The Maltase, Glucoamylase and Transglucosylase Activities of Acid α -Glucosidase from Rabbit Muscle

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1. The maltase and glucoamylase activities of acid α -glucosidase purified from rabbit muscle exhibited marked differences in certain physicochemical properties. These included pH stability, inactivation by thiol-group reagents, inhibition by $\alpha\alpha$ -trehalose, methyl α -D-glucoside, sucrose, turanose, polyols, glucono- δ -lactone and monosaccharides, pH optimum and the kinetics and pH-dependence of cation activation. 2. The results are interpreted in terms of the existence of at least two specific substrate-binding sites or sub-sites. One site is specific for the binding of maltose and probably other oligosaccharides. The second site binds polysaccharides such as glycogen. 3. The sites appear to be in close proximity, since glycogen and maltose are mutually inhibitory substrates and interact directly in transglucosylation reactions. 4. Acid α -glucosidase exhibited intrinsic transglucosylase activity. The enzyme catalysed glucosyl-transfer reactions from [14 C]maltose (donor substrate) to polysaccharides (glycogen and pullulan) and to maltose itself (disproportionation). The pH optimum was 5.1, with a shoulder or secondary activity peak at pH 5.4. The glucose transferred to glycogen was attached by α -1,4- and α -1,6-linkages. Three major oligosaccharide products of enzyme action on maltose (disproportionation) were detected. 5. The kinetics of enzyme action on [14 C]maltose showed that the rate of transglucosylation increased in a sigmoidal fashion as a function of substrate concentration, approximately in parallel with a decrease in the rate of glucose release. 6. The results are interpreted to imply competitive interaction at a specific binding site between maltose and water as glucosyl acceptors. 7. The results are discussed in terms of the possible existence of multiple subgroups of glycogen-storage disease type II.

The preceding paper (Palmer, 1971) described the purification and specificity of acid α -glucosidase from rabbit muscle. The enzyme was shown to possess constitutive α -1,4-glucosidase, α -1,6-glucosidase and glucoamylase activities. In this respect, the enzyme is similar to the acid α -glucosidase from other mammalian sources (Torres & Olavarria, 1964; Bruni, Auricchio & Covelli, 1969; Jeffrey, Brown & Brown, 1970a,b).

The present paper describes studies generally in support of a hypothesis, formulated by Jeffrey *et al.* (1970b) but supported by other studies (Rosenfeld & Belenki, 1968; Fujimori, Hizukuri & Nikuni, 1968), which proposes that acid α -glucosidases possess at least two catalytically active substrate-

binding sites. One site is supposedly specific for the binding of maltose as well, perhaps, as other oligosaccharides. The other site(s) is thought to bind polysaccharides such as glycogen. The sites would appear to be in close proximity and hence subject to mutual interaction. The differences in pH optimum and in the kinetics of cation activation of rabbit muscle acid α -glucosidase action on maltose and glycogen (Palmer, 1971) were consistent with the enzyme exhibiting such multiplicity in substrate-binding sites and prompted the present investigation.

Acid α -glucosidase is widely reported to catalyse transglucosylation reactions between maltose and a wide range of compounds that function as glucosyl acceptors (Hers, 1963; Lejeune, Thinés-Sempoux & Hers, 1963; Lukomskaya, 1962; Torres & Olavarria, 1964; Jeffrey *et al.* 1970a). The present paper

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describes studies on the specificity and reaction mechanism of transglucosylation reactions catalysed by rabbit muscle acid α -glucosidase.

MATERIALS AND METHODS

Carbohydrates. Shellfish glycogen was purchased from Mann Research Laboratories, New York, N.Y., U.S.A. and was further purified by treatment with 5% (w/v) trichloroacetic acid (to remove proteinaceous material) and repeated precipitation with ethanol. Maltose was purified by chromatography on charcoal-Celite (Whelan, Bailey & Roberts, 1953; Taylor & Whelan, 1962). D-Allose and D-allono-1,4-lactone were gifts from Dr E. E. Smith. Pullulan was a gift from Dr B. J. Catley. D-[U- 14 C]Glucose and [U- 14 C]maltose were purchased from Amersham/Searle, Des Plaines, Ill., U.S.A. and [14 C]maltose labelled only in the C-1 of the reducing glucosyl unit was a product of Calbiochem, Los Angeles, Calif., U.S.A. All other carbohydrates were obtained from commercial sources and were purified by paper chromatography or recrystallization when necessary.

Enzymes. Acid α -glucosidase was purified from rabbit muscle as described in the preceding paper (Palmer, 1971). Glucose oxidase and horseradish peroxidase, the enzymes comprising the glucose oxidase reagent (Lloyd & Whelan, 1969), were purchased from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. Sweet-potato β -amylase (crystalline) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Yeast amylo-1,6-glucosidase - oligo-1,4 $\rightarrow$ 1,4-glucantransferase (amylo-1,6-glucosidase/transferase) and *Aerobacter aerogenes* pullulanase were gifts respectively from Dr E. Y. C. Lee and Dr C. Mercier. *Aspergillus niger* amyloglucosidase was prepared by the methods in Fleming & Stone (1965) and Qureshi (1967).

