Urea-gradient gel electrophoresis studies on the association of procarboxypeptidases A and B, proproteinase E, and their tryptic activation products

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Received 12 August 1985; revised version received 11 September 1985

Monomeric procarboxypeptidase A (PCPA) and isolated proproteinase E (PPE), both from pig pancreas, were shown by means of electrophoresis on transverse urea gradients (0–9 M) to form a very stable complex, identical to their natural binary complex. Although the complex is maintained by the interaction of both active regions, the activation segment of PCPA participates directly in the binding. Procarboxypeptidase B (PCPB) also associates with PPE, but in this case the complex shows low stability. In contrast with carboxypeptidases A that strongly bind to their corresponding severed activation segments, no interaction was observed between carboxypeptidase B and its severed activation segment. The above results give some insight into several characteristics of the structure and activation properties of pancreatic PCPA and PCPB.

Procarboxypeptidase *Proproteinase E* *Activation product* *Association study* *Pig pancreas*

1. INTRODUCTION

PCPA occurs in pig pancreas both as a monomer and in binary association with PPE [1,2]. This association has a profound influence on the tryptic activation process of PCPA [2,3]. Similar effects have been reported for the complexes of procarboxypeptidases A and serine-proproteinases found in the pancreas of other species [4–6]. In addition, it is known that the isolated activation segment of PCPA strongly binds to and inhibits CPA [7], a fact which could slow down the tryptic activation of that proenzyme. Here the association of all the mentioned porcine proteins and their primary tryptic fragments was studied by electrophoresis on polyacrylamide gels with transverse urea gradients. The analysis was also extended to pig PCPB, a homologous protein found in monomeric form in pigs and other species [8–10]. The results throw some light on the activation properties of procarboxypeptidases A and B and on the quaternary structure of their complexes with PPE. In addition, the urea-denaturation behaviour of all the analysed proteins was examined.

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Abbreviations: PCPA, PCPB, CPA, CPB, procarboxypeptidases and carboxypeptidases A and B; PPE and PE, proproteinase E and proteinase E; asA and asB, severed activation segments of procarboxypeptidase A and B, respectively

2. MATERIALS AND METHODS

Monomeric PCPA, its natural binary complex with PPE and monomeric PCPB were isolated from the acetone powder of porcine pancreas as reported elsewhere [2,10,11]. Carboxypeptidases A_1, A_2 and B, the corresponding activation segments and PE were obtained by limited proteolysis of their monomeric precursors with trypsin, followed by DEAE chromatography in 7 M

