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Amylo-1,6-glucosidase/4-α-Glucanotransferase: Use of Reversible Substrate Model Inhibitors to Study the Binding and Active Sites of Rabbit Muscle Debranching Enzyme†

Baiba K. Gillard‡ and Thomas E. Nelson*

ABSTRACT: The mammalian glycogen debranching enzyme amylo-1,6-glucosidase/4-α-glucanotransferase is a eucaryotic enzyme which possesses two distinct activities on a single polypeptide chain. The transferase (1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and glucosidase (dextrin 6-α-glucosidase, EC 3.2.1.33) comprise the glycogen phosphorylase limit dextrin debranching system in muscle. The two activities appear to be located at separate catalytic sites on the enzyme molecule. The relationships between the catalytic and binding sites on this bifunctional enzyme have been investigated with the use of reversible substrate model inhibitors. Polyhydroxyamines were found to inhibit glucosidase activity. The best inhibitor was Nojirimycin (5-amino-D-glucose), with a $K_i = 3.9 \times 10^{-6}$ M, compared with a $K_m = 4.3 \times 10^{-2}$ M for glucose. The amine inhibitors are noncompetitive with phosphorylase limit dextrin, but competitive with glucose. The strength of binding indicates that the amines are transition state analogues and apparently mimic the structure of an activated glucosyl ion complex formed by the glucosyl-enzyme intermediate during hydrolysis. The effectiveness of inhibition increases with increasing acidity of the conjugate acid of the amine, with a Brønsted slope of 1.3. Binding of inhibitor to enzyme occurs with proton transfer from the conjugate acid of the amine to an enzyme amino acid residue whose pK_a is about 8.5. Transferase activity was not measurably inhibited by any of the compounds tested. Inhibition of glucosidase, without concomitant inhibition of transferase, provides further evidence for two distinct active sites on the debranching enzyme molecule. α-Schardinger-dextrin (cyclohexaamylose) and glycogen were both competitive inhibitors of debrancher action on phosphorylase limit dextrin. Apparently, polymer binding for transferase and glucosidase action does not occur at two independent binding sites, but rather at a single site or at sites which overlap or interact very strongly. Based on these results we propose a mechanism of action for the debranching enzyme in which polysaccharide binding serves to coordinate the action of the two catalytic sites on the enzyme, so as to allow a concerted degradation of the branched chain of the limit dextrin structure.

Rabbit muscle glycogen debranching enzyme (amylo-1,6-glucosidase/4-α-glucanotransferase) is a multicatalytic site protein which possesses two distinct activities on a single polypeptide chain (mol wt 160 000–170 000) and is the first such eucaryotic enzyme reported which is active as a monomer (White and Nelson, 1974, 1975; Taylor et al., 1975). The two activities, an oligotransferase (1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25) and a glucosidase (dextrin 6-α-glucosidase, EC 3.2.1.33), comprise the glycogen phosphorylase limit dextrin debranching system of skeletal muscle and are involved in a multienzyme complex with phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) in glycogen degradation (Nelson et al., 1972; Bates et al., 1975; Cohen et al., 1975). The transferase disproportionates the branched symmetric limit dextrin of glycogen formed by phosphorylase to form an asymmetric structure having a single glucosyl residue branch. This occurs by transfer of a maltotriosyl group from the side chain to the main chain. The glucosidase then removes the glucosyl branch with retention of anomeric configuration to produce free glucose and a debranched dextrin with an outer chain again susceptible to further degradation by phosphorylase (Brown and Brown, 1966; Nelson et al., 1969; Nelson and Larner, 1970a).

Studies on the purified rabbit muscle enzyme indicate that the two activities can be measured independently of each other

† From the Marrs McLean Department of Biochemistry (B.K.G.) and Fleming Department of Rehabilitation (T.E.N.), Baylor College of Medicine, Houston, Texas 77030. *Received March 18, 1977.* This work was supported in part by grants from the United States Public Health Service, National Institutes of Health, Grants AM-13950 and AM-17978, and The Robert A. Welch Foundation (Q-402). B.K.G. was a Postdoctoral Fellow of The Robert A. Welch Foundation and the National Institutes of Health (I-F02-AM-57255).

‡ Present address: Department of Pediatrics, School of Medicine, University of California, Los Angeles, California 90024.

as well as by their combined action on glycogen phosphorylase limit dextrin (ϕ-dextrin).[1] The glucosidase will remove glucose from single unit branched linear oligosaccharides such as the pentasaccharide, 6^3-α-glucosylmaltotetraose, "fast B_5" (Brown and Brown, 1966) and from single unit branched cyclic dextrins such as α-glucosyl α-Schardinger dextrin, α-(1→6)-glucosyl cyclohexaamylose (Taylor and Whelan, 1966). The glucosidase can also catalyze the reverse action of glucose incorporation into glycogen (Larner and Schliselfeld, 1956; Hers et al., 1967) as well as other carbohydrate acceptors (Nelson and Larner, 1970a,b; Stark and Thambyrajah, 1970). In both the forward and reverse directions, the glucosidase proceeds via a glucosyl-enzyme intermediate (Nelson and Larner, 1970a). The action of the transferase is also reversible. It will transglycosylate maltotriosyl and to a lesser extent maltosyl groups from the side chains of branched oligodextrins (Brown and Brown, 1966, 1967). The transferase will elongate the outer tier chains of glycogen, amylopectin, and ϕ-dextrin and can transfer maltotriosyl groups to cover up exposed glucosyl unit branches in glycogen (Brown et al., 1966; Brown and Brown, 1966; Hers et al., 1967; Nelson et al., 1970). The combined action of the two activities of purified debranching enzyme, when measured by glucose production, is neglible on amylopectin and only slight on glycogen compared with the action on ϕ-dextrin, indicating a high degree of specificity for the outer tier branch chain length of four glucosyl residues (Nelson et al., 1969; Lee and Carter, 1973). Various assay systems have been developed as a result of these findings which measure the individual transferase and glucosidase activities as well as the combined transferase-glucosidase activity (cf. Lee and Whelan, 1971).

Several lines of evidence indicate that the transferase and glucosidase activities occur at distinct catalytic sites. The pH optima of the two activities are different, suggesting that their active sites have different catalytic groups (Nelson et al., 1970). Also the two activities can be altered to different extents. Reversible inhibitors of the glucosidase have no effect on the transferase (Nelson et al., 1970). The apparent absence of transferase but not glucosidase activity has been proposed as a subclass of type III glycogen storage disease (Van Hoof and Hers, 1967). Brown et al. (1973) found that exposure of debranching enzyme to guanidine inhibited glucosidase activity on 6^3-α-glucosylmaltotetraose ("fast B_5") to a greater extent than the combined activity on 6^3-α-maltotriosylmaltotetraose (B_7). Nelson and Watts (1974) found that inactivation of the debrancher under various conditions by temperature and urea affects transferase activity on amylopectin to a greater extent than it affects glucosidase glucose incorporation into glycogen. Bates et al. (1975) have reported that partial proteolysis by exposure to trypsin or chymotrypsin destroys the combined activity to a greater extent than the glucosidase activity. These results suggest that the transferase and glucosidase activities are located at separate sites on the enzyme molecule.

