Isolation and re-association of the subunits from the pro-(carboxypeptidase A)–pro-(proteinase E) binary complex from pig pancreas

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The component subunits of the pro-(carboxypeptidase A)–pro-(proteinase E) binary complex from pig pancreas were separated with a high recovery (80–95%) of their original potential activity. The isolated subunits and the reconstituted complex have properties similar to those of the corresponding natural species. The tryptic activation course of the pro-(carboxypeptidase A) subunit is substantially modified when bound to pro-(proteinase E), whereas the activation of pro-(proteinase E) is not dependent on this association.

Mammalian pancreatic pro-(carboxypeptidases A) are found in quaternary association with zymogens of serine proteinases (Yamasaki *et al.*, 1963; Brown *et al.*, 1963; Yoneda, 1980; Kobayashi *et al.*, 1981). Comparative studies that can throw some light on the biological significance of such quaternary associations can be carried out with pig pancreatic pro-(carboxypeptidase A) because this proenzyme occurs in two different forms, one as a monomer and another as a binary complex with pro-(proteinase E) (Kobayashi *et al.*, 1978; Martínez *et al.*, 1981). However, the isolation and characterization of the subunits from the binary complex in a native state, together with reconstitution studies on their artificial complexes, is a prerequisite for the meaningful interpretation of such associations.

The high stability of the pig binary complex hinders the chromatographic separation of its subunits unless a strong dissociating medium is used. Kobayashi *et al.* (1978) isolated the subunits of the pro-(carboxypeptidase A)–pro-(proteinase E) complex from pig pancreatic secretion by incubating the complex in 2% (w/v) SDS at 100°C, followed by a long period of gel-filtration chromatography at 40°C in the presence of SDS. Unfortunately this method gives irreversibly denatured subunits. Among other methods previously used for the dissociation of related complexes, such as those formed by pro-(carboxypeptidase A) from bovine pancreas (Freisheim *et al.*, 1967; Puigserver *et al.*, 1972),

only the acylation of the complex with 2,3-dimethylmaleic anhydride followed by gel-filtration chromatography gives native subunits (Puigserver *et al.*, 1975). However, when this latter method was used on the pro-(carboxypeptidase A) binary complex from porcine pancreas, the yield of subunits was found to be very poor in our hands. For these reasons a new separation method based on DEAE-Sephacrose chromatography of the complex in the presence of 7 M-urea was developed. This allowed the preparative isolation of the subunits with high yield and a very high recovery of their initial activity and activation behaviour. In addition, a comparison of the activation properties of all the possible protomeric and associated forms of pig pancreatic pro-(carboxypeptidase A) and pro-(proteinase E) (the naturally occurring monomeric and binary forms, isolated subunits, and re-associated complexes) was carried out. From these results it was deduced that the behaviour of the re-associated complexes mimics that of the natural one. Moreover, interesting information was obtained about the activation pathway of the two zymogens and the functional effect of their association.

Materials and methods

The naturally occurring monomeric pro-(carboxypeptidase A) and the binary complex pro-(carboxypeptidase A)–pro-(proteinase E) were obtained from acetone-dried powder of pig pancreas, by the method described by Martínez *et al.* (1981).

Abbreviation used: SDS, sodium dodecyl sulphate.

Both proenzymes were treated with 1 mM-phenylmethanesulphonyl fluoride before being stored in the form of a precipitate, in $(\text{NH}_4)_2\text{SO}_4$, at 4°C. Carboxypeptidase A (EC 3.4.17.1) activities were measured against the substrate *N*-benzoylglycyl-L-phenylalanine at 1 mM concentration (Folk & Schirmer, 1963). Proteinase E activities were measured against *N*-succinyl-L-alanylalanylalanine *p*-nitroanilide at 1 mM concentration (Bieth *et al.*, 1974). The proenzymes, in the monomeric or in the associated forms, were activated at a concentration of 5.5 μM in 20 mM-sodium phosphate buffer, pH 6.5, by incubation with pig trypsin (2.6 μM final concn.), at 25°C. Samples were withdrawn at different times and assayed directly for carboxypeptidase A and proteinase E activities.