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00145793/85/$3.30

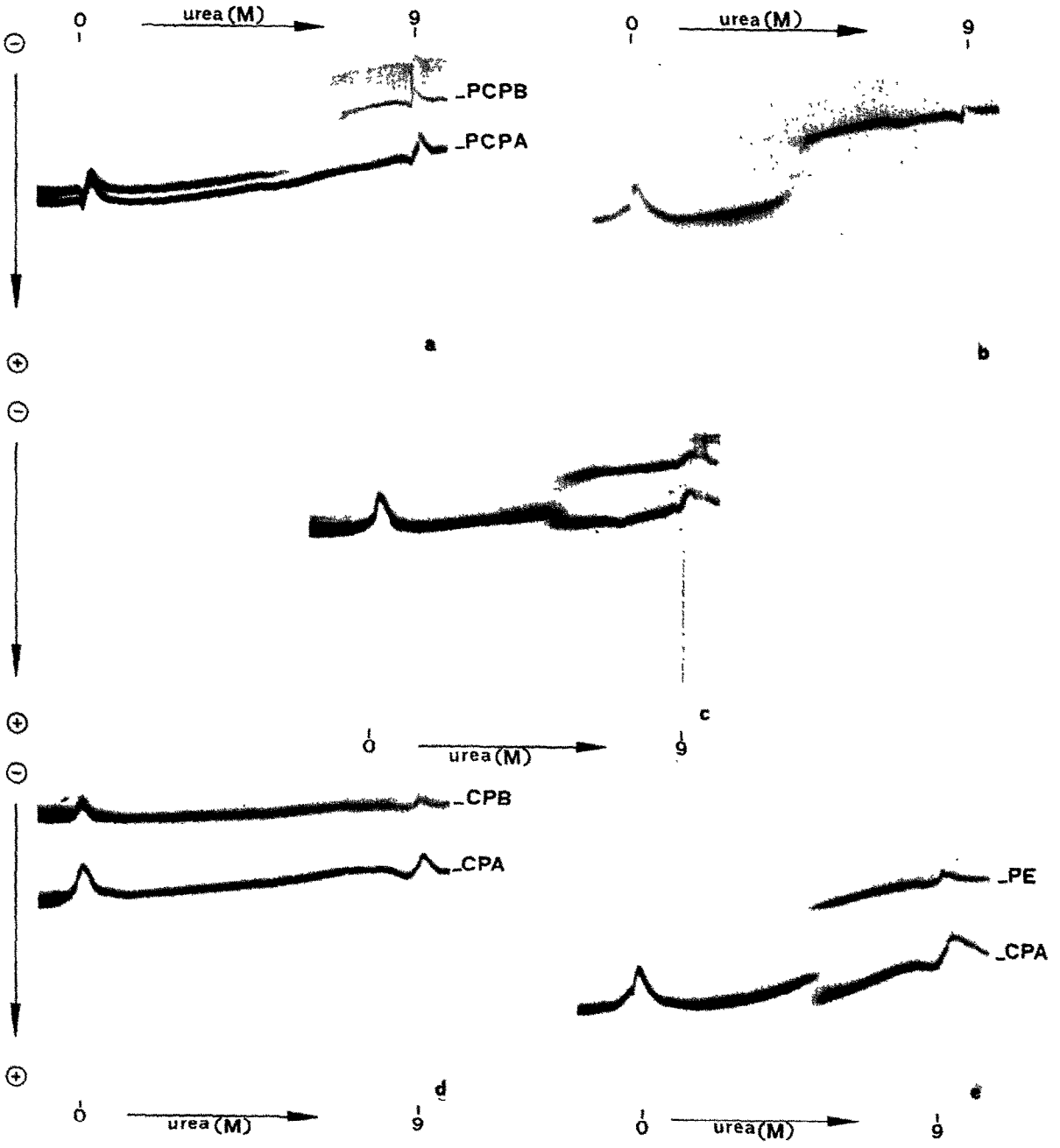

Fig. 1. Electrophoretic behaviour of pig pancreatic procarboxypeptidases and related proteins on urea-gradient (0–9 M) polyacrylamide gels: (a) co-electrophoresis of monomeric procarboxypeptidases A and B, (b) isolated PPE, (c) artificial complex of monomeric PCPA and PPE at equimolar ratio, (d) co-electrophoresis of carboxypeptidases A_1 and B, (e) artificial complex of carboxypeptidase A_1 and PE at equimolar ratio. Running time in the last electrophoresis was longer than in the others.

urea, according to [7,11]. Artificial complexes between the above proteins were prepared in 40 mM Tris-HCl (pH 8.6) by dropwise addition and stirring at 20°C. Alternatively, complexes between carboxypeptidases and their complementary activation segments were obtained by very mild treatment of their procarboxypeptidases with trypsin (40:1, w/w) for 2 min, at 25°C, in the above buffer, followed by the addition of the inhibitor Trasylol (10:1, w/w ratio to trypsin; kindly supplied by Dr Alvarez-Lage, from Química Farmacéutica Bayer). Polyacrylamide gels with transverse urea gradients (0–9 M urea) were prepared from 13% acrylamide solutions in 45 mM Tris, 25 mM boric acid, 0.78 mM EDTA (pH 8.6) by Creighton's procedure [12], slightly modified in order to place at the boundaries of the urea gradient a wide strip of gel containing the same concentration of urea as the adjacent region. Electrophoreses were carried out either at 80 V for 16 h or at 300 V for 5 h, at 20°C, with similar results. Gels were stained by diffusion with 0.1% Coomassie blue in 25% ethanol and 10% acetic acid and destained in 25% ethanol and 10% acetic acid.

3. RESULTS AND DISCUSSION

When pig monomeric PCPA is run in a polyacrylamide gel through a perpendicular continuous linear gradient of urea (0–9 M), a single and continuous band of protein is apparent after Coomassie staining (fig.1a). This indicates a lack of gross conformational change or unfolding of the protein along these concentrations of urea [12]. In contrast, under the same conditions, isolated PPE shows a sharp decrease in mobility above 4.5 M urea (fig.1b), which is probably due to the unfolding of the protein. Similar behaviour has been reported for another serine-proproteinase such as chymotrypsinogen A [12]. Moreover, an equimolar artificial mixture of monomeric PCPA and PPE gives a single and monotonous electrophoretic band of intermediate mobility between 0 and 5.7 M urea which is suddenly split into 2 lines, with mobilities corresponding to that of each component of the mixture, at higher urea concentrations (fig.1c). This behaviour indicates that natural monomeric PCPA strongly binds to PPE forming a stable and definite artificial complex, in agreement with our previous observations [3]. The naturally occurring binary complex between PCPA and PPE shows exactly the same electrophoretic behaviour with urea concentration as the above artificial complex. This is further evidence in favour of the identity between both complexes.

