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TWO-STEP DISSOCIATION OF BOVINE 6S PROCARBOXYPEPTIDASE A BY DIMETHYLMALEYLATION

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SUMMARY : Reversible condensation of the ternary complex form of bovine pancreatic procarboxypeptidase A with 2,3-dimethyl maleic anhydride was investigated at pH 9.0 and low concentration of reagent over the acylable amino groups. After subsequent modification of only a few lysyl residues, subunit III was found to have been released from the quaternary structure leading to the separation of an apparently native protein devoid of any contaminating subunit II, while dissociation of the remaining binary complex occurred upon further addition of the anhydride. This observation suggests that the electrostatic interactions existing between subunits I and III are more rapidly weakened than those between subunits I and II, probably because fewer lysyl residues are involved and/or there is greater accessibility to the chemical reagent. Although completely inactive on the specific substrates of trypsin, chymotrypsin and elastase, subunit III hydrolyzed p-nitrophenyl acetate at a rate similar to that of chymotrypsin but without any burst of p-nitrophenol, which indicates that the weakly functional active site of the subunit is not quite comparable to that of serine protease zymogens. Subunit III already has some of the functional characteristics of the corresponding active enzymes.

The disaggregation of bovine pancreatic 6S procarboxypeptidase A by reversible acylation of amino groups with 2,3-dimethylmaleic anhydride has been found to be the only way of obtaining the three subunits in native form (1). It has further been shown that subunit I, the immediate precursor of carboxypeptidase A, is likely to possess two independent and specific sites (2) interacting with complementary regions of subunit II, a chymotrypsinogen of the C-type (3,4) and subunit III. The latter, which certainly belongs to the serine protease family, contains a weakly functional active site reacting slowly with specific inhibitors or active site titrants (5,6), but seems so far to be devoid of any specific enzymatic activity before or after incubation with trypsin. Besides this noncovalent association of the three proteins, a binary complex with no subunit III has also been described in aqueous extracts of acetone powder of bovine pancreas (7). More recently, evidence has been provided for the existence in calf pancreatic secretion of a ternary complex containing in addition to subunits I and II the still poorly characterized zymogen E, which on activation with trypsin gives rise to protease E, an elastase-like protease (8). Besides the existence of monomeric procarboxypeptidase, an association with zymogen E has also been shown to exist in porcine

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pancreatic juice (9). However, in most animal species studied so far including dog (10), dogfish (11) and lungfish (12) procarboxypeptidase A is secreted as a single polypeptide chain and not as a noncovalent association of 2 or 3 chemically and/or functionally different subunits. Up to now, ternary complex forms of procarboxypeptidase A have only been found in the bovine species, so it is of interest to understand their biological significance. The highly specific interactions existing between subunits I and II on the one hand, and subunit I and III on the other hand, led us to further investigate the dissociation process in order to define more precisely both the structure and the function of this complex of secreted proteins. We report here that small amounts of 2,3-dimethylmaleic anhydride, as compared to protein concentration, induce a partial dissociation of the ternary complex, leading to the separation of subunit III in a very high state of purity. We used this finding to show that the latter failed to hydrolyze specific trypsin, chymotrypsin or elastase substrates although exhibiting a non-specific esterase activity.

EXPERIMENTAL PROCEDURES

Materials . Ultrogel AcA54 and DE-trisacryl were obtained from IBF (Villeneuve-la-Garenne, France) while 2,3-dimethyl maleic anhydride, hippuryl-L-phenyllactic acid, acetyl-L-tyrosine ethyl ester and diphenylcarbamyl-treated trypsin were Sigma products (St Louis , USA). Untreated trypsin used for procarboxypeptidase A activation was purchased from Opochimie (Monaco) and benzamidine from Fluka (Switzerland).

Preparation of 6S procarboxypeptidase A. Bovine ternary complex was purified from an acetone powder of pancreas in the presence of 2mM benzamidine (13). The procedure was slightly modified however since an ammonium sulfate precipitation (45% saturation in water at 0°C) was performed prior to chromatography on DE-trisacryl at pH 7.0. Protein concentrations were derived from the following molar extinction coefficient : $E^{1\%}_{1cm}$ = 18.8 at 280 nm.

Dissociation of the ternary complex. Dimethylmaleylation of 6S procarboxypeptidase A was performed at pH 9.0 as already described (1) in the absence of diisopropylfluorophosphate. The incubation medium was then concentrated with an Amicon cell (PM-10 membrane) before chromatography either on a Sephadex G-100 or ultrogel AcA54 column in 0.1 M bicarbonate, pH 9.0, containing 0.2 M NaCl and 2 mM benzamidine. Deacylation of proteins resulted from a 24 h dialysis at 0°C against a pH 7.0 buffer for subunit I and a pH 6.0 buffer for subunits II and III and the binary complex I-II.