Analysis of carbohydrates. Chromatography of maltosaccharides was performed on Whatman no. 3MM paper in solvent systems consisting of ethyl acetate-pyridine-water (10:4:3, by vol.), butan-1-ol-pyridine-water (6:4:3, by vol.) or propanol-nitromethane-water (5:4:3, by vol.). T.l.c. was performed on Eastman Chromatogram sheets (Cellulose 6064; Eastman-Kodak Co., Rochester, N.Y., U.S.A.). The spray reagent was AgNO_3 -NaOH (Trevelyan, Procter & Harrison, 1950).

^{14}C -labelled oligosaccharides were fractionated by paper chromatography and the chromatograms cut into sections corresponding to the position of the respective sugars on a guide strip run simultaneously. The sections were transferred to scintillation vials containing 0.5% (w/v) 2,5-diphenyloxazole and 0.1% 1,4-bis-(5-phenyloxazol-2-yl)-benzene in toluene (10ml) and counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer.

Glucose was determined by the specific glucose oxidase method (Lloyd & Whelan, 1969). The polyglucose content of maltosaccharides and polysaccharides was determined by the successive actions of *A. niger* amyloglucosidase and glucose oxidase (Lee & Whelan, 1966).

Determination of acid α -glucosidase activity. Acid α -glucosidase activity was determined by a modification of the method described in the preceding paper (Palmer, 1971). Unless specifically stated, enzyme (0.05–0.1 ml) was added to substrate (5mm-maltose or 100mg of glycogen/ml) in 10mm-sodium acetate buffer–200mm-

NaCl, pH4.5 (total volume 0.5ml). The reaction was allowed to proceed at 37°C and then stopped by the addition of glucose oxidase reagent (1ml). The E_{540} was read, after acidification (1ml of 9M- H_2SO_4), on a Gilson 240 spectrophotometer.

One unit of acid α -glucosidase activity is defined as the amount of protein that catalyses the hydrolysis of 1nmol of α -1,4-linkage/min at 37°C and at saturating cation concentration (200mm- Na^+).

RESULTS

pH stability of acid α -glucosidase and the effects of thiol-group reagents. The effect of pH on the stability of the maltase and glucoamylase activities of acid α -glucosidase was studied by incubating the protein (45 μg) for 1h at 22°C in 15mm buffers ranging from pH3.4 to pH10. The buffers used were sodium acetate (pH3.4–6.9), sodium phosphate (pH6.3–7.9) and glycine-NaOH (pH9.1–10). The enzyme was stabilized with human serum albumin (final concentration 0.001%). The pH-stability profiles are shown in Fig. 1.

Two regions of maximal pH stability were evident with both activities: one at neutrality, the

Fig. 1. pH-stability of the glucoamylase (Δ) and maltase ($\circ$) activities of acid α -glucosidase. Enzyme (45 μg) was incubated for 1h at 22°C in 15mm buffers ranging from pH3.4 to pH10.0. The buffers used were sodium acetate (pH3.4–6.9), sodium phosphate (pH6.3–7.9) and glycine-NaOH (pH9.1–10.0). Samples of the buffered enzyme solutions were transferred to 200mm-sodium acetate buffer–200mm-NaCl, pH4.5, containing either 100mg of glycogen/ml or 5mm-maltose, equilibrated at 37°C, and α -glucosidase activity on the two substrates was determined. Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

other at the pH optimum. Maximal recovery of activity was achieved at pH 4.5 (maltase activity) and pH 6.9 (glucoamylase activity). The relative activities of α -glucosidase action on maltose and glycogen varied significantly with the pH of enzyme preincubation. This variation in activity ratio implies that the interaction of acid α -glucosidase with maltose and glycogen involves different group(s) with markedly different pH stabilities. The role of cations in enzyme stabilization was not investigated.

Above pH 7.0 both activities decreased progressively and in parallel. Contrary to the report of Bruni *et al.* (1969), thiol-group reagents (2mM-iodoacetic acid, 2mM-iodoacetamide and 0.35mM-*p*-chloromercuribenzoate) did not protect against inactivation at alkaline pH. At pH 8.6 thiol-group reagents substantially increased the rate of inactivation by alkali. At pH 7.0 thiol-group reagents likewise caused enzyme inactivation. With iodoacetate and iodoacetamide at pH 7.0, however, such inactivation was selective. Enzyme action on glycogen was almost completely abolished (residual activity 10%) whereas action on maltose was only marginally decreased (residual activity 73–95%). It is concluded that a thiol or other reactive group(s) is implicated in interaction of α -glucosidase with the polysaccharide glycogen.