On the other hand, pig monomeric PCPB shows a clear electrophoretic transition at high urea concentration, the inflection point being at 6.5 M urea (fig.1a). This transition is probably due to a partial unfolding of the protein, since the mobility change is relatively small (30%) and the asB is fully denatured at high urea concentrations, while the complementary CPB shows no change in electrophoretic mobility under the same conditions (see below). When monomeric PCPB is mixed with isolated PPE, an artificial and previously not reported complex between both proteins is also formed. This is shown by the co-migration of both proteins on polyacrylamide gels of different pores (7.5–15% acrylamide) at 0 M urea where a single continuous band is formed. The mobility of this band is clearly different from those of the corresponding isolated proteins run under identical conditions. However, when run through a urea gradient, this complex dissociates at a lower urea concentration that that of monomeric PCPA and PPE (0.5 vs 5.7 M urea) – a clear indication of the weaker stability of the former complex (not shown). One question is raised from the above results: why is PPE found associated with only a fraction of PCPA molecules in porcine pancreas if all pancreatic procarboxypeptidases (both A and B) can clearly bind to PPE, although admittedly with different affinities?

The electrophoretic behaviour of the functional pieces derived from both procarboxypeptidases A and B (obtained by tryptic activation) is interesting as well. Neither CPA nor CPB shows unfolding transitions along the whole urea-gradient (fig.1d). We had previously reported that the asA is a globular polypeptide (about 100 residues long), which strongly binds to and inhibits CPA ($K_i \simeq$ 2 nM) [7,13]. When asA is run through a urea-gradient gel (fig.2a), a sigmoidal band covering a wide mobility range (typical of proteins devoid of S-S bridges and with a 2-state behaviour on urea denaturation) [12] is found. The denaturation inflection point is about 6.5 M urea, a value which