Zymogen activation and activity measurements. Subunits I and II were activated by a 60 min incubation in 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl at 37°C with 1/20 trypsin (weight ratio) and at 0°C with 1/100 tryspin (weight ratio), respectively. Resulting activities were measured potentiometrically at 25°C and pH 7.5 , using a 10 mM hippuryl-L-phenyllactic acid solution as substrate for carboxypeptidase A, and at pH 7.8 using a 10 mM acetyl-L-tyrosine ethyl ester solution as substrate for activated subunit II. Enzyme-catalyzed hydrolysis of p-nitrophenyl acetate at 25°C in a 0.1 M Tris-HCl buffer pH 7.5 , containing 4% acetonitrile, was measured spectrophotometrically at 410 nm using a molar extinction coefficient of 14,000 for the released p-nitrophenol.

Polyacrylamide gel electrophoresis. One dimensional electrophoresis in presence of 0.1% sodium dodecyl sulfate was carried out according to Laemmli

(14) using 10-12% polyacrylamide gels. Proteins were stained with a 0.25% Coomassie brilliant blue solution and destained in an acetic acid-ethanol-water mixture (1.5:1.0:17.5 vol./vol.).

RESULTS

Partial dissociation of 6S procarboxypeptidase A. Figure 1 compares elution profiles of the ternary complex acylated with 2,3-dimethylmaleic anhydride (left : 5 mg/mg of protein and right : 0.5 mg/mg of protein) through columns of Sephadex G-100 and ultrogel AcA54 at pH 9.0, respectively. The three peaks eluted from the former could easily be identified as undissociated complex, subunit I and a mixture of subunits II and III as clearly indicated in Figure 2A (see also reference 1). By contrast, only two peaks were obtained from the latter. As shown in Figure 2B, analysis of the material emerging from the ultrogel column by one dimensional polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicated that the first peak contained two proteins with apparent Mr of 40,000 and 29,000 identified as subunits I and II, respectively, whereas the protein of Mr 27,000 eluted under the second peak was found to be subunit III. This was also confirmed by activity measurements since the first-eluted material was potentially active on both carboxy-

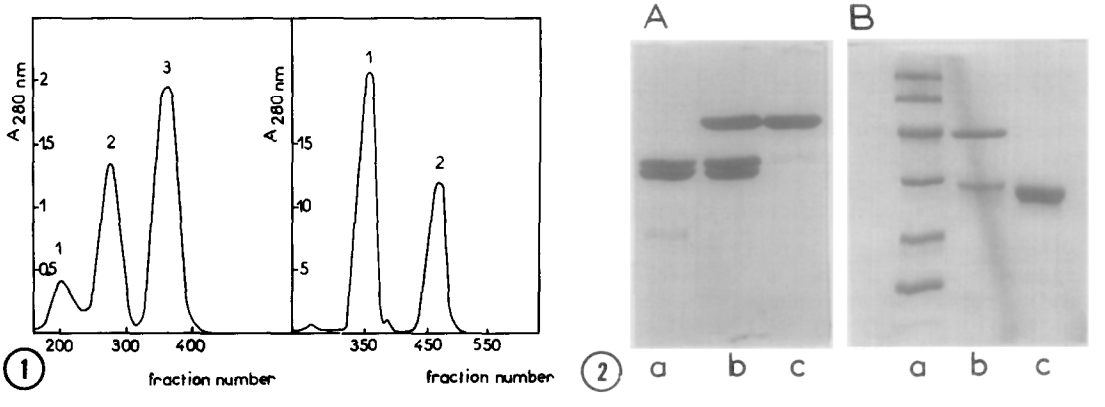

Figure 1 : Filtration through Sephadex G100 (left) or Ultrogel AcA54 (right) columns of acylated ' 6S procarboxypeptidase A. The columns (Sephadex : 3 cm x 200 cm and Ultrogel : 3.5 cm x 120 cm) were equilibrated with a 0.1 M sodium bicarbonate buffer, pH 9.0, containing 0.2 M NaCl and 2 mM benzamidine. Fraction volume : 2 ml and 2.5 ml and flow rate : 12 ml/h and 14 ml/h, respectively. The Sephadex column was loaded with 75 mg of the ternary complex acylated with 2,3-dimethyl maleic anhydride (5mg/mg protein) while 400 mg of the complex treated with 0.5 mg anhydride were used for the ultrogel column.

Figure 2: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate .
(A) Peaks eluted from the Sephadex G100 column. Slots a, b and c correspond to the material contained in peaks 3, 1 and 2, respectively.
(B) Peaks obtained from the Ultrogel AcA54 column. (a) Standard proteins of decreasing Mr : phosphorylase b (94,000); bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); α-lactalbumin (14,400). (b) and (c) stand for peaks 1 and 2, respectively.

