Heat-stable protein that stimulates acid α -glucosidase

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A hot-water extract of bovine spleen and guinea pig liver exhibited the ability to enhance acid α -glucosidase activity, with methylumbelliferyl α -glucoside, glycogen or maltose as substrate. The level of activator required for maximal stabilization was similar for all three substrates, indicating direct action on the enzyme rather than on substrate. The stimulator was partially purified by chromatography with gel-permeation (apparent M_r 20000–24000), ion-exchange and C_4 reverse-phase columns. It was retained by a narrow-pore dialysis tubing and destroyed by treatment with Pronase, and is presumably a protein. The stimulating protein protected the enzyme against denaturation by heat or incubation with a buffer of high ionic strength in the absence of substrate. RNA inhibited the enzyme, and the activator protein was able to counteract the effect. Activating material was found in a variety of mouse and rat tissues, as well as human urine.

INTRODUCTION

In the course of a study of endogenous inhibitors of glucosylceramide β -glucosidase (EC 3.2.1.45), we found that nucleic acids were strong inhibitors [1]. Several other lysosomal enzymes in a crude enzyme preparation were found to be inhibited also, but the measured activity for acid α -glucosidase (EC 3.2.1.20) showed confusing effects of nucleic acids and poor proportionality with respect to amount of enzyme. From the results, it appeared likely that a partly dissociated activator of the enzyme was present. When we heated the enzyme solution and tested the soluble product, we found that there was indeed a strongly stimulating material present. We report here on some properties of the activator (AGA) and the enzyme.

Lysosomal α -glucosidase hydrolyses the α -1,4- and α -1,6-glucosidic bonds present in glycogen, maltose and intermediate oligosaccharides, yielding free glucose [2,3]. The enzyme can also hydrolyse synthetic substrates, such as 4-methylumbelliferyl α -glucoside (MUG) [4]. Genetic lack of this enzyme results in Pompe's disease (glycogenosis type II), characterized by a fatal accumulation of glycogen particles in a lysosomal compartment [5].

EXPERIMENTAL

Materials

α -Glucosidase, purified approx. 2400-fold, was isolated from bovine spleen by the method of Murray *et al.* [6]. The portion of enzyme binding to Sephadex was used. Its specific activity was 1.5 μ mol/min per mg of protein, with MUG as substrate, in the assay system described below. Apparently neutral α -glucosidases were absent from this preparation, judged by the very low activity at pH 7. AGA did not stimulate this residual activity.

Oyster glycogen, maltose (grade I; < 2% maltotriose), Pronase E (nuclease-free; 4.7 units/mg in 25% calcium acetate) and other materials were from Sigma. Bovine spleens were from a slaughterhouse, frozen rapidly with solid CO_2 and stored at $-80^\circ C$. Guinea-pig livers

(frozen) were from Pel-Freez Biologicals, Rogers, AZ, U.S.A., and stored at $-20^\circ C$. Male mice of the ICR strain were from Harlan Sprague-Dawley. Two groups of six each were selected by a computer program [7] so that the mean weights were very similar (22.4 g) and the variances were minimal (0.5 g s.d.). For gel-permeation chromatography, a Superose 12 (Pharmacia) column (2.6 cm $\times$ 27 cm) was used, with Buffer A (50 mM-sodium phosphate, pH 7, 150 mM-NaCl, 0.02% NaN_3). Reverse-phase wide-pore butylsilylated silica gel was from J. T. Baker Chemical Co.

Glucosidase assays

Most assays for α -glucosidase activity were carried out with 4 mM-MUG in 20 mM-sodium acetate, pH 4.5, in a total volume of 0.2 ml. The incubations were run in disposable borosilicate glass tubes (10 mm $\times$ 75 mm), previously silicone-coated with Sigmacote and stored in vacuum over KOH. After 30 min at $37^\circ C$, 1 ml of 0.17 M-glycine in 0.5 M- Na_2CO_3 , pH 10, was added and the methylumbelliferone fluorescence was measured with excitation at 365 nm and emission at 450 nm. The amount of enzyme chosen was sufficient to hydrolyse ~ 1 nmol of substrate in 30 min. Under these conditions the observed activity was proportional to the amount of enzyme, and the rate of hydrolysis was constant.

