Analysis of the Activation Process of Porcine Procarboxypeptidase B and Determination of the Sequence of Its Activation Segment†

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ABSTRACT: The molecular events which lead to the proteolytic transformation of porcine procarboxypeptidase B (PCPB) in carboxypeptidase B (CPB) have been determined. Among pancreatic and other tested proteinases, trypsin is the only one capable of generating carboxypeptidase B activity from the zymogen, in vitro. In the first step of this process, trypsin produces cleavage at the boundary between the activation region and the CPB region. Subsequently, a definite sequence of cleavages occurs at the C-terminal end of the released activation segment of 95 residues, giving rise to characteristic intermediates and to a proteolytically resistant activation fragment of 81 residues. In this process, the newly formed CPB participates in the quick-trimming of the released activation peptides. Only a single CPB species is formed in the activation process. This fact and the inability of the released activation peptides to inhibit CPB—and, therefore, their inability to slow down the kinetics of appearance of CPB activity—are two important characteristics differentiating between the activation processes of procarboxypeptidases A and B. The sequence of the 95 residues (MW = 12 835) of the activation region of porcine PCPB has also been deduced, largely from the information obtained by Edman degradation of its fragments and in part by considerations of homology with the rat precursor. The porcine PCPB activation region contains a high percentage of acidic residues, lacks cysteines, methionines, and side-chain posttranslational modifications, and presents a low but significant homology (31%) with the corresponding sequence of porcine procarboxypeptidase A.

The present knowledge on the proteolytic process which generates pancreatic carboxypeptidases from their zymogens is poor when compared with the solid information available presently on the structure, function, and genetic codification of these enzymes (Puigserver et al., 1986; Auld & Vallee, 1987; Gardell et al., 1988). In the case of procarboxypeptidases A (PCPA),[1] the most studied forms, research was formerly focused on the first cleavages which occur during tryptic activation at the boundary between the long N-terminal activation segment and carboxypeptidase A (CPA), and on the concomitant release of the α and β fractions for this enzyme (Uren & Neurath, 1972). The slow kinetics of the proteolytic generation of CPA activity and the putative steps of the process were also studied in relation to the oligomeric complexes that these proenzymes establish with other proproteinases in many species (Brown at al., 1963; Freisheim et al., 1967; Lacko & Neurath, 1970; Uren & Neurath, 1972; Puigserver & Desnuelle, 1977; Kerfelec et al., 1985; Puigserver et al., 1986). Subsequently, attention has been paid to the dependence of the activation on the strong binding between CPA and the severed activation segment, and to the ability of this segment and fragments to inhibit CPA (SanSegundo et al., 1982; Vilanova et al., 1985a; Chapus et al., 1987). Sequence determination of the 94-residue activation segment of several procarboxypeptidases A (Quinto et al., 1982; Vendrell et al., 1986; Wade et al., 1988), and the characterization of the proteolytic fragmentation course, recently allowed one of our laboratories to propose a detailed model for the activation process of these proenzymes (Vendrell et al., 1990a).

In the case of procarboxypeptidase B (PCPB),[1] the activation process has been studied only in a few animal species and at an intermediate level; ox (Cox et al., 1963), lungfish (Reeck & Neurath, 1972), and human (Pascual et al., 1989). The general occurrence of PCPB as a monomer made it easier to characterize this process. In those reports, the kinetics of the activation of PCPB by trypsin were found to be quick, and the main region subjected to proteolysis—the activation segment (asB)—was only characterized at its N-terminal sequence for the last two species. The full sequence of the active product, carboxypeptidase B (CPB), was also determined for the bovine species (Titani et al., 1975). Only partial information had previously been obtained about the different cleavage points by the trypsin on the activation region of the proenzyme and about the order of these events. The recently solved gene structure of rat PCPB (Clauser et al., 1988) provided for the first time a putative 95-residue sequence for the activation region, a significant information for the activation studies.

The work reported here analyzes in detail at the qualitative, semiquantitative, and kinetic level the molecular events which occur in the generation of activity from porcine PCPB by trypsin. The preparative isolation and amino acid sequence determination of the activation segment and fragments derived from this proenzyme are also described for the first time. The

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[1] Abbreviations: PCPA and PCPB, procarboxypeptidases A and B, respectively; CPA and CPB, carboxypeptidases A and B, respectively, F1-F5, activation fragments isolated in the activation of porcine PCPB by trypsin; afBx, each one of the different fragments from the activation region of PCPB ending in a given C-terminal residue (x).

