Proteinase-Sensitive Release of Enzymes from Pancreatic Microsomal Fraction

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Suspensions of rat pancreatic microsomal fraction release α -amylase and ribonuclease on incubation at 37°C, but not at 2°C. The release is abolished by proteolytic enzymes. Ribonuclease associated with the microsomal fraction is protected from subtilisin BPN' attack, but is sensitive after release.

Pancreatic enzymes such as α -amylase are known to be synthesized in ribosomes attached to the rough endoplasmic reticulum and transferred across the membrane into the lumen of the endoplasmic reticulum (Palade, 1975). The mechanism of transport across the membrane is not understood, but strong evidence exists that the ribosomes are attached via nascent polypeptide chains. Many mRNA species for secretory proteins, when translated in heterologous systems in the absence of the microsomal fraction ('microsomes'), direct the synthesis of longer precursors with 15–30 extra amino acids. Blobel & Dobberstein (1975) have elaborated this into their elegant signal hypothesis.

In the present paper we report that when pancreatic microsomes are incubated in a simple buffer system, α -amylase (EC 3.2.1.1) and RNAase (EC 3.1.4.22) activities, as measured in the suspensions with high-molecular-weight substrates, increase over a 30 min period. This increase does not involve protein synthesis, but is due to the release of enzyme from the microsomes into the medium. Ribonuclease is protected from subtilisin BPN' digestion before release, but is susceptible once released, suggesting transfer of the enzyme from the interior of the microsome. The characteristics of this transfer are difficult to reconcile with non-specific enzyme leakage and raise the possibility of transport of completed proteins across the microsomal membrane.

Materials and Methods

Preparation of microsomes

Pancreatic microsomes were obtained from male Sprague-Dawley rats after deprivation of food overnight. The pancreas was excised after exsanguination, washed in cold 0.3 M-sucrose, pH 6.7, containing 0.2 mM-CaCl₂ and homogenized in 9 vol. of the same sucrose solution. Cellular debris and mitochondria were removed by centrifuging for 5 min at 500g

(r_{av} , 10.5 cm) and 15 min at 7000g (r_{av} , 7.1 cm) respectively. The postmitochondrial supernatant was centrifuged for 30 min at 105000g (r_{av} , 5.9 cm) to give a microsome pellet. All centrifugations were performed at 2°C.

The microsome pellet was resuspended in cold STKC buffer containing 300 mM-sucrose, 50 mM-Tris, 25 mM-KCl and 0.2 mM-CaCl₂ at pH 7.5 and at a protein concentration of approx. 5 mg/ml. Resuspended microsomes were incubated at 37°C in this buffer at a protein concentration of 0.2–0.3 mg/ml and samples were taken during the course of the incubation for enzyme assays.

Proteinase treatment

Chymotrypsin and 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one-treated trypsin were from Worthington Biochemical Corporation, Freehold, NJ, U.S.A., and subtilisin BPN' was from Sigma Chemical Co., St. Louis, MO, U.S.A. Chymotrypsin was inactivated with 2 mM-phenylmethanesulphonyl fluoride (Sigma).

Enzyme assays

α -Amylase was assayed with an insoluble substrate, purchased as Phadebas tablets from Pharmacia Diagnostics, Uppsala, Sweden. One tablet was suspended in 7.0 ml of buffer, pH 7.1, containing 10 mM-K₂HPO₄, 10 mM-NaCl and 0.2 mM-CaCl₂, and 1.4 ml samples were dispensed into clean tubes. After preincubation at 37°C, samples (100 μ l) of microsomal suspension were assayed for 1 min. The reaction was stopped by adding 0.2 ml of 0.5 M-NaOH and the A_{620} of the supernatant was measured after centrifugation. Under these conditions 50 ng of pig pancreatic α -amylase (Sigma) produced an absorbance change of 1.0 in 1 min and the assay was linear to 4.0 A_{620} units.

RNAase assays were performed by the method of Kraft & Shortman (1970) using an incubation time of 1 min. Under these conditions 200 ng of bovine

Abbreviation used: RNAase, ribonuclease.

pancreatic RNAase (Sigma) produced an absorbance change of 0.85 in 1 min.

