Autolysis of proproteinase E in bovine procarboxypeptidase A ternary complex gives rise to subunit III

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Received 24 July 1990; revised version received 9 October 1990

Extracts of bovine pancreatic tissue are shown by HPLC to contain two distinct ternary complexes of procarboxypeptidase A (subunit I), chymotrypsinogen C (subunit II) and either proproteinase E or subunit III. It is shown that proproteinase E in the complex generates subunit III by removal of 13 N-terminal residues when the former is allowed to autolyze in solution or when catalytic amounts of isolated active proteinase E are added to it. Autolysis of proproteinase E was accompanied by the loss of potential activity towards specific synthetic substrates and occurred at a higher rate in pancreatic juice than in pancreatic tissue extracts, even when both were processed in the presence of serine protease inhibitors. We conclude that subunit III (also called truncated protease E) is an autolytic product of proproteinase E and not an ab initio component of the native ternary complex.

Zymogen E; Bovine pancreas; Autolysis; Subunit III

1. INTRODUCTION

The so-called pancreatic procarboxypeptidase A-S6 in ruminants is a ternary complex in which the zymogen itself (subunit I) is tightly bound through noncovalent interactions to both subunits II (chymotrypsinogen C) and III [1,2]. A considerable literature exists relating to the occurrence and properties of subunit III [2-4]. Its rather high abundance in ruminant pancreatic secretion (about 6% by weight) and the lack of any defined enzymatic activity or activatability has made it a biological enigma for the past three decades. Enzymatic analysis [5] showed that subunit III has a weakly functional active site related to that of serine proteinases that results from the lack of two valine residues at the N-terminus of the polypeptide chain [2,6]. Moreover, on the basis of primary structure comparisons and molecular modeling, it has been suggested that bovine subunit III and proproteinase E, a pancreatic zymogen not previously found in ruminants, are naturally occurring related proteins that belong to a separate serine endopeptidase family [7].

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Abbreviations: PPE, proproteinase E; PE-α, active proteinase E generated by tryptic activation; PE-β, inactive proteinase E generated by N-terminal truncation of the zymogen by active proteinase E; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate

In nonruminants, the relationship between and simultaneous occurrence of proproteinase E and subunit III-like proteins has been a matter of discussion. For instance, proproteinase E is known to occur as a binary complex with procarboxypeptidase A in both pig [8] and human [9], although in the latter species a subunit III-like protein has also been found to be present either in the free form [10] or bound to procarboxypeptidase A [11]. On the other hand, we have recently shown that proproteinase E from both human and pig can readily undergo autolytic transformation into subunit III-like proteins [12].

In the present paper we show that proproteinase E is the natural ad initio component of the bovine ternary complex, besides subunits I and II, and that subunit III is generated from proproteinase E by autolysis within the complex through a mechanism similar to that observed in human and pig proteins. This artifactual transformation can be minimized under appropriate isolation conditions.

2. MATERIALS AND METHODS

2.1. *Preparation and processing of the samples*

Bovine pancreatic juice was collected over ice in the presence of 2 mM benzamidine and 0.3 mg/ml of bovine pancreatic trypsin inhibitor, and found to be devoid of any proteolytic activity. Pancreatic acetone powder was prepared as described in [13]. In order to minimize zymogen activation, aqueous extracts were obtained by suspending 0.1 g of either lyophylized pancreatic juice or pancreatic acetone powder in 1 ml of 20 mM Tris-HCl (pH 8.0) containing 0.3

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00145793/90/$3.50

mg/ml of bovine pancreatic trypsin inhibitor and 2 mM phenylmethylsulphonylfluoride. The suspension was briefly vortexed and centrifuged at 15000 rpm for 10 min. The resulting supernatant was collected and further centrifuged for 2 min prior to injection onto the HPLC column.