The interaction between these two active sites is not yet known. There is some evidence for cooperative conformational changes in the enzyme upon polymeric substrate binding (Nelson and Watts, 1974). To further define the relationship between the debranching enzyme active sites and binding sites, we have investigated the effect of a series of reversible inhibitors which were chosen to structurally define or "map" the glucose and polysaccharide binding sites of the glucosidase and transferase activities. Our results confirm that this enzyme possesses two distinct active sites for transferase and glucosidase action on ϕ-dextrin. They also indicate that the polysaccharide binding sites for the two activities are either the same site or two sites which overlap or interact very strongly. This close relationship between the polymer binding site(s) and the catalytic sites is consistent with cooperative action between the two activities.

Experimental Section

Methods

Determination of Enzymatic Activity. The combined activity on ϕ-dextrin was determined by the procedure described by Nelson et al. (1969), using the standard (static) enzyme assay. The static assay reaction mixture contains ϕ-dextrin in maleate buffer at pH 6.5. The enzymatic reaction is stopped by heat inactivation and the glucose produced detected using an enzymatic spectrophotometric method. In order to conserve on substrate, the coupled (dynamic) assay described by Nelson et al. (1969) was also used where noted. The coupled enzyme assay mixture contains both ϕ-dextrin and the enzymatic glucose detecting system in glycylglycine buffer at pH 7.2. In both the reaction mixtures, 7.5 mM DTT was used in place of the 10 mM 2-mercaptoethanol used by Nelson et al. (1969). Reactions were conducted at 30 °C in a thermostated bath or thermostated spectrophotometer cell compartment. The optical density was measured with a recording Gilford Model 240 spectrophotometer equipped with digital readout.

The glucosidase activity was determined by measuring [^{14}C]glucose incorporation into glycogen, following the procedure of Nelson and Larner (1970b). Again, 7.5 mM DTT was used in place of 10 mM 2-mercaptoethanol in the reaction mixture. Glucose having a specific activity of 20 μCi/μmol was used for total glucose concentrations of 1.5–30 mM in the reaction mixture. At glucose concentrations of 30–100 mM in the reaction mixture, the specific activity of the glucose was reduced to 1.5 μCi/μmol and at concentrations of 60–290 mM, to 0.52 μCi/μmol, in order to maintain low background counts at these higher glucose concentrations. The radioactivity incorporated into precipitated glycogen was proportional to the specific activity of the glucose in the reaction mixture, and the amount of glycogen retained on the filter paper was independent of the total glucose concentration. A Nuclear-Chicago Mark I scintillation counter equipped with an IBM 29 data keypunch was used to measure radioactivity. A computer program with 5-point instrument standardization written by Dr. Matthew W. Noall, Baylor College of Medicine, Houston, Texas, was used to analyze the scintillation counter data.

Transferase activity was determined by the modification of the iodine complex spectrum of amylopectin according to the procedure developed by Nelson et al. (1970). Optical density was measured with a Coleman Junior II spectrophotometer, Model 6/20.

Other Analytical Procedures. Protein was determined using an adaptation of the Miller (1959) modification of the Folin–Lowry method (Lowry et al., 1951), as described previously (Nelson et al., 1969). Carbohydrate concentration was determined by the phenol–sulfuric acid method of Dubois et al. (1956). All pH values given are those determined at 25 °C. All buffers and other salts were either in the sodium or the chloride counterion form.

[1] Abbreviations used: ϕ-dextrin, glycogen phosphorylase limit dextrin; DTT, dithiothreitol; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Nojirimycin, 5-amino-5-deoxy-D-glucopyranose, 5-amino-D-glucose; α-SD, α-Schardinger dextrin, cyclohexaamylose; Bistris, 2,2-bis(hydroxymethyl)-2,-2′,2″-nitrilotriethanol; Hydroxyethyltris, 2-(2-hydroxyethylamino)-2-hydroxymethyl-1,3-propanediol; Bis(tris)propane, *N,N′*-bis(tris[hydroxymethyl]methyl)-1,3-diaminopropane; HEPA, 2-hydroxyethyl-3-hydroxypropylamine; DAPH, 3-aminopropyl-2-hydroxyethylamine.

Materials

Debranching Enzyme. The debranching enzyme was purified as previously described (Watts and Nelson, 1972). The enzyme used in these studies came from preparations with specific activities of 6.5 to 8.9 units/mg. The enzyme was stored at 4 °C in 0.05 M Tris–0.005 M EDTA–0.001 M DTT, pH 7.2. For some experiments, the enzyme was dialyzed at 4 °C against 0.05 M maleate–0.005 M EDTA–0.001 M DTT, pH 6.5, to remove Tris prior to use.

Inhibitors. Nojirimycin bisulfite was kindly provided by Dr. S. Inouye (Niwa et al., 1970). D,L-Threitol and amylopectin were gifts from Dr. Betty Lewis, Cornell University, Ithaca, N.Y. Cyclic oxidized DTT and DTE were prepared by the method of Cleland (1964): cyclic DTT 45% yield, mp 127.5 °C uncorr (lit. 132 °C); cyclic DTE 50% yield, mp 126.5 °C uncorr (lit. 132 °C). α-Schardinger dextrin was a gift from Dr. Dexter French, Iowa State University, Ames, Iowa. 1-*S*-Dimethylarsino-1-thio-β-D-glucose was kindly provided by Dr. R. A. Zingaro (Zingaro and Thomson, 1973). Sigma type III rabbit liver glycogen was used after column treatment to remove ionic material (Nelson et al., 1969). All other inhibitors were the best available reagent grade and obtained either from Aldrich Chemical Co., Milwaukee, Wis., or Eastman Organic Chemicals. Inhibitors were made up in either H_2O or enzyme diluent (Nelson et al., 1969) and the solution was adjusted to pH 6.5. These solutions were stored at 4 °C, except for Nojirimycin bisulfate, which was stored at −20 °C.

Other Materials. Glycogen phosphorylase limit dextrin was available in the laboratory, prepared as described previously (Larner et al., 1952). Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim Corp., New York, N.Y. All other materials were of commerically available reagent grade or better. Uniformly labeled [^{14}C]glucose, ca. 200 mCi/mmol, was obtained from New England Nuclear and diluted to the appropriate specific activity.

