Reassessment of the Catalytic Mechanism of Glycogen Debranching Enzyme†

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ABSTRACT: The amylo-1,6-glucosidase catalytic activity of glycogen debranching enzyme allows it to hydrolyze α-D-glucosyl fluoride, in the absence or presence of glycogen or oligosaccharides, releasing equal amounts of fluoride and glucose at rates comparable to those seen with the natural substrates. 2-Deoxy-2-fluoro-α-D-glucosyl fluoride is found to be a poor substrate, rather than the covalent inhibitor that would be expected for a glucosidase which catalyzes hydrolysis of the glycosidic linkage with retention of anomeric configuration. In fact, analysis of the glucosidase reaction by NMR reveals that the debranching enzyme hydrolyzes the glycosidic linkage with inversion of configuration, releasing β-D-glucose from both α-glucosyl fluoride and its natural substrate, the phosphorylase limit dextrin. In contrast, its transferase activity necessarily proceeds with retention of configuration. As has been seen with other "inverting" glycosidases, the debranching enzyme releases β-D-glucose from β-D-glucosyl fluoride in the presence of oligosaccharides such as maltohexaose and cyclomaltoheptaose but, unlike the others, not in their absence. An intermediate glucosyl-α-(1,6)-cyclomaltoheptaose has been detected by NMR analysis. In the presence of a water-soluble carbodiimide, a single mole of glycine ethyl ester is incorporated into each mole of the debranching enzyme, resulting in its inactivation when measured by the combined assay for both transferase and glucosidase activities. Measurement of the latter two activities independently indicates that it is the transferase activity which is inactivated, while the glucosidase activity, measured with α-D-glucosyl fluoride as substrate, is unaffected. The results presented in this paper provide further evidence that the two reactions catalyzed by the debranching enzyme take place at two separate sites that utilize different catalytic mechanisms.

Glycogen debranching enzyme (amylo-1,6-glucosidase/4-α-glucanotransferase, EC 3.2.1.33 + EC 2.4.1.25) removes the α-(1,6) branch points in the limit dextrin remaining 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) links. This results in an elongated section of α-(1,4)-linked polymer, with a single glucose attached to it via an α-(1,6) linkage. It then cleaves the α-(1,6) linkage by which this glucose is attached. Both the glucosidase and transferase activities are found on the single polypeptide chain of this monomeric enzyme, which has a molecular mass of approximately 165 kilodaltons (Brown & Brown, 1966; Nelson et al., 1979). It may be added that recent thermodynamic studies by Tewari and Goldberg (1989) suggest that the free energy of hydrolysis of the α-(1,6) link is only −1.7 kcal mol^{-1}, which is 2.0 kcal mol^{-1} less than for the α-(1,4) link. This explains why relatively facile reversal of the glucosidase activity can be achieved under laboratory conditions (Larner & Schliselfield, 1956; Hers, 1959). It also explains why the debranching of glycogen requires two separate chemical reactions whereas the insertion of new branch points during glycogen synthesis can proceed via one thermodynamically favored step.

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 a single overlapping or interacting polymer binding site(s) flanked by a glucosidase site on one side and a transferase site on the other (Gillard & Nelson, 1979; Nelson et al., 1979; Gillard et al., 1980). Takrama and Madsen (1988) examined the binding of oligosaccharides, glucose, and glycogen to the debranching enzyme, with results consistent with the Nelson hypothesis. They also reviewed, in their introduction, the evidence that the glucosidase activity is similar to that of other glucosidases, especially with respect to the types of compounds that cause inhibition. Nelson et al. (1979) reported that debranching enzyme activity was inhibited by a water-soluble carbodiimide, suggesting participation of a carboxyl group, but no experimental details have been published. It would seem appropriate to pursue this finding in more detail.

The similarities between the α-1,6-glucosidase activity and other glycosidases suggest that substrates and inhibitors used for mechanistic studies on this large class of hydrolytic enzymes [for recent reviews see Lalegerie et al. (1982) and Sinnott (1987)] might usefully be applied to the debranching enzyme. In particular, since glycosidases hydrolyze artificial substrates, including glycosyl fluorides (Van Hofsten, 1961; Hehre et al., 1979; Kitahata et al., 1981), one predicts that α-glucosyl fluoride should be a substrate for the debranching enzyme. Indeed, a variety of "retaining" glycosidases (glycosidases that catalyze hydrolysis with retention of anomeric configuration) have been shown to operate upon their correct glycosyl fluorides, with one even also working upon its incorrect fluoride anomer (Hehre et al., 1990). In addition, it has been shown that a number of "inverting" glycosidases can utilize both anomers of their corresponding glycosyl fluorides. The same sugar anomer is the product in both cases; e.g., β-glucose is formed from α-glucosyl fluoride or β-glucosyl fluoride with the "inverting" glucoamylase (Kitahata et al., 1981). This has been rationalized as resulting from a direct attack of water on the "correct" glycosyl fluoride anomer, while the "wrong" anomer undergoes an initial attack at the active site by a second molecule of the glycosyl fluoride to give a disaccharide that is then rapidly hydrolyzed, again with inversion of con-

