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FURTHER KINETIC AND STRUCTURAL CHARACTERIZATION OF THE LYSOSOMAL α-D-GLUCOSIDE GLUCOHYDROLASE FROM CATTLE LIVER

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SUMMARY

1. The kinetic and structural properties of the lysosomal α-D-glucoside glucohydrolase (EC 3.2.1.20) from cattle liver have been further investigated. The purified enzyme is able to incorporate tracer quantities of glucose into glycogen starting either from glucose or maltose. The enzyme hydrolyzing a synthetic substrate is inhibited by glucose.

2. Ultracentrifugal analysis of the sedimentation of the enzyme in guanidine-HCl solutions shows that the protein is composed of subunits of similar molecular weight (25 000) held together by noncovalent bonds.

INTRODUCTION

It is now possible to obtain the acid α-D-glucoside glucohydrolase (EC 3.2.1.20) in a pure state and in large amounts[1]. Since the physiological significance of this enzyme—which is believed to play an important role in glycogen metabolism[2]—is still rather obscure, it seemed worth while to investigate further the kinetic and structural features of this protein. While the role of the acid α-glucosidase in glycogen degradation seems now well established, the possibility that it is also involved in glycogen synthesis needs investigation. The results here reported show that the purified enzyme is in fact able to incorporate tracer amounts of glucose into glycogen.

An ultracentrifugal analysis on the protein, which has a molecular weight of 107 000 (ref. 1), has also been carried out. The protein molecule is composed of subunits of similar molecular weight.

MATERIALS AND METHODS

Enzyme

The enzyme used throughout this study was prepared from cattle liver as previously described[1].

Chemicals

Maltose was obtained from Merck; shellfish glycogen from Sigma Chemical Co.;

uniformly ^{14}C-labeled-D-glucose (5 mC/mmole) from New England Nuclear Corp.; and uniformly ^{14}C-labeled maltose (5.7 mC/mmole) from the Radiochemical Centre, Amersham. Radioactive glucose was pure according to paper chromatography. 6-Bromo-2-naphthyl-α-D-glucopyranoside was obtained from Pierce Chemical Co., and Guanidine–HCl Ultrapure (M.A.) from Mann Research. All other reagents were of analytical grade.

Enzyme assays

The hydrolytic activity of α-glucosidase on either maltose or 6-bromo-2-naphthyl-α-D-glucopyranoside was measured as previously described[1]. In order to measure the incorporation of glucose into glycogen, starting either from radioactive glucose or maltose, a modification of the method of HERS[3] was used. In the standard assay 1 ml of the incubation mixture contained 116 mg of glycogen, 20 μmoles of acetate buffer (pH 5.0), 4 μg of enzyme and either 3 μC of radioactive maltose or 30 μC of radioactive glucose. The reaction was allowed to proceed at 37° for various times. Aliquots of 0.35 ml were added to 1 ml of 1 M trichloroacetic acid, and 2 ml of water were then added. The glycogen was precipitated with 4 ml of 95° ethanol and centrifuged at 2000 rev./min for 15 min. The pellet was resuspended in 2 ml of water and precipitated with ethanol once more. The sediment was resuspended in 2 ml of 20% KOH and heated at 100° for 30 min. The glycogen was precipitated twice more and finally redissolved in 0.3 ml of water. Aliquots of 0.1 ml were added to scintillation flasks containing 12 ml of Liquifluor (New England Nuclear Corp.) diluted 1:25 (v/v) with toluene, 2 ml of absolute ethanol and 0.3 ml of NCS solubilizer (Amersham-Searle), and counted for 10 min in a liquid scintillation spectrometer. Specific activity is expressed as nmoles of radioactive glucose incorporated per min per mg of protein. Protein concentration was measured by the microbiuret method of ZAMENHOFF[4].

Disk gel electrophoresis

Polyacrylamide gel electrophoresis at low pH was performed according to REISFELD *et al.*[5]. The acrylamide concentration was 7% in 0.04 M acetate buffer (pH 3.5). Electrophoresis was performed at 4° for 90 min with 7 mA per tube, in 0.04 M acetate buffer (pH 3.5). The gels were stained for proteins with 0.2% naphthalene black 12B in 7% acetic acid and destained with 7% acetic acid.

