The Substrate Specificity of Acid α -Glucosidase from Rabbit Muscle

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1. Acid α -glucosidase was purified 3500-fold from rabbit muscle. 2. The enzyme was activated by cations, the degree of activation varying with the substrate. Enzyme action on glycogen was most strongly activated and activation was apparently of a non-competitive type. With rabbit liver glycogen as substrate, the relative $V_{\max}$ increased 15-fold, accompanied by an increase in K_m from 8.3 to 68.6 mM-chain end over the cation range 2–200 mM- Na^+ at pH 4.5. Action on maltose was only moderately activated (1.3-fold, non-competitively) and action on maltotriose was marginally and competitively inhibited. 3. The pH optimum at 2 mM- Na^+ was 4.5 (maltose) and 5.1 (glycogen). Cation activation of enzyme action on glycogen was markedly pH-dependent. At 200 mM- Na^+ , the pH optimum was 4.8 and activity was maximally stimulated in the range pH 4.5–3.3. 4. Glucosidase action on maltosaccharides was associated with pronounced substrate inhibition at concentrations exceeding 5 mM. Of the maltosaccharides tested, the enzyme showed a preference for *p*-nitrophenyl α -maltoside (K_m 1.2 mM) and maltotriose (K_m 1.8 mM). The extrapolated K_m for enzyme action on maltose was 3.7 mM. 5. The macromolecular polysaccharide substrate glycogen differed from linear maltosaccharide substrates in the kinetics of its interaction with the enzyme. Activity was markedly dependent on pH, cation concentration and polysaccharide structure. There was no substrate inhibition. 6. The enzyme exhibited constitutive α -1,6-glucanohydrolase activity. The K_m for panose was 20 mM. 7. The enzyme catalysed the total conversion of glycogen into glucose. The hydrolysis of α -1,6-linkages was apparently rate-limiting during the hydrolysis of glycogen. 8. Enzyme action on glycogen and maltose released the α -anomer of D-glucose. 9. The results are discussed in terms of the physiological role of acid α -glucosidase in lysosomal glycogen catabolism.

The mammalian α -glucosidases are a group of exoenzymes that split individual glucose units from the non-reducing ends of α -1,4-glucans. α -Glucosidase (α -D-glucoside glucosylhydrolase; EC 3.2.1.20) with an acid pH optimum is widely distributed in mammalian tissues (Rosenfeld, 1964) and is almost certainly of lysosomal origin (Lejeune, Thinés-Sempoux & Hers, 1963). Acid α -glucosidase has been partially purified (Torres & Olavarria, 1964; Auricchio & Bruni, 1967; Auricchio, Bruni & Sica, 1968) and highly purified enzyme preparations have been obtained from bovine liver (Bruni, Auricchio & Covelli, 1969), the lysosomal fraction of rat liver (Jeffrey, Brown & Brown, 1970a) and bovine spleen (Fujimori, Hizukuri & Nikuni, 1968).

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The enzyme is a relatively non-specific α -glucosidase exhibiting hydrolytic activity towards maltose, isomaltose, nigerose, kojibiose and phenyl α -D-glucoside (Torres & Olavarria, 1964; Jeffrey *et al.* 1970a,b). The enzyme possesses constitutive glucoamylase activity (α -1,4-glucan glucosylhydrolase; EC 3.2.1.3) (Bruni *et al.* 1969; Jeffrey *et al.* 1970a,b). The coexistence of α -1,4-glucosylhydrolase, α -1,6-glucosylhydrolase and glucoamylase activities permits the total conversion of glycogen into glucose (Jeffrey *et al.* 1970b). The lysosomal α -glucosidase is also reported to possess intrinsic transglucosylase activity, catalysing glucosyl transfer from maltose (donor substrate) to a broad range of acceptors (Torres & Olavarria, 1964; Jeffrey *et al.* 1970a; Lukomskaya, 1962).