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full resolution of the activation pathway of a pancreatic PCPB is of interest on its own, given the important role played by CPBs in digestion. Also, there is increasing evidence of the structural and evolutionary relationships between these proteins and carboxypeptidase A and B like enzymes from other tissues and fluids (Skidgel, 1988; Rodriguez et al., 1989; Tan et al., 1989; Reynolds et al., 1989), enzymes of great biological interest, and some of them synthesized as zymogens of diverse structure. Cross-comparisons between the mechanisms for the inhibition and activation of all these carboxypeptidases should lead to interesting basic and applied conclusions.

Materials and Methods

Preparative Isolation of PCPB, CPB, and F3. Porcine PCPB was isolated from acetone pancreatic powder by anion-exchange chromatography on DEAE-Sepharose according to a previously reported procedure (Vilanova et al., 1985b). As a last purification step, an additional chromatography on the same support in 40 mM Tris-acetate (pH 5.7), with an elution gradient between 0.11 and 0.26 M NaCl, was included.

The 1–81-residue fragment (F3) of the activation segment was obtained from PCPB by limited proteolysis. To 20 mg of purified PCPB, at 1 mg/mL in 50 mM Tris-HCl and 10 μM $ZnCl_2$ (pH 7.5), was added 0.5 mg of TPCK-treated trypsin (Worthington), and the mixture was incubated for 15 min at 25 °C. The action of trypsin was stopped by the addition of 0.5 mg of aprotinin (Trasylol from Bayer), and the mixture was immediately chromatographed on a DEAE-Sepharose column, equilibrated with 40 mM Tris-HCl (pH 8.0). Elution of the proteins was performed with a linear 0–0.4 M NaCl gradient in the last buffer. Under these conditions, two clearly separated peaks containing active CPB and F3 were sequentially eluted and recovered in the pure state.

Proteolytic Activation of PCPB. For activation with trypsin, PCPB at 1 mg/mL in 50 mM Tris-HCl/10 μM $ZnCl_2$ (pH 7.5) was treated with trypsin (TPCK grade from Worthington) at ratios from 4/1 to 2000/1 (w/w), and at 0, 25, or 37 °C. At given times, aliquots were removed for activity measurements and for electrophoretic and HPLC analyses. For activity measurements, 10 μL of the activation mixture was added onto 190 μL of aprotinin at 0.1 mg/L in 50 mM Tris-HCl/10 μM $ZnCl_2$, and 10 μL of this new mixture was used to carry out spectrophotometric measurements on the substrate Bz-Gly-L-Arg according to Wolf et al. (1962).

For electrophoretic analysis, 50 μL of the activation mixture was added onto 50 μL of aprotinin at a variable concentration in water to reach a final aprotinin/trypsin ratio of 5/1 (w/w). Each sample was immediately mixed with electrophoretic loading buffer (containing 1% SDS and 3% β-mercaptoetanol), heated at 100 °C for 1 min, and stored at −20 °C until its analysis. Electrophoresis was carried out in polyacrylamide gels according to Laemmli (1970), in the presence or in the absence of 0.1% SDS, or in the presence of 0.1% SDS/7 M urea according to Hashimoto et al. (1983).

Activation studies with proteinases other than trypsin were carried out following the preceding procedures but only at 4/1 and 40/1 (w/w) PCPB/proteinases ratios, and at 37 °C. Bovine elastase and thermolysin were obtained from Sigma. Bovine chymotrypsin was from Merck. Porcine proteinase E was obtained in our laboratory according to Avilés et al. (1989). Clostripain, lysine endoproteinase, and arginine endoproteinase from submaximally gland were supplied by Boehringer. Proline endopeptidase and *Staphylococcus aureus* V8 proteinase were supplied by Miles. The proteolytic capability of all these proteinases was tested on denatured cytochrome *c* by electrophoresis.

Activation studies by HPLC were performed on Nucleosil C-4 reversed-phase supports (300-Å pore size; from Mackerey & Nagel), in 0.05% trifluoroacetic acid with an eluting linear gradient between this solvent (solvent A) and acetonitrile (solvent B), according to the following steps: 0% B from 0 to 5 min, 35% B at 45 min, and 65%, B at 130 min. Sixty-microliter samples (for analytical studies) or 600-μL samples (for preparative studies) were removed from the activation mixture, made 0.5% in trifluoroacetic acid to inhibit proteolysis, and immediately chromatographed or kept at −20 °C for subsequent analysis.

Inhibition and Binding Studies. The activation peptides, in 50 mM Tris-HCl/10 μM $ZnCl_2$ (pH 7.5), were added to CPB at 4 nM concentration in the same buffer to reach different molar ratios. The remaining CPB activity was measured with the substrate Bz-Gly-L-Arg, according to Wolf et al. (1962). The binding between CPB and activation peptides in the above mixtures was also investigated by electrophoresis in polyacrylamide gels in Tris/boric acid/EDTA (TEA) buffer in the absence of dissociating agents or in transverse gradients of urea (0–9 M), as previously described (Vilanova et al., 1985a).