The chromatography of the dissociated binary complex on DEAE-Sephacrose (Pharmacia Fine Chemicals) was carried out in a column (1 cm $\times$ 50 cm) equilibrated with 20 mM-sodium phosphate buffer, pH 6.5, containing 7 M-urea, at a flow rate of 20 ml/h. The urea solutions were prepared before each experiment and carefully purified with active charcoal and a mixed-bed ion-exchange resin [Bio-Rad AG 501-X8 (D)]. The binary complex was loaded on to the column in the same buffer after an extensive dialysis against it for 14 h at 4°C, and the subunits were eluted with a linear NaCl gradient from 0 to 0.35 M in the equilibration buffer (Fig. 1 below). The fractions containing the same subunit were pooled and dialysed. In the case of pro-(proteinase E), it was first dialysed against 20 mM-

phosphate buffer, pH 5.0 (containing 2 M-urea), followed by a dialysis with the same buffer without urea. In the case of the pro-(carboxypeptidase A) fraction, the dialysis was also stepwise with respect to urea concentration in 20 mM-phosphate buffer, pH 6.5, containing 0.01 mM- ZnCl_2 . The isolated zymogens were stored in solution at 4°C, or as a precipitate in 43% satd. $(\text{NH}_4)_2\text{SO}_4$.

Electrophoretic analysis of the fractions were performed in 12–19% (w/v) gradient polyacrylamide gels in the presence of SDS as described by Laemmli (1970). Protein concentration was evaluated spectrophotometrically at 280 nm (Martinez *et al.*, 1981).

Results and discussion

Preliminary studies showed that the acylating reagents maleic anhydride or 2,3-dimethylmaleic anhydride were not able to promote an efficient dissociation of the pro-(carboxypeptidase A) binary complex from pig pancreas, in contrast with the positive results obtained by Puigserver & Desnuelle (1975) with the latter reagent on related complexes of pro-(carboxypeptidase A) from bovine pancreas. After different trials, it was found that 7 M-urea was a good dissociating agent for the pig binary complex. The chromatography of this binary complex on DEAE-Sephacrose at pH 6.5 in the presence of 7 M-urea gives a complete separation of the pro-(carboxypeptidase A) and pro-(proteinase E) subunits (Fig. 1) as shown by electrophoretic analysis. After

Fig. 1. Isolation of pro-(proteinase E) (a) and pro-(carboxypeptidase A) (b) subunits from their natural binary complex from pig pancreas

DEAE-Sephacrose chromatography of the binary complex in the presence of 7 M-urea/20 mM sodium phosphate buffer pH 6.5, was carried out. The inset shows the electrophoretic analysis of the fractions: (1) natural binary complex; (2) fraction b; (3) fraction a. Abbreviations used: PCPA, pro-(carboxypeptidase A); PPE, pro-(proteinase E).

urea removal by means of stepwise dialysis, the potential specific activity of the isolated subunits was found to be approx. 60% for pro-(carboxypeptidase A) and 85% for pro-(proteinase E) with regard to their potential activity in the natural binary complex. However, after rechromatography of each subunit under the same conditions as before but in the absence of urea, they were obtained with an 85 and a 95–100% recovery of their potential activity in the binary complex respectively. A similar result was obtained by keeping the isolated subunits in the form of a precipitate in $(\text{NH}_4)_2\text{SO}_4$ for 7–15 days at 4°C, and eliminating any insoluble material by centrifugation when redissolved.

Direct mixing of equimolar amounts of the isolated subunits in 20 mM-sodium phosphate buffer, pH 6.5, reconstitutes the binary complex, as shown by the appearance of some distinctive properties of the binary complex, such as its elution behaviour on gel-filtration chromatography, its u.v. spectral properties, and the kinetics of generation of carboxypeptidase A activity after trypsin addition (Martínez *et al.*, 1981).

In order to investigate the functionality of the associated subunits and their re-associated complexes, the time course of their tryptic activation was studied (Figs. 2a and 2b). The activation of the natural monomeric pro-(carboxypeptidase A) and the hybrid complex of the last monomeric zymogen with isolated pro-(proteinase E) were also carried out for comparison.

