Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 501-509

INHIBITION OF THE PANCREATIC MICROSOME ENZYME RELEASE PHENOMENON BY INHIBITORS OF SIGNAL PEPTIDASE ACTIVITY

Linda M. Tabe, Brian K. May and William H. Elliott

Department of Biochemistry, The University of Adelaide, Adelaide, South Australia 5001

Received January 25,1980

SUMMARY: The protease-sensitive release of α-amylase from rat pancreatic microsomes, incubated at 37°C, was inhibited by protease inhibitors which have been reported to inhibit signal peptidase activity. Protease inhibitors which did not affect signal peptidase activity also failed to inhibit amylase release from microsomes. Although the observed amylase release was in the opposite direction to enzyme secretion and involved fully-synthesised proteins, rather than nascent peptides, it is proposed that the enzyme release phenomenon reported from this laboratory (Pearce *et al.* (1978) Biochem. J. *176*, 611-614) is related to the protein transporting mechanism involved in secretion.

INTRODUCTION

Most proteins destined to be transported across membranes are synthesised as precursors containing a short, amino-terminal extension of predominantly hydrophobic amino acids (1-5). This so-called signal sequence, which is proposed to act as a signal for membrane insertion of the peptide, is cleaved during, or soon after, translocation across the membrane (6).

Blobel's signal hypothesis (6) envisages that secretory proteins are translocated into the endoplasmic reticulum as extended, nascent, polypeptide chains which assume their native configurations after passage through the membrane. Evidence for the view that translocation is purely co-translational comes from reports of *in vitro* translation studies in which secretory enzymes were sequestered in microsomes only if the vesicles were present during protein synthesis (7). In the case of certain chloroplast (8) and mitochondrial (9) proteins, however, the completed pre-proteins are translocated across the organelle membranes, with concommitant processing, after cessation of protein synthesis.

Although there are conflicting hypotheses on the exact mechanism of trans-membrane translocation, it is agreed that a specific, membrane-

0006-291X/80/060501-09$01.00/0

associated signal peptidase must exist to account for the proteolytic processing of exported proteins to their mature forms. Ovalbumin appears to be an exception to this rule (10). Recent attempts to characterise the signal peptidase by the use of protease inhibitors of varying specificities, have revealed that the enzyme bears some relationship to both trypsin (11) and chymotrypsin (12).

In a previous communication from this laboratory (13), it was reported that active α-amylase (E.C. 3.2.1.1.) and ribonuclease (E.C. 3.1.4.22.) associated with rat pancreatic microsomes were released into free solution during incubation at 37°C, but not at 4°C. Protein synthesis was not involved in this phenomenon as release occurred in the presence of cycloheximide (13). Before release, the microsome-associated enzymes were protected from attack by exogenous protease, implying sequestration within the membrane vesicles.

The observed release of enzyme was in the opposite direction to that expected for secretion and involved fully-synthesised, enzymatically active proteins. The obvious trivial explanation of non-specific leakage is, however, difficult to reconcile with the fact that the release phenomenon was abolished by treatment of the microsomes with exogenous proteases. This implies that enzyme release may depend on the integrity of a membrane-associated protein.

Physiological concentrations of magnesium ions prevented the release phenomenon, therefore it was suggested that the outward movement of secretory proteins *in vitro* might be due to a non-physiological activity of a protein transport system which normally imports proteins *into* the endoplasmic reticulum *in vivo*. In the previous report, there was no experimental evidence to support the contention that the release phenomenon was, in fact, connected in any way with the accepted secretory process. In this paper, we demonstrate that those agents which have been shown to inhibit signal peptidase activity also inhibit the enzyme release phenomenon, while protease inhibitors which do not inhibit signal peptidase also fail to inhibit enzyme release.

MATERIALS AND METHODS

Protease Inhibitors. TLCK* and TPCK* were obtained from Worthington Biochemical Corp., Freehold, N.J.; soybean trypsin inhibitor, PMSF*, benzamidine HCl and 1,10 phenanthroline were from Sigma Chemical Co. Leupeptin and chymostatin were purchased from the Peptide Institute, Osaka, Japan, and elastinal was kindly donated by H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan). Procaine HCl, a Hoechst product, was a gift from Fisher and Co., Marrickville, N.S.W., Australia. Traysylol was obtained from Bayer, Australia.