Results

Three different reaction systems were used to characterize the combined and individual catalytic activities of mammalian debranching enzyme. The combined transferase–glucosidase action of the enzyme was studied by measuring the effect of inhibitors on the rate of formation of glucose from ϕ-dextrin (Nelson et al., 1969). The glucosidase action was studied by measuring the incorporation of [^{14}C]glucose into glycogen (Nelson and Larner, 1970b). The transferase action was followed by changes in the iodine–complex difference spectrum of amylopectin after exposure to debranching enzyme (Nelson et al., 1970). In all cases, the initial rate v was analyzed as a function of substrate and inhibitor concentrations, with the use of Lineweaver–Burke, Eadie, and Dixon plots (Laidler, 1958). The recently published methods of Cornish–Bowden (1974) and Eisenthal and Cornish–Bowden (1974) were also used. The latter method has the advantage of giving a quantitative measure of the goodness of fit of the date to the limiting cases of simple noncompetitive and competitive inhibition patterns. The K_m and K_i values obtained by the various methods of calculation agreed well within experimental error.

Combined Action on ϕ-Dextrin. The K_m value for ϕ-dextrin under the standard conditions was found to be 0.33 ± 0.08 mg/mL. Substrate concentration was varied from 0.5 to 5.0 mg/mL. This K_m value represents the mean and standard deviation for five separate experiments. It agrees well with the value of 0.30 mg/mL obtained by Nelson et al. (1969) under the same conditions.

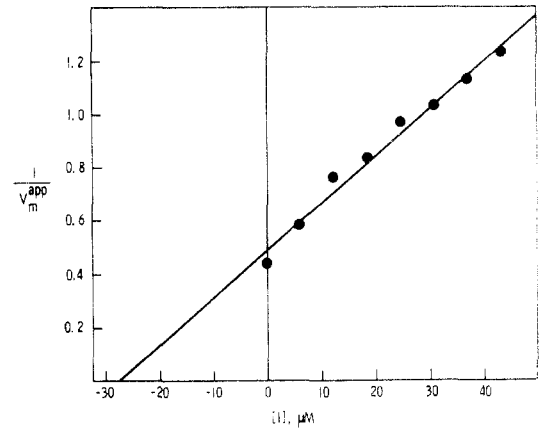


FIGURE 1: Nojirimycin inhibition of debranching enzyme combined activity. V_m^{app} dependence on inhibitor concentration. Reaction rates measured with standard assay procedure. Line represents linear least-squares fit to data points; $r = 0.992$.

Inhibition of Combined Action. Tris Analogues. Tris and structurally related hydroxyalkylamines inhibit the combined action of debrancher on ϕ-dextrin (Nelson et al., 1969). To further define the inhibition specificity of the debrancher, a series of Tris analogues was tested for their inhibitory effectiveness (Table I). Polyols, polyamines, and thiol derivatives were also tested (cf. Table I), as was D-glucano-1,5-lactone and found to have an effect on debranching enzyme. In addition to the compounds shown in Table I, from the effect on the combined activity, the following K_i values were obtained: DTE (100 mM), no inhibition; DTT (100 mM), no inhibition; putrescine, $K_i \simeq 300$ mM; cadaverine (50 mM), no inhibition; spermidine (50 mM), no inhibition; spermine (50 mM), no inhibition. For glucosidase activity, DTE (250 mM) and DTT (250 mM) were found to not inhibit at the noted concentrations. The relative effectiveness of these small molecular weight inhibitors provides information for mapping the glucosidase site.

Nojirimycin, Bistris, Bis(tris)propane, Tris, and erythritol were found to be simple noncompetitive inhibitors (Cleland, 1964) of ϕ-dextrin over a 50-fold range of substrate concentration. The K_i values listed in Table I for these compounds were obtained by an extension of the method of Eisenthal and Cornish–Bowden (1974) to noncompetitive inhibition, as follows: in general, the initial rate v is given by eq 1,

$$v = \frac{V_m^{app}[S]}{K_m^{app} + [S]} \quad (1)$$

where [S] is the substrate concentration, and V_m^{app} and K_m^{app} are the apparent maximum initial velocity and Michaelis constant for a given inhibitor concentration. For simple noncompetitive inhibition, the relationship between V_m^{app} and V_m, and K_m^{app} and K_m is given by eq 2:

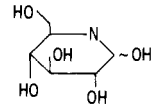
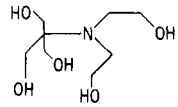
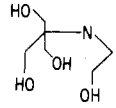
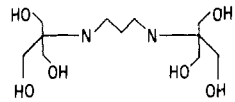
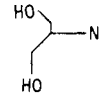
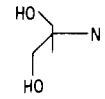
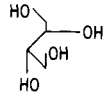
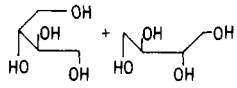
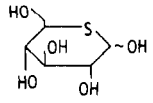
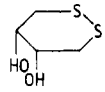
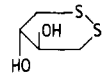
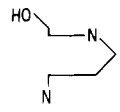
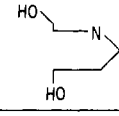
$$V_m^{app} = V_m/(1 + ([I]/K_i)) \quad (2a)$$

$$K_m^{app} = K_m \quad (2b)$$

A plot of $1/V_m^{app}$ vs. [I] gives $-K_i$ as the X-axis intercept (Figure 1).

The effect of some of the inhibitors on the combined activity was determined only at saturating substrate concentration, 5 mg/mL. For these inhibitors, the K_i values were obtained from Dixon (1953) and Cornish–Bowden (1974) plots. Noncompetitive inhibition was assumed for calculating K_i since these compounds are close structural relatives of the compounds determined above to be noncompetitive inhibitors over a 50-

TABLE I: Inhibition of Debranching Enzyme.

No.	Name	Structure[a]	pK_a[j]	Combined act.[b] K_i (mM)	Glucosidase act.[c] K_i (mM)
1	Nojirimycin, 5-amino-D-glucose		5.3	0.024 ± 0.004	0.0039 ± 0.0005
2	Bistris		6.48	1.66 ± 0.05 2.0 ± 0.2[e]	1.39 ± 0.20[d]
3	Hydroxyethyltris		7.83	4.2 ± 0.6[f]	1.7 ± 0.5
4	Bis(tris)propane		~6.9	14.8 ± 0.8	
5	Tris		8.08	11.8 ± 0.7[f] 23 ± 1[g]	6.3 ± 1.0
6	2-Amino-1,3-propanediol		8.4	19 ± 6[f]	3.8 ± 1.0
7	2-Amino-2-methyl-1,3-propanediol		8.80	64 ± 10[f]	27 ± 6
8	*m*-Erythritol			154 ± 11	86 ± 8
9	D,L-Threitol			No inhibition (100 mM)[h]	No inhibition (250 mM)[h]
10	5-Thio-D-glucose			~243[i] (25 mM)[h]	
11	Cyclic DTE			~150[i] (12.5 mM)[h]	No inhibition (31 mM)[h]
12	Cyclic DTT			~370[i] (25 mM)[h]	No inhibition (63 mM)[h]
13	DAPH			~220[i] (50 mM)[h]	
14	HEPA			~120[i] (50 mM)[h]	

[a] Drawn to mimic glucose structure. [b] Rates measured using the standard assay method, unless noted otherwise. [c] Rates measured using the [^{14}C]glucose incorporation assay. Except for Bistris, K_i values calculated from Dixon plots, assuming competitive inhibition. [d] Calculated by method of Eisenthal and Cornish-Bowden (1974). [e] Rate measured using coupled enzyme assay. [f] Calculated from Dixon plot assuming noncompetitive inhibition. [g] Value of Nelson et al. (1969). [h] Maximum concentration tested. [i] Calculated from relative activity in presence and absence of noted inhibitor concentration, assuming noncompetitive inhibition. [j] For dissociation of conjugate acid of amines. References to cited values are given in legend to Figure 4.

fold range of substrate concentration.