2.2. *Purification of procarboxypeptidase A complexes*

Pancreatic extracts were chromatographed on a HPLC TSK-DEAE column (from TosoHaas) as previously described [9]. Briefly, after sample loading and washing the column for 10 min with a 20 mM Tris-HCl buffer at pH 8.0, elution was performed for 120 min by a 0–0.8 M linear gradient of ammonium acetate in the buffer. Fractions were collected and tested for intrinsic and potential proteolytic activities [9], and analyzed by SDS-polyacrylamide gel electrophoresis [14].

2.3. *Subunit isolation*

Active bovine proteinase E was obtained following the procedure described for the porcine enzyme [12] after tryptic activation of the ternary complex containing potential proteinase E activity. For sequence studies, proproteinase E (PPE), active proteinase E (PE-α) and defective proteinase E (PE-β) were isolated from the corresponding ternary complexes by reversed-phase HPLC on a Vydac C4 column, using a 30–50% acetonitrile gradient in 0.1% trifluoroacetic acid during 40 min, at a flow rate of 0.5 ml/min.

2.4. *N-Terminal sequence determination*

Automatic sequencing was performed using PITC-based Edman degradation with an Applied Biosystems gas-phase sequencer. A C18 column (Brownlee, 5 μm, 2.1 × 220 mm) was used to analyze the phenylthiohydantoin derivatives of amino acids [15]. Proproteinase E samples were carboxymethylated prior to sequence analysis.

3. RESULTS

3.1. *Occurrence of ternary complexes in pancreatic tissue and juice*

Fig. 1 shows that HPLC anionic exchange chromatography of aqueous extracts of acetone powders from bovine pancreas, as well as of lyophilized juice yielded a number of well-resolved protein peaks. As expected, the pattern from bovine pancreatic secretion was significantly simpler. By means of enzyme activity measurements after tryptic activation and SDS-electrophoresis (data not shown), most of the eluted peaks were identified, in particular those containing potential carboxypeptidase activity. In contrast with procarboxypeptidase B, which eluted as a monomeric form, procarboxypeptidase A was present in a binary complex (eluting at about 70 min) with proproteinase A, a serine endoproteinase zymogen exhibiting potential activity towards Suc-(Ala)$_3$pNA, and additionally in two different ternary complexes (eluting roughly at 85 and 90 min). Both ternary complexes contained procarboxypeptidase A (subunit I) and chymotrypsinogen C (subunit II) but only the first-eluted complex contained proproteinase E while the second one contained the so-called subunit III. These compositions were confirmed by N-terminal sequence determination (see below).

It is worth stressing here that the amount of the ternary complex containing subunit III was higher in pancreatic juice than in pancreatic tissue extracts. Moreover, from both sources, its level increased when

