FEBS 15605

FEBS Letters 367 (1995) 211–213

Crystallization and preliminary X-ray analysis of the ternary complex of procarboxypeptidase A from bovine pancreas

F.X. Gomis-Rüth, M. Gómez, S. Ventura, J. Vendrell, F.X. Avilés*

Institut de Biologia Fonamental i Departament de Bioquímica i de Biologia Molecular, Facultat de Ciències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

Received 25 April 1995; revised version received 10 May 1995

Abstract **The ternary complex of procarboxypeptidase A, chymotrypsinogen C and proproteinase E from bovine pancreas has been crystallized using the sitting drop vapour diffusion method. The success in obtaining crystals has been found to be critically dependent on the prevention of autolysis of the complex. In preliminary stages, crystals twinned by merohedry were obtained from a solution containing $MgCl_2$ and polyethylenglycol 400 as precipitating agent. Later on, untwinned ones could be grown employing $CaCl_2$ instead of $MgCl_2$. These latter crystals belong to the rhombohedral system and to the spacegroup R3 with cell dimensions $a = b = 188.5$ Å and $c = 82.5$ Å. Consideration of the possible values of V_m accounts for the presence of one ternary complex molecule-oligomere per asymmetric unit. The crystals diffract beyond 2.6 Å resolution and are suitable for X-ray analysis.**

Key words: Protein crystallization; X-Ray crystallography; Ternary complex; Procarboxypeptidase A; Chymotrypsinogen C; Proproteinase E

1. Introduction

Most proteolytic enzymes are synthesized and secreted as inactive precursors. The occurrence of these proenzymes usually is a control mechanism for the biological activity, achieved by limited proteolysis of these proforms upon arrival at their operating milieu. This is the case for pancreatic carboxypeptidases A and B, digestive exopeptidases that belong to the family of metallo exoproteases [1]. They are involved in the hydrolysis of alimentary proteins from their C-terminal ends, and are produced in the pancreas of vertebrates as inactive precursor forms, the procarboxypeptidases A and B (PCPA and PCPB, respectively). One of the remarkable properties of pancreatic PCPA is its occurrence as a monomer, as a binary non-covalent complex with proproteinase E (BPE) or chymotrypsinogen C (CTGC), or as a ternary complex (TC) with both proenzymes [1].

The TC is the main form in which procarboxypeptidase A is found in bovine pancreas [2–5]. Formerly, this TC was characterized as a complex of PCPA with two unknown subunits called subunits II and III, both assignable to the serine proteinase family [2,4]. Based on sequence and activity analysis, subunit II was identified as chymotrypsinogen C (CGTC). In contrast, the identification of subunit III was more controversial because, although showing homology to elastases, it was recurrently found truncated at the N-terminus [6]. This truncation impedes activity and proteolytic activation. Subsequently, primary structure comparisons and molecular modelling suggested that subunit III is related to proproteinase E [7], a serine endopeptidase zymogen. Recently, we have shown that proproteinase E is a natural original component of the bovine ternary complex, besides PCPA and subunit II, and that subunit III is artificially generated from proproteinase E by autolysis of the complex [5].

The biological role of this TC is still not clear. Some hypotheses regarding the control of the timing and potentiation of the activation, the modulation of the activity of the subunits, and the proper co-ordination of the appearance of the proteolytical activities in the duodenum have been postulated [1,7]. Furthermore, it has been suggested to play a role in the protection of PCPA against inactivation by the acidic pH in the first track of the duodenum in ruminants [4]. No consensus has been reached about this issue so far.

Although many studies have been performed on the bovine TC at the biochemical and biophysical level [2–5,8,9], its detailed three-dimensional structure is unknown. The sequence of bovine PCPA shows a very high degree of homology to the porcine form and to porcine PCPB, whose structures have been recently solved [10,11]. None of them, however, has been isolated in a ternary complex. BPE closely resembles porcine proelastase, but only the active elastase structure is available [12], since the structure of the truncated, non-activatable form subunit III, recently published [13], can not be considered as a functional prototype for this proenzyme class. The sequence of CTGC more closely resembles the elastase sequence than that of chymotrypsinogen A, but the conserved five disulphide bonds (elastase and its proform display only four) account for the a priori classification of this proenzyme as a chymotrypsinogen.

2. Experimental

2.1. Materials

Phenylmethylsulphonylchloride, soybean trypsin inhibitor, and synthetic peptide substrates were obtained from Sigma. All other chemicals were purchased from Merck. For column chromatography, a FPLC system (Pharmacia) was used. The TSK-DEAE 5 PW column (10 μm particle size, 100 nm pore size, 0.75 × 7.5 cm) was supplied by Toso-Haas. *Chryschem* crystallization dishes were purchased from Charles Supper.