Isolation of Fragments from the Activation Segment. Analysis of N-terminal sequences only required the previous fragmentation of the protein and isolation of its peptides at the preparative level in the case of the trypsin-resistant N-terminal fragment (1–81 residues) F3. This peptide at 1 mg/mL in 0.2 M *N*-ethylmorpholine/4 M urea (pH 8.2) was fragmented by trypsin at a final 5/1 ratio (protein/trypsin, w/w) at 37 °C for 20 h (in two additions along time, the first at a 10/1 ratio at time zero and the second at an additional 10/1 ratio at time 4 h). Pepsin digestion of F3 was performed in 5% formic acid/1 mM HCl, at 25 °C for 4 h, with a 50/1 ratio (protein/pepsin, w/w). Fragmentation of F3 with BNPS-skatole was achieved in 80% acetic acid, at 37 °C for 18 h, with a 3/1 ratio (reagent/protein, w/w), followed by a 2-fold dilution with water, extraction of excess of reagent and byproducts with ethyl ether, and concentration of the remaining aqueous phase by a rotary evaporator before fractionation by HPLC.

The separation of the fragments of F3 obtained by enzymatic and chemical methods was performed by reversed-phase HPLC on the μBondapak C-18 column (300 × 4 mm, 10-μm particle size, 100-Å pore, from Waters Associates) for the trypsin-derived fragments, on a Novapak C-18 column (150 × 4.5 mm, 4-μm particle size, from Waters Associates) for the pepsin-derived fragments, and on a C4 Nucleosil column (300 × 4 mm, 7-μm particle size, 300-Å pore, from Mackerey & Nagel) for the fragments obtained by cleavage with BNPS-skatole. In all cases, chromatographies were carried out in 0.05% trifluoroacetic acid with an eluting complex gradient from 0% to 50% acetonitrile. The collected fractions were neutralized with ammonium hydroxide and freeze-dried. A similar reversed-phase HPLC fractionation on C4 Nucleosil was followed on other proteins subjected to N-terminal sequence determination and which did not require a previous fragmentation, such as PCPB, CPB, F1, F2, peptide F4, and peptide F5, in order to remove their accompanying salts.

Protein Sequence Determination. Amino acid analyses of the isolated peptides were carried out by the conventional ninhydrin system and by the HPLC/dabsyl chloride method previously reported by one of our laboratories (Vendrell & Avilés, 1986b). Provisional identification of many peptides and analysis of their purity were carried out by manual Edman degradation with 4-(*N*,*N*-dimethylamino)-4′-isothiocyanato-

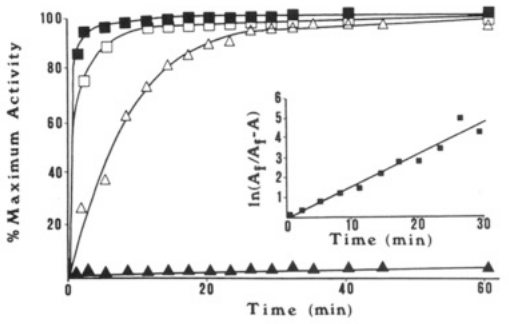


FIGURE 1: Activation of porcine procarboxypeptidase B by trypsin and other proteinases. The zymogen at 1 mg/mL in 50 mM Tris-HCl/10 μM $ZnCl_2$ (pH 7.5) was treated with trypsin at final ratios of either 4/1 (■), 40/1 (□), or 400/1 (△) (w/w), at 0 °C. Alternatively, it was treated with other proteinases (▲) (such as α-chymotrypsin, elastase, proteinase E, V8 proteinase, thermolysin, or lysine endoproteinase), in each case at a final ratio of 40/1 (w/w), at 37 °C. At controlled times, aliquots were withdrawn and analyzed for carboxypeptidase B activity. The insert shows the semilogarithmic representation of the tryptic activation process at a 400/1 (w/w) ratio. A_f, specific carboxypeptidase B activity at the end of activation by trpysin; A, specific carboxypeptidase B activity at each analyzed point of the activation course.