Temperature stability of acid α -glucosidase. At 55°C, acid α -glucosidase was rapidly heat-inactivated. Contrary to the report of Fujimori *et al.* (1968), of studies on the heat stability of crystalline acid α -glucosidase from bovine spleen, the activities of the rabbit muscle enzyme on maltose and glycogen were equally thermolabile at 55°C.

Selective inhibition of the action of acid α -glucosidase on glycogen. $\alpha\alpha$ -Trehalose and methyl α -D-glucoside are reported to inhibit selectively and competitively the action of rabbit liver acid α -glucosidase on glycogen and to be without effect on the enzymic hydrolysis of maltose (Rosenfeld & Belenki, 1968). Sucrose was also shown by these authors to exert a threefold greater inhibition of enzyme action on glycogen as on maltose. These studies were performed at an unspecified cation concentration. In view of the demonstration that acid α -glucosidase is subject to cation activation (Jeffrey *et al.* 1970a,b; Palmer, 1971), inhibition of rabbit muscle acid α -glucosidase by potentially selective inhibitors of the glucoamylase activity was investigated over a range of cation concentrations (2–200mM- Na^+).

At 200mM- Na^+ , $\alpha\alpha$ -trehalose, methyl α -D-glucoside and, in addition, sucrose and melizitose, were strictly selective in their inhibition of α -glucosidase action on glycogen. Enzyme action on maltose was not subject to inhibition. At non-saturating cation concentrations (2–25mM- Na^+) (Palmer, 1971), inhibition was not strictly selective

Fig. 2. Double-reciprocal plots showing the inhibition exerted by various concentrations of $\alpha\alpha$ -trehalose on the action of acid α -glucosidase on glycogen. ●, No addition; ○, with 2mM- $\alpha\alpha$ -trehalose; Δ, with 4mM- $\alpha\alpha$ -trehalose; □, with 20mM- $\alpha\alpha$ -trehalose. Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

in that the maltase activity was also marginally inhibited. Raffinose, melibiose, methyl β -D-glucoside, α - and β -lactose and cellobiose, regardless of cation concentration, were not inhibitory to α -glucosidase action on either maltose or glycogen.

To investigate the kinetics of inhibition of the glucoamylase activity by $\alpha\alpha$ -trehalose, methyl α -D-glucoside, melizitose and sucrose, the enzyme was incubated with glycogen in 10mM-sodium acetate buffer–200mM-sodium chloride, pH 4.5, in the presence of different concentrations of each inhibitor. The initial rates of glucose production were measured as a function of glycogen concentration and the result obtained for $\alpha\alpha$ -trehalose inhibition, which is typical of the inhibition exerted by this group of oligosaccharides, is shown in Fig. 2 in the form of double-reciprocal plots. In accord with results of Rosenfeld & Belenki (1968), inhibition was strictly competitive. The inhibition constants were 4.8mM ($\alpha\alpha$ -trehalose), 6.9mM (melizitose), 7.9mM (methyl α -D-glucoside) and 35mM (sucrose). At non-saturating cation concentrations (2–25mM- Na^+) inhibition of the glucoamylase and maltase activities was likewise of a competitive type.

Fig. 3. Double-reciprocal plots showing the inhibition exerted by various concentrations of turanose on the action of acid α -glucosidase on (a) glycogen and (b) maltose. ●, No addition; ○, with 2mM-turanose; △, with 4mM-turanose; □, with 7mM-turanose. Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

Inhibition of acid α -glucosidase by turanose. Turanose is a potent inhibitor of lysosomal acid α -glucosidase (Lejeune *et al.* 1963). Inhibition of

α -glucosidase action on maltose, dependent on the source of the enzyme, is variously described as non-competitive (rat liver; Lejeune *et al.* 1963), competitive (bovine liver; Bruni *et al.* 1969) and mixed (rat liver lysosomes; Jeffrey *et al.* 1970b). The last-named authors demonstrated that the type of inhibition exerted by the oligosaccharide was dependent on the substrate (glycogen, maltose or isomaltose). The kinetics of inhibition by turanose of rabbit muscle acid α -glucosidase action on glycogen and maltose are shown in Fig. 3.

In accord with the results of Jeffrey *et al.* (1970b), turanose inhibition was of a mixed type with either glycogen or maltose as substrate. However, the predominant effect of turanose inhibition on enzyme action on glycogen was on V_{max} . The K_m was only marginally increased and consequently inhibition tended towards a purely non-competitive type. This further strengthens the concept of multiplicity of substrate- and inhibitor-binding sites. Dixon plots gave curved lines from which approximate K_i values were extrapolated of 2mM-turanose (maltose) and 1.4mM-turanose (glycogen).

Acid α -glucosidase action on maltosaccharides is subject to substrate inhibition at concentrations exceeding 5mM (Palmer, 1971). At substrate-inhibitory concentrations of maltose, turanose inhibition was significantly and progressively decreased.