The enzyme activity on the gels was detected by a modification of the method of RUTENBURG *et al.*[6] for the histochemical demonstration of α-glucosidases. After the runs the gels were incubated for 30 min at 37° in 0.06 M acetate buffer (pH 4.5) containing 0.1 mg/ml of 6-bromo-2-naphthyl-α-D-glucopyranoside. At the end of the incubation the gels were briefly rinsed in cold water and stained with a solution of tetrazotized *o*-dianisidine (Fast Blue B) (2 mg/ml) in 24 mM $NaHCO_3$ (pH 8.1). The staining solution was replaced at 10 min intervals. The color developed in about 1 h.

Analytical ultracentrifugation

Ultracentrifuge studies were done in a Spinco Model E analytical ultracentrifuge at 20°. Sedimentation velocity experiments were performed at 56 100 rev./min. The s_{20} in 5 M guanidine– HCl were determined at different protein concentrations (4–10 mg/ml) and the s^0_{20} obtained by extrapolation of a plot of sedimentation co-

efficients to infinite dilution. The s observed were corrected to the standard conditions of water at 20° on the basis of (a) a density of 1.126 g per ml at 20° for the guanidine–HCl solution, (b) a viscosity for the guanidine–HCl solution of 1.169 cP (ref. 7) and (c) the assumption that the partial specific volume of the enzyme is 0.730 ml/g in the guanidine–HCl solution.

For determination of molecular weight, the enzyme (0.4–1.0 mg/ml) in 5 M guanidine–HCl was subjected to equilibrium centrifugation by the meniscus depletion method of YPHANTIS[8], with the use of Rayleigh interference optics and a double sector cell. The runs were performed either at 33 450, at 37 020 or at 42 040 rev./min. Fringe displacement on photographic plates was measured with a Nikon two-dimensional microcomparator.

Acylation of the protein

Enzyme was acylated with succinic anhydride as described by KLOTZ[9]. A 20-fold excess of reagent, with respect to the molarity of the amino groups in the protein, was used. The pH was kept constant at 8.5 with 2 M KOH using an Agla Syringe and miniature electrodes (Leeds and Northrup) connected to a Radiometer pH meter, Model PHM-26. The reagent was slowly added during 30 min; after 60 min standing the acylated protein was dialyzed overnight against 0.1 M phosphate buffer (pH 6.7) containing 25 mM NaCl and 1 mM EDTA and then subjected to analytical ultracentrifugation.

Exposure to alkaline pH

A 1% solution of the enzyme in 25 mM NaCl and 1 mM EDTA was brought to pH 10.5 with 1 M KOH and allowed to stand at room temperature for 60 min. After this treatment the enzyme was subjected to analytical ultracentrifugation.

Treatment with anionic detergent

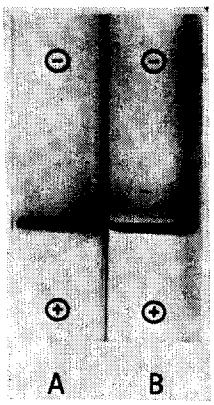
A 1% solution of the enzyme was dialyzed at 4° against 2% sodium dodecyl sulfate in 0.1 M phosphate buffer (pH 6.7).

Guanidine–HCl treatment

The enzyme at various concs. was dissolved in and dialyzed against 50 vol. of 0.1 M phosphate buffer *plus* 25 mM NaCl and 1 mM EDTA containing 5 M guanidine–HCl. The solution was adjusted to pH 6.7 with 2 M KOH. The dialysis time was at least 72 h. The protein was then subjected either to sedimentation velocity or to equilibrium centrifugation or to dextran gel filtration. The gel filtration experiments were carried out using a Sephadex G-100 column (1 cm diameter × 50 cm; total volume 40 ml) equilibrated and developed with the same guanidine–HCl solution. Fractions of 1 ml were collected. The absorbance at 280 nm was followed.

RESULTS

The enzyme used in all the experiments described below had a specific activity of 34 μmoles of maltose hydrolyzed per min per mg of protein and was homogeneous according to disk gel electrophoresis and analytical ultracentrifugation. Moreover, since the enzyme is able to hydrolyze the synthetic glucoside 6-bromo-2-naphthyl-



