Enzyme Activity in Partly Dissociated Fragments of Rat Intestinal Maltase/Glucoamylase

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Pure rat intestinal maltase/glucoamylase was partially inactivated in 1% sodium dodecyl sulphate by heating at 40–70°C for 5 min at pH 7.5, or by lowering the pH to 5.4–6.6 at 24°C. When these partially active preparations were electrophoresed in the presence of sodium dodecyl sulphate, a complicated protein band pattern of incompletely dissociated fragments of the enzyme was observed. Complete dissociation of the enzyme in sodium dodecyl sulphate, induced by boiling or by pH values below 5.4, was accompanied by total loss of enzyme activity and simplification of the protein pattern to five major species. Although the original enzyme band was absent from some partially dissociated preparations, enzyme activity was present and was associated with several transient protein bands on the gels. Maltase and α -glucosidase activities were detected in these bands, but glucoamylase activity was absent.

Intestinal maltase (α -D-glucoside glucohydrolase, EC 3.2.1.20)/glucoamylase (α -1,4-glucan glucohydrolase, EC 3.2.1.3) is an ectoenzyme of the brush-border membrane (Seetharam *et al.*, 1970; Kelly & Alpers, 1973; Galand & Forstner, 1974a; Maroux & Louvard, 1976). Although the enzyme exhibits a broad range of substrate specificity, hydrolysing many di- and poly-saccharides with α -1,4 and α -1,6 linkages, the best substrates are maltose and starch (Kelly & Alpers, 1973; Sivakami & Radhakrishnan, 1976). Maltase/glucoamylase is the largest component of the microvillous membrane (Galand & Forstner, 1974b; Mastracci *et al.*, 1975; Critchley *et al.*, 1975), and we have purified the rat enzyme to homogeneity (Flanagan & Forstner, 1978).

Several of the glycosidases of the brush border have been shown to exist in enzyme complexes containing several activities. These include the sucrase/isomaltase complex (Cogoli *et al.*, 1973), the lactase/phlorrhizin hydrolase or ' β -glycosidase complex' (Colombo *et al.*, 1973) and the maltase/glucoamylase complex (Kelly & Alpers, 1973; Sivakami & Radhakrishnan, 1976). Of these, separation of the activities into distinct subunits has only been convincingly shown for sucrase/isomaltase (Cogoli *et al.*, 1973; Conklin *et al.*, 1975). However, the existence of subunits in other macromolecular enzymes of the brush border is implied by the work of Green & Hadorn (1977). These workers separated and identified several of the enzyme activities of the guinea-pig brush-border membrane by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl

sulphate. However, membrane samples that had been boiled in sodium dodecyl sulphate before electrophoresis were devoid of enzymic activity and the bands to which enzyme activity had been ascribed previously were either absent or very much diminished (Green & Hadorn, 1977).

We have shown that pure rat intestinal maltase/glucoamylase was dissociated into five fragments with complete loss of enzymic activity by boiling in sodium dodecyl sulphate or by lowering the pH in the presence of the detergent (Flanagan & Forstner, 1978). In addition, preliminary experiments indicated that partial dissociation of the enzyme resulted in the production of several enzymically active fragments (Flanagan & Forstner, 1977). The present paper describes the latter phenomenon more completely.

Experimental

Rat intestinal maltase/glucoamylase was purified as described by Flanagan & Forstner (1978). Maltase, glucoamylase and α -glucosidase activities were determined by the methods of Dahlqvist (1968), Schlegel-Haueter *et al.* (1972) and Kolinska & Kraml (1972) respectively, and protein by the method of Lowry *et al.* (1951). The specific activities of maltase, glucoamylase and α -glucosidase in the pure enzyme, expressed as μ mol of glucose produced/min per mg of protein, were 152, 1.4 and 1.5 respectively.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out in 3.3% gels by using the Tris/EDTA/acetate system of Steck & Yu (1973). Maltase/glucoamylase (10–20 μ g of protein) was incubated in 5 mm-Tris/HCl, pH 7.5, at the temperatures indicated in the Figures or in

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5 mM-Tris/10 mM-acetate buffers at the pH indicated, and sodium dodecyl sulphate was added to give a final concentration of 1% (w/v). Mercaptoethanol was not added, since the enzyme can be fully dissociated in its absence (Flanagan & Forstner, 1978). Finally, 10 μ l of 50% (v/v) glycerol in 0.01% Bromophenol Blue was added to the samples (100 μ l) and electrophoresis was carried out at a current of 8 mA/gel. Electrophoresis was stopped after the dye had migrated 9 cm into the gel. Protein staining was carried out with Coomassie Blue (Flanagan & Zbarsky, 1977), and where necessary duplicate gels were sliced as follows: water was injected between the gel and the tube wall to free the gel from the glass; the tube was then placed in a Gilson Aliquogel Fractionator which, in a combined operation, sliced and homogenized the gel in 2 mm slices. Routinely, 45–46 fractions were obtained from the 9 cm-long gels. Water (0.5 ml) was added to each fraction, and they were then assayed for enzyme activity. Because of the large difference in activity between maltase,

on the one hand, and glucoamylase and α -glucosidase, on the other (see above), extended incubation periods were necessary during assay of the last two activities.