The assay for AGA activity used a stimulation unit: 1 unit of AGA is the amount that produces a doubling of the basal enzyme activity during the 30 min incubation with MUG.

Assays of glucosidase activity toward other substrates were carried out similarly, but with more enzyme and 2.5% (w/v) glycogen (incubated for 1 h) or 10 mM-maltose (30 min incubation). The reaction was terminated by boiling for 5 min and measuring the glucose formed by the linked-enzyme method (Boehringer Mannheim glucose-determination kit, using $NADP^+$, Mg^{2+} , ATP, hexokinase and glucose-6-phosphate dehydrogenase). The amount of glucosidase chosen produced 1–20 μ g of glucose from maltose.

Abbreviations used: AGA, α -glucosidase activator; MUG, 4-methylumbelliferyl α -glucoside

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Protein was determined with the very sensitive Quanti-gold reagent (Diversified Biotech, Newton Centre, MA, U.S.A.) with bovine serum albumin (50–200 ng) as standard [8].

Purification of the activator

All steps were carried out at room temperature. Frozen guinea-pig liver was cut into small pieces and homogenized in boiling water (5 ml/g), with an overhead homogenizer. The suspension was maintained at 100 °C for 10 min, cooled in ice, and centrifuged for 20 min at 10000 *g*. The supernatant fluid was freeze-dried and taken up in water (0.1 ml/g of tissue). The cloudy mixture was clarified by brief centrifugation, and 0.8 ml was added to the gel-permeation column, prepared in and eluted with Buffer A.

Pronase treatment

A sample of AGA after the gel-permeation step (0.46 µg) was treated with 46 ng of Pronase in 0.5 ml of 40 mM-potassium phosphate, pH 7.4, for 3 h at 37 °C. The mixture was boiled for 60 min to inactivate the proteases and assayed for glucosidase-stimulating activity. As controls, we used Pronase that had been boiled for 60 min before the preincubation with AGA, as well as an incubation tube lacking Pronase, and a sample of AGA which was not preincubated at all. Tests with a chromogenic protein (Azocoll; Calbiochem) showed that Pronase was not completely inactivated even with 30 min of heating.

RESULTS

AGA purification

The initial crude extract from 30 g of guinea-pig liver, after freeze-drying and reconstitution in water, contained 113000 units of AGA activity in 16.8 mg of protein. A preliminary test showed that freeze-drying did not cause any loss of activity. Passage of 30100 units of AGA through the Superose column yielded a peak of active material eluted in the region corresponding to an M_r of ~24 500, judged from a set of standard proteins (Fig. 1). Pooling the major fractions yielded 23300 units of AGA in 0.60 mg of protein (77% yield, enrichment factor of 5.8). One unit of Superose preparation corresponded to 0.26 mg of liver.

Earlier trials with bovine spleen showed that the stimulating activity could be purified to some extent by Sephadex gel permeation and ion exchange with DEAE-Sephacel in Buffer A, by using a 0–0.5 M-NaCl gradient. A 25% stimulation, in a slightly different assay system, could be achieved with 0.22–0.25 µg of protein. For the major fraction from the ion-exchange column, 0.25 µg corresponded to only 60 µg of spleen (wet wt.). Maximal stimulation was ~70% with additional AGA. It was also possible to achieve some purification with a column of C_4 reversed-phase resin, eluted with water/acetonitrile/trifluoroacetic acid. Both methods, including dialysis with Spectra/Por tubing (Type 3, M_r 3500 cut-off; Spectrum Medical Industries, Los Angeles, CA, U.S.A.), resulted in appreciable losses, possibly due to adsorption.