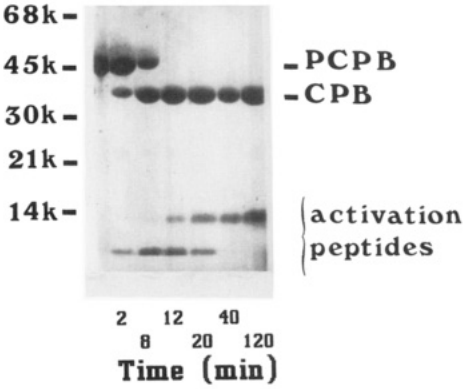


FIGURE 2: Electrophoretic analysis of the activation process of porcine procarboxypeptidase B by trypsin. Samples from a tryptic digest of the zymogen, at a 400/1 (w/w) ratio and at 0 °C, were withdrawn at fixed times, treated with aprotinin and electrophoretic loading buffer (containing SDS) to inhibit proteolysis, and analyzed in SDS/7 M urea electrophoresis gels. The relative molecular weights of protein markers run in parallel are indicated at the side.

azobenzene (DABITC), as recently described by us (Salva & Avilés, 1989). Extended N-terminal sequence determination of PCPB and fragments was performed by automated Edman degradation with phenyl isothiocyanate in an 890-M sequencer from Beckman, using the protein/peptide/microsequencing program. The released phenylthiohydantoins were analyzed by reversed-phase HPLC as previously described (Pascual et al., 1989). C-Terminal sequence determination of activation peptides was made by the use of carboxypeptidase Y. In this case, the peptides were dissolved at 0.1 μmol/mL in 50 mM mercaptoethanesulfonic acid, 1 mM EDTA, and 0.1% SDS (pH 8.0) and kept at 100 °C for 5 min. Then, the samples were equilibrated at 37 °C, and carboxypeptidase Y (from Boehringer) was added to reach a final concentration of 0.5 mg/mL. At different times, aliquots containing 2 nmol of peptide were removed, and their content in free amino acids was determined by following the HPLC/phenylthiocarbamyl derivative procedure of Bidlingmeyer et al. (1984).

Results

Proteolytic Activation of Procarboxypeptidase B. The analysis of the generation of carboxypeptidase B activity, using the synthetic substrate Bz-Gly-L-Arg, during the proteolysis of porcine PCPB at intermediate pH indicated that trypsin is an efficient activator at both 0 and 37 °C (see Figure 1 for the activation at 0 °C). Under the same conditions, other tested endoproteases, such as chymotrypsin, proteinase E, elastase, thermolysin, lysine endoproteinase, and *Staphylococcus aureus* V8 proteinase, were unable to perform this action at 37 °C (see Figure 1). These observations, taken together with the fact that clostripain and arginine endoproteinase showed similar effects to that of trypsin on PCPB (results not shown), suggested that the target region for the activation of this proenzyme should contain an Arg–X accessible peptide bond. The above results also substantiated the correctness of selecting trypsin as the proteolytic agent for detailed studies on PCPB activation.

The action of trypsin on PCPB at 37 °C and pH 7.5 is very quick since 100% activation of PCPB is achieved in 2 min at a 40/1 ratio (PCPB/trypsin, w/w) (not shown). To facilitate the characterization of the activation course as well as the identification and isolation of intermediates, subsequent studies were carried out at 0 °C and at different trypsin/PCPB ratios (from 1/4 to 1/400, w/w), as shown in Figure 1. In these conditions, the activation course is monotonic, and when a proper activation rate for analysis is selected, the step PCPB → CPB can be fitted to pseudo-first-order kinetics in which k = 0.12 min^{-1} and $t_{1/2}$ = 5.8 min (see Figure 1, insert, for the 1/400 trypsin/PCPB ratio). The electrophoretic analysis on SDS/7 M urea/polyacrylamide gels of the molecuolar species present during activation indicates that PCPB (M_r ~46 000) is progressively transformed in a single CPB (M_r ~36 000) without the appearance of intermediates (Figure 2). This important fact was confirmed by charge-dependent electrophoresis and isoelectrofocusing in different conditions (see Materials and Methods) and by HPLC studies which will be described later.

Two fragments with apparent molecular weights of approximately 14 000 and 11 000, which were subsequently assigned to activation peptides, also appear concomitantly with CPB during PCPB proteolysis. The kinetics of appearance–disappearance of these two fragments indicate that the one of lower mobility probably originates from the one of higher mobility. From this anomalous correspondence and from the fact that the apparent molecular weight of the fragment of lower mobility largely exceeds the different in molecular weight between PCPB and CPB, we concluded that the electrophoretic behavior of both activation peptides is anomalous, particularly for the one of lower mobility. This peptide probably shows a different resistance to unfolding in 0.1% SDS/7 M/urea than the other and/or binds a smaller number of SDS molecules. It is interesting to note that in SDS–polyacrylamide gels in the absence of urea both peptides run together and show a molecular weight of 11 000, which is close to the expected difference between PCPB and CPB.