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figuration. The usual test for the α-(1,6)-glucosidase activity of glycogen debranching enzyme is to measure the incorporation of [^{14}C]glucose into glycogen by reversal of the normal activity. However, measurement of the hydrolysis of α-D-glucosyl fluoride would obviate complications arising from the binding of glycogen and measure the intrinsic glucosidase activity of the purified enzyme. In addition, it would provide a technically simpler assay since liberated fluoride can be monitored with an ion-specific electrode in a continuous fashion.

Withers and his colleagues have recently introduced a new class of specific mechanism-based inactivators for retaining glycosidases in the form of 2-deoxy-2-fluoro-D-glycosyl fluorides (Withers et al., 1988). These inactivators work by generating a stable ($t_{1/2}$ > 500 h for hydrolysis) 2-deoxy-2-fluoro-D-glycosyl–enzyme intermediate, thereby inactivating the enzyme. In confirmation of this mechanism, Withers and Street (1988) have identified, by ^{19}F NMR,[1] a covalent 2-deoxy-2-fluoro-α-D-mannosyl enzyme formed from the corresponding 2-deoxy-2-fluoro-β-D-mannosyl fluoride with a β-glucosidase from *Alcaligenes faecalis*, and have subsequently identified the point of attachment to the enzyme (Withers et al., 1990). Since Nelson and Larner (1970b) reported that the free glucose formed by the debranching enzyme from the phosphorylase limit dextrin retains the α-anomeric configuration of the substrate, suggesting a β-glucosyl enzyme intermediate, one might also expect covalent inhibition by 2-deoxy-2-fluoro-α-D-glucosyl fluoride.

In this paper we describe experiments that provide evidence for the existence of two separate active sites on the enzyme, both through mechanistic studies and through specific inactivation of one activity but not the other.

Materials and Methods

The glycogen debranching enzyme was purified from rabbit muscle as described previously (Takrama & Madsen, 1988). The specific activity was 11 μmol min^{-1} mg^{-1}, measured by the coupled activity assay of Gillard and Nelson (1977). α-D-Glucosyl fluoride and β-D-glucosyl fluoride (Micheel & Klemer, 1961; Hayashi et al., 1984) and 2-deoxy-2-fluoro-α-D-glucosyl fluoride and 2-deoxy-2-fluoro-β-D-glucosyl fluoride (Adamson et al., 1970; Hall et al., 1971; Street et al., 1986; Withers et al., 1988) were synthesized as described. All other chemicals were used without further purification.

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 spectrum of the iodine–limit dextrin spectrum at 620 nm according to the method of Nelson et al. (1970). The glucosidase activity was usually assayed by measuring the fluoride released with α-D-glucosyl fluoride as the substrate in 0.1 M sodium phosphate and 1 mM EDTA buffer at pH 6.7. Glucosidase can also be measured by assaying the incorporation of [^{14}C]glucose into glycogen by the method of Nelson and Larner (1970a). Glucose release from the glucosyl fluorides was measured with a glucose diagnostic kit (No. 510) provided by Sigma, which utilizes glucose oxidase and horseradish peroxidase to oxidize *o*-dianisidine. Aliquots were taken from the same reaction mixture in which fluoride formation was being measured directly and added to one-third volume of 10% SDS to stop enzymic activity before the glucose was measured. All enzymic assays were carried out at room temperature (21 °C). A Beckman Model DU8 spectrophotometer with a kinetic module accessory and an Orion fluoride electrode with the PHM62 standard pH meter were used for assays.

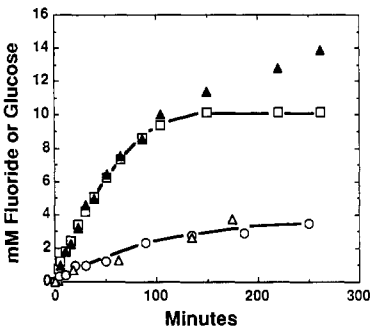

FIGURE 1: Release of fluoride and glucose from 10 mM α-glucosyl fluoride in the presence of 9.6% glycogen compared to the release of glucose from glycogen alone. The 2.5-mL reaction mixture contained 0.16 mg of debranching enzyme. (□) Fluoride; (▲) glucose; (○) glucose from glycogen alone; (△) difference between glucose and fluoride release from α-glucosyl fluoride with 86.5 min set to zero.