*Corresponding author. Fax: (34) (3) 581-2011.

Abbreviations: V_m, Matthews-parameter (protein volume per mass unit in the crystal); TC, ternary complex; PCPA, procarboxypeptidase A; CTGC, chymotrypsinogen C; BPE, proproteinase E; FPLC, fast performance liquid chromatography; PEG, polyethylenglycol.

SSDI 0014-5793(95)00533-1

2.2. Protein purification

Acetone powders from fresh bovine pancreas were used as the source for the TC. The acetone powders were extracted for 30 min in 20 mM Bis-Tris-HCl (pH 6.5) containing 0.3 mg/ml of soybean trypsin inhibitor and 2 mM phenylmethylsulphonylchloride, at 4°C. The crude extracts were centrifuged at 8000 rpm, the supernatants precipitated with 21% ammonium sulphate and left for 30 min at 4°C. The precipitate was recovered by centrifugation at 8000 rpm, redissolved in 20 mM Bis-Tris-acetate, pH 6.5 (buffer A) and equilibrated with this buffer by gel-filtration in small Sephadex G-25 columns. A TSK-DEAE 5PW column previously equilibrated with buffer A was used for the chromatography step. The proteins were loaded and then eluted by a 100-min linear gradient of this buffer and buffer B (20 mM Bis-Tris-acetate, 0.8 M ammonium acetate, pH 6.5) at 4 ml/min at room temperature. Under these conditions, TC eluted as a main peak at about 83 min. The fraction containing TC was desalted with a small G-25 column and immediately rechromatographed in the same conditions as above. The nature and purity of the eluted TC was confirmed by SDS-acrylamide gel electrophoresis and activity measurements with synthetic peptide substrates, as previously described [14]. The purified protein was immediately used for crystallization experiments to avoid autolysis.

2.3. Crystallization

Crystallization was carried out by the sitting-drop vapour diffusion method at 4°C and 20°C. A protocol using the incomplete factorial approach with 48 different crystallization conditions [15] in *Cryschem* dishes was applied for initial experiments. All reservoir solutions contained 0.02% (w/v) sodium azide. Drops were prepared by mixing 1 μl of TC solution (12 mg/ml) with 1 μl of the reservoir solution with equilibration against the same precipitating agent solution.

2.4. X-Ray diffraction analysis

Crystals were harvested using a buffer 1.5 times higher in the concentration of precipitating agents and buffers, and mounted in thin-walled glass capillary tubes. Diffraction data at −10 to −20°C (for preventing diffraction decay due to radiation damage) were collected at 120 mm crystal-to-detector distance on a 180-mm MAR-Research image plate detector attached to a Rigaku RU200 rotating Cu-anode X-ray generator operated at 120 mA and 45 kV. The data were evaluated with the MOSFLM package [16], and loaded, scaled and merged with PROTEIN [17].

3. Results and discussion

Obtaining large and well ordered crystals of bovine TC was found to be critically dependent on an efficient and fast purification performed immediately before crystallization. The tendency of this oligomeric complex to suffer quick autolysis of the BPE subunit in solution [18,19] can be minimized by using a fast purification scheme in the presence of inhibitors and by two rounds of anionic exchange FPLC in order to eliminate traces of active proteinase E. The autolytic process is stopped once the pure protein is crystallized, and the crystals can be safely stored for weeks in harvesting buffer at 4°C.

After a series of preliminary trials using the incomplete factorial approach, crystallization conditions using PEG 400 in the presence of 0.1 M divalent cations and 0.1 M HEPES (pH range 6.5–7.5) at protein concentrations from 5–20 mg/ml (in 10 mM Bis-Tris at pH 6.5) at 4°C provided best results. In a first set of conditions, crystals were obtained after 4–6 days from drops containing equal volumes of aqueous protein solution (15 mg/ml) and a solution 0.2 M in $MgCl_2$ and 30% (v/v) in PEG 400, buffered with 0.1 M HEPES to pH 7.1. These crystals (of 0.3 mm in the largest dimension) are well ordered and diffract beyond 2.8 Å resolution. Five complete data sets from one single crystal each were collected, loaded, scaled and merged. The crystals belong to the rhombohedral space group R3 with cell constants (hexagonal setting) varying between 187.6 and 188.5 Å (for $a = b$) and 80.5 and 82.1 Å (for c). Consideration of the possible values of V_m [20] accounts for the presence of one TC oligomer for each of the nine asymmetric units in the cell. However, these crystals displayed an almost perfect R32 symmetry, incompatible with the packing in the cell.