The activation process of PCPB by trypsin was also studied by HPLC in a wide-bore (300 Å) reversed-phase support, as shown in Figure 3. From the quantitative analysis of peak areas of the different molecular species (see Figure 4), it can be concluded that the rate of disappearance of PCPB and the appearance of CPB molecules again fits very well to pseudo-first-order reactions with identical kinetic constants [k_1(PCPB-HPLC) = 0.111 min^{-1}; k_1(CPB-HPLC) = 0.110 min^{-1}] and both fit reasonably well to the kinetic constant for the generation of CPB activity [k_1(CPB-act) = 0.12 min^{-1}] (this one derived from Figure 1).

Three different activation peptides, which we shall refer to in order of appearance as activation fragments 1 (F1), 2 (F2),

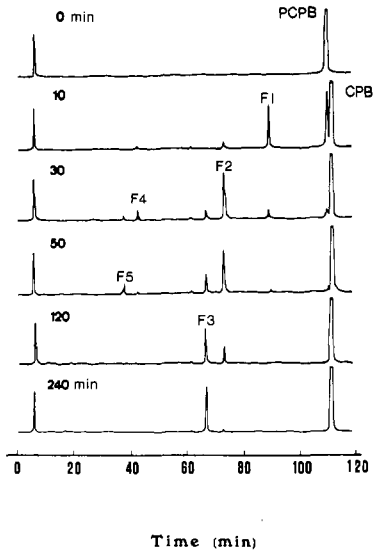


FIGURE 3: Analysis by reversed-phase HPLC of the protein fragments generated during tryptic activation of porcine procarboxypeptidase B. Samples from a tryptic digest of the zymogen at a 400/1 (w/w) ratio and at 0 °C, as described in Figure 1, were withdrawn at fixed times, made 0.1% in trifluoroacetic acid to inhibit proteolysis, and analyzed in a Nucleosil-C4 reversed-phase column. Elution was carried out with a water/acetonitrile gradient, in the presence of 0.1% trifluoroacetic acid. See text for details.

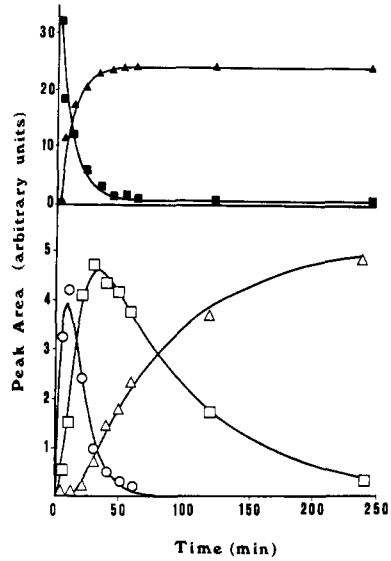
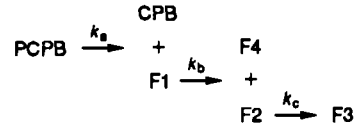


FIGURE 4: Quantification of the fragments generated in the activation of porcine procarboxypeptidase B by trypsin and comparison with the values derived from the proposed kinetic model. The peak areas deduced from Figure 3 were represented (open and closed symbols) together with the theoretical curves (solid lines) derived from the kinetic mathematical model which fits the molecular pathway proposed in the text. (Top panel) (■) PCPB; (▲) CPB. (Bottom panel) (○) F1; (□) F2; (△) F3.

and 3 (F3), were clearly separated by HPLC during activation. The activation fragment which first appeared, F1, was found (by electrophoresis) to correspond to the band of higher mobility in electrophoresis in SDS/7 M urea gels (Figure 2), while F2 and F3 had the same electrophoretic mobility as the band of lower mobility. Therefore, the lack of correlation between the electrophoretic mobility of these peptides and its expected relative size was again observed. Other minor peptides, labeled F4 and F5, appeared simultaneously with the transformation of F1 to F2 and subsequently disappeared from the chromatogram. Both peptides should have a small molecular weight since they are lost during SDS/urea electrophoresis. It is also

Scheme I



interesting to note that the terminal product of activation, F3, was found to be quite resistant to further proteolysis, particularly by proteinases other than trypsin.

The above suggested transformation pattern of the molecular species was confirmed by mathematical analysis of the evolution of the peak area pattern on the HPLC profiles. If we assume that the proteolytic fragmentation of PCPB occurs in accordance with Scheme I in which k_a, k_b, and k_c are the kinetic constants for the different pseudo-first-order reactions, the equations which define the changes in concentration of each molecular species in time would be

$$P = P_0 \exp(-k_a t)$$

$$C = P_0[1 - \exp(-k_a t)]$$

$$S1 = \frac{P_0 k_a}{k_b - k_a} [\exp(-k_a t) - \exp(-k_b t)]$$

$$S2 = k_a k_b P_0[(k_c - k_b) \exp(-k_a t) + (k_a - k_c) \exp(-k_b t) + (k_b - k_a) \exp(-k_c t)/(k_c - k_a)(k_c - k_b)(k_b - k_a)]$$