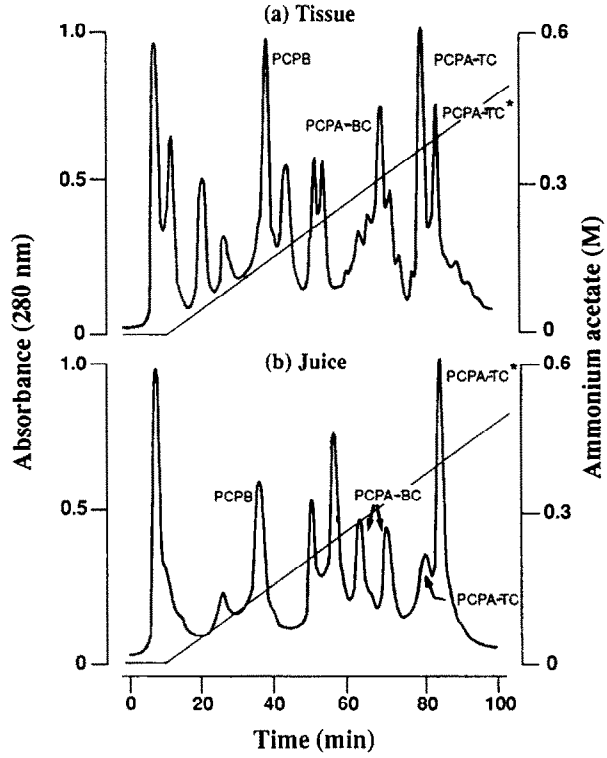

Fig. 1. Isolation of bovine pancreatic procarboxypeptidases by HPLC on a TSK-DEAE column. The aqueous extract from bovine pancreatic acetone powder (a) or from the freeze-dried bovine pancreatic juice (b) was made in 20 mM Tris-HCl (pH 8.0), loaded onto the column equilibrated with the same buffer and eluted using a 0–0.5 M ammonium acetate linear gradient. Procarboxypeptidase-containing peaks are marked: PCPB, procarboxypeptidase B; PCPA-BC, procarboxypeptidase A binary complex; PCPA-TC, procarboxypeptidase A ternary complex; PCPA-TC* defective procarboxypeptidase A ternary complex.

the sample incubation time prior to HPLC was longer, even in the presence of a covalent inhibitor (phenylmethylsulphonyl fluoride) and noncovalent inhibitors (bovine pancreatic trypsin inhibitor and benzamidine) of serine endoproteinases.

3.2. *Treatment with trypsin or active proteinase E*

Fig. 2a indicates that the proproteinase E subunit in the first-eluted ternary complex was rapidly activated upon addition of TPCK-treated trypsin (enzyme/substrate ratio of 1:40, w/w). The activation process was even faster than that previously observed with human and porcine proproteinases E in binary complexes with the corresponding procarboxypeptidase A [12]. The molar activity value (0.62 s^{-1}) for bovine active proteinase E was somewhat lower than that obtained for the human (1.26 s^{-1}) and porcine (1.38 s^{-1}) enzymes. In contrast, no significant level of activity could be detected in the subunit III-containing complex towards the proteinase E substrate Suc-(Ala)$_3$pNA either before or after addition of trypsin.

When the ternary complex with potential proteinase

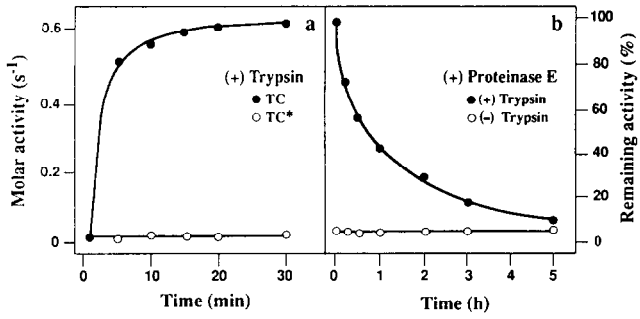

Fig. 2. Time course of proproteinase E activation or inactivation in the two procarboxypeptidase A ternary complexes isolated from bovine pancreas. (a) Activation with bovine trypsin: the samples were incubated with TPCK-treated trypsin at a 40:1 ratio (PCPA-TC- or PCPA-TC*/trypsin, w/w), at 25°C in a Tris-HCl buffer (pH 8.0), using Suc-Ala_3-pNA as a substrate for proteinase E activity (b). Inactivation by the addition of active proteinase E: the ternary complex showing potential proteinase E activity was incubated with active proteinase E at a 100:1 ratio (PCPA-TC/PE-α, w/w) for selected periods of time, and activity was measured with Suc-Ala_3-pNA. Samples were withdrawn from the incubation mixture at given times and treated with trypsin at a 1:4 ratio (PCPA-TC/trypsin, w/w). Activity was determined after 8 min incubation for each sample.

E activity was incubated with 1:100 (w/w) of PE-α, its ability to yield Suc-$(Ala)_3$pNA-hydrolyzing activity after a subsequent addition of trypsin suffered a continuous decrease with time, and it took about one hour to have the potential activity decreased by 50% (Fig. 2b). Control measurements in the absence of trypsin indicated that proteinase E activity remained quite low.

Fig. 3 shows that no apparent change in the molecular mass of any subunit could be detected by SDS electrophoresis after the proproteinase E-containing ternary complex was incubated with active proteinase E. However, HPLC analysis on TSK-DEAE of samples withdrawn from the reaction mixture clearly indicated that the first-eluted ternary complex was progressively replaced by the following one as the reaction proceeded. This demonstrates clearly for the first time that the ternary complex lacking potential proteinase activity, thus containing the so-called subunit III, was derived from that containing proproteinase E. Moreover, it can also be deduced that active proteinase E was responsible for the inactivation process.

