The Tryptic Activation Pathway of Monomeric Procarboxypeptidase A*

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Procarboxypeptidases are the remaining major digestive zymogens the activation process of which remains unsolved. Here it is shown that in the trypic activation of monomeric procarboxypeptidase A from porcine pancreas, the generation of carboxypeptidase A (CPA) activity parallels the limited proteolysis of the 94-residue activation segment. This degradation proceeds from the COOH-terminal end of the molecule, and CPA itself makes an important and unexpected contribution by exciting the COOH-terminal arginine residue of the released primary activation fragment. Successive cleavages at some of the peptide bonds of the activation segment nearest to the COOH terminus were found to be of prime importance in eliciting CPA activity, particularly those involving the carboxyl groups of Arg94 and Gly93 which were first cleaved. It is also shown that the rate of activation does not depend directly upon the generation of CPA-α and its conversion to CPA-β.

Compared with current understanding of the activation of other major digestive zymogens such as pancreatic trypsinogen (Huber and Bode, 1978; Rovery, 1988) and chymotrypsinogen (Miller et al., 1971; Rovery, 1988) or gastric pepsinogen (Foltmann, 1981; James and Sielecki, 1986; Foltmann 1988), data on the molecular events leading to the activation of pancreatic PCPA(s) are scarce (Keller et al., 1958; Brown et al., 1963; Uren and Neurath, 1972; Puigserver and Desnuelle, 1977; Chapus et al., 1987). The main unknowns are the cleavage points within the activation region and the subsequent conformational and functional effects. This scarcity of data is in sharp contrast to the extensive knowledge about their active forms, carboxypeptidases A (CPAs).

The molecular complexity of PCPA systems has hindered the detailed analysis of their activation process. First of all, these proenzymes contain a 94-residue activation segment whose sequence and conformational propensities in different species have been reported fairly recently (Quinto et al., 1982; Vendrell et al., 1986; Vilanova et al., 1987; Flogizzo et al., 1988; Wade et al., 1988; Gardell et al., 1988). This activation segment in the isolated state assumes a compact globular conformation (Avilés et al., 1982), fairly resistant to proteolytic hydrolysis, and behaves as a powerful competitive inhibitor of the active enzyme (K, ~ 2 nM) (SanSegundo et al., 1982). Moreover, in many species PCPA usually occurs in very stable oligomeric complexes with zymogens of endoproteases (Kerfelec et al., 1985; Puigserver et al., 1986; Chapus et al., 1987; Pascual et al., 1989). This has hindered direct study of the behavior of the monomer, which could be carried out in certain cases only after dissociation of the subunits or the weakening of their interaction by chemical modification (Freisheim et al., 1967; Puigserver and Desnuelle, 1977; Chapus et al., 1987). It is for these reasons that most of the previous analyses of the activation process of procarboxypeptidases had to focus primarily on the disaggregation of the quaternary complex rather than on the activation process itself (Brown et al., 1963; Uren and Neurath; 1972; Puigserver and Desnuelle, 1977; Chapus et al., 1987). The isolation and characterization of a natural monomeric form of PCPA from porcine pancreas (Kobayashi et al., 1978; Vendrell et al., 1982; Vilanova et al., 1985a, 1985b; Sanchez-Ruiz et al., 1988) together with the resolution of the sequence of its activation segment, reported recently by our group (Vendrell et al., 1986), established the basis for a direct study of the isolated monomer.

In this work we have approached the study of the trypic activation process of porcine monomeric PCPA through a detailed analysis of the structural changes undergone by the activation segment and CPA, and their correlation with the appearance of the biological activity of this enzyme. The primary structures and biological capabilities of the different fragments generated during the activation have been characterized as well as their ability to interact with each other. From this, the sequence of events in the activation of monomeric PCPAs by trypsin may be formulated.

EXPERIMENTAL PROCEDURES

Trypsin Activation—Porcine monomeric procarboxypeptidase A, obtained according to Vilanova et al. (1985a), was dissolved in 50 mM Tris-HCl, 0.1 mM ZnCl2 (pH 7.5) and adjusted to a concentration of 1 mg/ml. Trypsin was added to reach a final procarboxypeptidase A/trypsin ratio of 4:1 or 4:1 (w/w), and the temperature was kept at either 25 or 37 °C during activation. At controlled times, aliquots of 20 μl of activation mixture were withdrawn and analyzed for carboxypeptidase A activity using 1 mM benzoyl-Gly-L-Phe as substrate (Folk and Schirmer, 1963).