Development of the assay method

In the early stages of this project, we evaluated the AGA activity by its ability to stimulate α -glucosidase

Fig. 1. Size-exclusion chromatography of AGA from guinea-pig liver

Concentrated AGA solution, from 10 g of tissue, was added to a Superose 12 column (2.6 cm × 27 cm), which was eluted with 50 mM-sodium phosphate, pH 7, containing NaCl and NaN_3 . Fractions (5.2 ml) were collected, diluted, and assayed for AGA (□) and protein (○) content. The ordinates show contents per fraction. Arrows mark the elution points for standard proteins: A, bovine serum albumin (M_r 67000); B, carbonic anhydrase (M_r 30000); C, cytochrome *c* (M_r 16500).

Fig. 2. Stabilizing and stimulating action of AGA

Glucosidase was incubated for various lengths of time at 37 °C, with or without a saturating amount (92 ng of protein) of purified guinea-pig AGA in 0.2 ml of 100 mM-sodium acetate, pH 4.5 (●, ○) or preincubated for 30 min in 0.1 ml of 200 mM buffer (■, □). In the former case, the solution contained 4 mM-MUG; in the latter case MUG was added after the initial preincubation period. At each time point, alkaline buffer was added to enhance the methylumbelliferone (MU) fluorescence.

and used a more concentrated buffer (100 mM) in non-silicone-treated tubes. A time/activity study (circles, Fig. 2) showed that the enzyme gradually lost activity when incubated without AGA. In the presence of AGA, however, the activity remained constant. Evidently the factor acted as both a stimulator and a stabilizer, and the

degree of stimulation therefore depended on the duration of the incubation. The stimulation was already visible by 15 min of incubation, at which point loss of glucosidase activity in the control tubes was quite slight. In a different experiment, with bovine AGA, stimulation was seen as early as 7.5 min from the start.

The enzyme, preincubated at 37 °C without MUG, rapidly lost all activity in 200 mM-acetate within 30 min. This demonstrates the partially stabilizing action of the substrate. Inclusion of AGA during the initial 30 min period protected the enzyme completely, producing an activity similar to that obtained if the preincubation was omitted (squares, Fig. 2).

Although the literature on α -glucosidase has reported various findings on the stability of the enzyme under various conditions, no one seems to have studied the stability of the enzyme during assay incubation conditions. The possibility that buffer strength affected the enzyme's stability was tested by time trials in our incubation system. These showed that the enzyme was stable in 20 mM-acetate for > 90 min, but gradually lost activity in 100 mM buffer (our initial standard concentration). Tests with intermediate concentrations of acetate buffer, by preincubating for 30 min in the absence of substrate, followed by incubation for another 30 min with MUG, showed different rates of inactivation (Fig. 3). Citrate and succinate buffers showed a similar relationship.

Tests of the stimulating effect of AGA with various amounts of activator produced an S-shaped curve, with the lowest amounts yielding little or no stimulation. The weak effectiveness of AGA at low concentrations suggested that it is readily lost by adsorption on the glassware. Attempts to block the adsorption by including a small amount of acetonitrile, butanol, bovine serum albumin or other proteins, and the use of polypropylene centrifuge tubes for incubations, did not solve the problem. However, pretreating the glass reaction tubes

Fig. 3. Effect on α -glucosidase activity of incubation with buffers of differing concentrations

The enzyme was incubated in 0.1 ml of acetate buffer, pH 4.5, at 37 °C for 30 min without MUG or AGA, then diluted to 0.2 ml with 8 mM-MUG and incubated for a further 30 min. Maximal activity was retained with 20 and 40 mM buffer. The abscissa shows the buffer concentration during the preincubation.

with a siliconizing agent yielded stimulations that were proportional to small amounts of AGA. Under these conditions the enzyme, in the absence of AGA, underwent slow inactivation, but this effect was slight during the first 30 min of incubation (Fig. 4).