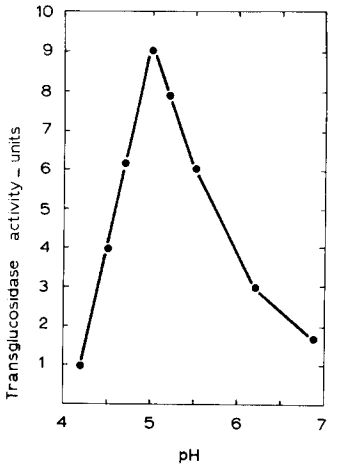


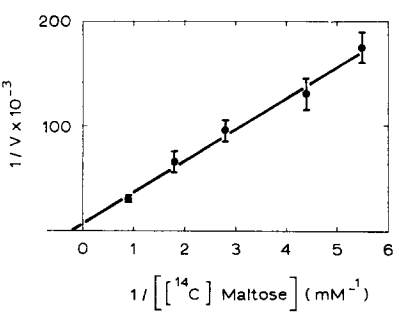
Fig. 1. Electrophoresis in polyacrylamide gel of acid α-glucosidase; 25 μg of enzyme in 100 μl of 0.04 M acetate buffer (pH 3.5), 7 mA per tube, 90 min at 4°. A. Gel stained for proteins. B. Gel stained for acid α-glucosidase activity. For further experimental details see MATERIALS AND METHODS.

Fig. 2. pH dependence of transglucosidase activity by purified α-glucosidase. [^{14}C] Maltose was used as glucosyl donor. The following buffers were used: 0.02 M acetate (pH 3.5–5.5) and 0.02 M phosphate (pH 6–7).

α-D-glucopyranoside[1], we detected the enzyme activity on disk gel electrophoresis by the use of this substrate. Fig. 1 shows that the purified enzyme migrated as a single band of protein, coincident with a band of activity.

When the transglucosidase activity was measured the incorporation of radioactivity into glycogen was linear up to 30 min and 4 μg of protein. Fig. 2 shows the pH dependence of enzyme activity: the pH optimum was 5.0. Several determinations of enzyme velocity against substrate concentration were carried out; Fig. 3 depicts one of these experiments. The K_m values obtained fell between 2 and 5 mM and averaged 3.4 mM.

The enzyme was also able to incorporate [^{14}C]glucose into glycogen, even though



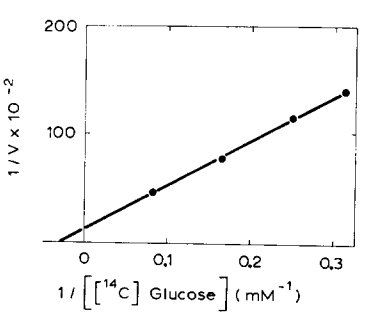
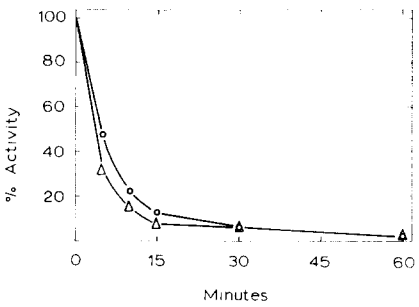


Fig. 3. Double reciprocal plot of transglucosidase activity of purified α-glucosidase, using maltose as glucosyl donor. Velocities (v) are expressed as nmoles of [^{14}C] glucose incorporated into glycogen per min per mg of protein.

Fig. 4. Double reciprocal plot of glucose into glycogen incorporation by purified α-glucosidase. Technical details as in Fig. 3.



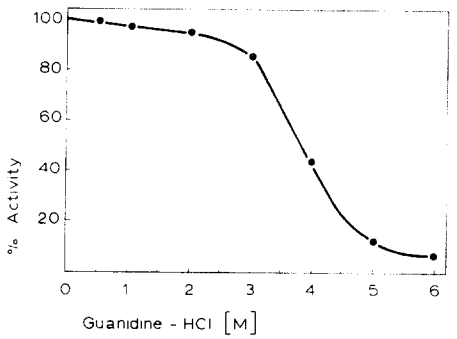


Fig. 5. Time dependence of inactivation of maltase activity of purified α-glucosidase by 6 M guanidine–HCl. The enzyme samples were incubated at both 0° (○) and 25° (△) in 6 M guanidine–HCl in 0.1 M phosphate buffer (pH 6.7). At various intervals of time aliquots were withdrawn and the maltase activity measured after appropriate dilution.

Fig. 6. Dependence of enzyme activity on guanidine–HCl concentration. The enzyme samples (protein concentration 1 mg per ml) were incubated at 25° in 0.1 M phosphate buffer (pH 6.7), containing increasing amounts of guanidine–HCl. After 30 min of incubation, aliquots were withdrawn and maltase activity measured after appropriate dilution.

to a much lower extent than maltose. The reaction was linear at least up to 60 min and 16 μg of enzyme. The pH optimum was also 5.0, and the K_m in mM 40 mM (Fig. 4).