For electrophoretic analysis, aliquots of 50 μl of the digest were withdrawn at fixed times and immediately mixed with 2 μl of 50 mM phenylmethylsulfonyl fluoride and 5 μl of soybean trypsin inhibitor at 1 mg/ml. Each sample was subsequently mixed with 57 μl of loading buffer for SDS-polyacrylamide electrophoresis (containing 1% SDS) and heated at 100 °C for 1 min. The electrophoretic analyses

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† The abbreviations used are: PCPA(s), procarboxypeptidase(s) A; CPA, carboxypeptidase A; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; TPCK, tosylphenylalanyl chloromethyl ketone.

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were carried out on 12.5% polyacrylamide-SDS gels containing 7 M urea (Hashimoto et al., 1985) for the visualization of activation peptides and on 9.5% polyacrylamide gels containing 7 M urea (in the absence of SDS) for the visualization of CPA-α and -β. The relative amounts of the different proteins in the gels were estimated by densitometry after Coomassie Blue staining.

**Porcine monomeric procarboxypeptidase A Activation Pathway**

Porcine monomeric procarboxypeptidase A was activated with trypsin according to the above mentioned procedure for activity measurements. At fixed times, 50-μl samples from the digest were removed, mixed with 5 μl of 10% trifluoroacetic acid, and injected onto a Nucleosil C4 reversed-phase column (wide pore, 25-cm length, from Macherey and Nagel, Düren, Federal Republic of Germany). Elution was carried out by a gradient between 0.1% trifluoroacetic acid in water (solvent A) and 80% acetonitrile, 0.08% trifluoroacetic acid (solvent B) and followed at 280 and 214 nm. The gradient applied was the following: initial equilibration, 33% solvent B; 17 min, 51% solvent B; 62 min, 89% solvent B. The collected fragments were adjusted to neutral pH by the addition of ammonium hydroxide and freeze-dried. Quantification of the eluted fragments was carried out by area measurement of the corresponding chromatographic peaks by electronic integration of the increment of absorbances at 280 and 214 nm and by amino acid analysis.

**Generation and Quantification of Proenthetic Fragments by HPLC**—Porcine monomeric procarboxypeptidase A was activated with trypsin in 50 mM Tris-HCl, 0.01 mM ZnCl₂ (pH 7.5) treated with porcine trypsin (TPCK-treated) at a ratio of either 40:1 (---) or 4:1 (----) (w/w). Temperature was kept at either 25°C (O) or 37°C (●) during activation. At controlled times, aliquots were withdrawn and analyzed for carboxypeptidase A activity using 1 mM benzoyl-Gly-L-Phe as substrate (Folk and Schirmer, 1963). Activities are expressed as μmol of substrate hydrolyzed/min/mg of protein. The inset shows the electrophoretic follow-up of the activation progress on SDS-polyacrylamide gels in the presence of 7 M urea.

**RESULTS AND DISCUSSION**

Initial studies confirmed that the generation of CPA activity from porcine monomeric PCPA by trypsin (see Fig. 1) is slow compared with the expected rates for in vivo digestion, as described previously for the bovine zymogen (Uren and Neurath, 1972; Puigserver and Desnuelle, 1977), although total activity was attained at shorter times in the porcine than in the bovine system. From the latter and other observations (Freisheim et al., 1967; Vendrell et al., 1982; Kerflec et al., 1985; Pascual et al., 1989) on the PCPA activation course, it seems that an *in vitro* model of activation in which trypsin is the only acting protease can only partially mimic *in vivo* conditions, particularly when PCPA is in a complex. The appearance and disappearance of the different peptides arising from proteolysis are a complex process from which some qualitative observations can be deduced. (i) Evidence for the conversion of fragment 1 to fragment 2 is only clear for the first 30 min, that is for the degradation of approximately 50% of the former fragment. (ii) It is difficult to define a sequence of clear-cut steps on going from approximately 43% (30-min activation) to 100% activation, as the primary and secondary activation peptides (fragments 1 and 2) appear to give rise to the other peptides simultaneously. (iii) There is a very good correlation between the combined disappear-
Activation segment of procarboxypeptidase A generated by trypsin activation and separated by reversed-phase HPLC. Values are the mean of four different determinations. The activation of procarboxypeptidase A by trypsin was performed at 25 °C as described in Fig. 1. Samples were removed from the digest at fixed times, mixed with 0.1 volume of 10% trifluoroacetic acid, and injected onto a Nucleosil C4 reversed-phase column. Elution was carried out with a water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The inset shows the elution pattern obtained at 1 h of digestion.