Results

Initially, the gel-electrophoretic patterns of maltase/glucoamylase were examined after partial heat inactivation of the enzyme. When samples of the pure glycoprotein were heated at temperatures ranging from 36 to 100°C for 5 min in 1% sodium dodecyl sulphate at pH 7.5, the gel patterns shown in Fig. 1(a) were obtained. By monitoring the maltase activity remaining in each sample a heat-inactivation curve for the enzyme was also produced. (Fig. 1b). The gels showed a gradual dissociation of the enzyme into a complicated series of bands at 40–60°C, which were resolved at higher temperatures into five major species. Although greater than 80% of the maltase activity persisted in 1% sodium dodecyl sulphate at temperatures up to 50°C, the enzyme was rapidly

Fig. 1. Dissociation and inactivation of maltase/glucoamylase by heat in the presence of sodium dodecyl sulphate. Samples of the pure enzyme (15 μ g) were heated for 5 min at the temperatures indicated in 0.15 ml of 5 mM-Tris/HCl, pH 7.5, containing 1% sodium dodecyl sulphate. After cooling to 24°C, samples (50 μ l) were removed, diluted 1000-fold with water and assayed for maltase activity (b). Activities were expressed as a percentage of the value obtained in the presence of 1% sodium dodecyl sulphate at 24°C. The remainder of each sample was electrophoresed as described in the Experimental section (a).

inactivated at temperatures above this value. By examining the inactivation curve and the gel-electrophoretic patterns, the interesting observation was made that although the original enzyme band was absent from the sample heated at 61°C, maltase activity persisted in samples heated at temperatures as high as 76°C (Fig. 1b). Therefore, maltase activity must have been present in some of the intermediate species formed in these experiments. This was also shown by heating maltase/glucoamylase at 55°C for periods up to 2h (Fig. 2). Quantification of the enzyme-protein band by densitometry showed clearly that it was destroyed at a rate faster than the enzyme was inactivated (Fig. 2), and when the enzyme band had disappeared altogether from the gels more than 20% of the original maltase activity remained.

Since we have previously shown that the enzyme can also be dissociated in sodium dodecyl sulphate simply by lowering the pH (Flanagan & Forstner, 1978), further attempts to characterize the partially dissociated species were carried out by using this

method, which was easier to control. Fig. 3 depicts the results of an experiment in which samples of maltase/glucoamylase were exposed to decreasing pH in the presence of sodium dodecyl sulphate. As with the heating experiments, a complex pattern of bands appeared in the initial stages of the dissociation process and was gradually resolved as the pH was lowered (Fig. 3a). Dissociation was again accompanied by progressive loss of maltase activity (Fig. 3b). In an attempt to determine if some of the intermediates possessed enzyme activity, three gels, containing samples treated at pH 6.2, 5.8 and 5.6, were sliced after electrophoresis and maltase activity was determined in the sliced fractions. The results are given in Fig. 4 and show that at least three transient intermediate bands possessed maltase activity. As the dissociation progressed the intermediates and their enzymic activities waxed and waned, this being most clearly seen in a band with an m_r value of 0.4. The transient species did not correspond to any of the final dissociated products, which were all enzymically inactive (Figs. 3 and 4).

Since maltase/glucoamylase exhibits several enzymic activities it was decided to investigate how partial dissociation of the enzyme affected these. Accordingly a sample of maltase/glucoamylase was partially dissociated at pH 5.7. After sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the gel was sliced and maltase, α -glucosidase and glucoamylase activities were determined in the fractions. Three coincident peaks of maltase and α -glucosidase activity were found on the gel, but glucoamylase activity was barely detectable, if at all, in the fractions (Fig. 5). This experiment suggested two things: (1) partial dissociation of the maltase/glucoamylase molecule did not result in separation of the maltase and α -glucosidase active sites, and (2) glucoamylase activity was either destroyed by the dissociation procedures used or was dependent on the interaction of two or more of the enzyme fragments.

Discussion

Several studies have demonstrated that the activities of many brush-border enzymes can survive polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, thereby enabling the appropriate bands to be identified afterwards (Maestracci *et al.*, 1975; Critchley *et al.*, 1975; Booth & Kenny, 1976; Green & Hadorn, 1977). In the reports of Maestracci *et al.* (1975) and Critchley *et al.* (1975) maltase activity was located in two places on the gels, one peak being associated with the largest component present, maltase/glucoamylase, and a second peak being due to the maltase activity of the sucrase/isomaltase complex. Green & Hadorn (1977) reported the presence of three peaks of maltase

Fig. 2. Effect of heating on the disappearance rates of maltase/glucoamylase protein band and maltase activity. A sample of the enzyme (120 μ g) was heated at 55°C in 1.2 ml of 5 mM-Tris/HCl, pH 7.5, containing 1% sodium dodecyl sulphate. At the times indicated, samples (150 μ l) were removed and a 50 μ l portion of each sample was diluted with water and assayed for residual maltase activity, and the remainder of the sample was electrophoresed. After electrophoresis and protein staining each gel was scanned in a Gilford spectrophotometer at 550 nm and the peak corresponding to the enzyme, the slowest-moving band on the gel (Fig. 1a), was cut from the chart and weighed. ●, Maltase activity; ■, enzyme protein band.