For carbodiimide inhibition, the debranching enzyme (8 mg mL^{-1}) was incubated with 50 mM 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and 25 mM glycine ethyl ester in 0.1 M sodium phosphate and 1 mM EDTA buffer at pH 6.7, 21 °C. Aliquots were taken at intervals and diluted at least 100-fold (in the appropriate buffer) to stop the reaction before measuring the residual enzymic activity. Measurement of the incorporation of [^{14}C]glycine ethyl ester into the protein required exhaustive dialysis of large aliquots of the reaction mixture into the above buffer at 4 °C before protein concentration, enzymic activity, and radioactivity were measured, the latter in a Beckman LS 6800 scintillation counter.

All NMR spectra were recorded on either a Varian XL 300 or a Bruker WH400 spectrometer using a 5-mm sample tube. Reactions were performed in a D_2O buffer containing sodium phosphate (100 mM) at pH 6.8. Values of pH quoted are uncorrected. All substrates and buffer materials were freeze-dried from D_2O at least twice prior to use, except for the extremely labile β-glucosyl fluoride, which was freeze-dried once for 1 h just prior to use. Enzyme was dialysed exhaustively against D_2O buffer to remove residual H_2O. Quantities of enzyme and substrate employed in each experiment are given in the legends to the figures. Values of pH for each NMR sample were measured after the completion of reaction (considerable care is required in the control of pH due to the fact that HF is released upon hydrolysis), and in each case the final pH was found to be within 0.5 pH unit of the starting pH.

Results and Discussion

α-Glucosyl Fluoride Substrates. α-Glucosyl fluoride is a good substrate for the debranching enzyme, releasing glucose and fluoride in equal amounts (data not shown) at a rate comparable to that measured with the standard substrate, limit dextrin. This rate is increased by the addition of glycogen to the mix. As is shown in Figure 1, even in the presence of excess glycogen the amount of glucose released is equal to the amount of fluoride until the glucosyl fluoride is completely hydrolyzed. Subsequently, glucose is released from the glycogen at a rate similar to that from glycogen alone, consistent with glycogen being a poor substrate as shown by Nelson et al. (1969). The

[1] Abbreviations: Bistris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; NMR, nuclear magnetic resonance.

Table I: Comparison of Glucose Substrates for Glucosidase Activities

substrate + additions	K_m (mM)	V_m (μmol min^{-1} mg^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
α-glucosyl fluoride	2.8	1.7	1619
α-glucosyl fluoride + glycogen[a]	8.1	12.6	4148
2-fluoro-α-glucosyl fluoride (2FGF)	>100	1.5	49[b]
2FGF + glycogen			25[b]
[^{14}C]glucose + glycogen	32	8.5	693[c]
ϕ-dextrin (combined assay)	0.063	11	465 000[d]

[a] 9.6% glycogen. [b] Estimated from v vs [S] plots since K_m's are too high to measure accurately. [c] From Nelson and Larner (1970); the estimate of V_m from their data is uncertain. [d] From Nelson et al. (1969); K_m is given in terms of branch points.

data are those expected for an enzyme acting consecutively on first a good substrate and then a poor substrate and indicate that no significant net transfer of glucose occurs from the glucosyl fluoride to a polymeric glucose acceptor. Maltopentaose also caused an equal acceleration of glucose and fluoride release but, unlike glycogen, no extra glucose was released after the glucosyl fluoride was all reacted.

Values of V_{max} and K_m determined are presented in Table I, and, as can be seen, the value of V_{max} for α-D-glucosyl fluoride is increased over 7-fold by the addition of glycogen, while the K_m increases some 3-fold. This increase in V_{max} is presumably a consequence of a favorable conformational change induced by the binding of the polysaccharide. The increase in K_m may well be due to competition of the glycogen for the glucosidase site.

The use of α-glucosyl fluoride provides a convenient method for measuring the glucosidase activity of the debranching enzyme without the complication of having to have glycogen present, as in the [^{14}C]glucose incorporation assay. In order to ensure that its hydrolysis was occurring at the glucosidase site and not the maltotriosyl transfer site, the effects of known specific inhibitors of the glucosidase activity were tested. Deoxynojirimycin is a reversible inhibitor of the glucosidase activity and, at 10 mM α-glucosyl fluoride, the concentration yielding 50% inhibition was 0.8 μM, in the same range as that obtained by Bollen and Stalmans (1989) using the [^{14}C]glucose incorporation assay. Kinetic analysis of the inhibition yielded complex patterns, suggesting binding of the inhibitor at a site additional to the one binding α-glucosyl fluoride, and making it difficult to calculate a conventional K_i. Gillard and Nelson (1977) showed that Bistris is another potent specific inhibitor of the glucosidase. This is also true of α-glucosyl fluoride hydrolysis, since a 10 mM concentration of Bistris caused 97% inhibition. These results are therefore consistent with the hydrolysis of α-glucosyl fluoride occurring at the site that normally hydrolyzes the α-(1,6) glucosyl linkage of the limit dextrin.

