The stability and aggregation properties of human liver acid β -D-galactosidase

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(Received 26 August 1980/Accepted 27 October 1980)

1. A method is described for following continuously the action of β -galactosidase on 4-methylumbelliferyl β -D-galactoside at pH 4.5, in which 4-methylumbelliferone production is measured at fluorescence excitation and emission wavelengths of 324 and 444 nm respectively. 2. Initial-rate studies show that the presence of salt activates β -galactosidase up to 100 mM, but is inhibitory above that concentration. The enzyme is very unstable at 37°C and low ionic strength, but stability increases with ionic strength. 3. The stability of the enzyme at 37°C decreases markedly with rising pH in the range 5.9–8.0. 4. Gel-filtration patterns demonstrate that there is a marked tendency to polymerization with increasing ionic strength. The gel-filtration pattern shows decreasing amounts of dimer with increasing pH. 5. The correlation between activity, stability and molecular form of β -galactosidase is discussed. It is suggested that the dimeric form of the enzyme is the most stable and active form. The implications of this finding for the assay of β -galactosidase under physiological conditions for prenatal diagnosis are discussed. 6. Evidence for the possible occurrence of a 36 000-mol.wt. form of β -galactosidase is presented. 7. A computer program for the calculation of initial rates has been deposited as Supplementary Publication SUP 50114 (4 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1981) 193, 5.

The existence of multiple forms of acid β -galactosidase (EC 3.2.1.23) in human liver has been reported by many workers (Hultberg & Ockerman, 1969; Cheetham & Dance, 1976; Frost *et al.*, 1978). These forms have been designated A₁, A₂ and A₃ and have molecular weights of 65 000, 150 000 and 660 000 respectively when determined by gel filtration under non-denaturing conditions (Cheetham & Dance, 1976; Frost *et al.*, 1978), and the data presented by these workers are consistent with the hypothesis that A₁, A₂ and A₃ are monomeric, dimeric and decameric forms of the same protein. The relative amounts of these forms of β -galactosidase in any preparation depend on the method of purification and the conditions used for the gel filtration. Preliminary experiments by both Hoeksema *et al.* (1979) and Cheetham & Dance (1976) showed that aggregation was probably salt-dependent and at least partially reversible, but no detailed analysis of the system has been presented.

It has been reported (Ho & O'Brien, 1970) that the activity of human liver acid β -galactosidase is both stimulated and stabilized by Cl⁻ ions. This is in

direct contrast with the activity of the neutral β -galactosidases of human liver, where at least one of the enzymes is inhibited by Cl⁻ ions (Cheetham & Dance, 1976). In all cases these observations have relied on a stopped assay system, where it is impossible to assess the relative contributions of stimulation and stabilization.

A knowledge of the stability and aggregation properties of β -galactosidase is crucial to our understanding of co-cultivation studies carried out between normal cells and cells deficient in β -galactosidase. Reuser *et al.* (1976) were unable to demonstrate intercellular exchange of β -galactosidase after co-cultivation under conditions where other lysosomal enzymes such as *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) were transferred. Very low extracellular activity of β -galactosidase has been shown in normal fibroblasts by Halley *et al.* (1978).

The present study was undertaken to determine the effect of various parameters on β -galactosidase activity, and to this end a flow system for the continuous measurement of fluorescence changes

and hence determination of initial velocity has been developed. The state of aggregation of β -galactosidase as a function of ionic strength and pH has been determined by gel filtration and the influence of aggregation state on the stability and activity of the enzyme assessed.

Experimental

Materials

Concanavalin A linked to Sepharose 4B and Sephadex G-200 were from Pharmacia (G.B.) Ltd., Hounslow, Middx. TW3 1NE, U.K. 4-Methylumbelliferyl β -D-galactopyranoside and 4-methylumbelliferone were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. DEAE-cellulose (DE52) was from Whatman, Maidstone, Kent, U.K. α -Methyl D-mannopyranoside was from Sigma (London) Chemical Co., Poole, Dorset, U.K., and the protein assay kit was supplied by Bio-Rad Laboratories, Bromley, Kent, U.K.

Human livers, which appeared normal on gross inspection, were obtained at autopsy and used fresh.

Partial purification of acid β -galactosidase

All stages were carried out at 4°C unless otherwise specified. The enzyme was extracted by macerating 150 g wet wt. of liver in 600 ml of 5 mM-sodium phosphate buffer, pH 6.0, containing 10 mM-NaCl and centrifuging at 24 000 *g* for 30 min.