Inhibition of acid α -glucosidase by glucono-(1→5)-lactone. The competitive inhibition of mammalian glycosidases by aldolactones is well documented (Levy, Hay & Conchie, 1964; Levy & Conchie, 1966). The kinetics of inhibition by glucono-(1→5)-lactone (glucono- δ -lactone) of acid α -glucosidase action on maltose and glycogen are shown in Fig. 4.

Inhibition with respect to both substrates was competitive. The inhibition constants were 1.2mM-glucono- δ -lactone (glycogen) and 0.6mM-glucono- δ -lactone (maltose). The differences in K_i , though small, were reproducible. As with inhibition by turanose, inhibition by glucono- δ -lactone of α -glucosidase action on maltose significantly decreased at substrate-inhibitory concentrations of maltose (exceeding 5mM). D-Glucuronic acid and D-glucuronic acid lactone were likewise potent inhibitors of α -glucosidase action. With glycogen as substrate, inhibition was competitive and the calculated K_i values were 3.1mM for D-glucuronic acid lactone and 6mM for D-glucuronic acid. D-Gluconic acid and D-allonic acid γ -lactone were weakly inhibitory and D-glucitol was without effect on enzyme action on glycogen and maltose.

Inhibition of acid α -glucosidase by polyols and tris. The inhibition of glycosidases by polyols is well documented (Kelemen & Whelan, 1966). Polyols are potentially multifunctional inhibitors of glyco-

sidases, and accordingly the kinetics of inhibition are relatively complex. Polyols may function as non-competitive inhibitors of enzyme substrate binding (Kelemen & Whelan, 1966). By displace-

ment of water in the liquid phase of the reaction system, polyols may equally exert inhibition by making water the rate-limiting substrate (Nelson, 1970) and, finally, polyols may exert apparent

Fig. 4. Double-reciprocal plots showing the inhibition exerted by various concentrations of glucono- δ -lactone on the action of acid α -glucosidase on (a) glycogen and (b) maltose. ○, No addition; △, with 2 mM-glucono- δ -lactone; □, with 5 mM-glucono- δ -lactone. Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

Fig. 5(a) and 5(b).

Fig. 5. Inhibition of acid α -glucosidase by polyols. (a) and (b) Double-reciprocal plots showing the inhibition exerted by various concentrations of erythritol on the action of acid α -glucosidase on (a) glycogen and (b) maltose. $\circ$, No addition; Δ , with 20 mM-erythritol; $\square$, with 100 mM-erythritol. (c) and (d) Double-reciprocal plots showing the inhibition exerted by various concentrations of glycerol on acid α -glucosidase action on (c) maltose and (d) glycogen. $\bullet$, No addition; $\circ$, with 50 mM-glycerol; Δ , with 200 mM-glycerol; $\square$, with 500 mM-glycerol. (e) Dixon plots showing the inhibition exerted by glycerol of acid α -glucosidase action on glycogen. The values adjoining each curve refer to glycogen concentration (mg/ml). Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

inhibition by functioning as glycosyl acceptors in transglycosylation reactions. The kinetics of inhibition of the action of acid α -glucosidase on glycogen and maltose by the polyols erythritol and glycerol are shown in Fig. 5.

Inhibition by erythritol was non-competitive (Fig. 5a and 5b). The K_i with respect to both glycogen and maltose was 0.125–0.133 M-erythritol. The kinetics of inhibition by glycerol were relatively complex. With maltose as substrate (Fig. 5c), inhibition was essentially non-competitive, except that at 0.5 M-glycerol a marginal increase in K_m was observed. The K_i was 0.37 M-glycerol. The kinetics of glycerol inhibition of α -glucosidase action on glycogen were dependent on glycerol

concentration (Fig. 5d). At 0.05 M-glycerol, inhibition was non-competitive. At 0.2 M-glycerol, inhibition was apparently of a mixed type, whereas at 0.5 M-glycerol, the double-reciprocal plot was non-linear. Dixon plots gave curved lines from which an approximate K_i of 0.26 M-glycerol was extrapolated (Fig. 5e).

In accord with results of Bruni *et al.* (1969), tris is a competitive inhibitor of acid α -glucosidase. The K_i with either glycogen or maltose as substrates was 7 mM-tris. At substrate-inhibitory concentrations of maltose exceeding 5 mM (Palmer, 1971), inhibition by tris, erythritol and glycerol was substantially decreased.