$$S3 = P_0 - P - S1 - S2$$

where P, C, $S1$, $S2$, and $S3$, are the concentrations of PCPB, CPB, F1, F2, and F3 at each point in time, P_0 is the initial concentration of PCPB, and t is the activation time. The theoretical curves for the evolution of the concentrations of these species as deduced from the above equations are shown in Figure 4, together with the experimental measurements for the same species taken from Figure 3. The good fitting between the experimental and theoretical curves supports the proposed pathway of transformation. The kinetic constants deduced from the fitting of the above equations to the experimental data by nonlinear regression are $k_a = 0.110$ min^{-1}, $k_b = 0.123$ min^{-1}, and $k_c = 0.013$ min^{-1}.

The small amount of peptides 4 and 5 and their transitory existence made it difficult to quantify them to be included in the above mathematical model. However, as we shall comment later on, sequence determination of both peptides confirmed the proteolytic path F1 → F2 + F4, peptide F5 being a primary degradation product of peptide F4.

In order to analyze the possible influence of the released activation peptides of PCPB on the activity of CPB, and therefore on the activation process, inhibition studies with this enzyme by F1, F2, and F3 were carried out at different concentrations of the enzyme, of these peptides, and of the synthetic substrate Bz-Gly-L-Arg used to measure carboxypeptidase activity. In no case were the activation peptides able to modify the activity of CPB, even at a 20/1 molar ratio. This is in contrast with the results previously reported by one of our groups for PCPA in which the activation peptides from this zymogen promoted a strong inhibition of CPA activity under similar experimental conditions as those described here (SanSegundo et al., 1982; Vendrell et al., 1990a). The inability of F1, F2, and F3 to bind to CPB was confirmed by charge-dependent electrophoresis (in the absence of dissociating agents), by gel filtration HPLC, and by electrophoresis in transverse urea gradients, according to a previously described procedure (Vilanova et al., 1985a). In the three cases, the activation segments and CPB were found to run or elute independently.

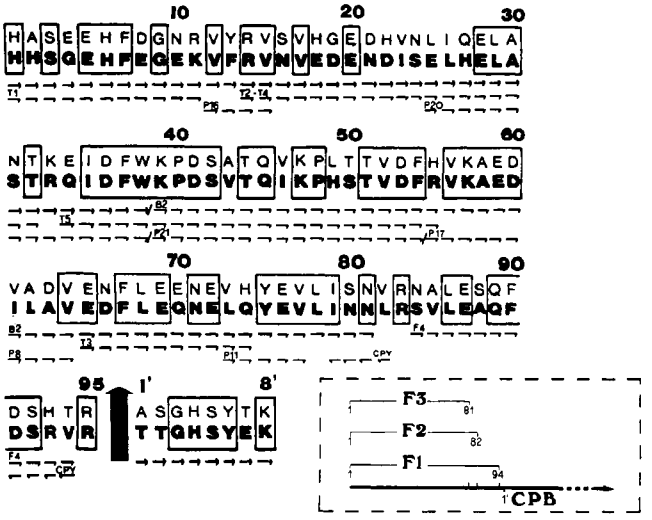


FIGURE 5: Determined primary structure of the activation region from porcine procarboxypeptidase B. Sequence data were obtained from the whole protein (→) or from peptides (—) derived from cleavages with trypsin (T or F), pepsin (P), and BNPS-skatole (B). Peptides were numbered according to their elution order when separated by reversed-phase HPLC. C-Terminal sequences were confirmed by digestion with carboxypeptidase Y and quantitative analysis of the released amino acid along time (—). The homologous sequence in rat procarboxypeptidase B, deduced from the gene structure by Clauser et al. (1988), is displayed in lighter symbols on top of the determined porcine sequence. Common sequences are indicated in boxes. The arrow indicates the primary tryptic cleavage point during activation at the boundary between carboxypeptidase B and the activation segment. A scheme of the large fragments generated from this region during tryptic activation, all of them used for sequence determination, is shown at the bottom.

Determination of the Sequence of the Activation Region. Sequence analysis by Edman degradation of the HPLC-isolated tryptic activation peptides from porcine PCPB (Figure 3) indicated that F1, F2, and F3 share the same N-terminal sequence, His-His-Ser-Gly-Glu-His, as the intact proenzyme. From this fact and from their high molecular weight, it was concluded that these activation fragments are consecutive proteolytic products differing in their C-terminal region and that they contain most of the activation region. Subsequently, F3 was purified at a preparative level by anion-exchange chromatography on DEAE-Sepharose and was used as the fundamental fragment for sequence determination.