Partial purification of the β -galactosidase in the supernatant was achieved by a batch separation on DEAE-cellulose, followed by chromatography on a concanavalin A-Sepharose column. DEAE-cellulose (DE52; 50 g) was pre-equilibrated with the extraction buffer and the resulting slurry (100 ml) was mixed with the supernatant, stirred for 30 min and then centrifuged at 20 000 *g* for 5 min. The supernatant, which contained only small amounts of β -galactosidase, was discarded and the DEAE-cellulose was resuspended in 250 ml of 10 mM-sodium phosphate buffer, pH 6.0, containing 500 mM-NaCl. The suspension was stirred for 30 min and the DEAE-cellulose sedimented by centrifugation as before. The extraction and sedimentation of the DEAE-cellulose was repeated, and the two supernatants were combined and dialysed exhaustively against 10 mM-sodium phosphate buffer, pH 7.0, containing 10 mM-NaCl. After centrifugation at 20 000 *g* for 5 min to remove any sediment formed, the supernatant (470 ml) was applied to a concanavalin A-Sepharose column and eluted with methyl mannoside as described by Norden & O'Brien (1974). The eluate had a specific activity of 145 units/mg of protein, was free of neutral β -galactosidase activity and was stored at -20°C.

Gel filtration

Packing and elution of a Sephadex G-200 column (1 cm $\times$ 30 cm) were performed under gravity at 4°C. The column was equilibrated by passage of at least 5 bed vol. of the desired buffer in each case. Routinely samples of the liver extract after partial purification by concanavalin A-Sepharose chromatography were adjusted to the desired buffer conditions and left at 4°C for 18 h. Then 100 μ l of the equilibrated extract was applied to the Sephadex column and 250 μ l fractions were collected. The fractions were assayed for β -galactosidase activity by the stopped-assay system. The columns were calibrated by the method of Andrews (1965), with as markers cytochrome *c* (mol.wt. 13 500), malate oxidoreductase (mol.wt. 70 000), lactate oxidoreductase (mol.wt. 120 000) and catalase (mol.wt. 230 000).

Protein determination

Protein was determined by the method of Bradford (1976) with a Bio-Rad protein assay kit and the freeze-dried protein supplied as standard.

β -Galactosidase assay

(a) *Stopped assay.* To assay acid β -galactosidase, 10 μ l of enzyme was added to 70 μ l of buffered substrate solution, giving a final concentration of 0.50 mM-4-methylumbelliferyl β -D-galactoside in 50 mM-sodium acetate buffer, pH 4.5, containing 100 mM-NaCl. After incubation at 37°C for 12 min, the reaction was stopped by the addition of 800 μ l of 0.1 M-2-amino-2-methylpropan-1-ol/HCl buffer, pH 10.4. Fluorescence was measured in circular micro-cells (500 μ l volume) on a Hitachi-Perkin-Elmer model 204 spectrofluorimeter, with excitation and emission wavelengths of 364 nm and 444 nm respectively. 4-Methylumbelliferone was used as a standard. The unit of enzyme activity is defined as the amount of enzyme that catalyses the production of 1 nmol of 4-methylumbelliferone/min under the above conditions.

Neutral β -galactosidase activity was measured by the method of Ho & O'Brien (1971).

(b) *Kinetic assay.* To allow continuous measurement of 4-methylumbelliferone production, a kinetic assay was developed. Preliminary experiments showed that, in buffer at pH 4.5, the wavelength of maximum excitation of 4-methylumbelliferone was shifted to 338 nm (Fig. 1), but the wavelength of maximum emission remained at 444 nm, as in alkaline solution. At 324 nm, at pH 4.5, 4-methylumbelliferone shows 86% of its maximum excitation, and at this wavelength the fluorescence of 4-methylumbelliferyl β -D-galactoside was negligible. Although the quantum efficiency was decreased to only 5% of that at alkaline pH, the use of these

Fig. 1. Fluorescence excitation spectra of 4-methylumbelliferone and 4-methylumbelliferyl β -D-galactoside at pH 4.5

Spectra were measured in 10 mM-sodium acetate buffer, pH 4.5, at an emission wavelength of 444 nm. —, 2.5 μ M-4-methylumbelliferone; ----, 1 mM-4-methylumbelliferyl β -D-galactoside.

wavelengths to determine 4-methylumbelliferone production continuously at pH 4.5 is feasible.