α-Schardinger Dextrin Inhibition. In contrast to the small molecular weight inhibitors, α-Schardinger dextrin (α-SD) was found to be a competitive inhibitor of the combined debrancher activity. The coupled assay (cf. Methods) was used in this case to conserve inhibitor. This assay method gave a K_i value for Bistris comparable to that obtained by the standard (static) method (Table I). For α-SD a value of K_i = 0.76 ± 0.05 mg/mL was obtained (Figure 2). The ϕ-dextrin concentration was varied from 0.1 to 5 mg/mL, while the α-SD concentration was varied from 0 to 5 mg/mL. The V_m^{app} values over this range of inhibitor concentration were all within

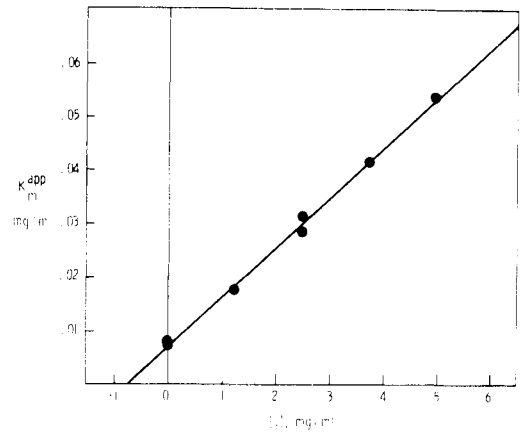


FIGURE 2: α-Schardinger dextrin inhibition of debranching enzyme combined activity. K_m^{app} dependence on inhibitor concentration. Reaction rates measured with coupled enzyme procedure. Line represents linear least-squares fit to data points; $r = 1.000$.

10% of each other, while K_m^{app} increased sevenfold. This behavior of K_m^{app} and V_m^{app} indicates α-SD is a competitive inhibitor of φ-dextrin (Eisenthal and Cornish-Bowden, 1974).

Glycogen Inhibition. The effect of glycogen on debrancher φ-dextrin activity was also determined. Analysis of the rate data is complicated by the fact that glycogen is a poor substrate for the debrancher (Brown et al., 1966; Nelson et al., 1969; Lee and Carter, 1973). To allow for this, reaction mixtures containing both φ-dextrin and glycogen were treated as two-substrate systems. The total observed initial rate of glucose production, v_T, is a sum of the rates of production from φ-dextrin, v_ϕ, and from glycogen, v_{Gn}: $v_T = v_\phi + v_{Gn}$.[2] To obtain v_ϕ values in the presence of glycogen, it was necessary to: determine v_{Gn} in the absence of φ-dextrin; estimate the effect of φ-dextrin on this rate; calculate v_{Gn}^{ϕ}, the rate of glucose production from glycogen in the presence of φ-dextrin; and subtract this rate from v_T to obtain v_ϕ.

Values of v_{Gn} were determined using the standard enzyme assay, with the substitution of glycogen for φ-dextrin in the reaction mixture, and a K_m value of 0.77 ± 0.06 mg/mL was obtained for glycogen. With the addition of φ-dextrin to the reaction mixture, the rate of glucose production from glycogen, v_{Gn}^{ϕ}, is decreased due to enzyme binding with φ-dextrin. The magnitude of this effect can be estimated if it is assumed that the enzyme-polysaccharide complex is in a steady state, and that the enzyme binds only one polysaccharide molecule at a time.[3] Based on this model, v_{Gn}^{ϕ} was calculated from v_{Gn} using eq 3, where K_m^{Gn} and K_m^{ϕ} are the Michaelis constants for glycogen and φ-dextrin, respectively. Values for v_ϕ were then obtained by subtracting the calculated v_{Gn}^{ϕ} from the total observed rate of glucose production, v_T. The v_{Gn}^{ϕ} values used were all less than 5% of v_T.

[2] Since only glucose production is being measured, debrancher binding with glycogen which results in transferase action but not glucosidase action would not contribute to the observed rate, but would contribute to the observed glycogen inhibition.

[3] The latter assumption is equivalent to assuming competitive inhibition of action on glycogen by φ-dextrin. It is supported by the observed competitive inhibition by both α-Schardinger dextrin and glycogen of debrancher action on φ-dextrin. Calculations which are based on assumption of enzyme binding to two polysaccharide molecules simultaneously (mixed or noncompetitive inhibition pattern) result in lower rates of glucose production from glycogen in the presence of φ-dextrin. The difference, however, in v_{Gn}^{ϕ} between the latter model and the competitive inhibition model is less than the experimental error in total rate measurements under our reaction conditions.

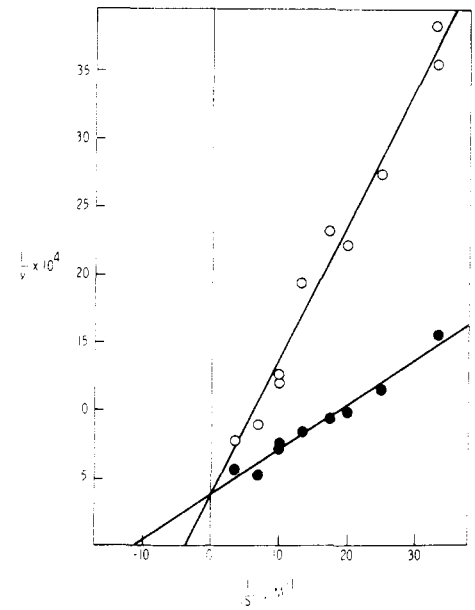


FIGURE 3: Bistris inhibition of debranching enzyme glucosidase activity. Lineweaver-Burk double-reciprocal plot. Reaction rates measured with [^{14}C]glucose incorporation assay procedure. Lines represent linear least-squares fit to data points. (●) With 0.0 mM Bistris, $r = 0.984$; (○) 2.0 mM Bistris, $r = 0.987$.