*2-Deoxy-2-Fluoro-*D*-Glucosyl Fluorides as Inactivators?* Since 2-deoxy-2-fluoroglucosyl fluorides have been shown to be good inactivators of retaining glucosidases, these compounds were tested with the debranching enzyme. If the glucosidase activity could be selectively inactivated, it might be possible to demonstrate directly whether the enzyme has a single active site or two by determining whether the transferase activity was inactivated or not. It might also be possible to use this reagent to identify glucosidase active-site residues. Instead of causing the anticipated inactivation, 2-deoxy-2-fluoro-α-D-glucosyl fluoride turned out to be a poor substrate; the kinetic parameters determined are shown in Table I. While normal saturation kinetics were seen with α-glucosyl fluoride, the 2-deoxy-2-fluoro-α-glucosyl fluoride showed such poor binding that only essentially first-order relationships between substrate concentration and rate could be observed at accessible substrate concentrations. The k_{cat}/K_m data recorded in Table I were therefore calculated from the slopes of these plots. These

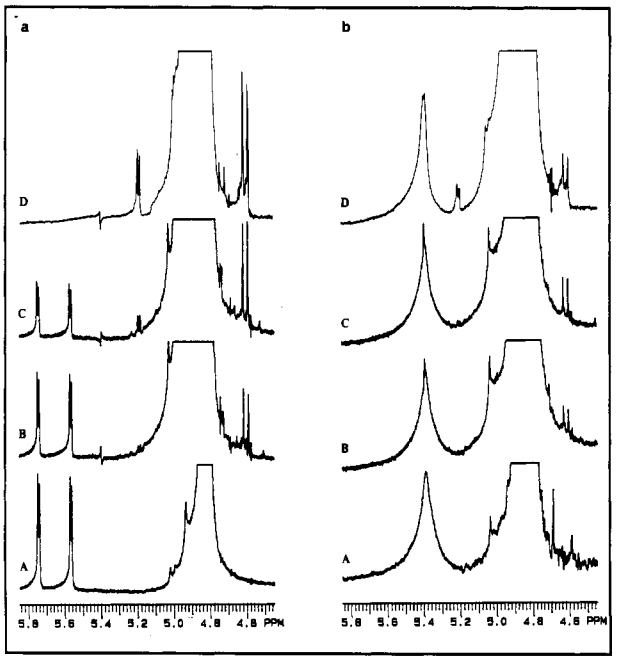

FIGURE 2: Determination of the stereochemistry of glycogen debranching enzyme action by ^{1}H NMR. (a) Hydrolysis of α-D-glucosyl fluoride (18 mM) by debranching enzyme (0.4 mg) in 100 mM phosphate buffer (0.5 mL), pH 6.8. Spectrum A, time = 0, before enzyme addition; spectrum B, time = 17 min; spectrum C, time = 33 min; spectrum D, time = 24 h. (b) Hydrolysis of limit dextrin (28 mg) by debranching enzyme (0.4 mg) in 50 mM phosphate buffer (0.5 mL), pH 6.8. Spectrum A, time = 0, before enzyme addition; spectrum B, time = 15 min; spectrum C, time = 30 min; spectrum D, time = 72 h.

results show that 2-deoxy-2-fluoro-α-glucosyl fluoride is a poor substrate by virtue of its poor binding since the turnover rate is reasonable compared to the other substrates. In order to further probe the binding of 2-deoxy-2-fluoro-α-glucosyl fluoride it was tested as an inhibitor of α-glucosyl fluoride hydrolysis. It was found that a 56 mM concentration of this compound caused only 17% inhibition of the hydrolysis of 8 mM α-glucosyl fluoride (K_m = 2.8 mM), confirming the poor binding of the inhibitor. The β-anomer of this compound was not a substrate (data not shown). For comparison with these artificial substrates, the superiority of limit dextrin is shown in Table I by data taken from the literature.