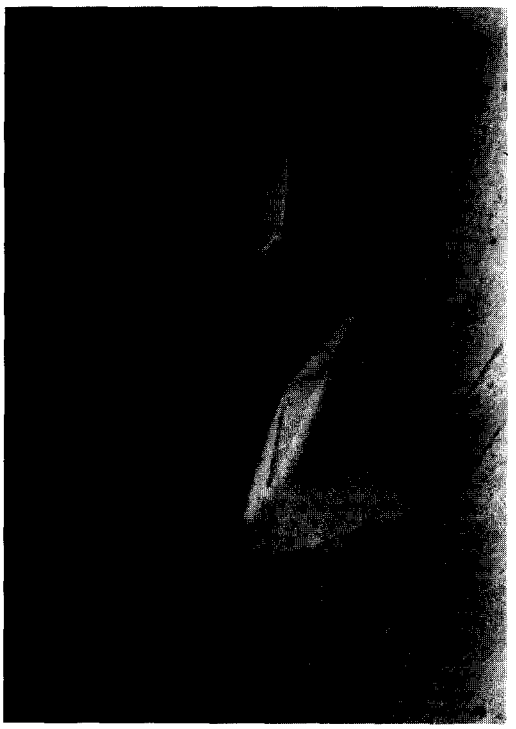

Fig. 1. Rhombohedral crystals of the ternary complex of procarboxypeptidase A. Biggest crystals are of approx. dimensions 0.5 × 0.5 × 0.2 mm.

In order to check if this R32 symmetry was due to a twinning operation by merohedry superimposed to the R3 lattice symmetry or was crystallographically correct but correlated with the presence of a binary complex (derived from the original TC and due to partial dissociation), N-terminal sequencing of washed and dissolved crystals was performed. Additionally, the crystal-volume determination procedure developed by Kiefersauer and Huber (unpublished) was applied to five different crystals whose amino-acid composition was determined afterwards. Both methods unequivocally showed: (a) the presence of the three molecules composing the TC; and (b) in equimolar ratios. This result confirmed therefore the hypothesis of hemihedral twinning, that is, the presence of a twinning axis along the diagonal between the crystallographic axes a and b.

Untwinned, well-ordered crystals were obtained from drops with equal volumes of protein solution and 0.1 M $CaCl_2$/0.05 M HEPES/15% (v/v) PEG 400. Only one or two crystals per drop of maximal dimensions 0.5 × 0.5 × 0.2 mm were obtained (see Fig. 1). These crystals diffract beyond 2.6 Å resolution and share the space group with the twinned ones, with just slightly modified cell constants ($a = b = 188.5$ Å, $c = 82.5$ Å), but the collected diffraction data display no R32 symmetry. Table 1 provides the statistics of the data-collection and evaluation.

The determination of the crystal structure of the bovine TC should be achieved by independent three-body Patterson search techniques, since the primary structures of PCPA, CTGC, and

Table 1
Data collection statistics and completeness

Number of measured reflections	97,896
Number of unique reflections	30,118
R_{merge}*	6.3%
Completeness	
for data (20.3–2.57 Å)	86.7%
for data (2.69–2.57 Å)	69.6%

*$R_{merge} = \Sigma\Sigma|I(h)_i - <I(h)>|/\Sigma<I(h)>$; $I(h)_i$ is the observed intensity of the ith measurement of reflection h, and $<I(h)>$ the mean intensity of reflection h; calculated after loading, scaling, and merging of Friedel pairs.

BPE closely resemble those of porcine PCPA, bovine chymotrypsinogen A, and porcine pancreatic elastase, respectively, proteins whose three-dimensional structures have been published [10,12,21] and are available from the Protein Data Bank.

In spite of the fact that the bovine TC was one of the first forms in which procarboxypeptidases, chymotrypsinogen C and proproteinase E were described more than 30 years ago [2], its detailed structure and biological role resisted resolution during many years. No doubt, the inability to obtain crystals for X-ray diffraction was one of the reasons of such difficulties. The presently described approach to obtain such crystals should permit the X-ray structure analysis of the TC, rendering significant information about the folding of the native proelastase-like serine proproteinase BPE.

Acknowledgements: We thank Prof. Robert Huber for the possibility of data-collection, Dr. K.H. Mann and Prof. F. Lottspeich for help in protein sequencing, so as Dr. T. Mather for careful reading. The financial help provided by a fellowship from the Human Capital and Mobility Programme of the European Union (ERBCHBICT920053) and by Grant BIO92-0458 is further acknowledged.

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