Acid α -glucosidase appears to play a vital role in glycogen metabolism *in vivo*, as evidenced by the severity of glycogen-storage disease type II

(Pompe's disease), in which the enzyme is specifically absent. Pompe's disease is typified by pronounced cardiac enlargement and extreme muscular weakness, which are invariably responsible for the premature death of the infant before the ninth month of life. The symptomatology of the disease strongly suggests a major impairment resulting from the congenital absence of lysosomal acid α -glucosidase in glycogen catabolism in skeletal and cardiac muscle. With this knowledge in mind, the purification of acid α -glucosidase from rabbit muscle was undertaken. A previous report (Abdullah, Taylor & Whelan, 1964) showed that rabbit muscle contained an acid α -glucosidase together with two glucosidases with neutral pH optima.

The present paper describes studies on the substrate specificity of the purified enzyme. The following paper (Palmer, 1971) deals in depth with evidence for the possession, by the enzyme, of multiple catalytically active substrate-binding sites.

MATERIALS AND METHODS

Carbohydrates. Maltosaccharides (Whelan, Bailey & Roberts, 1953; Taylor & Whelan, 1962), panose (Smith & Whelan, 1963), methyl β -maltoside (Brauns, 1929; Schoch, Wilson & Hudson, 1942) and *p*-nitrophenyl α -maltoside (Qureshi, 1967) were prepared by the documented methods. Rabbit liver glycogen (type III) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Shellfish glycogen was a product of 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. Amylose (degree of polymerization, D.P., 300) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Amylose partly oxidized with sodium metaperiodate (5% oxidation) was prepared as described by Drummond (1969). The β -amylase limit dextrin of the oxidized polysaccharide was prepared by the method of Walker & Whelan (1960). The phosphorylase limit dextrin and β -amylase limit dextrin of shellfish glycogen were gifts of Dr E. Y. C. Lee.

All other carbohydrates were obtained from commercial sources and were purified by recrystallization or paper chromatography when necessary.

Enzymes. Glucose oxidase and horseradish peroxidase, the enzymes comprising the glucose oxidase reagent, were purchased from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. *Aspergillus niger* amyloglucosidase was prepared by the method of Fleming & Stone (1965) and Qureshi (1967), and *Rhizopus niveus* amyloglucosidase (twice-recrystallized) was a product of Miles Laboratories Inc., Kankakee, Ill., U.S.A.

Enzyme purification. The purification procedure adopted was essentially that described by Auricchio and co-workers (Auricchio & Bruni, 1967; Auricchio *et al.* 1968; Bruni *et al.* 1969). Rabbit muscle was coarsely minced and suspended with stirring in 25 mM-NaCl-1 mM-EDTA adjusted with 2 M-NaOH to pH 6.7. The

suspension was filtered through muslin and the washings were discarded. The washing procedure served to remove most of the extraneous blood proteins. The washed muscle was homogenized in 25 mM-NaCl-1 mM-EDTA, pH 6.7 (2:1, w/v), frozen and thawed, and centrifuged for 30 min at 6000g. The precipitate was re-extracted with 25 mM-NaCl-1 mM-EDTA, pH 6.7 (2:1, w/v), a further four times.

The combined supernatants were acidified to pH 4.2 with M-acetic acid and then centrifuged. The supernatant was immediately adjusted to pH 6.7 with aq. M-NH₃ and the heavy precipitate removed by centrifugation. The supernatant was fractionated with solid (NH₄)₂SO₄ between 30% and 60% saturation. The precipitate was dissolved in a minimal volume of 25 mM-NaCl, pH 6.7, and extensively dialysed against frequent changes of 25 mM-NaCl, pH 6.7. The non-diffusible material was freeze-dried and redissolved in a minimal volume of 50 mM-sodium phosphate buffer-0.1 M-NaCl, pH 6.7. Insoluble material was removed by centrifugation at 12000g for 20 min.

A portion of the partially purified enzyme (25 ml) was applied to a column (2.5 cm $\times$ 100 cm; K 25/100 Sephadex column) of Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.) equilibrated in 50 mM-sodium phosphate buffer-0.1 M-NaCl, pH 6.7. Eighty fractions (10 ml each) were collected at a flow rate of 5 ml/h. Fractions were assayed for acid α -glucosidase activity. Protein was determined by the Lowry modification of the Folin method (Lowry, Rosebrough, Farr & Randall, 1951) with dried bovine serum albumin (Pentex Inc., Kankakee, Ill., U.S.A.) as standard.