The failure to incorporate the glucose moiety into polymeric glucose acceptors as well as the failure of 2-deoxy-2-fluoro-α-D-glucosyl fluoride to inactivate the enzyme are not what one would necessarily expect from a retaining glucosidase. We therefore reexamined the stereochemistry of the reaction. Figure 2a depicts the partial ^{1}H NMR spectra of α-glucosyl fluoride and its glucose product formed during the course of the debranching enzyme reaction. Spectrum A shows the anomeric proton region of the spectrum of α-glucosyl fluoride in buffer. The large double doublet centred at δ 5.66 arises from the anomeric proton while the broad resonance around

δ 4.84 is due to water. Spectra B, C, and D were recorded at different times after addition of glycogen debranching enzyme. As can be seen clearly, the decrease in intensity of the substrate anomeric proton resonance is accompanied by the rapid appearance of a resonance at δ 4.60 (doublet, J = 7.8 Hz) due to the anomeric proton of β-D-glucose. Subsequently a resonance appears at δ 5.20 (doublet, J = 3.75 Hz) from α-D-glucose that is formed by mutarotation of the initially formed β-D-glucose. Such mutarotation is complete within 24 h as shown in spectrum D, when the normal anomeric ratio (64% β:36% α) has been established. These data show unequivocally that the initial product of enzymic hydrolysis of α-D-glucosyl fluoride is β-D-glucose. In order to ensure that this result was not a peculiarity associated with the use of this activated artificial substrate, the experiment was repeated with the natural substrate, limit dextrin; the results are shown in Figure 2b. In this case the broad resonance at δ 5.39 is due to the anomeric protons of the α-(1,4) linkages. The resonance due to the α-(1,6) linkages is concealed under the water peak. Once again the initial product of enzyme action is seen to be β-D-glucose, as witnessed by the rapid appearance of the resonance at δ 4.62 and only subsequent appearance of the resonance due to α-D-glucose. It is not at all clear why this result is the opposite to that obtained by Nelson and Larner (1970b), using polarimetric methods, but the direct observation of the glucose species as it is formed suggests confidence in the NMR results. In addition, inversion of configuration is consistent with the results discussed above, since the 2-deoxy-2-fluoro-α-D-glucosyl fluoride cannot inactivate inverting enzymes since they have no intermediate to trap, nor can such enzymes act as transferases using substrates of the correct anomeric configuration. It is extremely interesting, therefore, that the two reactions catalyzed by the enzyme (glycosyl transfer and glycoside hydrolysis) occur with completely different stereochemistry, glycosyl transfer occurring with retention and glycoside hydrolysis with inversion. This therefore provides additional strong evidence for the hypothesis originated by Nelson and his colleagues and summarized in the review by Nelson et al. (1979) that the two activities occur at different catalytic sites, since it is highly unlikely that a single active site would be capable of catalyzing two mechanistically different reactions.

In a separate, related NMR experiment it was shown directly that hydrolysis of the limit dextrin under these conditions probably occurs only through cleavage of α-(1,6) linkages. This was achieved by setting up two separate reaction mixtures identical with those employed in the previous experiment, adding enzyme as before, and stopping them at time = 0 and 30 min by placing in a hot water bath (90 °C) for 5 min. ^{1}H NMR spectra of these samples were then recorded at a high temperature (80 °C) in order to sharpen lines and, more importantly, to move the water peak upfield and away from the resonance due to the anomeric protons of the α-(1,6) linked residues. Integration of the resonances due to the α-(1,4)- and α-(1,6)-linked anomeric protons revealed that the number of α-(1,6)-linked residues dropped significantly during the reaction since the ratio of α-(1,4):α-(1,6) increased from 8.2:1 at time = 0 to 11.4:1 at time = 30 min.

β-Glucosyl Fluoride. Hehre et al. (1979) have demonstrated that an inverting glycosidase such as β-amylase can utilize both the α- and β-anomers of its glycosyl fluoride. Thus β-amylase hydrolyzes α-maltosyl fluoride directly, generating β-D-glucose, but hydrolyzes β-maltosyl fluoride by first transferring the maltose moiety of β-maltosyl fluoride to another molecule of substrate, or to another suitable acceptor,