Results

Appearance of α -amylase activity in microsome suspensions

When suspensions of rat pancreatic microsomes were incubated at 37°C in STKC buffer, the α -amylase activity in samples of the suspension increased, reaching a maximum after 20–30 min incubation (Fig. 1). Occasionally a 5–10 min lag phase in α -amylase release has been observed. After incubation, no evidence of major vesicle disruption was evident from visual inspection of electron micrographs.

Appearance of α -amylase was not affected by cycloheximide, added before incubation as an aqueous solution, to a final concentration of 100 μ g/ml, the amount being 98% of that in absence of the drug. At 2°C, appearance of α -amylase was only 3% of that at 37°C.

Effect of proteinases on α -amylase appearance

When chymotrypsin (at a final concentration of 10 μ g/ml) was added to the microsomes at the start

Fig. 1. *Effect of chymotrypsin on appearance of α -amylase in microsome suspensions*

Microsome suspensions were incubated in STKC buffer at 37°C in the absence (O) or presence of chymotrypsin at a final concentration of 10 μ g/ml (Δ) or 100 μ g/ml ($\square$) and in the presence of phenylmethanesulphonyl fluoride-inactivated chymotrypsin at a final concentration of 10 μ g/ml ($\bullet$). α -Amylase was measured in the suspensions with an insoluble substrate.

of the incubation, the appearance of α -amylase was inhibited (Fig. 1). The proteolytic action of chymotrypsin was responsible for this since the addition of phenylmethanesulphonyl fluoride-inactivated chymotrypsin to microsomes did not affect α -amylase appearance (Fig. 1). Trypsin or subtilisin BPN' at final concentrations of 100 μ g/ml and 1 mg/ml respectively similarly inhibited the appearance of α -amylase.

The inhibitory effect of proteinases on α -amylase appearance was not due to proteolytic digestion of the α -amylase appearance, as addition of proteinase after incubation of the microsomes had no effect on the α -amylase activity during a further 30 min of incubation. Addition of proteinase after 15 min incubation of the microsomes prevented further α -amylase release, thus eliminating the possibility that proteinase inhibits by destroying, for example, a phospholipase that renders the membrane permeable.

Redistribution of α -amylase between microsomal pellet and supernatant during incubation

Microsomes were incubated with and without chymotrypsin (final concentration 100 μ g/ml) before being separated into soluble and pellet fractions by centrifuging for 30 min at 105000g (r_{av} , 5.9 cm). In the absence of proteinase, α -amylase activities in the soluble fraction increased during incubation matched by decreased activities in the pellet fraction [measured after solubilizing the membrane with 0.5% (w/v) deoxycholate] (Fig. 2). If chymotrypsin was present during the incubation, α -amylase activities in each fraction remained relatively constant (Fig. 2). These results show a proteinase-inhibited transfer of α -amylase from the microsome to the soluble fraction. Total α -amylase activities (i.e. soluble plus pellet) remained constant during the incubation period, confirming that α -amylase was not synthesized or destroyed.

Effect of Mg^{2+} on α -amylase release

The inclusion of 5 mM- Mg^{2+} in the incubation buffer inhibited the release of α -amylase (Fig. 3) and also prevented the detachment of ribosomes, which occurred during incubation in the STKC buffer alone, as judged by electron microscopy. To determine whether Mg^{2+} exerted its effect on α -amylase release through ribosome binding, or in some other manner, microsomes were incubated in an STKC buffer containing 750 mM-KCl either with or without 5 mM- Mg^{2+} . Adelman *et al.* (1973) showed that incubation in high KCl buffers led to the detachment of ribosomes from liver rough microsomes. In the present work, incubation of microsomes in the high-KCl buffer caused complete detachment of the

Fig. 2. Effect of chymotrypsin on redistribution of α -amylase between pellet and supernatant during incubation at 37°C

Microsome suspensions were incubated in STKC buffer at 37°C with and without chymotrypsin for the times indicated. α -Amylase was measured in the pellets and supernatants after centrifugation and treatment with 0.5% (w/v) deoxycholate. No added chymotrypsin: α -amylase activity in supernatant (○) and in pellet (●). Chymotrypsin at a final concentration of 100 μ g/ml: α -amylase activity in supernatant (□) and in pellet (■).