The fractions containing the most highly retarded acid α -glucosidase activity were combined and concentrated by ultrafiltration. All steps in the purification procedure were performed at 4°C.

Enzyme assay procedure. Acid α -glucosidase activity, unless otherwise stated, was determined by addition of enzyme (0.1 ml) to 10 mM-maltose in 50 mM-sodium acetate buffer, pH 4.5 (0.9 ml). The reaction was allowed to proceed at 37°C for 0.5-3 h and was then stopped by the addition of 0.16 M-ZnSO₄·7H₂O (0.3 ml) and 0.32 M-NaOH (0.3 ml). Glucose was determined in portions of the protein-free solution by the specific glucose oxidase method (Fleming & Pegler, 1963; Catley, 1967; Lloyd & Whelan, 1969). In studies with the purified enzyme, the volume of the reaction mixture was halved (0.5 ml) and the reaction was stopped by direct addition of the glucose oxidase reagent. The buffer used in the preparation of the glucose oxidase reagent [0.3 M-tris adjusted to pH 7.0 with 9 M-H₃PO₄ and containing 40% (v/v) glycerol] completely inhibits acid α -glucosidase activity. One unit of α -glucosidase activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 nmol of maltose/min at 37°C (Torres & Olavarria, 1964).

Neutral α -glucosidase activity was determined essentially as described above except that 10 mM-maltose in 50 mM-imidazole-HCl buffer, pH 7.0, served as substrate.

Polyacrylamide-disc-gel electrophoresis. Disc-gel electrophoresis was performed as described by Davis (1964) at pH 8.5. The degree of polymerization of the gel was 7%.

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.) or propanol-nitromethane-water (5:4:3, by vol.). The spray reagent was AgNO_3 -NaOH (Trevelyan, Procter & Harrison, 1950).

The polyglucose content of maltosaccharides and polysaccharides was determined by the successive actions of *A. niger* amyloglucosidase and glucose oxidase (Lee & Whelan, 1966). The average chain length of glycogens was estimated by the method of Lee & Carter (1971) and the degree of β -amylolysis by the method of Walker & Whelan (1960).

The anomeric form of D-glucose released by enzyme action on maltose and glycogen was ascertained by the method of Semenza, Curtius, Raunhardt, Hore & Muller (1969) with the modification that the buffer used in preparation of the glucose oxidase reagent was 0.3M-tris adjusted to pH 5.0 with 3M-acetic acid and containing 40% (w/v) glycerol.

RESULTS

Purification of acid α -glucosidase. The purification procedure adopted was essentially that described by Auricchio & Bruni (1967). The method takes advantage of the pronounced retardation of acid α -glucosidase on Sephadex G-100. The retardation is specific and apparently results from enzyme-substrate interaction in the form of enzyme binding to the α -1 $\rightarrow$ 6-linked D-glucose units of the dextran gel.

Enzyme activity after gel filtration on Sephadex G-100 was extremely labile. Storage or dilution

produced progressive and irreversible loss of enzyme activity. This instability prevented further purification. Subsequent chromatography on Bio-Gel P-60 or DEAE-cellulose led to more than 80% loss of enzyme activity. Accordingly, the enzyme studied was that of highest specific activity obtained from Sephadex G-100, i.e. the most highly retarded (designated B in Fig. 1). A typical purification is summarized in Table 1. Final products with a specific activity of approx. 6000 units/mg of protein were usually obtained. Acid α -glucosidase is a trace activity in skeletal muscle: approx. 70000 units/kg wet wt. were generally obtained.

Enzyme stabilization. 10mM-Sodium phosphate buffer, pH 6.7, containing 0.1% human serum albumin protected the enzyme against irreversible inactivation on storage at 4°C. All reaction mixtures, unless otherwise indicated, contained a final concentration of 0.01% human serum albumin.

Criteria of purity. On disc-gel electrophoresis at pH 8.5 the enzyme migrated as a single protein band. Further, the purified acid α -glucosidase was essentially free of α -amylase activity as judged by the following criteria.