Preparation of Microsomes. Pancreatic microsomes were obtained from male Porton rats weighing between 200 g and 300 g as previously described (13). Differences in the total number of A_{620} units of amylase released by different preparations of microsomes were due to differences in the ages and weights of the rats from which they were obtained. After isolation, the microsomes were resuspended at a total protein concentration of approximately 5 mg/ml in cold STKC buffer (300 mM sucrose, 50 mM Tris HCl, 25 mM KCl, 0.2 mM $CaCl_2$ at pH 7.5). For convenience in the enzyme assay, this suspension was diluted, with STKC buffer, to a protein concentration of 0.2-0.3 mg/ml for incubation at 37°C in the presence or absence of protease inhibitors. Samples were taken during the course of the incubations for enzyme assay.

Enzyme Assay. α-Amylase activity was assayed, as described (13), using an insoluble substrate purchased as Phadebas tablets from Pharmacia Diagnostics, Uppsala, Sweden. 50 µl samples of microsome suspension were assayed, the assay being linear to 1.0 A_{620} unit.

RESULTS

Trypsin Inhibitors

It has been reported that the trypsin inhibitors tosyl-l-lysine chloromethyl ketone (TLCK) and benzamidine and several local anaesthetics, including procaine HCl, inhibit processing of the precursor of an outer membrane protein of *Escherichia coli* (11). When added to pancreatic microsome suspensions TLCK, benzamidine and procaine all inhibited the appearance of α-amylase activity in the suspending medium (Fig. 1). The amylase could subsequently be released from the microsomes by disruption of the vesicles with the detergent, sodium deoxycholate. The concentrations of these compounds, which inhibited amylase release, were similar to those which inhibited pre-protein processing in *E. coli*.

The effects of some other known trypsin inhibitors were also investigated. The naturally-occurring proteins traysylol and soybean trypsin inhibitor, and a relatively non-specific inhibitor of serine proteases,

*The abbreviations used are:- TLCK, tosyl-l-lysine chloromethyl ketone; TPCK, L-1-tosylamide-z-phenylethylchloromethyl ketone; PMSF, phenylmethane sulphonyl fluoride; A_{620}, Absorbance at 620 nm.

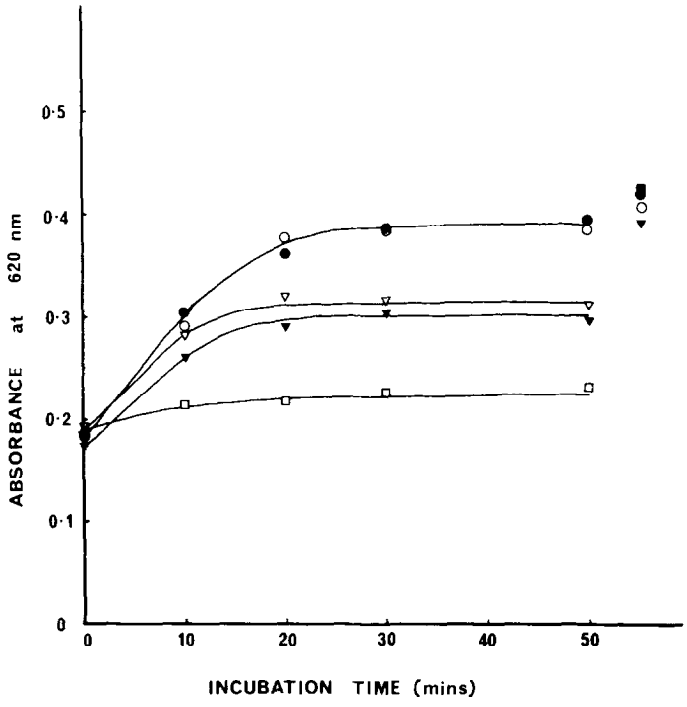

Fig. 1: Appearance of α-amylase in microsome suspensions incubated in the presence of procaine, benzamidine or TLCK. Microsomes were incubated at 37°C in STKC buffer containing: no additions (●); 1% w/v procaine HCl (▽); 20 mM benzamidine HCl (▼); 5 mM TLCK and 2% v/v ethanol (□); 2% v/v/ ethanol (○). 50 µl aliquots of each suspension were assayed for amylase activity at 10 minute intervals. After 50 mins of incubation, deoxycholate was added to each suspension to a final concentration of 1% w/v and amylase activity was assayed after 5 mins further incubation.

phenylmethane sulphonyl fluoride (PMSF),all diminished the release of amylase from rat pancreatic microsomes (Fig. 2).

