it is both a product and a substrate (in the reverse direction) for the glucosidase reaction. It is logical to conclude, then, that maltose and maltotriose have their reducing glucose units binding in the same site, with their remaining glucose units also finding significant binding to adjacent subsites since they bind progressively more tightly. Yet the overall site extending from the position of the scissile bond cannot be exactly the same as that which binds the α-1,6-linked four-glucose unit side chain in the transferase reaction because, were that true, maltotetraose would also

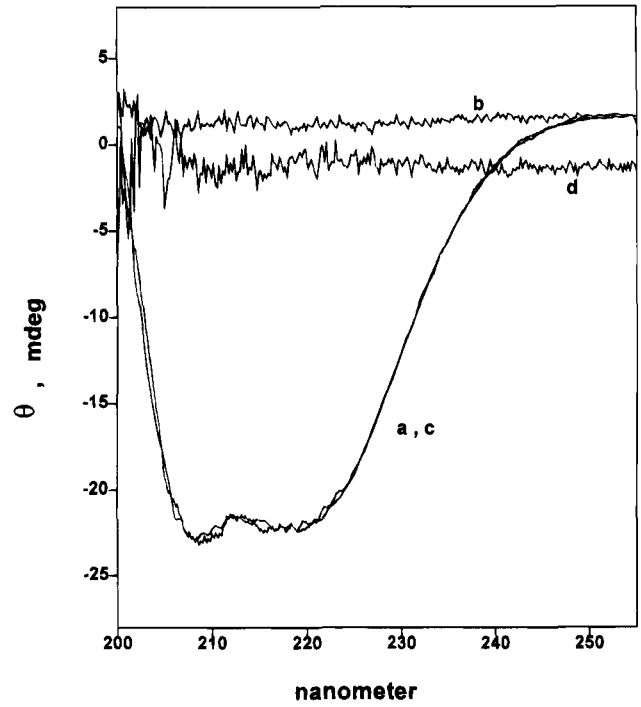

FIGURE 7: Circular dichroic spectra of glycogen debranching enzyme with and without the presence of β-cyclodextrin. Curve a represents the native debranching enzyme. Curve b is the spectrum of β-cyclodextrin (20 mM) in the buffer without the enzyme. Curve c is the spectrum of the enzyme in the presence of β-cyclodextrin (20 mM). Curve d is the result of subtracting curve a from curve c.

Table 2: Structure Content (%) of Glycogen Debranching Enzyme from Rabbit Muscle as Estimated by Circular Dichroism and of α-Amylase from Hog Pancreas as Determined by the X-ray Structure

	α-helix	β-structure	random structure
debranching enzyme (this work)	29	28	43
α-amylase (Buisson et al., 1987)	18	22	60

compete with α-glucosyl fluoride instead of being an activator. As noted in the introduction, Nelson and his colleagues proposed overlapping polymer sites or interacting polymer sites with the α-1,6-glucosyl unit rotating after the transferase reaction to move into the glucosidase site. This proposal for two distinct catalytic sites was supported by our earlier finding (Liu et al., 1991) that the transferase activity could be eliminated by reaction with a water-soluble carbodiimide and an amine without affecting the glucosidase activity. The results in this report further distinguish the two sites.

The activation of glucosidase activity by all saccharides containing four or more glucose units makes sense if one assumes that they bind to the position normally occupied by the main chain of the natural substrate so that the combination of α-gluocosyl fluoride and glucose oligomer resembles the natural substrate. There would be no steric hindrance since the fluoride atom on the artificial substrate is no larger than a hydrogen atom. Furthermore, we know that both maltohexaose and β-cyclodextrin bind in close proximity to β-glucosyl fluoride because the enzyme transfers the glucosyl unit of the latter to the oligomer, forming an α-1,6 bond, before hydrolyzing it (Liu et al., 1991). The increasing efficiency of activation with increasing chain length merely

points to the extended nature of this site, while the advantage of the cyclized glucose oligomers suggests that the site is curved to fit the natural helical shape of an extended α-1,4-linked glucose polymer, as was found for the "glycogen storage site" of phosphorylase (Goldsmith & Fletterick, 1983). Now we must try to explain the obvious paradox that, according to a strict interpretation of the kinetics, the activating saccharides do not bind to the enzyme until after the binding of the substrate, α-glucosyl fluoride, yet there is ample evidence that all of these saccharides bind to the free enzyme. Thus all of those tested are competitive inhibitors for the transferase activity, they affect the reactivity of the enzyme's sulfhydryl groups, they promote a different crystal habit than that obtained in their absence, and as shown in this study, they decrease the fluorescence emission of the protein. One could dismiss the uncompetitive activation as a kinetic artifact as yet not recognized, but on the other hand the results could be indicative of successive events during catalysis.