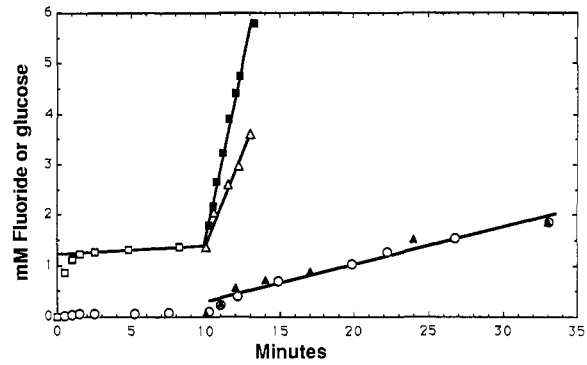

FIGURE 3: Production of fluoride and glucose from β-glucosyl fluoride and α-glucosyl fluoride in the presence of maltohexaose. Solid glucosyl fluorides were added at 0 min to yield calculated concentrations of 12.5 mM. Solid maltohexaose was added at 6 min to yield approximately 25 mM. Debranching enzyme (0.117 mg) was added to the 2.0 mL incubation mixtures at 10 min. The total measured fluoride concentration is shown but the glucose concentrations were corrected to equal the fluoride concentrations at 10 min. (□) Fluoride released spontaneously from β-glucosyl fluoride, rate after lag period is the equivalent of 0.3 μmol min^{-1} mg^{-1}; (■) fluoride released from β-glucosyl fluoride after addition of enzyme, rate = 22.4 μmol min^{-1} mg^{-1}; (△) glucose released from β-glucosyl fluoride after addition of enzyme, rate = 12.0 μmol min^{-1} mg^{-1}; (○) mM fluoride released from α-glucosyl fluoride, rate after addition of enzyme = 1.3 μmol min^{-1} mg^{-1}; (▲) glucose released from α-glucosyl fluoride after addition of enzyme.

and then rapidly hydrolyzing the newly formed α-anomeric bond. The ultimate product is again β-glucose. We therefore tested the activity of the debranching enzyme with β-glucosyl fluoride. The addition of enzyme to a buffered sample of β-D-glucosyl fluoride did not increase the rather large rate of spontaneous hydrolysis of this labile compound, but the further addition of maltopentaose to this sample caused a very large increase in the rate of fluoride release (data not shown). The rate of glucose release was found to be only 53% of the rate of fluoride release, as is shown in Figure 3. For comparison, the rates of release of fluoride and glucose from α-glucosyl fluoride in the presence of maltohexaose are also shown in Figure 3, and these are found to be exactly equivalent. This suggests that the debranching enzyme indeed hydrolyzes the β-glucosyl fluoride by a two-step mechanism and utilizes the oligosaccharide as an initial acceptor of the glucose moiety, forming a new α-(1,6) link which it subsequently hydrolyzes. It may be added that in the final stages of the progress curve, when all the β-glucosyl fluoride is gone, the glucose and fluoride level off at approximately the same value (data not shown).

The data in Figure 3 suggest that not only is the hydrolysis of the presumed branched maltoheptaose intermediate slower than the initial transfer reaction but also a significant proportion of the intermediate accumulates in the earlier stages of the reaction. Attempts were made to observe this intermediate directly by ^{1}H NMR, but these were unsuccessful, largely due to the number of resonances in this region, especially the anomeric proton resonances of maltopentaose. Our attention therfore turned to the use of another type of oligosaccharide on which the enzyme acts, the cyclodextrins, since such compounds have no reducing (hemiacetal) anomeric centers and therefore have less cluttered NMR spectra.

Taylor and Whelan (1966) demonstrated that the debranching enzyme will hydrolyze glucose from glucosyl-α-(1,6)-cyclomaltohexoase (Schardinger α-dextrin), and one would therefore predict that the cyclodextrin would act as an acceptor for glucose from β-glucosyl fluoride. We chose to use cyclomaltoheptaose (β-cyclodextrin) instead of the α-cyclodextrin because the resonance due to the glycosidic anomeric

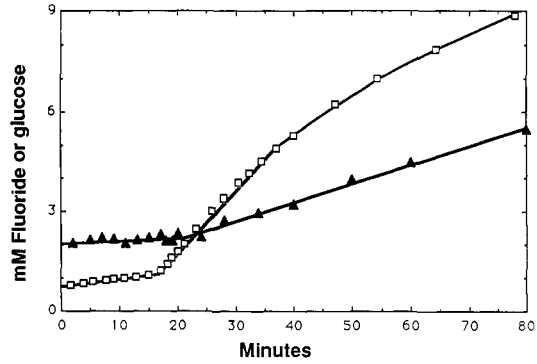

FIGURE 4: Production of fluoride and glucose from β-glucosyl fluoride in the presence of β-cyclodextrin. The incubation mixture of 2.0 mL contained 15 mM β-glucosyl fluoride, dissolved at zero time in 0.1 M sodium phosphate and 1 mM EDTA buffer, pH 6.7. Debranching enzyme (0.16 mg) was added at 6 min and solid cyclomaltoheptaose was added at 16 min to yield 9.4 mM. Values for fluoride and glucose are uncorrected. (□) fluoride; (▲) glucose.

protons of β-cyclodextrin occurs at slightly lower field than that for α-cyclodextrin and thus slightly further away from the presumed resonance position of the α-(1,6) linkage being sought. Figure 4 shows the effect of the β-cyclodextrin on the rate of production of fluoride and glucose from β-glucosyl fluoride in the presence of glycogen debranching enzyme. In this case the rate of release of glucose was considerably lower than the rate of release of fluoride, providing a more favorable situation for observing an intermediate than had been seen with maltopentaose. Differences between these two reactions might possibly be due to the putative glucosyl-α-(1,6)-cyclomaltoheptaose not being as suitable a substrate for the debranching enzyme as the linear analogue.

