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Identification of a procarboxypeptidase A-truncated protease E binary complex in human pancreatic juice

Maxime Moulard, Brigitte Kerfelec, Bernard Mallet+ and Catherine Chapus

Centre de Biochimie et de Biologie Moléculaire du CNRS, Boîte Postale 71, F-13402, Marseille Cedex 9 and *+Laboratoire de Chimie Biologique (Faculté de Médecine), 27 Bd J. Moulin, 13385 Marseille Cedex 5, France*

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The characterization, in human pancreatic juice, of a binary complex associating procarboxypeptidase A with a 32 kDa inactive glycoprotein (G32) is reported in this paper. Free G32 was isolated after dissociation of the binary complex. N-terminal sequence analysis revealed a complete homology between this protein and human protease E (HPE 1), except for the two strongly hydrophobic N-terminal residues (Val-Val) which are missing in G32. This protein might be a truncated protease E highly analogous to the subunit III of the ruminant procarboxypeptidase A-S6 ternary complex. The analogy with bovine subunit III is further supported by interspecies reassociation experiments showing that bovine procarboxypeptidase A can specifically bind human G32.

Protease E; Subunit III; Binary complex; Procarboxypeptidase A; Truncated protease; (Human)

1. INTRODUCTION

Pancreatic procarboxypeptidase A presents the interesting property of forming complexes with other pancreatic proteins. In ruminant species, for example, it is mainly secreted as a ternary complex (proCPA-S6) [1–3] in association with a chymotrypsinogen of the C-type [4,5] and subunit III [6,7], an inactive truncated protease E [8]. In porcine pancreas, proCPA is found under two forms, monomeric [9–11] and associated with zymogen E [12,13].

Recently, Pascual et al. [14] have reported, besides several monomeric forms of proCPA, the presence in human pancreas of a proCPA-zymogen E binary complex. Interestingly, Guy-Crotte et al. [15] identified in the human pancreatic secretion a glycoprotein of 35 kDa (P35) with a truncated protease E, highly analogous to bovine subunit III. However, it has not been shown whether this protein is secreted free or associated with proCPA, as in bovine pancreas.

In this paper, we report the characterization of a proCPA-32 kDa protein (G32) binary complex in human pancreatic juice and the identification of this 32 kDa protein with a truncated protease E highly analogous to bovine subunit III. The analogy of G32 to human P35 is also discussed.

Correspondence address: C. Chapus, Centre de Biochimie et de Biologie Moléculaire du CNRS, Boîte Postale 71, F-13402, Marseille Cedex 9, France

Abbreviations: proCPA-S6, procarboxypeptidase A-S6 ternary complex; proCPA, procarboxypeptidase A; HPE, human protease E; G32, 32 kDa protein associated with human procarboxypeptidase A; Mes, 2[*N*-morpholino]ethanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Materials*

Lyophilized human pancreatic juice devoid of any proteolytic activity was a generous gift from Dr Lombardo, Marseilles. Ultrogel AcA 54, DE trisacryl and CM Sepharose were purchased from IBF (Villeneuve la Garenne, France). 2,3-Dimethylmaleic anhydride, hippuryl-L-phenyllactic acid, *N*-acetyl-L-tyrosine ethyl ester, *N*-acetyl-L-trialanine methyl ester, *N-t*-Boc-L-alanine *p*-nitrophenyl ester, phenylmethylsulfonyl fluoride and Mes were from Sigma (St. Louis, MO, USA). Benzamidine was from Fluka (Zürich, Switzerland).

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2.2. *Methods*

2.2.1. Filtration of lyophilized human pancreatic juice on AcA 54 Ultrogel

4.8 g of lyophilized pancreatic juice, collected from a single individual, were dissolved in 25 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 0.4 M NaCl and 2 mM benzamidine, loaded on an AcA 54 Ultrogel column (3 cm × 4 m) and eluted with the same buffer.

2.2.2. Activities measurements

After tryptic activation, the carboxypeptidase A activity was measured as previously described [16] using a 1 mM hippuryl-L-phenyllactic acid as substrate. The protease E activity was determined either potentiometrically at pH 7.5 and 25°C using a 10 mM *N*-acetyl-trialanine methyl ester solution as substrate in a 1 mM Tris-HCl buffer containing 0.1 M NaCl or spectrophotometrically at pH 6.0 and 25°C using as substrate *N-t*-Boc-alanine *p*-nitrophenyl ester as previously described [17].