The use of relatively concentrated AGA solutions, diluted minimally in silicone-treated test tubes and portioned out with silicone-treated micro-syringes, seemed to minimize losses. Standardization curves for the initial hot extract, the freeze-dried extract and the AGA purified

Fig. 4. Activity of α -glucosidase as a function of time in silicone-treated tubes

The enzyme was incubated in 20 mM-acetate, with (■, □) and without (●, ○) a saturating amount of AGA, for the times shown. Tubes: ●, ■, plain glass; ○, □, silicone-treated.

Fig. 5. Relationship between enzyme activity and amount of AGA

Glucosidase was incubated for 30 min under the standard conditions with various amounts of AGA. The AGA solutions were made up from the original samples by dilution with water to give (○) crude hot extract (10.3 μ g of protein/ml), (●) freeze-dried extract (16.5 μ g of protein/ml) and (□) Superose-purified AGA (4.6 μ g of protein/ml). The concentrations of the materials were adjusted to bring their curves on to the same small graph. The specific activities were 6920, 6710 and 39 140 units/mg of protein for the three preparations respectively.

by gel permeation are shown in Fig. 5. The maximum amount of stimulation was unaffected by the concentration and purification steps. Thus it would appear likely that the purification did not remove an enzyme inhibitor or a second, qualitatively different, stimulator.

In view of the ready adsorption of AGA by glassware, the possibility had to be considered that assays of tissues for α -glucosidase activity give low values owing to dissociation and loss of AGA. A test was made with 0.08–1.7 mg of guinea-pig liver, by using the tissue-handling procedure of Shin *et al.* [9], comprising homogenization in water, brief sonication, and preparation of a 10000 *g* supernatant. This yielded a proportional relationship between liver weight and observed MUG hydrolysis. The activities were not affected by adding a large amount of purified AGA, indicating that tissues normally contain an ample supply of the stimulator protein.

Demonstration that AGA is a protein

Incubation with Pronase destroyed 87% of AGA's stabilizing activity. Heat-inactivated Pronase (100 °C, 60 min) had no effect, showing that the loss of activity was not due to a heat-stable component of the protease preparation. Comparison of freshly added AGA with the AGA that had been incubated with denatured Pronase showed that the stabilizing activity was unchanged. It is evident also that AGA is quite stable to heat.

Similar inactivation was obtained with the spleen AGA, which was incubated with only 1% of its weight of Pronase but for 2 days.

Several lysosomal enzymes are known to be stimulated considerably by acidic lipids, such as bile salts or phosphatidylserine. To check the possibility that part or all of the AGA activity was due to a lipid, a portion of the Superose-purified activator was extracted with 33 vol. of chloroform/methanol (2:1, v/v), sonicated for 5 min, and filtered through a Celite-coated glass filter. Evaporation of the filtrate to dryness and suspension in water at the original volume yielded an inactive preparation. This is further support for the conclusion that AGA is a protein.

Neither glucosidase nor the stimulatory action of AGA was appreciably affected by including up to 5 mM-dithiothreitol.

Distribution of AGA activity

The stimulating material seems to occur in many organs, as shown by testing hot-water extracts of mouse organs. Two groups of six mice each were killed in the morning, but one group was fasted overnight (16 h) to decrease their stores of glycogen. The tissues were frozen rapidly after dissection, in dry ice-cooled pentane. Extracts were made by heating and centrifuging to remove denatured protein, as in the isolation procedure, and tested at various levels with the spleen α -glucosidase (Table 1). Maximal specific activity in the control mice was seen in heart, and minimal activity in brain. This can be contrasted with the distribution of glucocerebrosidase activator in mice, which occurs at the lowest concentrations in heart and lung and the highest in liver [19].

Mouse blood was found to contain a relatively low but distinct amount of AGA, 9.3 units/ μ l.

Fasting produced a 42% decrease in the weight of the liver and a 22% decrease in weight of kidney. Although the specific activity for AGA increased by 26% in fasted-

Table 1. Distribution of α -glucosidase-stimulating protein in mouse tissues

The conditions for assaying AGA activity are described in the Experimental section. Each value is derived from six individually analysed tissues from weight-matched mice by measuring the slope of a plot of extract volume versus observed increase in glucosidase activity and is the mean $\pm$ S.D.