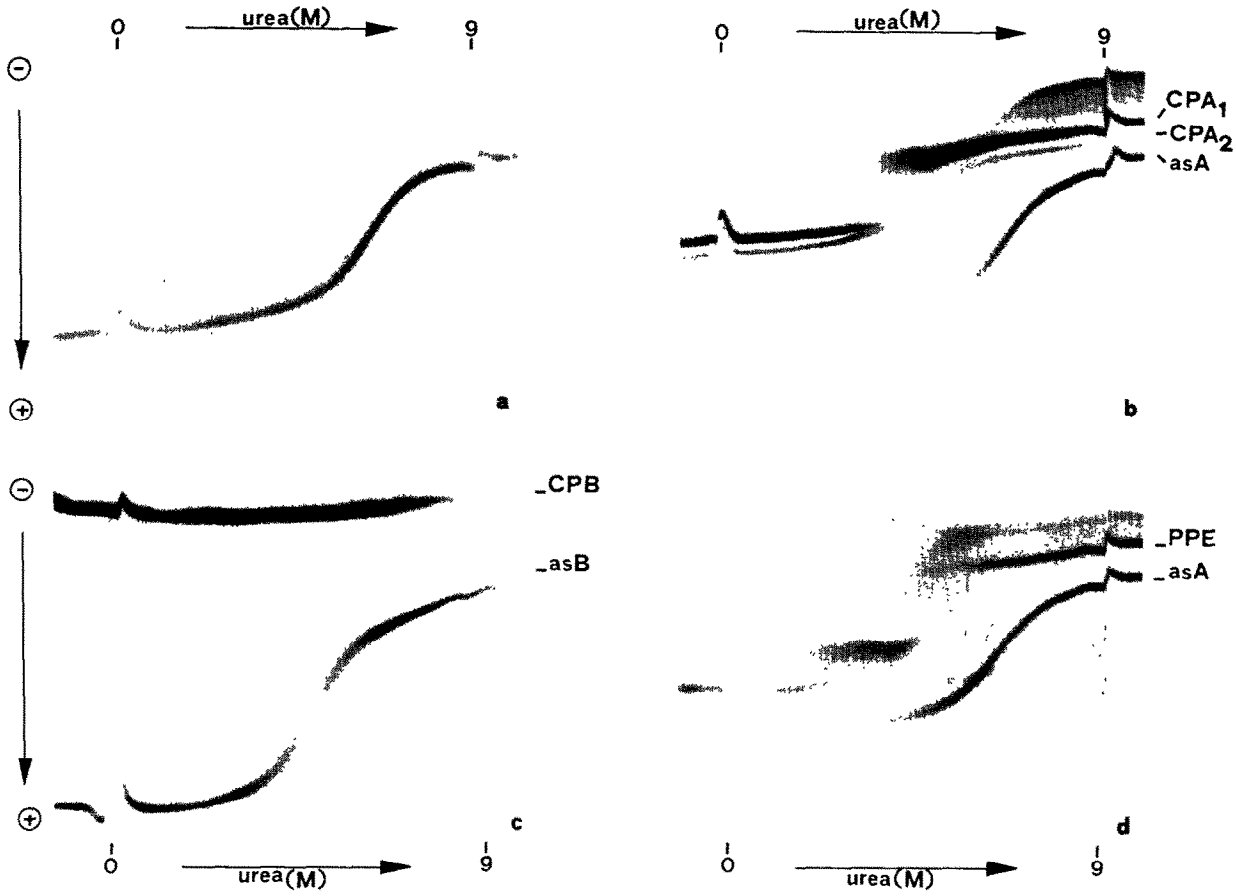

Fig.2. Electrophoretic behaviour of the isolated activation segments of pig pancreatic procarboxypeptidases and of their complexes with related proteins on urea-gradient (0–9 M) polyacrylamide gels: (a) asA, (b) equimolar complex between a mixture of carboxypeptidases A ($A_1 + A_2$) and the corresponding severed activation segment, (c) equimolar complex between CPB and asB, (d) equimolar artificial complex between PPE and PCPA. See text for more details.

is in agreement with that found in parallel denaturation studies followed by means of NMR spectroscopy (Vendrell et al., unpublished). In the presence of active CPA (1:1 molar ratio in the mixture) the denaturation transition of that activation segment cannot be seen below 4 M urea (fig.2b). Instead, a band of intermediate mobility ascribable to a stable complex of both proteins is apparent between 0 and 4 M urea. In the experiment shown in fig.2b, the complex was obtained by a short and mild trypsin treatment of PCPA and contains a mixture of carboxypeptidases A_1 and A_2, and the complementary severed activation segment [7,11].

asB also shows a sigmoidal electrophoretic transition similar to that of the homologous asA, but with a denaturation inflection point at a lower urea concentration (5 vs 6.5 M urea). However, this activation segment does not bind to the complementary CPB, as deduced by the observed independent electrophoresis of both proteins along the whole urea-gradient when they are run as a 1:1 molar ratio mixture (fig.2c).

The inability of asB to bind to its active enzyme, in contrast to the strong binding of asA to its enzyme, could help to explain the great differences in the proteolytic activation process of both proenzymes, which requires a few minutes in the former case and several hours in the latter [3,5,8,9]. Thus, during the action of trypsin asB would be quickly

released from CPB, the activity of which would be expressed very early. In contrast, asA would remain bound to CPA, delaying the appearance of its activity.

The relative spatial position of PPE with respect to the functional regions of PCPA in the natural binary complex of both proenzymes is unknown. Yet, this is an important feature since it has been reported that the tryptic activation process of mammalian PCPA is influenced by the accompanying proteins in oligomeric complexes [3,5]. By means of urea-gradient electrophoresis, we have observed that an equimolar mixture of active carboxypeptidase A_1 and PE shows an electrophoretic behaviour identical to that of a complex of their proenzymes (see fig.1c and e); i.e., a strong binding between both active proteins is observed. Moreover, PPE and the isolated asA, after mixing, run together in the gradient between 0 and 2 M urea but run independently above 2 M urea (fig.2d). From these results it can be concluded that the strong association between PPE and PCPA is due to the interaction of their active enzyme regions, although the asA proenzyme also participates 'directly' in the binding with PPE. This binding probably alters the conformation of the asA and/or its accessibility to trypsin, thus affecting the proteolytic activation of PCPA, as reported [3].

ACKNOWLEDGEMENTS

This work has been supported by grant 0338/81 from CAICYT, Ministerio de Educación (Spain). M.V. and F.J.B. acknowledge a fellowship from FPI and FIS, Ministerios de Educación y Sanidad (Spain), respectively. We are indebted to Fundació M.F. Roviralta for kind support.

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