In previous experiments, inhibition of enzyme activity was observed at high substrate (maltose) concentrations. The double reciprocal plot of enzyme velocity *versus* substrate concentration suggested a product inhibition[1]. We used the synthetic substrate 6-bromo-2-naphthyl-α-D-glucopyranoside to investigate the effect of glucose on the enzyme activity. Inhibitions of 50 and 100% were observed with 10 and 100 mM glucose, respectively.

Several attempts were made to test whether the enzyme molecule is composed of subunits. When the protein was exposed to alkaline pH, the enzyme activity was totally lost and the ultracentrifugal analysis showed that a heavy aggregation had occurred. Furthermore, after acylation of the enzyme with succinic anhydride the sedimentation coefficient remained unchanged with respect to that of the native protein. A massive precipitation occurred when the enzyme was dialyzed against 2% sodium dodecyl sulfate.

We also studied the effect of guanidine–HCl on both activity and structure of the α-glucosidase. Fig. 5 shows the effect of 6 M guanidine–HCl at two different temperatures as a function of time. Almost 100% inactivation was reached after 15 min both at 0 and 25°. We then tested whether lower concentrations of guanidine–HCl were also effective in inactivating the enzyme. Fig. 6 shows the behavior of enzyme activity with respect to guanidine–HCl concn. Up to 3 M the enzyme was stable: then it critically lost activity, which was reduced to about 10% at 5 M concentration.

In order to clarify whether the loss of enzyme activity at the highest concentrations of the unfolding agent was or was not associated with structural changes, ultracentrifugal analyses of the protein in 5 M guanidine–HCl were carried out. Fig. 7 shows the sedimentation pattern of the enzyme in 5 M guanidine–HCl. Besides

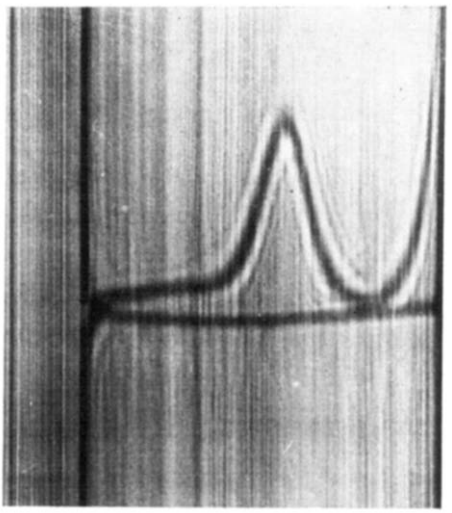
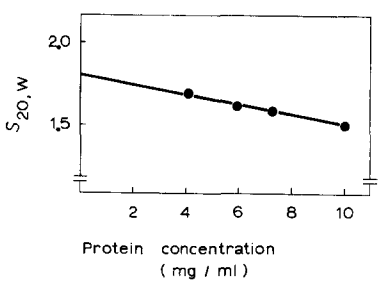


Fig. 7. Sedimentation velocity pattern of purified α-glucosidase in 5 M guanidine–HCl. Protein concentration 10 mg per ml in 5 M guanidine–HCl in 0.1 M phosphate buffer containing 25 mM NaCl and 1 mM EDTA (pH 6.7). The picture was taken at a bar angle of 60°, 6 h after reaching maximal speed (56 100 rev./min).

some light material, a major component with a sedimentation coefficient of 1.5 S was present. In order to study the nature of the light material a Sephadex gel filtration in guanidine–HCl was performed. Besides a broad peak composed of dialyzable material, which appeared with the total volume of the column, a major peak which exhibited a molecular weight of 26 000—as determined by calibration of the column with trypsin and bovine serum albumin[10]—was detectable. It seems likely that the dialyzable material recovered by gel filtration corresponded to the light substance observed in the sedimentation velocity pattern.



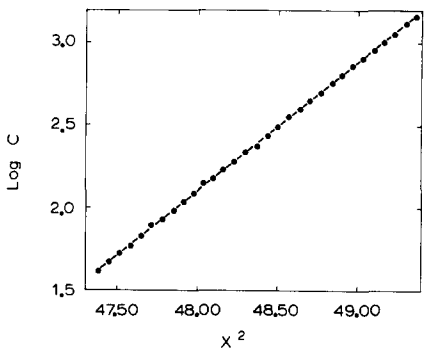


Fig. 8. Dependence of sedimentation coefficient on protein concentration for α-glucosidase in 5 M guanidine–HCl. The $s_{20,w}$ value extrapolated to zero protein concentration is 1.8. Technical details are as described in MATERIALS AND METHODS.