Fig. 3. Effect of Mg^{2+} in the presence and absence of 750mM-KCl on appearance of α -amylase in microsome suspensions

Microsome suspensions were incubated in STKC buffer alone (○), STKC buffer containing 5mM- Mg^{2+} (□), and STKC buffer containing KCl at a final concentration of 750mM with (●) and without (Δ) 5mM- Mg^{2+} . α -Amylase was measured in the suspensions with an insoluble substrate.

ribosomes in the presence or absence of Mg^{2+} . However, release of α -amylase during incubation in the high-KCl buffer was prevented in the presence of Mg^{2+} (Fig. 3) showing that the metal ion has an effect independent of its action in stabilizing ribosome attachment. (This does not preclude the possibility that ribosome detachment may be a prerequisite for enzyme release.) The reason for microsomes releasing rather less α -amylase during incubation in high-KCl buffers is not known.

RNA release from microsomes coincides with loss of protection from subtilisin BPN' digestion

It was of interest to see whether enzyme associated with the microsome was protected from proteolytic attack, but α -amylase proved to be unsuitable because of its inherent resistance to proteolytic digestion. RNAase is released from microsomes and, as with α -amylase, the release is inhibited by chymotrypsin. RNAase is resistant to chymotryptic attack, but is readily inactivated by subtilisin BPN' allowing one to test whether enzyme release is accompanied by loss of protection from proteolysis.

The addition of subtilisin BPN' (final concentration

Fig. 4. Effect of subtilisin BPN' on released RNAase and on RNAase release

Microsome suspensions were incubated in STKC buffer at 37°C in the absence (○) or presence of subtilisin BPN' at a final concentration of 1mg/ml added at zero min (□) and at 30min (●). At the times indicated, RNAase was measured in the microsome suspension with yeast RNA as a substrate.

1 mg/ml) to microsomes, previously incubated for 30 min, inactivated over 85% of the released RNAase within 2 h (Fig. 4). Addition of the proteinase before incubation prevented the appearance of RNAase (Fig. 4), as might be expected from the result with chymotrypsin. In samples of microsomes incubated with and without subtilisin BPN' for 180 min, total RNAase activity was measured after phenylmethane-sulphonyl fluoride-inactivation of the proteinase and solubilization of the microsomes with 0.5% (v/v) Triton X-100. The activity of total RNAase present in suspensions incubated with subtilisin BPN' was 75% of that measured in those incubated without the proteinase. Since released RNAase is destroyed by subtilisin BPN' (Fig. 4), this experiment showed that most of the releasable RNAase was initially inaccessible to the proteinase.

Discussion

From the evidence presented here it is reasonable to conclude that enzymes within the pancreatic microsomes are transferred to the outside. Protection against proteolysis is regarded as the only rigorous criterion that a polypeptide chain is sequestered within the microsome (Blobel & Dobberstein, 1975), and the results in the present paper show that microsomal RNAase is so protected. The alternative explanations that RNAase is adsorbed to the external surface, and in this form is subtilisin-resistant, requires that desorption of the enzyme is prevented by proteolysis, which seems extremely improbable.

The main question is whether the enzyme release observed has a trivial explanation, such as vesicle lysis or non-specific leakage, but again it is extremely difficult to reconcile such explanations with the inhibitory effect of proteinases. The hypothesis that seems best able to explain the observations is that the enzymes are traversing the membranes by a process dependent on the presence of one or more proteins readily destroyed by proteinases.

Such a hypothesis poses many problems. The apparent transport we observe is outwards and not

inwards as expected for a physiological process (Palade, 1975), although Rothman (1975) has proposed reversible movement of proteins across the endoplasmic reticulum based on work later criticized by Palade (1976). In any case, since Mg^{2+} at cellular concentrations inhibits the release *in vitro* of microsomal enzymes, the process we observe would not be expected to occur *in vivo*. It also remains to be determined whether release of enzymes observed here depends on prior detachment of the ribosomes.

The possibility exists that we are observing an unphysiological activity of a transport system which *in vivo* is normally involved in the inward vectorial transport of completed proteins into the endoplasmic reticulum. This obviously conflicts with that part of the signal hypothesis which envisages polypeptide transport only in the nascent form. However, recently Highfield & Ellis (1978) demonstrated transport of a completed protein across the chloroplast membrane.

Until more work is done it cannot be decided whether the observed release of enzyme is related to the physiological transport of proteins across membranes. However, the phenomenon seems to be of sufficient interest to warrant consideration and further investigation.

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