2.2.3. Zn^{2+} content determination

An aliquot (500 μl) of each fraction obtained during pancreatic juice Ultrogel filtration was directly analyzed by flame atomic absorption spectrophotometry using a spectrophotometer Varian model 1275. Measurements were performed at 213.8 nm (slit 0.5).

2.2.4. Purification of the proCPA-G32 binary complex

The fractions containing the proCPA-G32 binary complex eluted from the above-mentioned gel filtration column were pooled as indicated in fig.1 (pool 1), dialyzed against 25 mM Tris-HCl, pH 8.0, containing 25 mM NaCl and 1 mM benzamidine and chromatographed on a DE trisacryl column (2 cm × 15 cm) in the same buffer. Elution was performed with a linear NaCl gradient (25–150 mM).

2.2.5. Dissociation of the proCPA-G32 binary complex

The dissociation of the binary complex (20 mg) was performed at pH 6.0 using a two steps procedure: chromatography on CM Sepharose followed by AcA 54 Ultrogel filtration. The CM Sepharose column (2 cm × 13 cm) was equilibrated in a 10 mM Mes buffer, pH 6.0, 50 mM NaCl and 2 mM benzamidine. The proteins were eluted by a linear NaCl gradient (50–250 mM) in the same buffer (total volume of the gradient, 300 ml). The eluted asymmetric peak (not shown) was concentrated in an Amicon cell equipped with a PM 10 membrane and loaded on an AcA 54 Ultrogel column (1 cm × 2 m) equilibrated in a 10 mM Mes buffer, pH 6.0, containing 0.2 M NaCl and 2 mM benzamidine. The elution was achieved using the same buffer. Protein concentrations were estimated assuming a mean value of 1.8 for $E^{1\%}$ at 280 nm.

2.2.6. Gel electrophoresis

Electrophoresis in the presence of SDS was carried out in 10–15% polyacrylamide gels as described by Laemmli [18] using a PHAST gel apparatus (Pharmacia).

2.2.7. N-terminal sequence analysis

N-terminal sequence analysis was performed by a stepwise Edman degradation using a gas-phase sequencer (Applied Biosystems, model 470 A). The resulting phenylthiohydantoins were analyzed by HPLC using a C 18 column (Brownlee 5 μm, 2.1 × 220 mm). They were eluted by a methanol gradient (10–46%) in a 7 mM sodium acetate buffer (pH 4.84).

2.2.8. Carbohydrate content of G32

Neutral sugar content was estimated using the phenol sulfuric acid method of Dubois et al. [19].

2.2.9. Reassociation experiments

Bovine proCPA (0.17 mg) was mixed at pH 7.2 (25 mM Tris-HCl buffer containing 50 mM NaCl) with a slight molar excess of human G32. The resulting mixture was then filtered through a Superose 12 analytical column using a FPLC apparatus (Pharmacia). The elution was performed using the same buffer.

3. RESULTS AND DISCUSSION

3.1. *Identification of free and associated forms of proCPA in human pancreatic juice*

The elution pattern resulting from gel filtration of lyophilized human pancreatic juice is shown in fig.1.

Different molecular forms of proCPA were detected by activity measurements and Zn^{2+} content analysis (about 1 mol Zn^{2+}/mol protein). SDS gel electrophoresis revealed that proCPA occurred in human pancreas both as binary complexes, in association with a 32 kDa (G32) (pool 1) or 28 kDa (pool 2) protein and as a monomer (pool 3). The different forms of proCPA were eluted as expected from their M_r values. No chymotrypsin or protease E activity was detected under the two complexes even after tryptic incubation. No investigation was performed on procarboxypeptidase B.

SDS gel analysis of the purified binary complex proCPA-G32 is presented in fig.2.

3.2. *Dissociation of the proCPA-G32 binary complex*

Dissociation of the binary complex was achieved at pH 6.0. CM Sepharose chromatography at pH 6.0 alone failed to completely separate proCPA from G32 probably because of their similar p*I* values (5.2–5.4). A complete separation was performed by gel filtration on AcA 54 Ultrogel at pH 6.0. This finding leads to the conclusion that the two partners are mainly bound through ion pairing, as observed for the bovine proCPA-subunit III interactions. As a matter of fact, the dissocation of the complex can also be achieved using 2,3-dimethylmaleic anhydride as for the bovine proCPA-S6 complex [20]. The binary complex