A flow cell was constructed to replace one of the standard rectangular fluorimeter cells (Fig. 2). An aluminium block (50 mm $\times$ 12 mm $\times$ 12 mm) was drilled in the centre of a square side to give a 2 mm circular hole along the long axis of the block. Slits (10 mm $\times$ 2 mm) for excitation and emitted light were cut into the block on adjacent sides at a height of 20 mm from the base of the block. A groove (4 mm deep) was cut at the base of the block to connect with the hole. A micro-cell was constructed from Spectrosil precision tubing of diameter to give a loose fit in the central hole of the block. The tube was bent at 90° at one end to fit the groove and constrictions were made at lengths just outside those covered by the slits. The total volume of the cuvette was approx. 50 μ l. All internal and external surfaces

Fig. 2. Flow cell for continuous fluorimetric measurement
For further details of construction see the text.

of the block were treated with matt black to avoid reflections.

The reaction was carried out in a vessel with a water jacket so that a constant but variable temperature could be maintained. The incubation mixture was stirred by a magnetic stirrer and circulated through the flow cell by coupling with fine polypropylene tubing to a peristaltic pump. The pumping rate was 5 ml/min and the mixture was returned to the incubation vessel from the output of the flow cell. The fluorimeter was connected to a recorder and the system was calibrated with standard solutions of 4-methylumbelliferone.

In a typical kinetic assay, 5 ml of a solution containing 0.5 mM-4-methylumbelliferyl galactoside in 10 mM-sodium acetate buffer, pH 4.5, was placed in the reaction vessel maintained at 37°C and circulated until a stable recorder trace was obtained. The reaction was started by adding 10 μ l of an appropriate dilution of enzyme solution and the fluorescence recorded continuously for 30 min. Fig. 3 shows a typical experiment. In control experiments it was found that non-enzymic hydrolysis of substrate and photo-decay of the 4-methylumbelliferone were small and mutually cancelling effects. The ionic strength of the incubation mixture was varied either by including different concentrations of NaCl in the buffered substrate or by varying the concentration of the acetate buffer.

The rate of release of 4-methylumbelliferone in the enzymically catalysed reaction (Fig. 3) shows an exponential decay. The initial velocity of the reaction and the final product concentration were computed by using a Gauss-Newton iteration method for

Fig. 3. *Kinetic assay of β -galactosidase*
Continuous trace of 4-methylumbelliferone production measured at 324 nm excitation wavelength and 444 nm emission wavelength in the presence of 100 mM-NaCl. For other details see the text. ----, Initial rate of reaction computed from the continuous trace.

$y = k_1 e^{-k_2 x}$ and by using a least-squares method applied to a Taylor-series expansion of this equation to calculate the correction factors. This computer program in FORTRAN (Supplementary Publication SUP 50114) has been deposited with the British Library Lending Division. The limit was usually set so that the program terminated when the correction was less than 0.1% of the calculated factor. The data used were readings of the recorder trace at 1 min intervals. Estimates for final product concentration and initial velocity supplied to the computer were very approximate, and a 10-fold overestimate or underestimate could be tolerated by the program.

Results

Effect of ionic strength on activity at 37°C

The initial velocity of the reaction catalysed by β -galactosidase as measured at 37°C by kinetic assay is dependent on the ionic strength of the incubation mixture. Maximal activity is observed when the reaction is carried out in 100 mM-sodium acetate buffer, pH 4.5, and the activity falls to only 66% of the maximal value in 1 M-sodium acetate buffer. Similar results are obtained if the ionic strength is increased by adding NaCl to a constant sodium acetate buffer concentration.

Fig. 4. *Effect of preincubation at 37°C at different ionic strengths on the stability of β -galactosidase*

After preincubation for various periods of time in 10 mM-sodium acetate buffer, pH 4.5, containing various amounts of NaCl (●, 1 M; ○, 100 mM; ▲, 10 mM; △, none), the enzyme activity remaining was measured by stopped assay as described in the text.

Stability to preincubation at various ionic strengths at 37°C

The effect of preincubation at 37°C and various ionic strengths (obtained by addition of NaCl to 10 mM-sodium acetate buffer, pH 4.5) for times in the range 0–20 h before normal stopped assay for 12 min are shown in Fig. 4. With no added salt, the enzyme activity decreases to about 33% of its original value after 4 h. The loss of activity is progressive, and after 20 h only 14% of the activity remains. Stability increases as the ionic strength during preincubation is increased up to 100 mM-NaCl, when 80% and 50% of the activity remain after 4 h and 20 h respectively. Further increases in ionic strength have little effect on stability.