$$v_{Gn}^{\phi} = v_{Gn}\left[\frac{1 + \frac{K_m^{Gn}}{[G_n]}}{1 + \frac{K_m^{Gn}}{[G_n]}\left(1 + \frac{[\phi]}{K_m^{\phi}}\right)}\right] \quad (3)$$

Analysis of the v_ϕ values obtained in this manner indicates that glycogen is a competitive inhibitor of debranching enzyme catalyzed glucose production from φ-dextrin. For the six sets of data obtained, V_m^{app} decreased at most by 22%, while K_m^{app} increased by more than tenfold over the range of 0 to 5 mg/mL glycogen in the reaction mixture. A value of 0.56 ± 0.14 mg/mL was obtained for the K_i for glycogen. It is of interest to note that debranched φ-dextrin has previously been found to be a competitive inhibitor of debrancher action on φ-dextrin with a K_i of 0.55 mg/mL (Larner and Schlisefeld, 1956). Debranched φ-dextrin would be expected to have a structure analogous to glycogen with the outer tiers removed (cf. Lee and Whelan, 1971).

Glucosidase Action. Glucosidase activity of the debrancher was determined using the [^{14}C]glucose incorporation assay (Nelson and Larner, 1970b). The K_m value obtained for glucose was 43 ± 10 mM. This is comparable to the value of 32 mM reported by Nelson and Larner (1970b) for the same reaction conditions.

The glucose incorporation assay is normally run under first-order conditions with respect to glucose to minimize background radioactivity. Under these conditions, competitive and noncompetitive inhibition cannot be distinguished. In order to determine its mode of inhibition, the effect of Bistris was determined over a glucose concentration range of 30 to 300 mM (Figure 3). In contrast to its noncompetitive effect relative to φ-dextrin, Bistris was found to be competitive with glucose.

Transferase Activity. Transferase activity of mammalian debranching enzyme was followed by changes in the iodine-complex difference spectrum of amylopectin after action by the enzyme (Nelson et al., 1970). None of the compounds tested measurably inhibited the transferase activity at con-

centration levels which inhibit glucosidase activity. Neither 1.25 mM Nojirimycin, 10 mM Bistris, 25 mM hydroxyethyltris, 100 mM Tris, 100 mM 2-amino-2-methylpropanediol, 30 mM 2-amino-1,3-propanediol, 100 mM glucose, 12.5 mM cyclic DTE, 25 mM cyclic DTT, 100 mM D,L-threitol, nor 250 mM erythritol inhibited transferase action on amylopectin. Nelson et al. (1970) also found that Tris does not appreciably inhibit transferase activity.

Discussion

Inhibition Patterns. The small molecular weight inhibitors tested were found to be noncompetitive with ϕ-dextrin, but competitive with glucose. The noncompetitive inhibition observed for the combined action of transferase and glucosidase on ϕ-dextrin indicates that an enzyme–inhibitor complex [EI] and an enzyme–substrate–inhibitor complex [ESI] are formed with equal facility, i.e., that the small molecule inhibitor and ϕ-dextrin are bound at different sites. In the case of glucose incorporation, where only the glucosidase action is measured, the kinetic evidence indicates that inhibitor and substrate (glucose) compete for enzyme to form either an enzyme–inhibitor complex [EI] or an enzyme–substrate complex [ES]. Binding of one compound prevents binding of the other, so that an enzyme–substrate–inhibitor complex [ESI] does not occur. The effectiveness of the inhibitor appears to be related to the stereochemical structure, to the functional groups, and to the basicity of the compound. The inhibition pattern indicates that these small molecular weight compounds block glucose binding, but do not affect polysaccharide binding.

The competitive/noncompetitive inhibition pattern observed here is similar to that of D-glucono-1,5-lactone inhibition of polysaccharide phosphorylase (Tu et al., 1971). In that case, gluconolactone was found to be competitive with glucose 1-phosphate but a noncompetitive inhibitor with both glycogen and arsenate. Based on this inhibition pattern and additional data, Tu et al. (1971) concluded that glucono-1,5-lactone competes for the glucosyl transfer site of polysaccharide phosphorylase. Likewise, D-glucono-1,5-lactone inhibition of debranching enzyme was found to be noncompetitive with ϕ-dextrin ($K_i \simeq 2.2$ mM) and competitive with glucose ($K_i \simeq 0.24$ mM) (White and Nelson, unpublished results). The similar pattern of the other small molecular weight inhibitors tested here suggests that they too bind to the glucosidase glucosyl site, but not to the polymer site. This is confirmed by the observed inhibition of the debrancher by the irreversible inhibitor 1-*S*-dimethylarsino-1-thio-β-D-glucose (Gillard et al., 1973). Preincubation with this inhibitor destroys glucosidase but not transferase activity. The rate of loss of glucosidase activity is proportionately decreased in the presence of Bistris, indicating that the two inhibitors bind at the same or interacting sites (Gillard and Nelson, in preparation).

Enzyme Inhibitor Specificity. A variety of small molecular weight inhibitors were tested to determine the specificity of the debranching enzyme glucosidase activity. Glucosidases in general have been found to be susceptible to competitive inhibition by Tris (Larner and Gillespie, 1956; Halvorson and Ellias, 1958; Jørgensen and Jørgensen, 1967; Semenza and von Balthazer, 1974). Glucosidase inhibition has been attributed to specificity for both the amino group (Larner and Gillespie, 1956) and the hydroxyl groups (Keleman and Whelan, 1966). Our data indicate that both functional groups contribute to inhibitor binding.

Compounds whose only functional groups are amino groups, such as ethylamine (Nelson et al., 1969) and putrescine (1,4-diaminobutane), are weak inhibitors. Larger polyamines such as cadaverine, spermidine, and spermine may fail to inhibit due to their high charge, and/or lack of conformational orientation at the glucosyl site. Substitution of hydroxyl groups into these structures as in HEPA and DAPH (**13** and **14**) increases their effectiveness as inhibitors.

The polyol *m*-erythritol (**8**) inhibits the debrancher, while D,L-threitol (**9**) does not. This can be attributed to the similarity in structure of erythritol and glucose at C_6, C_5, C_4, and C_3 (Keleman and Whelan, 1966). Threitol can be superimposed on glucose at C_2 through C_5 or C_1 through C_4, but not in a configuration which includes C_6. The sulfur analogues, DTE and DTT, do not inhibit at 100 mM, perhaps because the thiol group is larger and less basic than the preferred hydroxyl group. The thiol compounds cyclic DTT, cyclic DTE (**11** and **12**), and 5-thio-D-glucose (**10**) inhibit only slightly, while 1-thio-β-D-glucose at 50 mM did not inhibit. These polyol and thiol inhibitors indicate that the glucosidase site has a strong preference for the D-glucosyl 6-CH_2OH group and for oxygen over sulfur in the ring heteroatom position.