Fig. 9. Sedimentation equilibrium of purified α-glucosidase in 5 M guanidine–HCl. Double sector cell, rotor speed 37 020 rev./min. Equilibrium time 72 h; temperature 20°. Protein 0.5 mg per ml in 5 M guanidine–HCl in 0.1 M phosphate buffer (pH 6.7), containing 25 mM NaCl and 1 mM EDTA. Abscissa, square of distance from the center of rotation (X); ordinate, logarithm of protein concentration (c).

Fig. 8 shows the dependence of sedimentation coefficient of the enzyme in 5 M guanidine–HCl on protein concentration. The sedimentation constant ($s^{\circ}_{20,w}$) was 1.8 S. The enzyme, exhaustively dialyzed against 5 M guanidine–HCl, was subjected to equilibrium centrifugation by the meniscus depletion method of YPHANTIS[8]. The molecular weight from several experiments averaged 25 000 ± 3.000. Fig. 9 depicts one of these experiments. A small amount of a heavier component (mol. wt. 50 000 ± 5000) was sometimes detectable at the bottom of the cell. When the peak that exhibited a molecular weight of 26 000 on Sephadex was subjected to equilibrium centrifugation, a picture superimposable on that reported in Fig. 9 was obtained.

The effect of β-mercaptoethanol (0.14 M) on both the kinetic and structural properties of the enzyme in guanidine–HCl was also investigated. No differences either in the rate of inactivation with respect to time and to the guanidine–HCl concns., or to the sedimentation coefficient and the molecular weight, were observed.

DISCUSSION

A simultaneous absence of both acid maltase and acid transglucosidase activities has been reported to occur in tissues of patients with Type II glycogenosis[3]. On the basis of this observation it has been postulated that both activities are related to the same enzyme[2]. The possibility, however, that Type II glycogenosis could be due to a multi-enzyme defect, could not be ruled out[2].

The results now obtained show that pure α-glucosidase actually possesses not only hydrolytic but also transglucosidase activity; in fact the enzyme is able to incorporate glucose into glycogen. Lack of this enzyme in Type II glycogenosis can well account for loss of both activities. The K_m for maltose in the transfer reaction (3.4 mM) is similar to the K_m for maltose in the hydrolytic reaction (10 mM). Furthermore, the enzyme is also able to incorporate [^{14}C]glucose into glycogen with a K_m and a v_{max} that are about one tenth of the values obtained using maltose as glucosyl donor. This difference can be due to the fact that the enzyme has more affinity for maltose than for glucose. The physiological significance of this transglucosidase activity, *i.e.* the maltose reaction, requires further investigation. However, since large amounts of glycogen are needed to start the reaction, it is very unlikely that such a transferase activity is significant *in vivo*.

Some authors suggest that different acid α-glucosidases could be present in different tissues[2,11]. The method reported here to reveal glucosidase activity on disk gels should enable a search to be made for electrophoretic differences between acid α-glucosidases from various tissues.

Mild experimental conditions such as alkaline pH and acylation failed to dissociate the α-glucosidase. However, treatment with guanidine–HCl was able to dissociate the protein. The sedimentation constant of the enzyme in guanidine–HCl was very low (1.8 S) as compared with that of the native enzyme (5.7 S) and cannot readily be accounted for on the assumption that the enzyme is unfolded but not dissociated into subunits in the guanidine–HCl solution. Moreover, the sedimentation equilibrium experiments of the protein (mol. wt 107 000) in 5 M guanidine–HCl showed the presence of a single component with a molecular weight of 25 000. The heavier component (mol. wt. 50 000) occasionally found could represent either a different subunit

or the product of an incomplete dissociation. The more likely model, however, for the structure of the acid α-glucosidase is that of a molecule composed of subunits of similar molecular weight held together by non-covalent interaction.

After we had sent this paper to Biochim. Biophys. Acta, a report on kinetic characterization of a lysosomal α-glucosidase purified from rat liver appeared in Biochemistry (refs. 12, 13). The rat liver enzyme can also act as a transglucosidase at acid pH and, like the cattle enzyme[1], has isomaltase activity.

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