Inhibition of acid α -glucosidase by monosac-

charides. In accord with the results of Torres & Olavarria (1964), acid α -glucosidase was subject to product inhibition by D-glucose. To test for such inhibition, α -glucosidase was incubated, under initial rate conditions, with D-glucose (0–8.2 mM, unlabelled) and 7.9 mM-[U- 14 C]maltose (1 μ Ci/ml) in 10 mM-acetate buffer–200 mM-sodium chloride, pH 4.5. The 14 C-labelled reaction products were fractionated by paper chromatography as described in the Materials and Methods section. The results (Fig. 6) indicate that glucose is primarily inhibitory to the hydrolytic conversion of maltose into glucose. Only at glucose concentrations exceeding 5 mM was the formation of 14 C-labelled oligosaccharide (transglucosylation) affected. In a parallel experiment in which D-[14 C]glucose and unlabelled maltose constituted the reaction mixture, effectively no 14 C-labelled oligosaccharide was detected. (The significance of these results in terms of the reaction mechanism of transglucosylation is discussed below.)

Secondary to product inhibition by glucose, the following monosaccharides (at a concentration of 25 mM) were inhibitory to α -glucosidase action on glycogen and maltose: D-allose, D-mannose, D-xylose, D-fructose (in order of decreasing inhibition). D-Arabinose and D-galactose were not inhibitory and it was tentatively concluded that, for binding at the monosaccharide inhibition site(s), there was an absolute structural requirement for an equatorial hydroxyl group at C-4.

The kinetics of monosaccharide inhibition of α -glucosidase action on maltose and glycogen were examined only in reference to D-allose (Fig. 7). Allose inhibition with both substrates was uncompetitive and apparently more pronounced with glycogen as substrate.

Mixed-substrate inhibition studies. Mixed-substrate inhibition studies with glycogen and various concentrations of maltose, and vice versa, showed that maltose and glycogen were mutually inhibitory of the hydrolysis of each other.

Transglucosylase activity of acid α -glucosidase in mixed-substrate systems. It is reported that acid α -glucosidase shows, in addition to glucanohydrolase activity, a transglucosylase activity towards maltose, disproportionating the disaccharide with the formation of glucose and tri- and higher-oligosaccharide products (Lukomskaya, 1962; Torres & Olavarria, 1964; Jeffrey *et al.* 1970a). Other oligo- and poly-saccharides will substitute for maltose as glucosyl acceptors in transglucosylation reactions from maltose (donor substrate) (Torres & Olavarria, 1964; Hers, 1963). As an example of this type of mixed-substrate transglucosylation, the incorporation of [14 C]glucosyl units from [U- 14 C]maltose into the polysaccharides glycogen and pullulan was examined. The trans-

Fig. 6. Inhibition exerted by various concentrations of D-glucose on the action of acid α -glucosidase on [14 C]-maltose (7.9 mM). Activity was determined as initial rates of [14 C]maltose disappearance (O), D-[14 C]glucose release (Δ) and formation of [14 C]oligosaccharide (transglucosylation) ($\square$). Activity is expressed as a percentage of the uninhibited reaction.

glucosylation reaction was studied by incubation of the enzyme with polysaccharide (80 mg/ml) and [U- 14 C]maltose (1 mM, 10 μ Ci/ml) in 20 mM-sodium acetate buffer, pH 4.8. At various times, samples of the reaction mixtures were removed and the radioactivity incorporated into the polysaccharides was counted by the method of Thomas, Schlender & Lerner (1968). The time-course of [14 C]glucosyl incorporation is shown in Fig. 8. The initial rate of incorporation into glycogen was approximately twofold that into pullulan. The peak 14 C-incorporation (expressed as a percentage of the total [U- 14 C]maltose radioactivity) was 1% (glycogen) and 0.3% (pullulan).

Replacement of [U- 14 C]maltose by D-[14 C]glucose or [14 C]maltose labelled exclusively in the C-1 of the reducing glucosyl unit led to no incorporation of radioactivity into glycogen under the experimental conditions adopted. In agreement with results of Hers (1963), it was concluded that the reaction was a *de facto* transglucosylation with transfer of the non-reducing glucosyl moiety of maltose into the polysaccharide. Thus as acid α -glucosidases from other sources (Hers, 1963; Lejeune *et al.* 1963; Torres & Olavarria, 1964; Jeffrey *et al.* 1970a), the rabbit muscle enzyme possesses constitutive transglucosylase activity.

pH-dependence of transglucosylation from [U- 14 C]-maltose into glycogen. The pH-dependence of the transglucosylase activity of acid α -glucosidase, determined by incorporation of radioactivity from [U- 14 C]maltose into glycogen, was studied under initial-rate conditions in 20 mM-acetic acid–sodium acetate buffers, pH 2.5–6.6. The pH profile is

Fig. 7. Double-reciprocal plots showing the inhibition exerted by various concentrations of allose on the action of acid α -glucosidase on (a) glycogen and (b) maltose. ●, No addition; ○, with 5 mM-allose; △, with 10 mM-allose; □, with 20 mM-allose. Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

Fig. 8. Time-course of incorporation of ^{14}C -radioactivity (transglucosylation) from $[\text{U-}^{14}\text{C}]$ maltose into the polysaccharides glycogen (○) and pullulan (△).

shown in Fig. 9(a). The pH optimum was 5.1, identical with that for enzymic hydrolysis of glycogen at low cation concentrations (Palmer, 1971). The pH profile was characterized by a steep decrease in transglucosylase activity below pH 4.7. Unlike the hydrolytic activities of the enzyme (Palmer, 1971), the enzyme-catalysed transglucosylation was only weakly activated by 200 mM- Na^+ at pH 4.8.