Gel filtration of β -galactosidase at different ionic strengths

The concentration of Cl^- ions in the eluting buffer has a marked effect on the properties of β -galactosidase on gel filtration (Fig. 5). With no Cl^- in the eluting buffer, three peaks of activity are resolvable, corresponding to mol.wts. 36 000, 67 000 and 130 000 (Fig. 5a). In the presence of 1 M-NaCl, a major peak of activity of mol.wt. greater than 400 000 is seen, with shoulders corresponding to the lower-molecular-weight forms (Fig. 5b). At intermediate ionic strengths there is a progressive decrease in the amounts of low-molecular-weight forms observed and an increase in the high-molecular-weight form as ionic strength increases. Cl^- ion therefore increases the aggregation of β -galactosidase to polymeric forms.

Ionic strength has a considerable stabilizing effect on the β -galactosidase activity. In the absence of Cl^-

80% of the activity is lost during the gel filtration, but in 1 M-NaCl only 20–40%. There was no loss of activity in either case during the pre-equilibration at 4°C.

Stability to preincubation at various pH values at 37°C

The effect of preincubation at 37°C in 20 mM-Pipes (1,4-piperazinediethanesulphonic acid) buffer of various pH values containing 100 mM-NaCl for times in the range 0–7 h before normal stopped assay for 12 min are shown in Fig. 6. Typically, at pH 5.9, only 25% of the activity is lost over 6 h, whereas at pH 8.0 93% of the activity is lost in 2 min. This decrease in stability is progressive over the pH range 5.9–8.0. When the percentage of activity remaining after 30 min preincubation is plotted against pH of preincubation, a sharp discontinuity is seen in the pH range 6.8–7.3 (Fig. 7).

Fig. 5. Gel filtration of β -galactosidase on Sephadex G-200 in the presence of various concentrations of NaCl. Elution was at 4°C with 10 mM-sodium acetate buffer, pH 4.5, containing added NaCl as specified. Fractions (250 μ l) were assayed by the stopped assay technique. (a) ○, 10 mM-NaCl; ●, No added salt. (b) □, 1 M-NaCl; ■, 100 mM-NaCl.

Fig. 6. Effect of preincubation at 37°C and various pH values on the stability of β -galactosidase.

After preincubation for various periods of time in 20 mM-Pipes buffer containing 100 mM-NaCl, the enzyme activity remaining was measured by stopped assay as described in the text. ○, pH 5.9; ■, pH 6.5; □, pH 7.0; ●, pH 8.0.

Fig. 7. Effect of pH on the percentage of the enzyme activity remaining after preincubation at 37°C for 30 min. For further details see the text.

Fig. 8. Gel filtration on Sephadex G-200 at various pH values

Elution was at 4°C with 20mM-Pipes buffer containing 100mM-NaCl. O, pH 5.9; ●, pH 7.0; Δ, pH 8.0.

Gel filtration of β -galactosidase at different pH values

The gel-filtration pattern of β -galactosidase at constant ionic strength is influenced markedly by the pH of the eluting buffer (Fig. 8). At pH 6.0, the molecular weight of the predominant species is 130 000. On increasing the pH to 7.0, this 130 000-mol.wt. peak disappears and is replaced by two peaks of mol.wts. 67 000 and >400 000. On further increase to pH 8.0, the high-molecular-weight material is not seen and a single peak of mol.wt. 67 000 is observed. There was no loss of activity during pre-equilibration at 4°C for 18 h, but the recoveries from the column were 84%, 36% and 4.5% for pH 6.0, 7.0 and 8.0 respectively.

Discussion

Our results show clearly the potential pitfalls encountered in β -galactosidase assay. At 37°C the time course of the enzymically catalysed reaction departs markedly from linearity, and gross errors may be introduced if times over 15 min are used in a stopped-assay system. This is particularly relevant for clinical biochemistry, where β -galactosidase activity is assayed routinely for pre-natal diagnosis of certain lysosomal storage diseases, and the few cells available and weak enzyme activity necessitate extended incubation times.

The influence of ionic strength on the activity and stability of β -galactosidase is complex. The finding that the enzyme activity increases up to 100mM-acetate and Cl^- concentrations is similar to the results of Ho & O'Brien (1970), although they did not examine the even higher inhibitory salt concentrations.