Tissue	AGA concn. (units/mg of tissue)
Heart	26.0 $\pm$ 1.3
Small intestine	20.0 $\pm$ 1.8
Spleen	19.9 $\pm$ 2.9
Thigh muscles	16.9 $\pm$ 0.3
Liver	14.3 $\pm$ 0.9
Kidney	14.2 $\pm$ 1.4
Brain	11.8 $\pm$ 2.9

Table 2. Inhibition of α -glucosidase by RNA and its reversal by activator protein from bovine spleen

Glucosidase measurements were done as in the standard procedure, but with 100 mM-acetate buffer, in non-silicone-treated tubes, incubated for 15 min. Yeast RNA and/or purified AGA was added before the addition of enzyme, and liberated methylumbelliferone was measured.

RNA (μ g)	Stimulator protein (μ g)	Observed activity (nmol/15 min)	Stimulation (%)
0	0	0.40	—
10	0	0.22	—45
0	0.06	0.47	+16
10	0.06	0.24	—40
10	0.13	0.28	—30
10	0.63	0.33	—19
10	1.25	0.53	+32
0	1.25	0.57	+40

mouse liver ($P < 0.005$, Student's *t* test), the total AGA content decreased by 27%. Evidently, during the loss of tissue weight, the AGA is conserved somewhat, compared with other proteins. In kidney, the AGA concentration rose by only 5% ($P > 0.05$), so there is no discrimination during the decrease in organ size.

Rat tissues, as well as human urine, were also found to contain AGA. The latter exhibited ~ 5 units/ μ l, and gel permeation of this active material showed that it was eluted in the same position as the guinea-pig liver factor. It is important to boil the urine like the other sources, as appreciable α -glucosidase activity is present.

Inhibition of α -glucosidase by nucleic acid

Yeast RNA did indeed inhibit α -glucosidase (Table 2). This observation, made also with other lysosomal enzymes [1] and with a ganglioside sialidase [10], adds this enzyme to the list of hydrolases blocked by strongly acidic polymers. The inhibition was 45% with 50 μ g of RNA/ml; this could be decreased and even overcome by adding sufficient bovine AGA.

Effect of AGA on the hydrolysis of natural substrates

Guinea-pig AGA stimulated maltose hydrolysis up to

Fig. 6. Stimulation of glucosidase activity on maltose and glycogen

The enzyme was incubated with both substrates as described in the text, with various amounts of purified AGA at a concentration of 4.6 $\mu\text{g}/\text{ml}$. Non-silicone-treated tubes were used here.

608 % and glycogen hydrolysis up to 100 % (Fig. 6). It is significant that the amount of AGA required for maximal stimulation was similar for the three substrates tested (compare the $\square$ in Fig. 5).

DISCUSSION

The newly discovered activator for α -glucosidase (AGA) appears to be a protein, judging by its inactivation by Pronase, retention during dialysis, and binding to an anion-exchange resin. The protein appears to resemble other activators of lysosomal enzymes in its stability to heat, strong binding to an anion-exchange column, and resistance to protease action [11]. Publications on α -glucosidase purification have not remarked on the presence of a second, smaller protein, suggesting that the stimulatory protein is not essential for basal activity and can be readily dissociated from the enzyme. Gel-permeation chromatography with Toyopearl HW-55 has indeed been found to yield a stabilizer of maltase activity, eluted later than the enzyme (H. Matsui, personal communication). It may be relevant that recently described isolation procedures [6,12,13] yielded only 7–18 % of the glucosidase activity present in the initial homogenate. This could in part be due to dissociative loss of the AGA originally present, owing to dilution for the assay. Our own enzyme preparation, on heating to 100 °C to inactivate it, did not exhibit any AGA activity, as expected from the above observations.