(1) Glycogen (0.5%, w/v, in 0.1M-sodium acetate buffer-0.1M-sodium chloride, pH 5.0) was incubated with enzyme (19 units) at 37°C. When 23% hydrolysis had occurred, on the basis of glucose release,

Fig. 1. Gel filtration of rabbit muscle α -glucosidase activities on a Sephadex G-100 column (2.5cm $\times$ 100cm) equilibrated with 50mM-sodium phosphate buffer-100mM-NaCl, pH 6.7. The eluate was examined for protein ($\square$), neutral α -glucosidase activity ($\circ$) and acid α -glucosidase activity ($\triangle$). The acid α -glucosidase activity peak was divided into two fractions, designated A and B, on the basis of specific activity. The most highly retarded fraction B, of highest specific activity, was used in subsequent studies.

Table 1. *Purification of acid α -glucosidase from rabbit muscle*

Results are for a typical purification by the steps described in the Materials and Methods section. The crude extract was the supernatant from 1670 g of rabbit muscle.

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification	Yield (%)
Crude extract	4500	99300	56500	1.75	1	100
Acid supernatant	4980	107700	49000	2.19	1.3	108
30–60%-(NH ₄) ₂ SO ₄ precipitate	260	87700	17400	5.04	2.9	88
Dialysed freeze-dried enzyme	162	75500	5900	12.8	7.3	76
Sephadex G-100 filtrate	91	22200	3.6	6167.0	3524	22

the reaction mixture was heat-inactivated (3 min, 100°C) and partitioned by dialysis against water. The non-diffusible polysaccharide fraction (re-precipitated twice with ethanol) was treated successively with *A. niger* amyloglucosidase and glucose oxidase. The polysaccharide accounted for 76.5% of the original glycogen. The diffusible material, shown by chromatography to be exclusively glucose, was equivalent to 21.6% of the original polysaccharide.

(2) Enzyme action on the β -dextrin of periodate-oxidized amylose (Smith, Drummond, Marshall & Whelan, 1970) resulted in the release of only a trace of glucose. The degree of hydrolysis was comparable with that produced by the action of *R. niveus* amyloglucosidase, an enzyme known to be essentially free of α -amylase activity (Marshall & Whelan, 1970).

Dependence of acid α -glucosidase activity on salt concentration. Purified acid α -glucosidase, desalted by gel filtration on Sephadex G-25 equilibrated in water, rapidly lost activity, despite the presence of 0.1% human serum albumin. Addition of 0.1M-sodium chloride stabilized the enzyme on storage at 4°C. The effect of sodium chloride was not confined to stabilization of the enzyme. Fig. 2 shows the effect of increasing concentrations of sodium chloride on enzyme action on maltose, maltotriose and glycogen at pH 4.5.

As demonstrated by Jeffrey *et al.* (1970*a,b*), for the enzyme from the lysosomal fraction of rat liver, acid α -glucosidase is subject to activation by sodium chloride. Activation was strikingly differential with respect to substrate. Enzyme action on glycogen was most markedly subject to activation by sodium chloride. Activity increased 2.4-fold over the concentration range 2–200 mM-sodium chloride. The maltase activity was only moderately activated (1.3-fold) and enzyme action on maltotriose was slightly inhibited.

It was considered essential to investigate the specificity of salt activation to ascertain whether the activation was specifically mediated by anions,

Fig. 2. Effect of NaCl concentration on the activity of acid α -glucosidase on shellfish glycogen (200 mg/ml) (Δ), on 5 mM-maltose ($\circ$) and on 5 mM-maltotriose ($\square$). The buffer used was 2 mM-sodium acetate buffer, pH 4.5.

cations or was determined by the ionic strength of the medium. The chloride salts of Na⁺, K⁺, Mg²⁺, Ca²⁺, Mn²⁺, NH₄⁺, Li⁺ and Co²⁺, in equimolar cation concentrations, were approximately equally effective in the activation of enzyme action on glycogen, as were sodium bromide, sodium fluoride and sodium sulphate. Arginine hydrochloride, histidine hydrochloride and glucosamine hydrochloride were only slightly activatory. In accord with the results of Jeffrey *et al.* (1970*a,b*), it was concluded that salt activation was largely a function of cation concentration. Sodium nitrite was completely ineffective in enzyme activation. Some measure of specificity must therefore exist as regards the anion.