Figure 5 shows the anomeric proton regions of a series of ^{1}H NMR spectra of an incubation mixture similar to that analyzed chemically in Figure 4. These spectra were recorded at different times after addition of enzyme. Spectrum A is that before addition of enzyme, and the principal peaks are as follows. The large double doublet centered around δ 5.22 (J = 54 and 3 Hz) is due to the anomeric proton of β-glucosyl fluoride, and the small resonances at δ 5.20 and 4.62 are due to α-D-glucose and β-D-glucose, respectively, which arise from decomposition of the β-glucosyl fluoride. The large resonance at δ 5.06 arises from the anomeric protons of the α-(1,4) linkages of the β-cyclodextrin, while that at 4.72 is due to water. Addition of debranching enzyme brings about the rapid appearance of a new resonance at δ 4.93 (doublet, J = 3.7 Hz), which we assign as the newly formed α-(1,6) linkage between the glucose and the β-cyclodextrin. The coupling constant and chemical shift are essentially identical with those determined both in this work and previously (Gidley, 1985; Withers, 1990) for the anomeric protons of suitable reference materials such as isomaltose, glycogen, and glycogen limit dextrin. In addition, the resonance at δ 4.62 due to β-D-glucose has increased significantly, with relatively little change in the resonance due to α-D-glucose. As the reaction progresses (spectra B–E), there is at first a buildup of the resonance at δ 4.93 due to the intermediate, as well as the resonance at δ 4.62 due to the β-D-glucose, and then the resonance due to the intermediate decreases and finally, after 24 h (spectrum F), has disappeared completely. At the same time, there is a rapid buildup of the β-glucose resonance, but then ultimately this resonance decreases, with concomitant increase in the resonance due to α-D-glucose such that after 24 h the expected equilibrium ratio (64:36) of the two glucose anomers is observed. These data therefore clearly indicate the initial formation of an α-(1,6)-linked glucosyl transfer product from the β-glucosyl

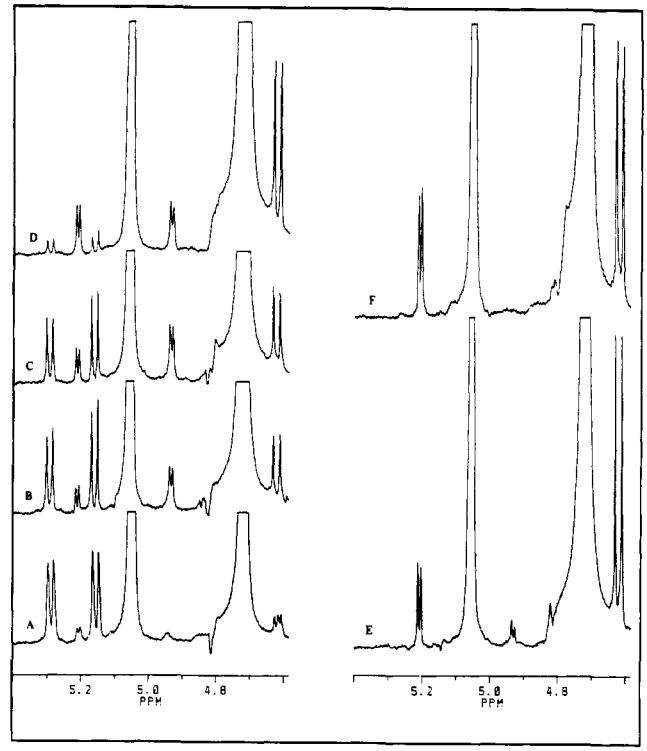

FIGURE 5: ^{1}H NMR investigation of the action of debranching enzyme on β-glucosyl fluoride in the presence of β-cyclodextrin. (A) β-Glucosyl fluoride (40 mM) plus β-cyclodextrin (18 mM) in 150 mM phosphate buffer (0.5 mL), pH 6.8, just prior to addition of enzyme. (B) 16.5 min after addition of debranching enzyme (0.1 mg). (C) 26 min after addition of enzyme. After 30 min a second aliquot (0.16 mg) of debranching enzyme was added and spectra D and E were collected after a total of 40 and 52 min from the first enzyme addition. Spectrum F was recorded after 24 h.