Different sets of peptides (Figure 5) were obtained by fragmentation of F3 using trypsin (in the presence and absence of 4 M urea), pepsin, and BNPS-skatole, followed by reversed-phase HPLC. The harsh conditions required for fragmentation with trypsin gave rise to some anomalous cleavages (i.e., peptides T2 and T3). Other cleavage agents (thermolysin, V8 proteinase, clostripain, *N*-bromosuccinimide, etc) were also tested but found to be unsuitable for obtaining peptides from F3, mainly due to its particularly strong resistance to fragmentation even in the presence of denaturing agents (i.e., 4 M urea). From the overlapping of sequences of the isolated peptides, mainly determined by automated Edman degradation, the complete sequence of F3 was deduced. As shown in Figures 5 and 6, F3 is composed of 81 residues, with a single Trp (W-38) and Tyr (Y-75) and lacking Met and Cys residues and side-chain posttranslational chemical modifications. The deduced sequence of F3 has been recently fully checked by 2D NMR assignments (Vendrell et al., 1990b). On the other hand, N-terminal sequence determinations, trypsin peptide maps, and C-terminal analysis using carboxypeptidase Y indicate that F2 is identical with F3 but has one more residue (leucine) at its C-terminal end. It is

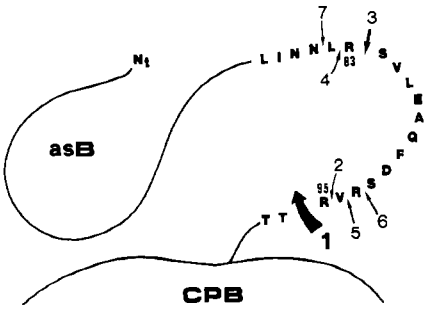


FIGURE 6: Schematic representation of the main cleavage points observed in the activation of porcine procarboxypeptidase B by trypsin. 1 and 2 refer to the primary cleavages, which give rise to the generation of carboxypeptidase B activity and to the release of activation fragment F1 (residues 1–94). 3 and 4 refer to the secondary cleavages giving rise to undecapeptide 84–94 and to activation fragment F2 (residues 1–82). Trypsin is responsible for cleavages 1 and 3, while the generated carboxypeptidase B is responsible for cleavages 2 and 4–7. The latter three cleavages produce the last definite activation fragments, nonapeptide 84–92 and fragment F3 (residues 1–81).

therefore a proteolytic precursor of F3.

Peptides F4 and F5 (Figure 3) were found to arise from the same region, the latter covering the first nine N-terminal residues of the former, which is an undecapeptide. At first glance, F4 seemed to cover the C-terminal end of F1, the region which differentiates this fragment from F2. This hypothesis was proved by the observation that cleavage of isolated F1 by trypsin generated only peptide F4 and a large fragment with an HPLC elution similar to F2. Direct amino acid analysis of the solution after cleavage proved that no free amino acids were released in this process. The C-terminal location of F4 was also confirmed by C-terminal sequence analysis by carboxypeptidase Y digestion, which released the same amino acids from F1 and from F4 (see Figure 5). At this stage, doubts remained whether both F1 and F4 contained the whole connecting region with the active enzyme.

On the other hand, the N-terminal sequence of the complementary CPB isolated by HPLC was also determined (Figure 5) and found to be homologous with that of ox (Titani et al., 1975) and rat (Quinto et al., 1982) CPB. Comparison of the deduced sequences of F1, F2, F4, and porcine CPB with the sequence of the activation region of rat PCPB, recently deduced from analysis of the DNA (Clauser et al., 1988), made it clear that F1 most probably contained the whole activation region of porcine PCPB except the last C-terminal residue. This comparison also confirmed that peptide F4 is the linking region between F2 and CPB (except for the last C-terminal residue). Efforts to show experimentally this linking by isolation of a fragment of PCPB overlapping the C-terminal end of F1 and the N-terminal end of CPB failed due to the resistance of PCPB to limited cleavages with proteinases other than trypsin and to the lack of a nearby potential cleavage point using chemical reagents.

The above assumption on the sequence of the linking region between F1 and CPB was also proved indirectly by analysis of the free amino acids released to the activation solution during the activation process. This analysis showed that no other fragments than CPB, F1, and free arginine are generated from PCPB in the first stages of the activation and that three arginines, one leucine, and one valine are released from PCPB at the end of its activation. These amino acids are exactly the ones to be expected if arginine residues are placed at positions 83 and 95, that is, at the C-terminal end of fragments F2 and F1, and if the generated CPB hydrolyzes the putative fragments after trypsin cleavages at these positions. These as-

signments of Arg-83 and Arg-95 are in full homology with the corresponding sequence of rat PCPB.