The finding that the stimulator stabilized the enzyme against thermal denaturation suggests that the stimulator acts by binding to the enzyme at some effector site. The same conclusion can be drawn from our observation that all three substrates required the same concentration of AGA for maximal stimulation of their hydrolysis. We previously found that a spleen activator for β -glucosidase, which binds to the glucosidase in conjunction with an acidic lipid, protects this enzyme against inactivation by thiol-binding agents [14].

The physiological roles of AGA and additional in-

formation on its properties remain to be determined. The spleen activator(s) for β -glucosidase increase in concentration in liver rapidly when the amount of substrate (glucosylseramide) increases [15], and it seems possible that the role of enzyme activators is to make rapid adjustments in the effective activity of their corresponding enzymes as the need fluctuates. Our finding that the highest concentration of AGA is in heart is relevant here, since heart failure is a major problem in patients with glycogenosis II.

Human genetic disorders have been found in which the activator protein, not the enzyme itself, was inactive or absent [11]. Several patients exhibiting symptoms of glycogen storage disease II have indeed been found to have a normal level of the enzyme [16], and it is possible that the problem lies in their AGA.

Our finding that AGA binds to glucosidase may have therapeutic relevance, since infusing glycogenosis patients with purified glucosidase has not proved helpful [17]. We have shown that β -glucosidase is taken up by neuroblastoma cells more effectively if it is first complexed with its activator protein and an acidic lipid [18]. It is possible that infusing a complex of α -glucosidase with AGA (possibly coupled chemically) would help the enzyme reach its required location.

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REFERENCES

1. Sano, A. & Radin, N. S. (1988) *Biochem. J.* **254**, 297–300
2. Hers, H. G. (1964) *Adv. Metab. Disord.* **1**, 1–44
3. Brown, B. I., Brown, D. H. & Jeffrey, P. L. (1970) *Biochemistry* **9**, 1423–1428
4. Dreyfus, J. C. & Alexandre, Y. (1972) *Biochem. Biophys. Res. Commun.* **48**, 914–920
5. Hers, H. G. (1963) *Biochem. J.* **86**, 11–16
6. Murray, A. K., Brown, B. I. & Brown, D. H. (1978) *Arch. Biochem. Biophys.* **185**, 511–524
7. Radin, N. S. & Klinger, P. (1986) *Comput. Appl. Biosci.* **2**, 107–109
8. Stoschek, C. M. (1987) *Anal. Biochem.* **160**, 301–305
9. Shin, Y. S., Endres, W., Unterreithmeir, J., Rieth, M. & Schaub, J. (1985) *Clin. Chim. Acta* **148**, 9–19
10. Van Dessel, G., De Wolf, M., Lagrou, A., Hilderson, H., & Dierick, W. (1984) *J. Biochem. (Tokyo)* **96**, 937–947
11. Conzelmann, E. & Sandhoff, K. (1987) *Adv. Enzymol.* **60**, 89–197
12. Tashiro, K., Iwamasa, T., Kato, H., Ogata, S. & Anai, M. (1986) *J. Biochem. (Tokyo)* **99**, 693–701
13. Bruni, C. B., Auricchio, F. & Covelli, I. (1969) *J. Biol. Chem.* **244**, 4735–4742
14. Berent, S. L. & Radin, N. S. (1981) *Biochim. Biophys. Acta* **664**, 572–582
15. Datta, S. C. & Radin, N. S. (1986) *Lipids* **21**, 702–709
16. Tripathy, D., Coleman, R. A., Vidaillet, H. J., Jr., Steenbergen, C., Hirschhorn, K. & Packer, D. L. (1988) *Ann. Intern. Med.* **109**, 985–987
17. Lauer, R. M., Mascarinas, T., Racela, A. S., Diehl, A. M. & Brown, B. I. (1968) *Pediatrics* **42**, 672–676
18. Datta, S. C., Snider, R. M. & Radin, N. S. (1986) *Biochim. Biophys. Acta* **877**, 387–398
19. Datta, S. C. & Radin, N. S. (1984) *Anal. Biochem.* **142**, 196–203