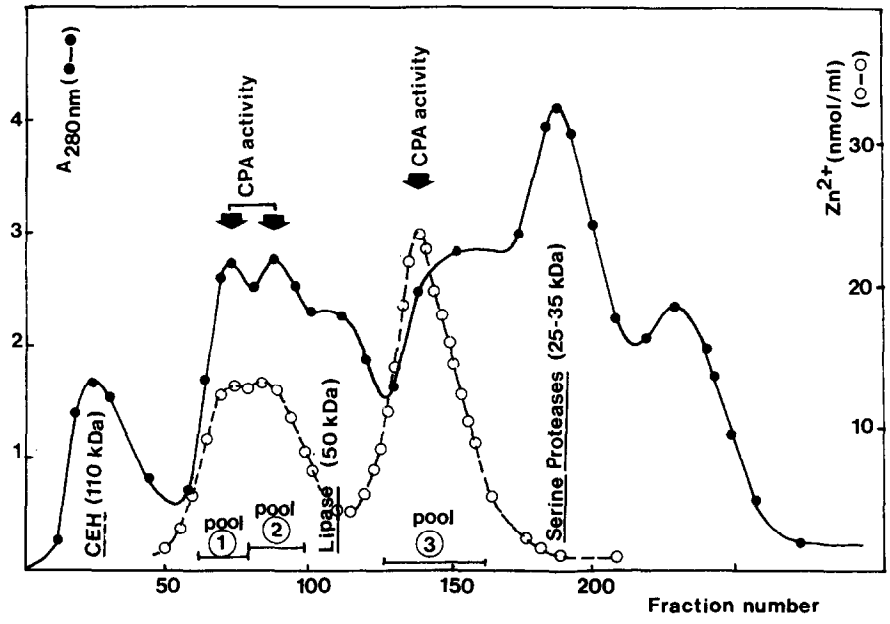

Fig.1. Filtration of lyophilized pancreatic juice on AcA 54 Ultrogel (see section 2). Volume fraction, 3.2 ml. Flow rate, 32 ml/h. The peaks in which a potential carboxypeptidase A activity was detected are indicated by arrows. CEH, carboxylic ester hydrolase.

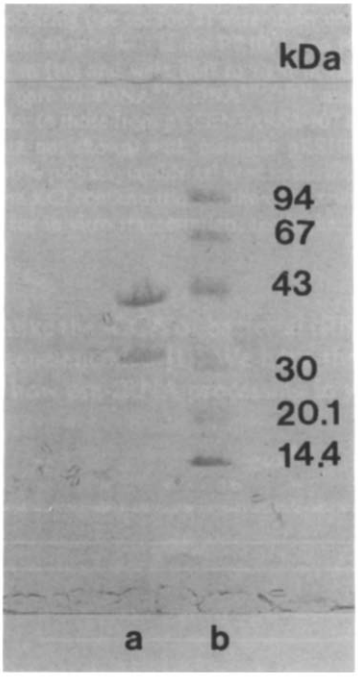

Fig.2. SDS gel electrophoresis of the purified binary complex proCPA-G32. Lanes: a, purified binary complex; b, molecular mass markers.

proCPA-28 kDa protein was also dissociated using the latter method.

3.3. *Characterization of G32*

Isolated G32 appears to be devoid of any chymotryptic or elastolytic activity since it failed to hydrolyze *N*-acetyl tyrosine ethyl ester, trialanine methyl ester and *t*-Boc-alanine *p*-nitrophenyl ester even after trypsic incubation.

The N-terminal sequence of G32 is reported in table 1 and compared to the sequences of human P 35 [15], protease E isoenzymes (HPE1, HPE2) [21,22] and to bovine subunit III [23]. Human G32, like bovine subunit III and human P 35, lacks the N-terminal dipeptide Val-Val. Apart from this difference, a complete identity can be observed between the N-terminal sequences of HPE1 and this protein. Interestingly, reassociation experiments clearly demonstrated that human G32 was able to bind to monomeric bovine proCPA (data not shown). This finding emphasizes the homology between human G32 and bovine subunit III. It must be pointed out that carbohydrates are not likely to be involved in the interaction proCPA-G32, since bovine subunit III is not glycosylated.

Table 1

N-terminal sequences of human 32 kDa protein (G32), P 35 [15], HPE 1 [21,22], HPE 2 [21] and bovine subunit III [23]

	1	5	10	15	20
HPE 1	V V N G E D A V P Y S			W P W Q V S L Q Y E K	
HPE 2	V V H G E D A V P Y S			W P W Q V S L Q Y E K	
Human G32	– – N G E D A V P Y S			W P W Q V S L Q Y E K	
Human P 35	– – X G E D A V P Y S			W P W Q V S L Q Y E K	
Bovine subunit III	– – N G E D A V P Y S			W S W Q V S L Q Y E K	

(–) Missing residue; (X) unknown residue. The residues different from the G32 protein sequence have been underlined

Since the N-terminal residue of human P 35 has not been determined, the identity of G32 with P 35 cannot be definitely ascertained. However, carbohydrate analysis further supported an identity between both proteins. G32 was shown to contain 6.8% carbohydrates which is in good agreement with the results obtained by Guy-Crotte et al. [15] for human P 35.