The observed preference for O over S in the ring position has been reported for other glucose binding enzymes. For example, Hoffman and Whistler (1968) have reported that 5-thio-D-glucose is neither a substrate nor inhibitor of glucose oxidase, and that it also does not inhibit yeast hexokinase.

The Tris analogues (inhibitors **2**–**7**) contain both hydroxyl groups and amine groups. Their K_i values are comparable to or smaller than the K_m for glucose. The good inhibition of Bis(tris)propane (**4**), compared with similarly sized spermidine and spermine could be due to enzyme interaction with the hydroxyl groups, as well as to the difference in the proton affinities of the compounds.

A position of prime importance in substrate binding at the glucosidase site appears to be the ring oxygen position. The very good inhibition by Nojirimycin indicates that nitrogen substitution at the ring oxygen leads to stronger interaction between enzyme and inhibitor. For the Tris analogues, such positioning of the nitrogen also leads to similarities in structural conformation between the hydroxyalkyl groups and the glucopyranose ring. In all cases, positioning of the nitrogen on the ring oxygen leads to greater structural similarities with glucose than does positioning the nitrogen on the 1-C oxygen of the α-glucosidic linkage. In this regard 5-amino-D-glucose (Nojirimycin) is a much more potent inhibitor of simple glycosidases than is 1-amino-β-D-glucose (Nina et al., 1970; Reese et al., 1971; Lai and Axelrod, 1973). The hydroxyalkylamines may be most effective when they can bind to the enzyme with the N positioned to mimic the glucosyl ring oxygen.

Brønsted Relationship. It was of interest to determine if the protonated, unprotonated, or both forms of the amine bound to the enzyme. Tris inhibition of yeast α-glucosidase (Halvorson and Ellias, 1958) and intestinal maltase and oligo-1,6-glucosidase (Larner and Gillespie, 1956) has been found to increase with pH, suggesting that the free base is the inhibitory species. A similar conclusion was reached by Jørgensen and Jørgensen (1967) with malt α-glucosidase and Semenza and von Balthazar (1974) in the case of intestinal sucrase. However, these experiments were conducted at pH values at which only the protonated form of Tris ($pK_a = 8.1$) is present in significant concentrations. In contrast to these studies, Nelson et al. (1969) obtained the same K_i value for Tris at pH 6.5 and 7.2, and concluded that inhibition of the debrancher is due to binding of the protonated, conjugate acid form of the amine and involved the hydroxyalkyl substituents. In order to obtain more direct evidence as to whether the protonated or unprotonated species of the amines is inhibitory to the debrancher, the degree of inhibition has been related to the acidity of the conjugate acid of the amine.

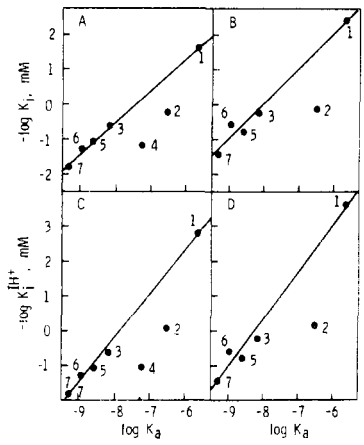


FIGURE 4: Brønsted plots of inhibition constants. Lines represent linear least-squares fit to points for inhibitors 1, 3, 5, 6, and 7, with slope $\alpha \pm$ standard deviation, and correlation coefficient, r. Numbers refer to inhibitors in Table I. (A) Combined activity; K_i values based on total inhibitor concentration; $\alpha = 0.85 \pm 0.07$; $r = 0.98$. (B) Glucosidase activity, K_i values based on total inhibitor concentration; $\alpha = 0.93 \pm 0.15$; $r = 0.96$. (C) Combined activity; K_i values based on concentration of protonated amine; $\alpha = 1.2 \pm 0.1$; $r = 0.98$. (D) Glucosidase activity; K_i values based on concentration of protonated amine; $\alpha = 1.3 \pm 0.2$; $r = 0.97$. Inhibitor K_a values were obtained from the following sources: (1) Inouye (1968); (2) Bates (1970); (3) Lewis (1966); (4) calculated using $\rho^*\sigma^*$ relationship (Wells, 1968), based on Tris pK_a value; (5) Bates and Pinching (1949); (6) calculated using $\rho^*\sigma^*$ relationship (Wells, 1968); values derived from Tris and 2-amino-2-methyl-1,3-propanediol pK_as agree within 0.04 pK unit; (7) Hetzer and Bates (1962). K_a values were corrected for symmetry effects (Wells, 1968).

Our results indicate that the degree of inhibition by Tris analogues increases with increasing acidity of the conjugate acid of the amine inhibitor. A Brønsted plot for the amine inhibitors is given in Figure 4 (Brønsted and Pederson, 1924; Hammett, 1970). In the Brønsted equation, the acid coefficient α (the slope of the plot) relates inhibitor binding to enzyme to inhibitor proton donation to solvent. One can use this linear free energy relationship (Leffler and Grunwald, 1963) to compare the equilibrium constants K_i to the dissociation constants K_a of the conjugate acid of the inhibitors. This relationship can indicate whether or not inhibitor binding to the enzyme occurs with proton transfer and whether proton transfer occurs in the same or the opposite direction as in the acid dissociation where proton transfer occurs from the conjugate acid of the amine to solvent. The observed positive correlation of $-\log K_i$ vs. $\log K_a$ indicates that inhibitor binding to the enzyme occurs with proton donation from inhibitor to enzyme, and, therefore, that the binding species is the protonated or conjugate acid form of the amine, IH^+.

A rough estimate of the acidity of the enzyme residue accepting the proton can be obtained from consideration of the following thermodynamic cycle, in which K_1 and K_3 are dissociation constants for IH^+ and EIH^+, respectively, and K_4 and K_2 are dissociation constants for protonated and unprotonated inhibitor from enzyme.

$$\begin{array}{ccc} E + I + H^+ & \underset{K_2}{\rightleftharpoons} & EI + H^+ \\ K_1 \uparrow\downarrow & & K_3 \uparrow\downarrow \\ E + IH^+ & \underset{K_4}{\rightleftharpoons} & EIH^+ \end{array}$$

The desired dissociation constant, K_3, for the EIH^+ complex, is seen to be related to the other three equilibrium constants by:

$$K_3 = K_1K_4/K_2$$

For Nojirimycin inhibition of the combined activity, $K_4 = K_i = 2.4 \times 10^{-5}$ M and $K_1 = K_a = 5.0 \times 10^{-6}$ M. K_2 represents enzyme binding to neutral inhibitor. K_4 represents enzyme binding to protonated (charged) inhibitor. If enzyme binding to unprotonated (neutral) inhibitor can be approximated by enzyme binding to glucose, then use of the glucose K_m value for K_2 gives $K_2 \simeq 3 \times 10^{-2}$ M. These values give $K_3 \simeq 4 \times 10^{-9}$ M, and p$K_3 \simeq 8.4$. Use of Tris K_a and K_i values in place of Nojirimycin gives p$K_3 \simeq 8.6$. Using Nojirimycin and Tris K_i values for glucosidase inhibition gives p$K_3 \simeq 9.1$. These calculations indicate that the enzyme residue accepting the transferred proton appears to have an acid dissociation constant (pK_a = pK_3) in the range of 8.5–9.1, similar to the pK_a for cysteine, suggesting the presence of this residue at the glucosidase active site.[4] It should be noted that these calculations do not take into account any differences in enzyme conformation on binding the protonated and unprotonated species.