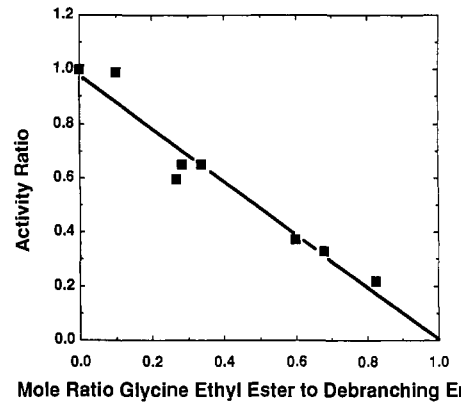

FIGURE 6: Relationship between the incorporation of [^{14}C]glycine ethyl ester, in the presence of carbodiimide, into debranching enzyme and the loss of activity when measured by the combined assay.

fluoride and cyclodextrin, followed by the hydrolysis of this intermediate, again with inversion of anomeric configuration, to give β-D-glucose. These data are exactly in line with those anticipated on the basis of the results obtained by Kitahata et al. (1981), where they were able to demonstrate the formation of α-glucosyl glucosides from β-glucosyl fluoride and an acceptor methyl α-D-glucopyranoside by two inverting glucosidases, glucoamylase, and glucodextranase.

Carbodiimide Inhibition. Using the combined glucosidase–transferase assay, we confirmed the unpublished results of Nelson et al. (1979) that the debranching enzyme can be inactivated by a water-soluble carbodiimide in the presence of an amine. Figure 6 shows that incorporation of a single mole of glycine ethyl ester per mole of enzyme results in complete inactivation. To determine which catalytic activity

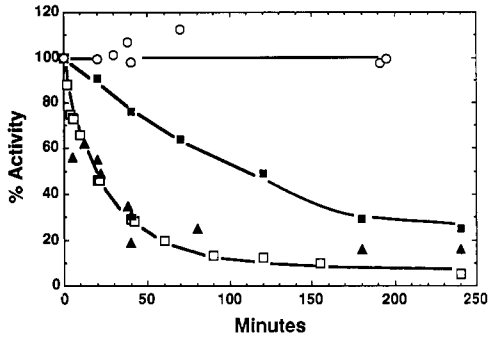

FIGURE 7: Inhibition of debranching enzyme by carbodiimide and glycine ethyl ester as measured by four different assay methods. (○) Glucosidase activity measured by the release of fluoride from α-glucosyl fluoride; (■) glucosidase activity measured by the incorporation of [^{14}C]glucose into glycogen; (▲) transferase activity; (□) combined assay method.

was being affected, we assayed the enzyme by four different methods during the reaction with carbodiimide plus glycine ethyl ester. Figure 7 shows that inactivation of the transferase activity was paralleled by the loss of the combined activity, whereas the glucosidase activity, as measured by the hydrolysis of α-glucosyl fluoride, was unaffected. The results suggest the reaction of a single carboxyl group at the catalytic site responsible for the transferase activity. This experiment provides the first example of covalent modification of the transferase site only, clearly differentiating it from the glucosidase site. When we examined the effect of the carbodiimide on the glucosidase activity as measured by [^{14}C]glucose incorporation into glycogen, we observed a slow inactivation. Under the conditions of this experiment, the apparent first-order rate of inactivation of transferase was identical with that of the loss of the combined activity with a constant of 0.036 min^{-1}, while the glucosidase inactivation as measured with [^{14}C]glucose had an apparent k_{1st} of 0.0067 min^{-1}. All three activity losses followed apparent first-order kinetics for at least the first 75% of the reaction, followed by a gradual decrease in rate. The decreased rate of [^{14}C]glucose incorporation into glycogen suggests that glycogen binding might be affected by the modification of the transferase site. Glycogen (2%) and amylopectin (1.7%) provided slight protection against inhibition by carbodiimide, reducing the rates of inhibition to 83% and 62%, respectively.

Conclusions. The results described in this paper expand our understanding of the mechanism of action of this enzyme and provide substantial support on two fronts for the notion that the enzyme has two separate active sites rather than a single one that performs two different functions. First, the fact that the two reactions proceed with quite different stereochemical outcomes (retention in the case of the transferase and inversion in the case of the glucosidase) argues strongly for two different active sites since two entirely different mechanisms must obtain. Second, since the transferase activity can be inactivated without significant effect upon the glucosidase activity by treatment with a water-soluble carbodiimide plus an amine, it is again unlikely that the two reactions occur at the same site.

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