The enzyme is reasonably stable at both -20°C and 4°C at all ionic strengths, but at 37°C and pH 4.5, it is progressively unstable as ionic strength decreases.

Stability is also pH-dependent. In the pH range 5.9–8.0 the enzyme is stable at 4°C, but at 37°C is very unstable at pH 8. At lower pH values it is more stable.

This complex activity and stability pattern is explicable by considering the aggregation state of the enzyme under various conditions. It is compatible with a model where the monomer (mol.wt. 67 000) has negligible activity, the dimer (mol.wt. 130 000) is fully active and the decamer (mol.wt. 660 000) shows only half the activity of the dimer. Under the normal conditions of stopped assay, i.e. 100mM-NaCl and pH 4.5, the dimer predominates and maximal activity is seen. Chang (1978) has previously shown that renal β -galactosidase is reversibly aggregated by NaCl.

The stability results indicate an equilibrium between monomer, dimer and decamer dependent on pH and ionic strength. At 4°C all the forms are stable even at low ionic strength, so that full activity is seen when the equilibrium is shifted by the addition of high salt to the assay medium. At 37°C the monomer, present at low ionic strength or high pH, is rapidly denatured and the equilibrium is disturbed irreversibly. The explanation of the low recovery on gel filtration at 4°C is less obvious. Probably, when monomer is separated from the other forms it is destabilized, and denaturation is rapid and irreversible. The finding that the dimer virtually disappeared on raising the pH from 5.9 to 7.0 was unexpected. Equally unexpected was the disappearance of decamer between pH 7.0 and 8.0. If the monomer is denatured rapidly at 37°C, the fact that it is by far the predominant species at this pH explains the extreme instability of β -galactosidase at pH 8.0. An alternative explanation is that β -galactosidase is degraded by some contaminating enzyme in the preparation, but this is unlikely, since even highly purified preparations obtained by affinity chromatography show this extreme instability at pH 8.0 (C. M. Heyworth, unpublished work).

Under physiological conditions within the lysosome, i.e. at pH 5.9 and ionic strengths corresponding to 100 mM-NaCl, β -galactosidase is at its most stable. On its release into the extracellular environment it will be rapidly inactivated by the sharp rise in pH. This would explain the low extracellular activities of β -galactosidase observed by Halley *et al.* (1978) and the failure to show intercellular exchange of β -galactosidase on co-cultivation studies (Reuser *et al.*, 1976).

It has been assumed previously (Cheetham & Dance, 1976; Frost *et al.*, 1978) that the smallest subunit of β -galactosidase has a mol.wt. of 65 000. Our finding of a peak of activity corresponding to a mol.wt. of 36 000 on gel filtration was surprising. This peak is most evident when Cl^- ions are absent from the eluting buffer, under which conditions gel filtration is difficult and somewhat unreliable (Morgan & Ramsden, 1978), and this peak of activity may be an artefact of interaction between column material and enzyme. Previous studies (e.g. Cheetham & Dance, 1976; Frost *et al.*, 1978; Hoeksema *et al.*, 1979) employed moderate ionic strength or neutral pH, when this low-molecular-weight form would be negligible.

Several reports associate low-molecular-weight proteins with purified β -galactosidase. Lo *et al.* (1979) showed bands corresponding to mol.wts. 22 000 and 31 000 on electrophoresis of purified human placental β -galactosidase on polyacrylamide gels containing guanidine hydrochloride and sodium

dodecyl sulphate. Holmes & O'Brien (1979) found minor bands at mol.wts. 29 000 and 21 000 on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of purified cat liver β -galactosidase. Kress & Miller (1978) saw an acid β -galactosidase of mol.wt. 20 000–27 000 when human urine was fractionated on Sepharose 6B columns at low pH values.

Further support for a smaller subunit of β -galactosidase comes from the studies of Wit *et al.* (1979). They found a dual location of acidic β -galactosidase on chromosomes 3 and 22 by an analysis of cell hybrids and concluded that both genes were necessary for full expression of its activity. Possibly two such subunits come together to form the so-called monomeric species of mol.wt. 67 000.

We thank Professor H. Galjaard and his colleagues of Erasmus University, Rotterdam, and Professor D. Robinson and his colleagues of Queen Elizabeth College, London, for many helpful discussions. C. M. H. is grateful for the award of an S. R. C. studentship.

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