The magnitude of the Brønsted α is related to the extent of proton transfer from inhibitor to enzyme in the enzyme–inhibitor complex. Since we are dealing here with species at potential minima and not with a transition state, the transferred proton would be expected to be covalently bonded to one base rather than being only partially transferred. In our case a value of $\alpha = 1$ would be expected, consistent with complete proton transfer from the inhibitor N to a S, O or N atom on the enzyme. However, the enzyme also interacts with hydroxyl substituents, as evidenced for example by erythritol inhibition. For the Tris analogues the two effects are in the same direction: the number of hydroxyl groups increases with increasing K_a (except for inhibitor **4**). Thus stabilizing interactions in the enzyme–inhibitor complex increase both with increasing K_a and increasing hydroxyl substitution. The extra interaction with hydroxyl groups could lead to values of $\alpha > 1$. Such "abnormal" values of α are observed for cases in which the studied reaction contains interactions not present in the reference acid dissociation reaction (Kresge et al., 1971; White and Jencks, 1976). The value observed here for α suggests complete proton transfer from inhibitor to enzyme, with an additional unknown contribution to α from the interaction of hydroxyl groups with the enzyme, which causes the value of α to deviate from 1.0 (Figure 4C,D).

The inhibitors Bistris (**2**) and Bis(tris)propane (**4**) inhibit to a lesser extent than expected based on inhibitors **1, 3, 5–7** (Figure 4). The former two inhibitors differ from the latter group in that they have substituents which cannot fit into a glucose-like conformation and project into the region of the α-(1 → 6)-branch linkage of ϕ-dextrin. Since this linkage is located at the active site of the enzyme–substrate complex, close interaction between substrate and enzyme residues would

[4]Our data indicate that it is the protonated (charged) amine, not the neutral species, which acts as the transition state analogue; therefore, glucose binding seems a valid approximation for binding of the neutral amine. It is not unreasonable to assume, however, that enzyme affinity for the neutral inhibitor is in the range from enzyme affinity for glucose (substrate-like) to that for gluconolactone (transition state-like). To accommodate the possibility that the neutral amine may bind as a transition state analogue, pK_3 was calculated using gluconolactone K_i values for both combined and glucosidase inhibition to approximate neutral amine binding. In this case values of 6.7–7.4 were obtained for pK_3. The glucose and gluconolactone models give a combined range for pK_3 of 8 ± 1 which should account for substrate-like to transition state-like binding of the neutral amine.

be expected. Therefore, the added substituents on inhibitors **2** and **4** which protrude into this region may result in nonfavorable steric interactions between enzyme and inhibitor and lead to the observed decrease in inhibitory effectiveness.

The Brønsted plot of K_i values for glucosidase activity has a slightly higher, although not significantly different, slope than that for the combined activity. The glucosidase K_i values for the Tris analogues are about one-half the K_i values for the combined activity. For Nojirimycin, the glucosidase K_i is one-sixth that for the combined activity. This increase in inhibitory effectiveness could reflect a slight conformational change in the enzyme–inhibitor complex under the two sets of reaction conditions. Conformational changes upon substrate binding are suggested by the differences in susceptibility of the glucosidase and the transferase to denaturants in the presence of substrate (Brown et al., 1973; Nelson and Watts, 1974).

Glucosidase Mechanism. The inhibitory effectiveness of the small molecular weight inhibitors tested, Nojirimycin, the Tris analogues, D-glucono-1,5-lactone, the polyols, and cyclic thiols, is related to the degree to which they can "mimic" a glucose-like structure, which enables binding to the glucosyl site, and to their acidity, which enhances the binding. The strength of binding of the better inhibitors suggests that they may represent "transition state" analogues (Wolfenden, 1972), and that a charged glucosyl structure may be an intermediate in the enzyme-catalyzed hydrolysis. The occurrence of proton transfer from inhibitor to enzyme during binding indicates that the glucosidase site possesses at least one basic residue which could act as a nucleophile in a double-displacement mechanism (Koshland, 1959) or could participate in ion pair formation in a stereospecific carbonium–oxonium ion mechanism (Mayer and Larner, 1959; Koshland et al., 1962). Either mechanism for glucosidase action is consistent with the evidence that the debrancher hydrolysis proceeds via a glucosyl-enzyme intermediate and that the α-(1 → 6) bond is cleaved with retention of anomeric configuration (Nelson and Larner, 1970a). Both types of hydrolytic mechanism have been proposed as plausible alternatives for certain carbohydrases that retain configuration (cf. Raftery and Rand-Meir, 1968) and the present results cannot distinguish between these possibilities.

Transferase. Transferase activity was not measurably inhibited by any of the Tris analogues tested at concentrations which inhibit glucosidase activity. As noted in the introductory section, the transferase requires at least an α-(1 → 4)-linked maltosyl glycone group for action and prefers a maltotriosyl group. This requirement for a minimum substrate size has also been documented for other carbohydrases, such as α-amylase (Robyt and French, 1970). Small reversible inhibitors such as Nojirimycin or Tris have been found to be ineffective against such carbohydrases (Reese et al., 1971; Gillard et al., 1976). The present observations of glucosidase inhibition without concomitant transferase inhibition are consistent with these earlier observations of inhibitor specificity. They also provide strong additional evidence for the existence of two distinct active sites on the debranching enzyme molecule.