The different fragments from the activation segment isolated by HPLC during activation were submitted to Edman degradation. In every case, the sequence Lys-Glu-Asp-Phe-Val was obtained, a sequence that coincides with that of the NH2-terminal end of intact activation segment and PCPA treated trypsin (from Worthington) retreated with TPCK in our own laboratory and purified by reversed-phase HPLC (Titani et al., 1982) was used throughout. Furthermore, it was shown that purified bovine and porcine trypsins generated the same cleavages. However, the other two secondary cleavages, at positions 87 and 65 of the sequence, do not correlate with the classical action of trypsin (Walsh, 1970; Blackburn, 1976) since a Gln-Met and an Ala-His bond are hydrolyzed. Although minimal contamination of trypsin with other proteases could be responsible for these anomalous cleavage points, we are reluctant to believe it since high quality TPCK-treated trypsin (from Worthington) retreated with TPCK in our own laboratory and purified by reversed-phase HPLC (Titani et al., 1982) was used throughout. Furthermore, it was shown that purified bovine and porcine trypsins generated the same cleavages. In the light of the low amounts of fragments 3 and 5 generated, we suggest that they are the result of minor nonspecific trypsin cleavages derived from the long digestion times and due to a particularly favorable spatial structure around these cleavage points. They are also probably favored by the existence of exposed and loose secondary structures in the region around them (Vilanova et al., 1987).

It is worth remembering that in spite of its usual extreme specificity, trypsin can act in proteins at peptide bonds other than lysyl or arginyl (Plapp et al., 1967; Bachmayer et al., 1987). It is worth remembering that in spite of its usual extreme specificity, trypsin can act in proteins at peptide bonds other than lysyl or arginyl (Plapp et al., 1967; Bachmayer et al., 1987; Walsh, 1970).

To check the inhibitory capabilities of the different activation fragments, we proceeded to their isolation and carried

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**Fig. 2.** Quantification of the five large fragments of the activation segment of procarboxypeptidase A generated by trypsin activation and separated by reversed-phase HPLC. **Fig. 3.** Schematic representation of the main cleavage points observed in the activation of porcine monomeric procarboxypeptidase A by trypsin. 1, 2, and 4 refer to the cleavages that generate the major fragments of the activation segment. The numbers also refer to the corresponding fragments generated by the cleavages. 1' indicates the peptide bond split in the conversion of carboxypeptidase A-a to -a, activation segment. The NH2- and COOH-terminal sequences of the different activation fragments were characterized by Edman degradation, tryptic peptide maps, amino acid analysis of the peptides, and analysis of the released amino acids after carboxypeptidase Y digestion.
Procarboxypeptidase A Activation Pathway

The activation pathway of porcine procarboxypeptidase A (PCPA) by trypsin proceeds in three main steps corresponding to cleavages at positions 94, 93, and 69 of the activation segment. The minor cleavages at positions 87 and 65 or around them could have a greater importance in activation. The limited proteolysis of the activation segment leaves some questions that will require further study. Thus, the unexpected cleavages at residues 87 and 65 by trypsin should be investigated in PCPAs from other species such as rat or ox. In addition, reasons underlying the bimodal activation process and degradation of fragment 1 and its quantitative analysis merit further attention.

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REFERENCES


Gardell, S. J., Craik, C. S., Clauser, E., Goldsmith, E. J., Stewart, C.-
The tryptic activation pathway of monomeric procarboxypeptidase A.
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