Position of ^{14}C glucosyl incorporation into glycogen. The mode of attachment of ^{14}C glucosyl units incorporated by transglucosylation into glycogen was examined in a glycogen (16 mg) rendered radioactive by incubation with enzyme and $[\text{U-}^{14}\text{C}]$ maltose (0.4 μmol ; specific radioactivity 10 $\mu\text{Ci}/\mu\text{mol}$) in 20 mM-sodium acetate buffer,

pH 4.5 (total volume 200 μl), for 24 h. The ^{14}C -glycogen, after extensive dialysis, was incubated separately with amylo-1,6-glucosidase/transferase, β -amylase and pullulanase. The radioactivity remaining in the degraded polysaccharides so produced was determined by the method of Thomas *et al.* (1968).

In the action of α -glucosidase on glycogen in the presence of ^{14}C maltose, hydrolysis and transglucosylation proceed simultaneously and presumably are in continuous equilibrium. The major reaction is hydrolysis. Consequently the ^{14}C -labelled polysaccharide isolated will have been extensively degraded by the hydrolytic action of the enzyme. In that cleavage of the α -1,6-linkages (debranching) is rate-limiting in the total conversion of glycogen into glucose (Jeffrey *et al.* 1970a,b; Palmer, 1971), it is predicted that the partially degraded ^{14}C -labelled polysaccharide will be densely branched and have short outer A and B chains. These predictions are borne out by the results. In agreement with the results of Hers & van Hoof (1968) a significant proportion (50%) of the ^{14}C -glucosyl units incorporated were liberated by amylo-1,6-glucosidase/transferase and were therefore attached by α -1,6-linkages. Pullulanase, the α -1,6-linkage-specific glucanohydrolase, liberated 25% of the radioactivity incorporated. Pullulanase releases α -1,6-linked maltosyl, maltotriosyl and higher maltosaccharyl residues but not glucosyl residues. Accordingly, 25% of the ^{14}C glucosyl units are presumably distributed in the outer A chains. The manner by which they are attached is not clear. β -Amylase, the exo-acting glucanohydrolase which catalyses stepwise cleavage of α -1,4-linked maltosyl units from the outer A and B chains of branched α -1,4-glucans, liberated 24% of the radioactivity incorporated. This radioactivity may well be largely distributed in the outer A chains since specificity studies with model

Fig. 9. pH dependence of the transglucosylase activities of acid α -glucosidase. (a) pH-dependence of incorporation of radioactivity from [U- 14 C]maltose into glycogen (Δ). (b) pH-dependence of [14 C]oligosaccharide formation from [U- 14 C]maltose ($\circ$). Activity is expressed as a percentage of the maximum activity at the pH optimum.

oligosaccharides suggest preferential attack by the enzyme on the B chains (Jeffrey *et al.* 1970a). If this supposition proves to be correct, then the radioactivity released by β -amylase and pullulanase is derived from α -1,6-linked maltotetraosyl or higher maltosaccharyl residues containing α -1,4-linked [14 C]glucosyl units presumably at their non-reducing ends.

In summary, the transglucosylase activity of acid α -glucosidase catalyses glucosyl-transfer reactions to form α -1,4- and α -1,6-linkages. The

possibility of glucosyl-transfer reactions to form α -1,2- and α -1,3-linkages cannot be precluded.

Disproportionating activity of acid α -glucosidase towards maltose. The preceding discussion outlined the specificity of the transglucosylase activity of acid α -glucosidase as expressed in a mixed-substrate system. As an expression presumably of the same catalytic site(s), the enzyme is reported to catalyse disproportionation of maltose (Torres & Olavarria, 1964; Lukomsкая, 1962; Jeffrey *et al.* 1970a). To investigate the disproportionating activity, the enzyme was incubated overnight with [U- 14 C]-maltose (40mM, specific radioactivity 0.05 μ Ci/ μ mol) in 10mM-sodium acetate buffer-200mM-sodium chloride, pH 5.0. The reaction mixture was heat-inactivated (3min, 100°C) and fractionated by paper chromatography.