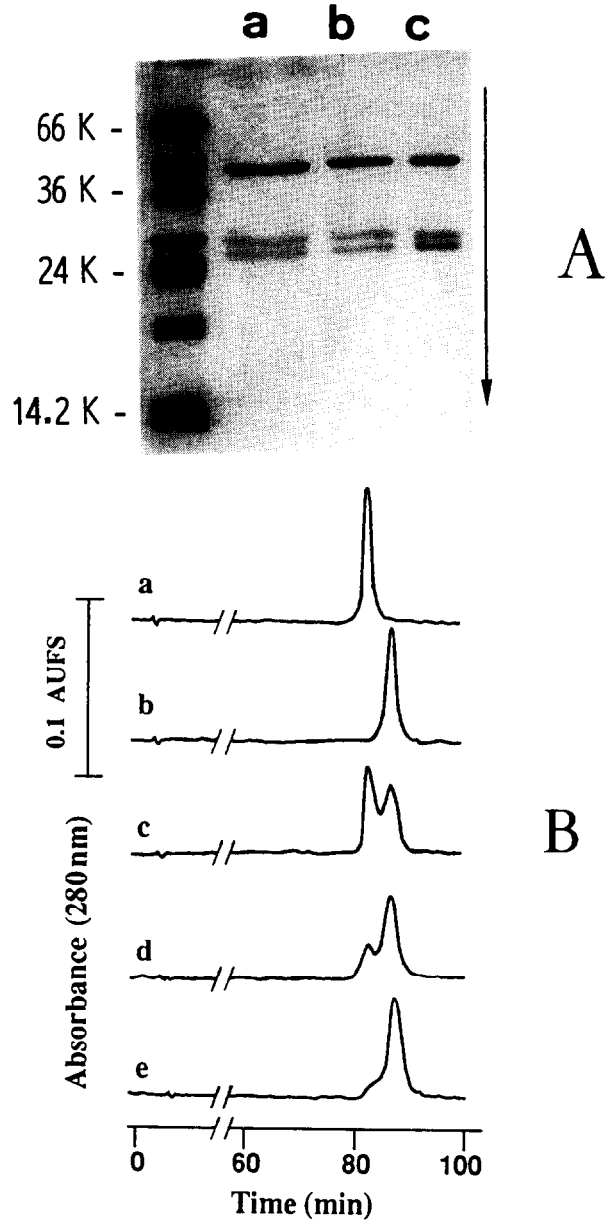

Fig. 3. (A) Electrophoretic analysis of the procarboxypeptidase A ternary complexes from bovine pancreas: (a) activatable ternary complex; (b) defective, trypsin-unactivatable ternary complex; (c) activatable ternary complex after incubation with PE-α (PCPA-TC/PE-α, 100:1, w/w ratio). (B) Chromatographic analysis of the transformation of the activatable ternary complex into the defective form: (a) activatable ternary complex; (b) defective ternary complex; (c, d and e) samples withdrawn from the incubation mixture of Fig. 2b at times 5 min, 30 min, and 1 h, respectively.

3.3. *N-Terminal sequence analysis*

Proproteinase E (PPE), active proteinase E (PE-α) and inactive proteinase E (PE-β) were isolated by reversed-phase HPLC from the corresponding ternary complexes, i.e. from potentially active, trypsin-activated, and proteinase E-treated complexes, respectively.

Fig. 4 shows that a high degree of identity exists between the N-terminal sequences of bovine PPE and those of the corresponding human and porcine proteins [12], particularly from Val-13 onwards in the region corresponding to the N-terminus of PE-α [8,12]. Of particular interest was the finding that the N-terminal sequence of bovine PE-β was exactly that of the so-called bovine subunit III, i.e. it lacks the first two valine residues of the corresponding PE-α.