The pH-dependence of acid α -glucosidase action on maltose and glycogen at 2 mM-sodium chloride

and 200mM-sodium chloride is shown in Fig. 3. The pH optimum of enzyme activity at 2mM-sodium chloride was 4.5 (maltose) and 5.1 (glyco-

Fig. 3. Dependence of acid α -glucosidase activity on pH. (a) Enzyme action on shellfish glycogen (200mg/ml) at cation concentrations of 2mM-Na⁺ (Δ) and 200mM-Na⁺ (▲). (b) Enzyme action on maltose (5mM) at cation concentrations of 2mM-Na⁺ (○) and 200mM-Na⁺ (●). The buffers used were sodium acetate (pH 2.5–5.6), sodium citrate (pH 3.2–6.2) and sodium phosphate (pH 5.7–6.8).

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gen). The activation by sodium chloride of enzyme action on glycogen was strikingly pH-dependent. There was a shift in the pH optimum to pH 4.8 and activity was maximally stimulated in the range pH 3.3–4.5. In contrast, activation by sodium chloride of enzyme action on maltose was largely independent of pH.

Action of acid α -glucosidase on maltosaccharides. The maltosaccharides, maltose, maltotriose, maltotetraose, maltopentaose and maltohexaose, were incubated separately with purified acid α -glucosidase in 10mM-sodium acetate buffer–200mM-sodium chloride, pH 4.5, at 37°C and the initial rates of glucose formation were measured as a function of substrate concentration. Fig. 4 shows the results in the form of double-reciprocal plots. The reaction velocities are expressed as nmol of α -1,4-linkage hydrolysed/min. With the maltosaccharides maltotriose to maltohexaose, calculation of such reaction velocities requires prior assumption of a multichain reaction mechanism. In such a mechanism, only the non-reducing α -1,4-linkage of the maltosaccharide is subject to enzymic hydrolysis in the initial phase of the reaction. Thus the rate of glucose release is a direct expression of the rate of α -1,4-linkage hydrolysis. Chromatographic evidence tentatively supports a multichain action pattern.

Pronounced substrate inhibition was observed with all maltosaccharide substrates at concentrations exceeding 5mM. The extrapolated values for K_m and relative V_{max} are summarized in Table 2. The K_m for maltose was 3.7mM. Accepting the restrictions imposed on the interpretation of the kinetic results by the incidence of substrate inhibition and by the assumption of a multichain action pattern, it is nevertheless possible to conclude that maltotriose is the preferred maltosaccharide substrate.

Fig. 4. Double-reciprocal plots showing the effect of maltosaccharide concentrations on the activity of acid α -glucosidase. The maltosaccharide substrates examined were maltose (●), maltotriose (▲), maltotetraose (Δ), maltopentaose (○) and maltohexaose (■). Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

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Table 2. *Kinetic parameters of acid α -glucosidase action on maltosaccharides*

Maltosaccharide	K_m (mM)	Relative maximal velocity ($V_{max.}/V_{max.}$ of maltose)
Maltose	3.7	1.0
Maltotriose	1.8	1.4
Maltotetraose	3.2	1.6
Maltopentaose	3.7	1.3
Maltohexaose	3.4	1.2

Fig. 5. Double-reciprocal plot showing the dependence on Na^+ concentration of the rate of hydrolysis of maltose: 2mM- Na^+ ($\circ$), 25mM- Na^+ (Δ) and 200mM- Na^+ ($\square$). Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

The dependence of the kinetics of hydrolysis of maltose on the concentration of sodium chloride is shown in Fig. 5. Activation was non-competitive. The maximum velocity increased 1.2-fold at 25mM-sodium chloride and 1.3-fold at 200mM-sodium chloride. In contrast, enzyme action on maltotriose was inhibited in a competitive fashion by cations. The K_m increased from 1.7mM (2mM- Na^+) to 1.8mM (25mM- Na^+) and 2.2mM (200mM- Na^+).