Chymotrypsin Inhibitors

It has been suggested that the signal peptidase enzyme resembles chymotrypsin because processing of pre-human placental lactogen can be inhibited by chymostatin, a peptide inhibitor of chymotrypsin and similar endopeptidases (12). Leupeptin and elastinal, similar peptides with different specificities had no detectable effect on processing. When added to rat pancreatic microsome suspensions, chymostatin caused marked inhibition of amylase release, however leupeptin and elastinal caused only slight inhibition comparable to that produced by the solvent, dimethylsulphoxide, alone (Fig. 3).

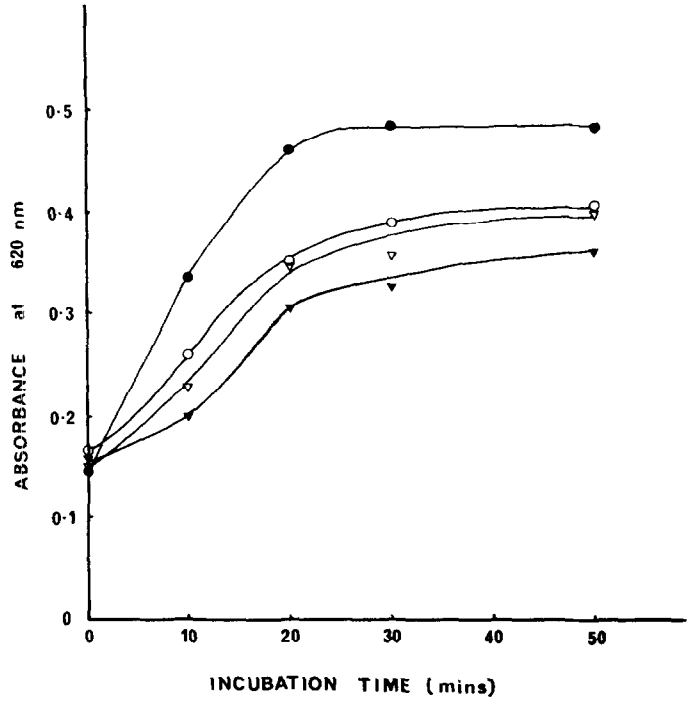

Fig. 2: Appearance of α-amylase in microsome suspensions incubated in the presence of traysylol, soybean trypsin inhibitor or PMSF. Microsomes were incubated at 37°C in STKC buffer containing: no additions (●); 100 Kallikrein inactivator units/ml traysylol (○); 0.5 mg/ml soybean trypsin inhibitor (▽); 0.5 mg/ml PMSF, 1% v/v ethanol (▼). (For ethanol control see Fig. 1.) 50 µl aliquots of each suspension were assayed for amylase activity at 10 minute intervals.

The chymotrypsin inhibitor L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK), which has been reported to inhibit the processing of pre-growth hormone in rat pituitary tumor cells (14), also inhibited the amylase release phenomenon. This finding was complicated by an inhibitory effect of TPCK on the α-amylase activity itself as well as on the release process (results not shown).

O-Phenanthroline

Another report on the nature of the pre-protein processing enzyme has suggested that it is a metallo-protease because its activity is inhibited by high concentrations of the chelating agent 1,10 phenanthroline (15). Relatively high concentrations of 1,10 phenanthroline inhibited the release of amylase from rat pancreatic microsomes (Fig. 4).