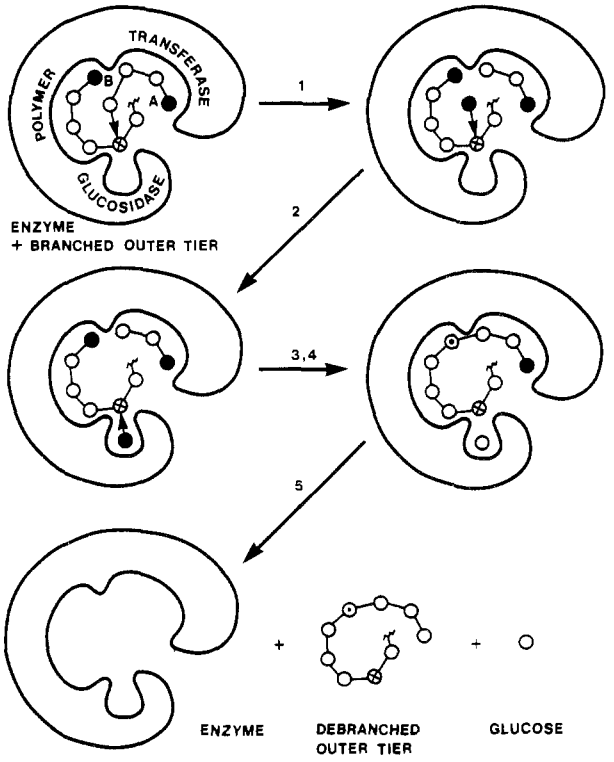
Polymer Binding Site(s). The polysaccharide inhibition patterns were determined to obtain information on the debrancher's polysaccharide binding site(s). The polysaccharide binding site(s) for the two activities could be: (1) two independent sites; (2) two interacting sites; (3) two overlapping sites; or (4) a single site. Case 1 predicts that a polysaccharide inhibitor would have a simple noncompetitive inhibition pattern. Case 2 is consistent with a mixed or competitive pattern. The competitive pattern would indicate that interaction between sites is so strong that binding at one site totally prevents binding at the second site. Case 3 is consistent with mixed or noncompetitive inhibition if substrate and inhibitor can be accommodated simultaneously, or with competitive inhibition if the two cannot be bound simultaneously. Case 4 predicts simple competitive inhibition.

Both α-Schardinger dextrin and glycogen inhibition was found to be competitive with ϕ-dextrin. The observed competitive inhibition pattern indicates that the debrancher does not have two independent polysaccharide binding sites but in fact can only accommodate one polysaccharide molecule at a time. This rules out case 1. Further work with substrates and inhibitors of specified size is necessary to map the polymer binding site to determine definitively if the two activities have a single polymer binding site (case 4), or interacting and/or overlapping sites (cases 2 and 3).

The relative effectiveness of glycogen and α-Schardinger dextrin inhibition provides some information on the shape of the polysaccharide binding site. α-Schardinger dextrin (cyclohexaamylose) is much less effective than glycogen, suggesting that the binding site can accommodate more than six residues of helical polysaccharide structure, or that the bound region of the polysaccharide is not in a rigid helical conformation. Since binding of the α-Schardinger dextrin blocks binding of ϕ-dextrin, it appears that blockage of even a portion of the polymer binding site is sufficient to disrupt effective binding of another polymer. Again, this indicates either a single site for polymer binding, sharing of portions of an overlapping site, or a very strong interaction between separate polymer binding sites.

Model for Action of Debrancher. The debranching enzyme is one of the few enzymes known to possess two distinct active centers on a single polypeptide chain. The observed specificity of the debranching enzyme requires that the enzyme possess at least: a maltotriosyl binding site associated with the transferase active site; a glucosyl binding site associated with its respective active site; and a polymer or aglycone binding site to accommodate polysaccharide chains which can act as donors or acceptors of these glycone moieties. We would like to propose a mechanism for the debranching of ϕ-dextrin that takes advantage of a close proximity between the two active sites and their respective glycone and aglycone binding sites. The evidence indicates that the catalytic portions of the two active sites are distinct, while the polymer binding site(s) may be a set of subsites which either overlap or interact very strongly with each other and with the active sites. It seems unlikely that the transferase product would dissociate completely from the enzyme before action by the glucosidase took place since then the advantage of a bifunctional enzyme would be dissipated (White and Nelson, 1974). A mechanism is therefore suggested in which the transferase product acts in a manner similar to that of the initial product of multiple attack carbohydrases. In these cases, after initial cleavage, the enzyme–product does not dissociate but instead the product shifts to occupy the substrate binding subsites vacated by the initial cleavage product and, in so doing, exposes new bonds to attack (cf. Thoma et al., 1971; French, 1975). In a similar manner, after initial transferase action, in which the maltotriosyl group is removed from the branch chain to leave a single glucosyl moiety, the newly exposed residue may shift or rotate into the glucosidase binding site. This shift could even be one which orients the nonreducing terminal residue of the main chain of the dextrin to allow it to function as an acceptor of the reducing terminal of the maltotriosyl group, thereby facilitating transfer and producing an elongated linear outer chain. Thus it is conceivable that the final maltotriosyl group transfer step and the glucosyl group debranching step could occur in the same enzyme–substrate configuration without a significant shift of

SCHEME I: Action of Debrancher on Limit Dextrin.[a]



[a] Legend: (○) glucosyl residue; (●) nonreducing terminal; (—) α-(1 → 4) linkage; (↓) α-(1 → 6) linkage. Proposed mechanism: (1) transferase removes maltotriosyl group; (2) glucosyl residue shifts to glucosidase site; (3 and 4) maltotriosyl group transferred to nonreducing terminal and (1 → 6) linkage cleaved; (5) release of products.

the order tier main chain in the process.

The transfer of the maltotriosyl group to the nonreducing terminal of the main chain could occur in a configuration analogous to the glucanosyl transfer catalyzed by the *Bacillus macerans* amylase (1,4-α-D-glucan 4-α-glycosyltransferase (cyclizing), EC 2.4.1.19) which forms cyclic or linear dextrins from helical amylodextrins depending upon the length of the chain (cf. French, 1975). Iodine–complex difference spectra suggest a helical configuration for the two φ-dextrin outer tier chains (A and B chains) (Hers et al., 1967; Nelson et al., 1970). A space-filling model of a left-handed double helix configuration of the φ-dextrin outer tier chains indicates that the nonreducing terminal of the main chain (B chain) can be in reasonably close proximity to the reducing terminal of the branch chain (A chain) maltotriosyl group so that transfer could occur without a shift of the entire dextrin molecule.

The essentials of the proposed mechanism are visualized in Scheme I. The coordinated mechanism proposed here, although hypothetical, is consistent with the evidence that the debrancher has two distinct catalytic sites and a polymer binding site or sites which overlap or interact strongly. The model accounts for the observed inhibition patterns and permits simultaneous binding of glucosidase inhibitors and polymer. It is also consistent with separate transferase action and reverse glucosidase action occurring independently of each other. It will be of interest to see if further evidence can be obtained to support such an efficient cooperativity between enzyme catalytic sites and binding sites.

Conclusions

Mammalian debranching enzyme possesses two distinct active sites, a glucosidase and a transferase, on a single polypeptide chain. The glucosidase but not the transferase is inhibited by polyhydroxyamines. Inhibition occurs by binding of the protonated amine, with transfer of the proton from the amine to an enzyme residue with an apparent pK_a of about 8.5. The debrancher does not possess two independent polymer binding sites, but instead either a single site or overlapping or strongly interacting sites. This is consistent with a coordinated interaction between the two activities of the bifunctional enzyme.

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