Action of acid α -glucosidase on p-nitrophenyl α -maltoside and methyl β -maltoside. Both p-nitrophenyl α -maltoside and methyl β -maltoside were

Fig. 6. Double-reciprocal plots showing the dependence on substrate concentration of the rates of hydrolysis of (a) methyl β -maltoside and (b) p-nitrophenyl α -maltoside. The buffer used was 10mM-sodium acetate-200mM- NaCl , pH4.5. Continuous lines without experimental points refer to control experiments in which maltose was the substrate. Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

substrates for the purified enzyme. The initial rates of glucose formation were measured as a function of substrate concentration and the results

in the form of double-reciprocal plots are shown in Fig. 6. Substrate inhibition was apparent with both compounds at concentrations exceeding 5 mM. The extrapolated values for K_m were 1.2 mM (*p*-nitrophenyl α -maltoside) and 4.2 mM (methyl β -maltoside) and the maximum velocities relative to maltose (1.0) were 1.4 and 0.8 respectively.

Under the experimental conditions adopted for the determination of the kinetic constants for enzyme action on *p*-nitrophenyl α -maltoside, *p*-nitrophenol release was not detectable.

Substrate inhibition with maltosaccharide substrates. Substrate inhibition is not readily amenable to kinetic analysis. Some information, however, can be gained by plotting the reciprocal of the initial velocity against substrate concentration at inhibitory concentrations of substrate (Dixon, 1953; Jeffrey *et al.* 1970b). Such plots, as shown in Fig. 7, define straight lines that can be extrapolated to the abscissa to give relative values for K_i (K_s).

Maltotriose, maltotetraose and *p*-nitrophenyl α -maltoside all gave an extrapolated K_i of 10 mM. With the exception of maltotetraose, the high affinity of these maltosaccharides as inhibitors of enzyme action parallels their high affinity (low K_m) as substrates (Table 2). The K_i for maltose in inhibition of its own hydrolysis was approx. 80 mM. Maltopentaose, maltohexaose and methyl β -maltoside were likewise only weakly inhibitory.

Action of acid α -glucosidase on glycogens. The kinetics of action of acid α -glucosidase on polysaccharides were investigated by using as sub-

strates two glycogens of markedly different structure. Rabbit liver glycogen (average chain length 16; end-group content 6%) and shellfish glycogen (average chain length 10; end-group content 10%) possessed average outer-chain lengths of 10 and 6 respectively. The glycogens were incubated with acid α -glucosidase in 10 mM-sodium acetate buffer, pH 4.5, containing various concentrations of sodium chloride. The initial rates of glucose formation were measured as a function of glycogen concentration and Fig. 8 shows the results in the form of double-reciprocal plots.

The macromolecular polysaccharide substrate differs markedly from linear maltosaccharide substrates in its kinetics of interaction with acid α -glucosidase. Enzyme action was not subject to substrate inhibition. With both glycogens, the kinetics of activation by sodium chloride were of an apparently uncompetitive type. With increasing salt concentration, there was an almost parallel increase in K_m and V_{max} . (Fig. 8 and Table 3). In all experiments there was no indication of a predominantly non-competitive activation at low cation concentrations as reported by Jeffrey *et al.* (1970b) in studies on acid α -glucosidase from rat liver lysosomes.

The extrapolated values for K_m and V_{max} for enzyme action on rabbit liver glycogen and shellfish glycogen are shown in Table 3. The K_m values are expressed both as mg of glycogen/ml and as concentration (mM) of non-reducing chain ends. Maximal velocity V_{max} is expressed relative to

Fig. 7. Dixon plots showing the substrate inhibition exerted on acid α -glucosidase activity by maltose (○), maltotriose (Δ), maltotetraose (□), methyl β -maltoside (▲), *p*-nitrophenyl α -maltoside (●) and maltopentaose (■). The reciprocals of the initial velocities are plotted against substrate concentration at inhibitory concentrations of each substrate (exceeding 5 mM). Initial velocity v is expressed as nmol of α -1,4-linkage hydrolysed/min.

Fig. 8. Double-reciprocal plots showing the dependence on polysaccharide concentration of the rates of hydrolysis of (a) shellfish glycogen and (b) rabbit liver glycogen at various concentrations of NaCl: 2 mM- Na^+ (○), 25 mM- Na^+ (△) and 200 mM- Na^+ (□). Initial velocity is expressed as nmol of α -1,4-linkage hydrolysed/min.

that for maltose, which is taken as unity. The K_m values for enzyme action on the long-chain rabbit liver glycogen were as much as an order of magnitude higher than the K_m values for action on linear maltosaccharides. Enzyme action on this glycogen was subject to markedly pronounced cation activation. Over the range 2–200 mM- Na^+ ,

the K_m and $V_{\max}$ increased 8.3-fold and 13-fold respectively to 37 mM-non-reducing chain end (196 mg/ml) and a $V_{\max}$ 7.3-fold greater than that for action on maltose.