The conversion of maltose (the loss of radioactivity) was 55.5%. Of this maltose converted, 76.5% was liberated as D-glucose. The remaining radioactivity was located in three oligosaccharides designated I, II and III, which contained respectively 12.7, 8.7 and 2.1% of the radioactivity converted. Traces of slower-moving oligosaccharides (0.1%) were also detected. On chromatography in butan-1-ol-pyridine-water, oligosaccharide I migrated approximately with maltotriose. Oligosaccharide II migrated in a position intermediate between maltotriose and maltotetraose and fractionally ahead of panose. Oligosaccharide III migrated as a broad spot marginally behind maltotetraose. T.l.c. in propanol-nitromethane-water indicated trace amounts of an oligosaccharide with a higher chromatographic mobility than maltose. This oligosaccharide was presumed to be nigerose (Lukomsкая, 1962).

The pH-dependence of 14 C-labelled oligosaccharide formation was studied, under initial-rate conditions, by incubating the enzyme with [14 C]-maltose (50mM, specific radioactivity 0.02 μ Ci/ μ mol) in 20mM-acetic acid-sodium acetate buffer, pH 3.2-6.5, containing 200mM-sodium chloride. The 14 C-labelled products were fractionated by paper chromatography and the radioactivity was counted as described in the Materials and Methods section. The pH profile of 14 C-labelled oligosaccharide formation (disproportionation) (Fig. 9b) was complex, with an activity peak at pH 5.1 and a secondary activity peak at pH 5.4.

The pH profile was identical in all significant respects with that of the glucosyl-transfer reaction from [14 C]maltose to polysaccharides (Fig. 9a). Both activities exhibit a pH optimum at pH 5.1, a sharp decrease in activity on the acid side of the pH optimum and a shoulder or secondary activity peak at pH 5.3-5.6. Neither activity was significantly activated by 200mM- Na^+ at pH 4.8. The co-identity of pH profiles suggests that, whereas

multiple substrate-binding sites specific for binding of oligo- and poly-saccharides may exist (see the Discussion section), transglucosylation, whether it be to polysaccharides or to maltose, involves a common catalytic site(s).

The reaction mechanism of transglucosylation is assumed to be bimolecular and involve glucosyl transfer between enzyme-bound donor and acceptor molecules (the formation of a ternary complex is a highly probable event in the transglucosylation reaction mechanism). It is noteworthy that although glucose causes product inhibition of the hydrolytic action of acid α -glucosidase (Fig. 6), the monosaccharide does not function to any appreciable extent as an acceptor substrate in transglucosylation reactions from maltose.

Kinetics of the transglucosylase activity of acid α -glucosidase on maltose. An approach to the reaction mechanism of transglucosylation was made by investigating the kinetics of acid α -glucosidase action on [14 C]maltose. [U - 14 C]Maltose (0.8–80 mM,

specific radioactivity 0.034 μ Ci/ μ mol) was incubated, under initial-rate conditions, with α -glucosidase in 10 mM-sodium acetate buffer–200 mM-sodium chloride, pH 5.1. The reaction mixtures were heat-inactivated and the 14 C-labelled products fractionated and counted for radioactivity as described in the Materials and Methods section. The kinetics of hydrolysis and transglucosylation are shown in Fig. 10.

Up to 4.2 mM-maltose, the reaction stoichiometry was consistent with a strictly hydrolytic reaction mechanism. Above 4.2 mM-maltose, there was an apparent sigmoidal increase in the rate of 14 C-labelled oligosaccharide formation (transglucosylation) coupled with a parallel decrease in the rate of glucose production. The curve of the rate of maltose disappearance versus maltose concentration was hyperbolic up to 48 mM-maltose, at which point the rate decreased. The results are highly consistent with a substrate-concentration-dependent transition from a strictly hydrolytic

Fig. 10. Kinetics of action of acid α -glucosidase on [U - 14 C]maltose. (a) Dependence on maltose concentration of the initial rates of [14 C]maltose disappearance ($\circ$), D-[14 C]glucose release (Δ) and [14 C]oligosaccharide formation ($\square$). (b) Double-reciprocal plots showing the dependence on substrate concentration of action of acid α -glucosidase on maltose. α -Glucosidase activity was determined as initial rates of [14 C]-maltose disappearance ($\circ$) and as initial rates of D-[14 C]glucose release (Δ). Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

reaction mechanism to one involving transglucosylation. There was apparent reciprocity above 4.2mM-maltose between the rate of glucose release and the rate of transglucosylation, implying a competitive mechanism whereby water and maltose compete as reactants in glucosyl-transfer reactions. It should be noted that the extrapolated K_m , whether based on maltose hydrolysis or glucose release, was approx. 4mM (Fig. 10b).

As discussed in the preceding paper (Palmer, 1971), the kinetics of maltosaccharide hydrolysis by acid α -glucosidase, based on the measurement of initial rates of glucose formation, show pronounced substrate inhibition at maltosaccharide concentrations exceeding 5mM. In the light of the present experiment, such substrate inhibition is more apparent than real and simply reflects a transition in reaction mechanism. At substrate-inhibitory concentrations of maltose exceeding 5mM, it was shown that inhibition of α -glucosidase action by turanose, glucono- δ -lactone, polyols and tris was progressively decreased with increasing maltose concentration. This would suggest that the rate-limiting step in transglucosylation is catalysed by a site(s) not subject to inhibition by such conventional inhibitors and hence distinct from the site(s) that binds maltose in the hydrolytic reaction. The present studies suggest that this site is identical with that implicated in the catalytic event(s) involving water, presumably a nucleophilic attack on an enzyme-glucosyl intermediary complex. The proposed model visualizes maltose as competing with water for binding at this site and that such binding is rate-limiting to the overall transglucosylation reaction.