	1				5					10					15					20					25	
Bovine PPE	F	S	Q	P	F	S	R	P	S	S	R	V	V	N	G	E	D	A	V	P	Y	S	W	S	W
Porcine PPE [a]	C	G	R	P	S	Y	N	P	A	S	R	V	V	N	G	E	D	A	V	P	Y	S	W	P	W
Human PPE [a]	Y	G	P	P	S	S	R	P	S	S	R	V	V	N	G	E	D	A	V	P	Y	S	W	P	W
Bovine PE-α												V	V	N	G	E	D	A	V	P	Y	S	W	S	W
Porcine PE [b]												V	V	N	G	E	D	A	V	P	Y	S	W	P	W
Bovine PE-β														N	G	E	D	A	V	P	Y	S	W	S	W
Bovine subunit III [c]														N	G	E	D	A	V	P	Y	S	W	S	W
Porcine PE-β [a]														N	G	E	D	A	V	P	Y	S			
Human PE-β [a]														N	G	E	D	A	V	P	Y	S	W	P	W

Fig. 4. N-Terminal sequences of proproteinase E and derived forms associated with procarboxypeptidases A. (a) Taken from Avilés et al. [12]; (b) taken from Kobayashi et al. [8]; (c) taken from Venot et al. [6]. Others from this work.

4. DISCUSSION

The biological significance of the trypsin nonactivable subunit III in the ternary complex with bovine pancreatic procarboxypeptidase A has long been a matter of controversy. A possible approach to the understanding of its origin was recently suggested by the observation that subunit III-like proteins can be generated from both human and porcine procarboxypeptidase A/proproteinase E binary complexes by addition of proteinase E [12]. This protease E-catalyzed transformation consisted of the autolysis of proproteinase E with subsequent release of the first 13 residues of the zymogen including the N-terminal dipeptide Val-12/Val-13 from the corresponding active enzyme. The importance of this dipeptide for the stabilization of the active site of serine endoproteinases has been known for a long time [16]. Since there is no evidence as to whether a comparable inactivation process occurs in the bovine ternary complex, we wished to determine if the inactivation occurs during the isolation procedure or if the N-terminally truncated protein is encoded at the DNA level and results from some deletion in the corresponding gene.

The present study shows unambiguously that the so-called subunit III of bovine procarboxypeptidase A ternary complex is not the native subunit and is derived from proproteinase E by the same autolytic process previously described [12] for the human and porcine forms. The N-terminal sequence of the protein generated by proteinase E-catalyzed autolysis of proproteinase E (now designated as PE-β) is exactly that of the protein known as subunit III of the procarboxypeptidase A ternary complex. The isolation of the autolyzed form of the protein instead of the native form for more than 25 years is now readily explained by the extreme lability of bovine proproteinase E in the complex (see both Figs 2 and 3). The fate of these ternary complexes in the intestine under environmental conditions quite different from those in vitro is unknown up to now. This lack of knowledge also applies to the confirmation of the current hypothesis which attributes a protective function [2,11,17] or a multienzymatic role [9] to the association of procarboxypeptidases with zymogens of serine proteinases. In any case, the experimental conditions used throughout the present study represent a positive step towards the development of an efficient procedure for the isolation of the native ternary complex of bovine procarboxypeptidase A and towards its functional characterization.

Procarboxypeptidase A also occurs in binary complexes in bovine pancreas although their nature, stability and origin have been subjected to certain controversy [1,2,18]. Using our isolation conditions, we have detected these binary complexes both in pancreatic tissue and juice (Fig. 1), and postpone the determination of their characteristics to further studies.

Acknowledgements: We are indebted to Prof. C. Crane-Robinson for kindly revising this manuscript. R. Pascual is a recipient of a FIS fellowship (Ministerio de Educación y Ciencia-Spain). This work was supported by Acción Integrada España/Francia 220/04, by Grant BIO88/0456 from CICYT (Ministerio de Educación y Ciencia-Spain) for the Spanish group, and by a grant from the Ministère de la Recherche et de la Technologie for the French group.

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