By contrast, the K_m values for enzyme action on the short-chain shellfish glycogen were only moderately higher than the K_m values for action on linear maltosaccharides. In fact, at 2 mM-sodium chloride, the K_m was 4.0 mM-non-reducing chain ends, compared with 3.7 mM for enzyme action on maltohexaose (the average outer chain of shellfish glycogen is 6 glucose units). In parallel with the relatively low K_m values for enzyme action on shellfish glycogen, the $V_{\max}$ values were equally low.

It appears that acid α -glucosidase possesses greater substrate-binding affinity for glycogens with short outer chains but that catalytic activity increases with increasing outer-chain length. This might be a negative expression of steric hindrance, which presumably is greatest in a densely structured short-chain glycogen.

Action of acid α -glucosidase on panose. Panose (6²- α -glucosylmaltose) is an ideal substrate for the investigation of the α -1,6-glucanohydrolase activity of acid α -glucosidase (Abdullah *et al.* 1964). Panose was incubated with the enzyme in 10 mM-sodium acetate buffer, pH 4.5, containing 0.2 M-sodium chloride. The results, in the form of a double-reciprocal plot, are shown in Fig. 9. The K_m for enzyme action on panose was 20 mM and the $V_{\max}$ relative to maltose ($V_{\max}$ for maltose taken to be unity) was about 0.1. Slight substrate inhibition was apparent at concentrations exceeding 85 mM. Purified acid α -glucosidase thus possesses constitutive α -1,6-glucosidase activity.

Extent of hydrolysis of polysaccharides by acid α -glucosidase. In view of the demonstration of constitutive α -1,4- and α -1,6-glucosidase activities, it was pertinent to establish whether the enzyme could catalyse the total conversion of glycogen into glucose. Total hydrolysis of glycogen by acid α -glucosidase from rat liver lysosomes was reported by Jeffrey *et al.* (1970b). Enzyme (30 units) was incubated under toluene with rabbit liver glycogen (1.94 mg), shellfish glycogen (2.16 mg), shellfish glycogen β -limit dextrin (2.34 mg) and shellfish glycogen phosphorylase-limit dextrin (2.11 mg) in 10 mM-sodium acetate buffer, pH 4.8, containing 0.2 M-sodium chloride. The results (Fig. 10) show that the enzyme catalysed more than 95% conversion into glucose with all four polysaccharides. The relative initial rates of hydrolysis (%) were in the descending order: shellfish glycogen (100): rabbit liver glycogen (84): phosphorylase-limit dextrin (70): β -limit dextrin (55). The slow rates of hydrolysis of the limit dextrins implies that hydrolysis of the α -1,6-linkages (branch-points) is

Table 3. *Kinetic parameters of acid α -glucosidase action on glycogens*

Glycogen source	Concn. of NaCl (mm)	K_m		$V_{max.}$ (relative to maltose)
		mg of glycogen/ml (expressed as mg of glucose)	mm-chain end	
Rabbit liver	2	24	8.3	0.5
	25	100	35.3	2.4
	200	196	68.6	7.3
Shellfish	2	7.1	4.0	0.3
	25	13	7.4	0.8
	200	18	10.4	1.1

Fig. 9. Double-reciprocal plot showing the dependence on substrate concentration of the rate of hydrolysis of panose (●). The broken line refers to a control experiment in which maltose was the substrate. Initial velocity is expressed as nmol of α -1,6-linkage hydrolysed/min.

rate-limiting in the conversion of glycogen into glucose.

Anomeric form of D-glucose released by acid α glucosidase action. The anomeric form of D-glucose released by enzyme action on maltose and glycogen was investigated by the method of Semenza *et al.* (1969), which is based on the known specificity of fungal glucose oxidase for β -D-glucose. Enzyme action on glycogen and maltose, regardless of cation concentration, released α -D-glucose.