4. CONCLUSION

We have shown that human pancreatic proCPA is secreted, for one part, in association with a glycosylated 32 kDa protein (G32). This protein is likely to be identical with the human P 35 described by Guy-Crotte et al. [15] and could be identified as an inactive truncated protease E. Besides this binary complex, another complex in which proCPA is associated with a 28 kDa protein has been found. This protein, which has not yet been identified, might be either a different protein or a degraded product.

The occurrence of a truncated protease E associated with proCPA was first described in bovine pancreas [8]. The finding of such an association in other species (man) added to interspecies conservation of the proCPA-truncated protease E binding site raises the question of the physiological significance of the association. Preliminary studies performed on the bovine proCPA-S6 complex have shown that such an association results in a stabilization and an improvement of the catalytic efficiency of proCPA [7,16].

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REFERENCES

[1] Keller, P.J., Cohen, E. and Neurath, H. (1956) J. Biol. Chem. 223, 457–467.
[2] Yamasaki, M., Brown, J.R., Cox, D.J., Greenshields, R.N., Wade, R.D. and Neurath, H. (1963) Biochemistry 2, 859–866.
[3] Kerfelec, B., Chapus, C. and Puigserver, A. (1985) Eur. J. Biochem. 151, 515–519.
[4] Peanasky, R.J., Gratecos, D., Baratti, J. and Rovery, M. (1969) Biochim. Biophys. Acta 181, 82–92.
[5] Keil-Dloua, V., Puigserver, A., Marie, A. and Keil, B. (1972) Biochim. Biophys. Acta 276, 531–535.
[6] Puigserver, A. and Desnuelle, P. (1975) Proc. Natl. Acad. Sci. USA 72, 2442–2445.
[7] Kerfelec, B., Cambillau, C., Puigserver, A. and Chapus, C. (1986) Eur. J. Biochem. 157, 531–538.
[8] Cambillau, C., Kerfelec, B., Sciaky, M. and Chapus, C. (1988) FEBS Lett. 232, 91–95.
[9] Marchis-Mouren, G., Charles, M., Ben Abdeljlil, A. and Desnuelle, P. (1961) Biochim. Biophys. Acta 50, 186–188.
[10] Lacko, A.G. and Neurath, H. (1970) Biochemistry 9, 4680–4690.
[11] Reeck, G.R., Winter, W.P. and Neurath, N. (1970) Biochemistry 9, 1398–1403.
[12] Kobayashi, R., Kobayashi, Y. and Hirs, C.H.W. (1978) J. Biol. Chem. 253, 5526–5530.
[13] Martinez, C.M., Avilès, F.X., Sansegundo, B. and Cucillo, C.M. (1981) Biochem. J. 197, 141–147.
[14] Pascual, R., Burgos, F.J., Salva, M., Soriano, F., Mendez, E. and Avilès, F.X. (1989) Eur. J. Biochem. 179, 606–616.
[15] Guy-Crotte, O., Barthe, C., Basso, D., Fournet, B. and Figarella, C. (1988) Biochem. Biophys. Res. Commun. 156, 318–322.
[16] Chapus, C., Kerfelec, B., Foglizzo, E. and Bonicel, J. (1987) Eur. J. Biochem. 166, 379–385.

[17] Largman, C., Brodrick, J.W. and Geokas, M.C. (1976) Biochemistry 15, 2491–2500.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, G. (1956) Anal. Chem. 28, 350–356.
[20] Kerfelec, B., Chapus, C. and Puigserver, A. (1984) Biochem. Biophys. Res. Commun. 121, 162–167.
[21] Shirasu, Y., Takemura, K., Yoshida, H., Sato, Y., Iijima, H., Shimada, Y., Mikayama, T., Ozawa, T., Ikeda, N., Ishida, A., Tamai, Y., Matsuki, S., Tanacha, J., Ikenaga, H. and Ogawa, M. (1988) J. Biochem. 104, 259–264.
[22] Shen, W.F., Fletcher, T.S. and Largman, C. (1987) Biochemistry 26, 3447–3452.
[23] Venot, N., Sciaky, M., Puigserver, A., Desnuelle, P. and Laurent, G. (1986) Eur. J. Biochem. 157, 91–99.