Fig. 3. *Dissociation and inactivation of maltase/glucoamylase by pH in the presence of sodium dodecyl sulphate* Samples of the enzyme (15 μ g) were incubated at 24°C in 0.15 ml of buffers containing 5 mM-Tris, 10 mM-acetate and sufficient HCl or NaOH to obtain the pH value indicated. Sodium dodecyl sulphate was also present at a final concentration of 1%. After 2 min a 50 μ l portion from each sample was removed, diluted 1000-fold with water and assayed for residual maltase activity (b). The remainder of each sample was electrophoresed (a).

activity on gels after electrophoresis of guinea-pig brush-border membrane. In addition to the complexes described above, a third peak contained maltase, sucrase and glucoamylase activities, and Green & Hadorn (1977) suggested that this combination may be due to a previously undescribed glycosidase complex. The interpretation of these experiments is complicated by the presence in the membrane of several enzymes of overlapping specificities. Our present results show that even for a single, pure, glycosidase (Flanagan & Forstner, 1978), multiple peaks of enzyme activity are possible, depending on the degree of dissociation of the enzyme. Presumably, this phenomenon may also occur in crude membrane preparations, depending on the temperature and pH at which preincubation with sodium dodecyl sulphate is carried out. Additionally, caution is necessary when activities are ascribed to protein bands in crude preparations, since a single band may contain several superimposed bands and a small amount of active enzyme may not be detected by the protein stain used (cf. Fig. 4). Therefore the results of Green & Hadorn (1977) may also be explained by part-

dissociation of some of the glycosidases and by co-electrophoresis of some of the resultant partly dissociated fragments.

Although Kelly & Alpers (1973) have suggested that maltase/glucoamylase contains a single active site, the work of Sivakami & Radhakrishnan (1976) and de Burlet & Sudaka (1977) supports the concept of at least two interacting sites on the enzyme. Our results also favour the latter model, since the activities of maltase and α -glucosidase appeared to persist in partially dissociated fragments, whereas glucoamylase activity was not present. In agreement with this finding, human kidney glucoamylase activity has been shown to be inactivated by heat more rapidly than maltase activity (de Burlet & Sudaka, 1977).

A major difference between the human and rat maltase/glucoamylases has been noted in their behaviour on electrophoresis. The enzymes from human intestine (Kelly & Alpers, 1973) and human kidney (de Burlet & Sudaka, 1976) have been isolated in two forms, whereas that from rat intestine was homogeneous (Flanagan & Forstner, 1978). Since we have shown in the present paper that partly dis-

Fig. 4. Maltase activity in partly dissociated fragments of maltase/glucoamylase

Duplicate gels corresponding to those shown in Fig. 3(a) at pH 5.6, 5.8 and 6.2 were sliced immediately after electrophoresis as described in the Experimental section and maltase activity, expressed in arbitrary units, was determined in the sliced fractions (●). m_F is the electrophoretic mobility relative to that of Bromophenol Blue.

Fig. 5. Maltase, α -glucosidase and glucoamylase activities in partly dissociated fragments of maltase/glucoamylase. A sample of the enzyme ($10\mu\text{g}$) was incubated at pH 5.7 in the presence of 1% sodium dodecyl sulphate as described in the legend to Fig. 3 and then electrophoresed. The gel was sliced and residual enzyme activity was determined in the fractions and expressed, arbitrarily, in terms of the amount of glucose released/fraction per min for maltase (●) and amount of glucose released/fraction per 100 min for α -glucosidase (■) and glucoamylase (▲). m_F is the electrophoretic mobility relative to that of Bromophenol Blue.

about half that of the rat enzyme (Kelly & Alpers, 1973; Flanagan & Forstner, 1978) it is tempting to speculate that the dual forms of human maltase glucoamylase may be 'half-molecules' of an enzyme that is in fact similar in size to the rat maltase/glucoamylase. In support of this, Maestracci *et al.* (1975) have demonstrated a maltase/glucoamylase in human brush-border membrane preparations with a mol.wt. of 440000, which is close to the value of 500000 obtained for the isolated rat enzyme by Flanagan & Forstner (1978).

Finally, the experiments demonstrating gradual dissociation of maltase/glucoamylase in the present paper show that a considerable rearrangement of the initial breakdown fragments occurred before the final products were formed (Fig. 3a). In several cases the initial fragments appeared to aggregate, giving higher-molecular-weight bands, which eventually persisted as the final dissociated forms. This occurred with all the transient species that exhibited enzymic activity. Although the nature of the final products of

sociated fragments of rat maltase/glucoamylase are enzymically active and since the molecular weight of the human enzyme, when isolated, was found to be

dissociation is at present unclear (Flanagan & Forstner, 1978), the present experiments suggest that the bands may be aggregated forms of one or more true subunits of the enzyme.

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