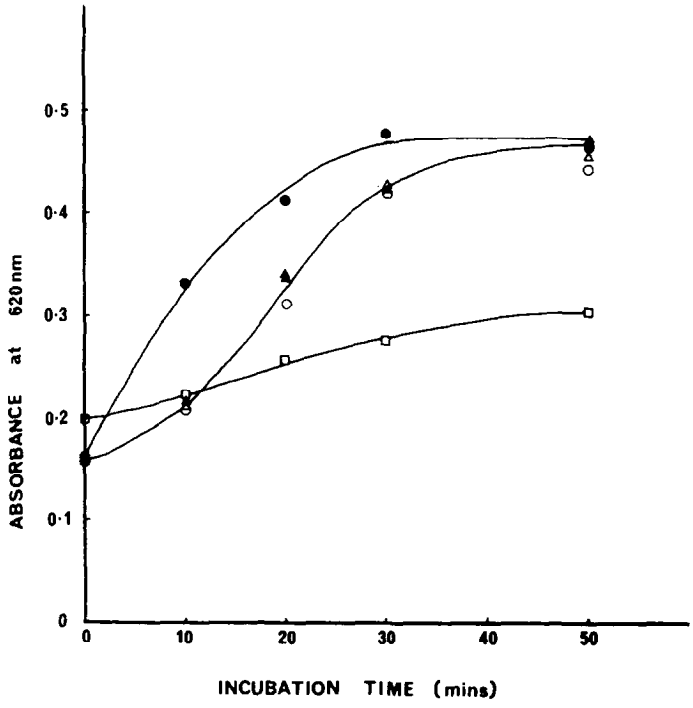

Fig. 3: Appearance of α-amylase in microsome suspensions incubated in the presence of chymostatin, leupeptin or elastinal. Microsomes were incubated at 37°C in STKC buffer containing: no additions (●); 0.6 mg/ml chymostatin, 6% v/v DMSO (□); 0.6 mg/ml leupeptin, 6% v/v/ DMSO (○); 0.6 mg/ml elastinal, 6% v/v DMSO (▲); 6% v/v DMSO (△). 50 µl aliquots of each suspension were assayed for amylase activity at 10 minute intervals.

DISCUSSION

It is clear that there exists a remarkably close correlation between the effects of various protease inhibitors on signal peptidase activity and the effects of the same concentrations of the same compounds on the release of α-amylase from rat pancreatic microsomes. This evidence suggests that the enzyme release phenomenon reported from this laboratory (13) is related to the protein transporting mechanism involved in secretion despite the fact that the two processes operate in opposite directions.

The simplest explanation of the data is that there exists, in pancreatic microsomal membranes, a transport system which allows the movement of secretory proteins across the membrane and that this system is destroyed by exogenous proteases and inhibited by inhibitors of signal peptidase activity. The essential difference between such a system and Blobel's 'secretory pore'

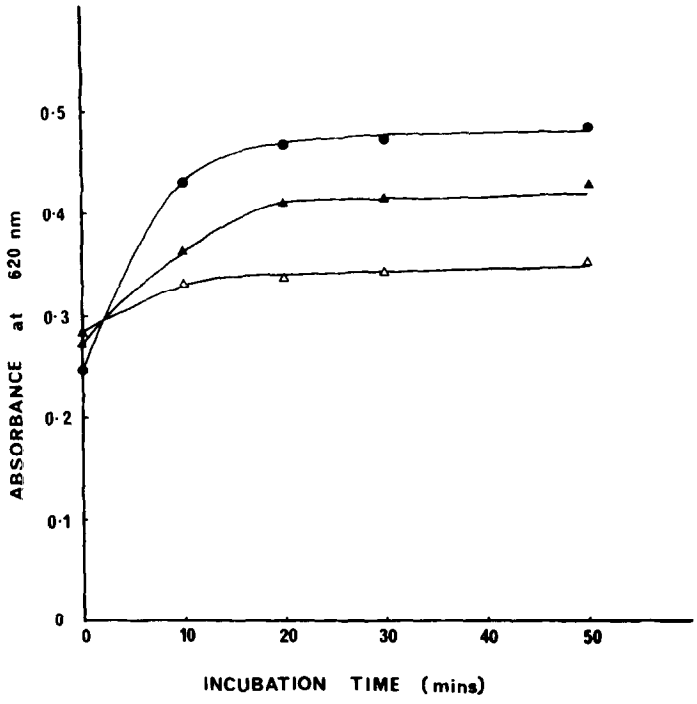

Fig. 4: Appearance of α-amylase in microsome suspensions incubated in the presence of 1,10 phenanthroline. Microsomes were incubated at 37°C in STKC buffer containing: no additions (●); 10 mM 1,10 phenanthroline, 1% v/v ethanol (▲); 20 mM 1,10 phenanthroline, 2% v/v ethanol (△). (For ethanol control see Fig. 1.) 50 µl aliquots of each suspension were assayed for amylase activity at 10 minute intervals.