If when the α-glucosyl fluoride has bound to its active site the series of subsites for the main chain are no longer in the correct conformation to orient the oligomeric glucose next to the glucosidase site, then there has to be a reorientation of the oligomer before it can be an activator. Kinetically, then, it would act as if it had not been bound before. In other words, the polymer binding site is not the same for the two reactions. Similar to the present finding, Northrop and Cleland (Northrop & Cleland, 1974) noted that while uncompetitive inhibitors appear to "bind" only to an enzyme–substrate complex, an inhibitor, however, need only exert its effect within the complex—even if it actually binds before the substrate. In the case of the glycogen debranching enzyme, the completion of the transferase reaction presumably triggers a conformational change to create the overall glucosidase site (including both main chain and single glucose side chain). α-Glucosyl fluoride by itself can bind to the catalytic site, possibly even inducing its correct conformation, but this is not the complete binding site for the natural substrate. Thus the unusual kinetics may be an indication that not only is the binding site for the side chain different for the two reactions of the enzyme but also is the binding site for the main chain. This need not imply that there is a complete dissociation of the natural limit dextrin substrate from the enzyme between the two reactions since the enzyme catalyzes both intra- and intermolecular transfers when acting on small branched glucose polymers (Brown & Illingworth, 1962). The very large limit dextrin may well have additional saccharide binding sites well away from the catalytic sites, as has been shown for both phosphorylase (Fletterick et al., 1976) and glycogen synthase (Larner et al., 1976). This may account for the tighter binding of the large polymers, especially the limit dextrin with a k_m of 0.063 mM branch points (Nelson et al., 1969), and would permit an intramolecular transfer of a triose unit from the side chain to the adjacent main chain, followed by hydrolysis of the glucose residue from the same branch point, even if there is reorientation of the substrate between the two catalytic events. Furthermore, there is no need to suggest that the product of the transferase reaction moves to a completely new location on the enzyme since this speculation would not be the most parsimonious interpretation of currently available data. Rather, a major conformational change in the binding site for the main chain is envisaged.

In conclusion, the results discussed in this paper suggest an extension of the original model of Nelson et al. (1979) to include a reorientation of the main polysaccharide chain between the two catalytic events in order to properly reposition the single glucosyl side chain for the glucosidase reaction. Therefore, there is a different binding mode for the main chain in the two events. These possibilities will need to be borne in mind when interpreting the forthcoming structure derived from ongoing X-ray crystallographic studies.

ACKNOWLEDGMENT

We are grateful to Mrs. Shirley Shechosky, in the Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, for preparing the glycogen debranching enzyme and the limit dextrin, and to Dr. Stephen G. Withers for donating the α-glucosyl fluoride. Their support made this work possible. The authors thank Ms. Lucy A. Hunsaker for her expert technical assistance on the fluorescence measurements.

REFERENCES

Bates, E. J., Heaton, G. M., Taylor, C., Kernohan, J. C., & Cohen, P. (1975) *FEBS Lett. 58,* 181–185.
Brown, D. H., & Illingworth, B. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48,* 1783–1787.
Brown, D. H., & Illingworth, B. (1964) in *Ciba Foundation Symposium on the Control of Glycogen Metabolism* (Whelan, W. J., & Cameron, M. P., Eds.) pp 139–150, Little, Brown & Co., Boston.
Brown, D. H., & Brown, B. I. (1966) *Methods Enzymol. 8,* 515–524.
Brown, D. H., Gordon, R. B., & Brown, B. I. (1973) *Ann. N.Y. Acad. Sci 210,* 238–253.
Buisson, G., Duee, E., Haser, R., & Payan, F. (1987) *EMBO J. 6,* 3909–3916.
Fletterick, R. J., Sygusch, J., Semple, H., & Madsen, N. B. (1976) *J. Biol. Chem. 251,* 6142–6148.
Frieden, C. (1964) *J. Biol. Chem. 239,* 3522–3531.
Gillard, B. K., & Nelson, T. E. (1977) *Biochemistry 16,* 3978–3987.
Gillard, B. K., White, R. C., Zingaro, R. A., & Nelson, T. E. (1980) *J. Biol. Chem. 255,* 8451–8457.
Goldsmith, E., & Fletterick, R. J. (1983) *Pure Appl. Chem. 55,* 577–588.
Larner, J., Takeda, Y., & Hizukuri, S. (1976) *Mol. Cell. Biochem. 12,* 131–136.
Liu, W., Madsen, N. B., Braun, C., & Withers, S. G. (1991) *Biochemistry 30,* 1419–1424.
Liu, W., De Castro, M. L., Takrama, J., Bilous, P. T., Vinayagamoorthy, T., Madsen, N. B., & Bleackley, R. C. (1993) *Arch. Biochem. Biophys. 306,* 232–239.
Nelson, T. E., & Watts, T. E. (1974) *Mol. Cell. Biochem. 5,* 153–159.
Nelson, T. E., Kolb, E., & Larner, J. (1969) *Biochemistry 8* , 1419–1428.
Nelson, T. E., Palmer, D. H., & Larner, J. (1970) *Biochim. Biophys. Acta 212,* 269–280.
Nelson, T. E., White, R. C., & Gillard, B. K. (1979) *ACS Symp. Ser. 88,* 131–162.
Northrop, D. B., & Cleland, W. W. (1974) *J. Biol. Chem. 249,* 2928–2931.
Osterlund, B. R., Hayakawa, K., Madsen, N. B., & James, M. N. G. (1984) *J. Mol. Biol. 174,* 557–559.
Saxena, W. P., & Wetlaufer, D. B. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 969–972
Segal, H. L., Kachmar, J. F., & Boyer, P. D. (1952) *Enzymologia 5,* 187–198.
Takrama, J., & Madsen, N. B. (1988) *Biochemistry 27,* 3308–3314.
Tewari, Y. B., & Goldberger, R. N. (1989) *J. Biol. Chem. 164,* 3966–3971.

BI9429400