Table I
Influence of 2,3-dimethyl maleic anhydride concentration on the extent of dissociation of procaboxypeptidase A

Protein forms %	Dimethyl maleic anhydride (mg/mg protein)			
	0.5	1.0	2.5	5
Dimer I-II	95	85	70	20
Subunit I	5	15	30	80

peptidase and chymotrypsin specific substrates. As expected, the potential specific activities on hippuryl-L-phenyllactic acid and acetyl-L-tyrosine ethyl ester (90-100 units/mg protein) were higher than those of the ternary complex (70-80 units/mg protein), which is consistent with a stoichiometric association of subunits I and II. None of these enzymatic activities could be detected in the material eluted under the second peak. It should also be pointed out here that under experimental conditions leading to partial dissociation of the ternary complex, less than 5% of either free subunits I or II was obtained.

Table I indicates that increasing acylating reagent concentration from 0.5 to 5.0 mg/mg of protein in the reaction mixture resulted in decreasing amounts of the noncovalent association of subunits I and II. The possibility of an association between subunits I and III or II and III could definitely be ruled out in this dissociation process.

Esterase activity of isolated subunit III. Taking advantage of the fact that subunit III was isolated in a very high state of purity, it was attempted to characterize more precisely its weakly functional active site. Indeed it was interesting to see whether the low hydrolyzing activity of subunit III towards acetyl-L-tyrosine ethyl ester previosuly described (1) might result from contaminating subunit II. In fact, subunit III was found to be quite unable to hydrolyze either the specific substrates of chymotrypsin (acetyl-L-tyrosine ethylester), trypsin (benzoyl-L-arginine ethylester), or elastase (acetyl-L-trialanyl methylester), or elastin and casein prior or subsequent to tryptic activation. However, as indicated in Figure 3, subunit III readily hydrolyzed p-nitrophenylacetate although it did so more slowly than activated subunit II and without exhibiting any burst of p-nitrophenol. Kinetic parameters of the subunit III-catalyzed hydrolysis of this ester substrate (k_{cat} = 0.12 min^{-1} and Km app = 2.2 ± 0.2 mM), as compared to the values (0.36 min^{-1} and 1.6 μM) reported for chymotrypsin (15) have indicated that the affinity of subunit III for this substrate is several orders of magnitude

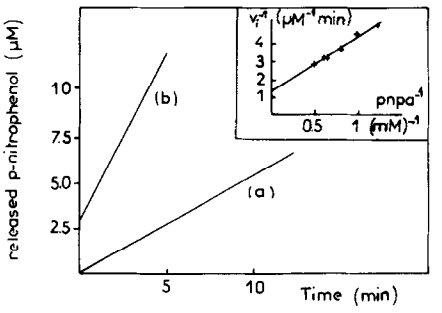

Figure 3 : Hydrolysis of p-nitrophenyl acetate at 25°C, pH 7.5, catalyzed by subunit III (a) and activated subunit II (b). Inset : Lineweaver-Burk plot corresponding to subunit III.

lower than that of chymotrypsin, suggesting that the binding site of subunit III, if formed, possesses a different structure.

DISCUSSION

The present study demonstrates that dissociation of 6S procarboxypeptidase A by reversible condensation with 2,3-dimethylmaleic anhydride is a two-step process since subunit III is first released from the quaternary structure and then followed by disaggregation of the remaining binary complex. These sequential events, which have never been pointed out in previous studies (1, 13, 16, 17), depend on the acylating reagent concentration. The fact that subunit III is released first and selectively from the ternary complex might result from the electrostatic interactions being weaker between subunits I and III than between subunits I and II. According to this hypothesis, a high number of lysyl residues might be either involved or protected against the acylating reagent in the specific interacting site of subunits I and II. However, the fact that the chemical modification of a few lysyl residues in subunits I, II or III, inducing some conformational changes in the protein, might weaken interactions between the complementary regions of subunits I and III rather than between I and II should not be ruled out. It can also be assumed that the binding between subunits I and III mainly involves electrostatic interactions, whereas the binding between subunits I and II involves both electrostatic and other noncovalent interactions.

Another point of interest worth stressing here is the rather simple means of obtaining pure and apparently native subunit III without any residual contamination of subunit II. This is not possible once the complete dissociation of the ternary complex has been achieved since subunits II and III are closely related chemically (1,6). Using this purified protein, it has now been established that subunit III does not hydrolyze any of the specific substrates of the pancreatic serine proteases (trypsin, chymotrypsin

and elastase). The lack of hydrolytic activity towards casein, elastin and acetyl-L-tri-alanine methyl ester indicates that subunit III is distinct from zymogen E and the corresponding enzyme, protease E, described in bovine and porcine pancreatic secretion and tissue (8, 9, 18).

Subunit III-catalyzed hydrolysis of p-nitrophenyl acetate, a non-specific substrate for serine proteases which is not hydrolyzed at a significant rate by their parent zymogens, is a noteworthy finding. Furthermore, subunit III has been shown to slowly but significantly hydrolyze titrants directed towards the active site of serine proteases (6). All these findings strongly suggest that the weakly functional active site of subunit III might already share some common features with those of active serine proteases. There is actually no answer to the intriguing question as to the possible functional significance of subunit III, whether free or bound in the ternary complex. Work is currently in progress to define more precisely its active site as well as its interactions with procarboxypeptidase A itself.

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