(6) is that the former allows release of completed proteins whereas the latter is thought to mediate transport of nascent peptides only.

The molecular mechanism of the amylase release phenomenon described here is not yet understood. If cleavage by signal peptidase is required for amylase release, it implies that virtually all of the amylase initially associated with microsomes is unprocessed before release. It has not yet been determined whether or not pre-amylase is present in pancreatic microsomal preparations, but on current views it would be surprising if all the completed amylase of microsomes is unprocessed. Alternatively, actual signal peptidase activity may not be required for amylase release. If the signal peptidase formed an integral part of a structure permitting protein movement, inhibition of the enzyme may affect the whole structure, or block a receptor site, so that enzyme translocation would be prevented.

The enzyme release phenomenon may also be considered in terms of Wickner's "trigger hypothesis" (16). It is generally assumed that secretory enzymes found in association with microsomes exist in aqueous solution in the lumen of the vesicles. As far as we are aware, there is no unequivocal evidence for this assumption; the alternative, that the enzymes exist within the membrane structure, sequestered from exogenous proteolytic attack, cannot be eliminated. Our data could equally well account for the release of amylase from such a position if escape from the membrane was dependent on signal peptidase activity.

Although the nature of the enzyme release phenomenon is, as yet, a matter for speculation, the evidence presented in this report suggests that it is closely connected with enzyme secretion. The pancreatic microsomes from which enzymes are released may therefore constitute a simple, convenient system whose further study will possibly contribute to understanding the precise mechanism of protein translocation across membranes.

REFERENCES

1. Devillers-Thiery, A., Kindt, T., Scheele, G. and Blobel, G. (1975). Proc. Nat. Acad. Sci. (USA) *72*, 5016-5020.
2. Lingappa, V.R., Devillers-Thiery, A. and Blobel, G. (1977). Proc. Nat. Acad. Sci. (USA) *74*, 2432-2436.
3. Habener, J.F., Rosenblatt, M., Kemper, B., Kronenberg, H.M., Rich, A. and Potts, Jr., J.T. (1978). Proc. Nat. Acad. Sci. (USA) *75*, 2616-2620.
4. Thibodeau, S.N., Palmiter, R.D. and Walsh, K.A. (1978). J. Biol. Chem. *253*, 9018-9023.
5. Haugen, T.H. and Heath, E.C. (1979). Proc. Nat. Acad. Sci. (USA) *76*, 2689-2693.
6. Blobel, G. and Dobberstein, B. (1975). J. Cell Biol. *67*, 835-851.
7. Dobberstein, B. and Blobel, G. (1977). Biochem. Biophys. Res. Commun. *74*, 1675-1682.
8. Highfield, P.E. and Ellis, R.J. (1978). Nature *271*, 420-424.
9. Maccecchini, M-L., Rudin, Y., Blobel, G. and Schatz, G. (1979). Proc. Nat. Acad. Sci. (USA) *76*, 343-347.
10. Lingappa, V.R., Lingappa, J.R. and Blobel, G. (1979). Nature *281*, 117-121.
11. Gayda, R.C., Henderson, G.W. and Markovitz, A. (1979). Proc. Nat. Acad. Sci. (USA) *76*, 2138-2142.
12. Strauss, A.W., Zimmerman, M., Boime, I., Ashe, B., Mumford, R.A. and Alberts, A.W. (1979). Proc. Nat. Acad. Sci. (USA) *76*, 4225-4229.
13. Pearce, P.D., May, B.K. and Elliott, W.H. (1978). Biochem. J., *176*, 611-614.

14. Sussman, P.M., Tushinski, R.J. and Bancroft, F.C. (1976). Proc. Nat. Acad. Sci. (USA) *73*, 29-33.
15. Steiner, D.F. (1979). Nature *279*, 674-675.
16. Wickner, W. (1979). Ann. Rev. Biochem. *48*, 23-45.