DISCUSSION

Acid α -glucosidase, purified from rabbit muscle, exhibits intrinsic α -1,4-glucosidase (α -D-glucoside glucohydrolase, EC 3.2.1.20), α -1,6-glucosidase and glucoamylase (α -1,4-glucan glucohydrolase, EC 3.2.1.3) activities. Consistent with such specificity,

Fig. 10. Time-course of the hydrolysis by acid α -glucosidase of shellfish glycogen (○), rabbit liver glycogen (Δ), shellfish glycogen phosphorylase-limit dextrin (▲) and shellfish glycogen β -amylase-limit dextrin (●).

the enzyme will catalyse the complete conversion of glycogen into glucose. Thus the enzyme is a relatively non-specific glucosidase and thereby fulfills its physiological role in lysosomal degradation of glycogen.

Acid α -glucosidase is primarily an oligosaccharase with a preference for short-chain malto-

saccharides. The lack of inversion at the anomeric carbon atom on enzymic hydrolysis is compatible with both a 'frontal attack' and a 'double-displacement' mechanism. With maltosaccharides, pronounced substrate inhibition was apparent at concentrations exceeding 5mM, irrespective of whether the anomeric C-1 was blocked with a methyl or *p*-nitrophenyl group. Relative to maltosaccharides, enzyme action on panose (α -1,6-glucosidase activity) was weak.

The physiological role of acid α -glucosidase requires that *in vivo* the enzyme functions primarily as a glucoamylase. Enzyme action on glycogen is markedly influenced by univalent and bivalent cations. The glucoamylase activity is subject to pronounced uncompetitive cation activation, particularly in the acid pH range (pH 3.3–4.8). Cation activation is non-specific with respect to cation and maximal at cation concentrations exceeding 0.1M. Thus it appears highly improbable that a cation-enzyme complex is the functional catalyst in the mechanism of polysaccharide hydrolysis. One possible mechanism whereby cations might exert their activation effect would be through potentiation of the C–O linkage at the anomeric C-1 of the glucosidic bond to be hydrolysed, thereby facilitating nucleophilic attack by the enzyme at the C-1 position. It is a strong possibility, as proposed by Jeffrey *et al.* (1970a), that intralysosomal glycogen catabolism *in vivo* is regulated by cation concentration or pH or both.

At saturating cation concentrations, enzyme action on glycogen is markedly dependent on polysaccharide concentration and structure. In general, maximal catalytic activity requires elevated glycogen concentrations. There is no reason to presuppose, however, that such elevated concentrations should not exist within the micro-environment of the lysosome. In the light of the broad specificity of the acid α -glucosidase, a point worthy of future consideration is the possible secondary function of the enzyme in the hydrolysis of 'aberrant structures' in native glycogens. The specificities of the enzymes of glycogen biosynthesis are not absolute with respect to their accepted physiological precursors. For example, Cabantchik & Schramm (1965) have reported the isolation of highly branched macrodextrins from glycogen. After treatment of glycogens from various sources with α -amylase they were able to isolate large fragments of the inner core structures containing 80–280 glucose units, which were relatively resistant to α -amylase and which had a high content of branch points (22–25%). Such structures are presumably relatively inert biochemically and are not converted completely into glucose 1-phosphate and glucose by the combined actions of glycogen phosphorylase and amylo-1,6-glucosidase/trans-

ferase. An alternative route for the catabolism of maltosaccharides may well involve lysosomal acid α -glucosidase. It is not unlikely that native glycogens might contain, in addition to macrodextrin, other structures with aberrant glycosidic linkages. One possible function of acid α -glucosidase might be to hydrolyse such aberrant structures.

As opposed to their uncompetitive activation of enzyme action on glycogen, cations are non-competitive activators of α -glucosidase action on maltose and marginally inhibit, in a competitive fashion, action on maltotriose. The substrate-dependency of the cation-activation effect is consistent with the hypothesis, advanced by Jeffrey *et al.* (1970b), that acid α -glucosidase may have multiple catalytically active substrate-binding sites. Further evidence in support of this hypothesis is presented in the following paper (Palmer, 1971).

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