From these results it is apparent that the isolated subunits and the re-associated complexes display, from a qualitative point of view, a similar ability to that of the corresponding natural species for the generation of active forms. The carboxypeptidase A activity originating from the artificial species attains 80–95% of that for the corresponding natural species at intermediate and long digestion times (under the chosen experimental conditions). The reasons for these small dissimilarities are unknown at present, but could probably be due to some conformational differences between the activation segment of natural monomeric pro-(carboxypeptidase A) and isolated pro-(carboxypeptidase A) subunit obtained from the binary complex and/or to the way in which they interact with the pro-proteinase E subunit. In contrast, pro-(proteinase E) shows exactly the same activation behaviour in the artificial complexes as in the natural one. It should be noticed that in the above reasoning it has been assumed that the natural monomeric pro-(carboxypeptidase A) and the isolated pro-(carboxypeptidase A) subunit from the natural binary complex are identical. However, this is at present an undemonstrated working hypothesis that awaits confirmation by sequence studies, although some evidence, including the present activation curves, favours it (Kobayashi *et al.*, 1978; Martínez *et al.*, 1981).

The above results also give valuable information about the activation path of the studied zymogens and their complexes. Thus it is observed that the

Fig. 2. Tryptic activation course of the different species derived from the natural monomeric pro-(carboxypeptidase A) and natural pro-(carboxypeptidase A)–pro-(proteinase E) binary complex, both from pig pancreas

■, Natural binary complex; ○ and ●, isolated pro-(carboxypeptidase A) from the binary complex and its re-associated complex with pro-(proteinase E); △ and ▲, natural monomeric pro-(carboxypeptidase A) and its derived hybrid complex with pro-(proteinase E); □, isolated pro-(proteinase E) subunit. (a) Generation of carboxypeptidase A activity measured against *N*-benzoylglycyl-L-phenylalanine. (b) Generation of pro-(proteinase E) activity measured against *N*-succinyl-L-alanylalanylalanine *p*-nitroanilide. Activations were carried out at 5.5 μM-zymogen with 2.6 μM-pig trypsin in 20 mM-sodium phosphate buffer, pH 6.5 at 25°C.

'initial' activation of pro-(carboxypeptidase A) by trypsin is much faster in the monomeric than in the complex forms (either natural or re-associated). However, after 1–2 h the activity generated in the latter forms is higher than in the former ones. This fact is very apparent between 3 and 7 h of activation. Afterwards, the generation of activity proceeds smoothly until a similar activity is reached for all studied species at 18–24 h. In the case of pro-(proteinase E), its activation by trypsin proceeds at a similar rate both for the isolated pro-(proteinase E) and for its natural or artificial complexes with pro-(carboxypeptidase A).

Our experimental observations are somewhat different from those reported by Puigserver & Desnuelle (1977) on the activation of the re-associated complexes between pro-(carboxypeptidase A) (subunit I) and the other two accompanying proenzymes of serine proteinases (subunits II and III) in the bovine pancreatic ternary complex. According to these authors, the tryptic activation rate of bovine pro-(carboxypeptidase A) subunit is increased on binding to subunit II and decreased on binding to subunit III. However, either in the isolated or in the complex forms, the activation of bovine pro-(carboxypeptidase A) seems to follow a similar pathway, as judged by the similar shape of the activation courses. This is in contrast with our observations about the different shape of the tryptic activation course of pig pro-(carboxypeptidase A) in the binary complex with pro-(proteinase E) compared with that of the monomeric form.

The results shown in Fig. 2(a) clearly indicate that the presence of pro-(proteinase E) delays the initial activation of pro-(carboxypeptidase A), although once the activation is initiated, it proceeds more efficiently. This behaviour suggests that the binding of pro-(proteinase E) to pro-(carboxypeptidase A) could have at least two biological advantages: (i) the pro-(carboxypeptidase A) would be protected against a 'premature' proteolytic activation; (ii) the generation of carboxypeptidase A activity would be hastened in the binary complex, probably to adapt to the high-velocity requirements for the intestinal digestion.

The greater ease with which pro-(carboxypeptidase A) becomes fully activated after pro-(proteinase E) binding suggests that the activation segment of the

former zymogen is more labile to compete proteolysis after complex-formation, probably due to some conformational constraints or to a displacement from the enzyme domain. Preliminary electrophoretic analyses carried out in this laboratory substantiate this hypothesis.

It is worth indicating that the separation method described in the present work greatly improves a previously reported preparative method for pro-(proteinase E) (Kobayashi *et al.*, 1978), since that method yields irreversibly denatured subunits. Besides, from the pro-(proteinase E) isolated by the present method, it is very easy to obtain pure active proteinase E simply by trypsin addition followed by the same chromatographic procedure in the absence of urea. Further physico-chemical and functional characterization of this proenzyme and of its active form could, therefore, be facilitated by the use of these preparative methods.

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