DISCUSSION

Evidence is presented in this and the preceding paper (Palmer, 1971) which clearly indicates that acid α -glucosidase may be regarded as possessing at least three specific substrate-binding sites. One site is regarded as specific for binding of the disaccharide maltose, and perhaps for other oligosaccharides. The second site is implicated in the catalytic event(s) involving water, presumably a nucleophilic attack on an enzyme-glucosyl intermediary complex. Maltose and presumably other maltosaccharides may function as alternative glucosyl acceptors and compete accordingly with water at this site. The replacement of water by maltose leads to a transition from a hydrolytic to a transglucosylation reaction mechanism. A third site(s) binds polysaccharides such as glycogen. This model is basically in accord with the hypothesis of Jeffrey *et al.* (1970b), which recognizes separate and catalytically active binding sites for maltose and polysaccharide.

The differences in the pH-stability profiles of the maltose and glucoamylase activities of α -glucosidase suggest that enzyme interaction with the two substrates involves different group(s) with markedly different pH stabilities. A multiplicity of functional groups is also suggested by the pH optimum and the kinetics and pH-dependence of cation activation (Palmer, 1971). Such groups may be catalytic, implicated in substrate binding or possess a critical conformational role. Selective inactivation at pH7 by iodoacetate or iodoacetamide of enzyme action on glycogen suggests that one such group is readily alkylated and will be a thiol or other reactive group (the possibility of side reactions with lysine, methionine, tyrosine or histidine residues is not precluded).

Certain specific structural requirements for binding at the glycogen site can be deduced from inhibition studies. The glucoamylase activity is, at saturating cation concentrations (200mM- Na^+), selectively and competitively inhibited by α -trehalose, melizitose, methyl α -D-glucoside and sucrose. Common to the four inhibitory oligosaccharides is a terminal non-reducing α -glucosyl group. It is tentatively proposed that this is the functional group in enzyme inhibition and hence in binding at the polysaccharide-binding site. Of specific reference is a possible absolute requirement for an equatorial hydroxyl group at C-4 of the non-reducing hexosyl moiety, since those oligosaccharides tested that terminated in a galactosyl unit were not inhibitory. Likewise, there would appear to be an absolute requirement for an α -configuration at the C-1 glycosidic bond.

Studies on the inhibition of acid α -glucosidase by turanose, glucono- δ -lactone, glycerol and monosaccharides, specifically D-allose, reveal additional differences between the maltase and glucoamylase partial activities and suggest multiple sites of interaction of inhibitor with enzyme. Nevertheless, that these inhibitors affect both activities implies some measure of structural similarity or interrelationship or both between the maltose- and polysaccharide-binding sites. In agreement with results of Jeffrey *et al.* (1970b), the polysaccharide- and maltose-binding sites must be regarded as being in close proximity and subject to a high degree of interaction. This is substantiated by the fact that maltose and glycogen are mutually inhibitory substrates and interact directly in transglucosylation reactions.

It is premature to propose a reaction mechanism for acid α -glucosidase that encompasses the results on multiple substrate-binding sites. Conceivably, the maltose- and polysaccharide-binding sites are associated with structurally separate catalytic sites. An alternative mechanism more in line with experimental results would be one in which a single

active site is composed of multiple sub-sites, analogous to the situation in lysozyme (see review by Chipman & Sharon, 1969).

The multiplicity of substrate-binding sites and hence the multiplicity of functional groups involved in enzymic catalysis might provide a molecular basis for the possible existence of subgroups of glycogen-storage disease type II (Pompe's disease). Hers & van Hoof (1968) presented results pertaining to two atypical cases of glycogen-storage disease type II, in which tissue homogenates showed very low transglucosylase activity ($[^{14}\text{C}]$ maltose $\rightarrow$ glycogen) but were relatively active in maltose hydrolysis (50% of normal controls). Such a situation might theoretically arise by a mutation(s) which results in a specific modification at a sub-site involved in transglucosylation (glycogen-binding sub-site?). In the event of this modification not affecting the catalytic site(s), the hydrolysis of maltose, bound at a separate sub-site, would be influenced only to a secondary and minimal extent. Whether the multiple substrate-binding site structure of acid α -glucosidase will allow such multiple sites of genetic modification and hence lead to subgroups of glycogen-storage disease type II awaits more rigorous studies on type II glycogen-storage disease patients.

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