As shown in Figure 5, of the 95 residues of the porcine and rat PCPB activation region, 57 shows identities in sequence. This level of homology (60%) is much higher than the one observed between the activation region sequences of porcine PCPB and PCPA, the later taken from Vendrell et al. (1986a), in which only 29 identities are found (31% homology).

Definition of Cleavage Sites on PCPB during Activation by Trypsin. From the above-described results, it is clear that the detected (and isolated) activation fragments by HPLC and electrophoresis are not the only intermediates produced during activation. The release of free amino acids during the activation process and the inability to detect the precursors of F1 and F2 indicate the combined action of trypsin and CPB in the processing of activation fragments and the very efficient action of the latter enzyme in this task. From the kinetics of the generation of fragments and amino acids, the pathway of activation of porcine PCPB by trypsin has been deduced at the molecular level, and it is shown in Figure 6. According to this, the initial cleavage occurs at the Arg-95 (asB)–Thr-1 (CPB) peptide bond and promotes the release of the whole activation segment from PCPB and, most probably, the full generation of CPB activity. Immediately, the generated CPB removes the C-terminal arginine from the activation segment, giving rise to fragment F1.

At present, it is not clear whether the whole activation segment (residues 1–95) is able to bind and inhibit CPB, as the corresponding segment of CPA does with this enzyme (Vendrell et al., 1990a), but it has been shown that fragment F1 (residues 1–94) has already lost this ability. Kinetic analysis of the tryptic activation process of PCPB in the presence of inhibitors of CPB activity (Villegas et al., unpublished results) seems to favor the hypothesis that the whole activation segment of PCPB fully loses its inhibitory ability after being severed from the proenzyme.

Subsequently, a C-terminal undecapeptide is removed by the action of trypsin on the Arg-83–Ser-84 peptide bond of F1, giving rise to peptide F4 and to the 1–83-residue fragment. Arg-83 is immediately trimmed by CPB from the latter giving rise to fragment F2. Both cleavages and the following ones have no influence on the generation of further CPB activity.

In the next step, the Ser-84–Val-94-released undecapeptide F4 is transitorily trimmed to the Ser-84–Ser-92 nonapeptide F5. This action is probably also performed by CPB because of the nature and position of the released residues. Much later, CPB fully degrades F5 to amino acids.

In the last major and definite step, fragment F2 is converted to F3 by the slow removal of the C-terminal leucine by CPB. The feasibility of this fact was experimentally proved by mixing isolated F2 and CPB and showing that F3 and leucine are released, although at a lower rate than from the proenzyme. Also, the action of CPB on the C-terminal of F2 and on peptide F4 was indirectly proved by the observation that both fragments appear as terminal ones and were not transformed to shorter ones when a powerful inhibitor of carboxypeptidases, such as that isolated from potatoes (Hass & Ryan, 1981), was added to PCPB before this protein was activated by trypsin (Villegas et al., unpublished results). Amino acid analysis of the first chromatographic peak of the HPLC profiles of Figure 3 (the peak at 6 min, containing unretained material) confirmed the sequential and estequiometric release of Arg, Arg, Val, Arg, and Leu from the C-terminal regions of the large fragments generated from the activation region of PCPB during trypsin action (data not shown). Finally, it is interesting to remember that the remaining F3 is resistant to further proteolysis.

Discussion

The ability of trypsin to activate porcine PCPB was an expected observation in agreement with previous reports on other PCPBs (Cox et al., 1963; Reeck & Neurath, 1972; Pascual et al., 1989). In contrast, the great resistance shown by porcine PCPB to activation by many proteinases of different specificities was surprising, particularly if one takes into account the existence of several bonds susceptible to the cleavage by these proteinases in the target region for activation in PCPB (residues 82–95). Probably, the conformation of this region protects it against proteolysis. From this point of view, and in vitro conditions, the range of putative activators for porcine PCPB is much more restricted than for PCPAs from the same or other species for which an easy activation by different proteinases has been reported (Uren & Neurath, 1972; Fresheim et al., 1967).

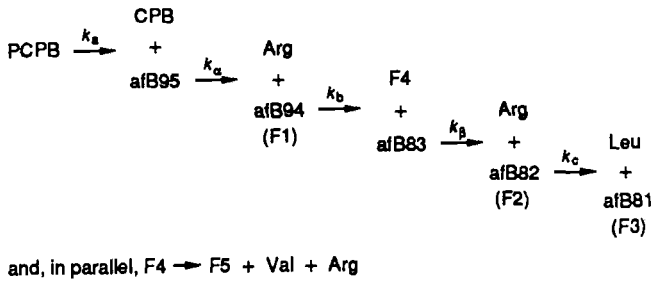
The activation of porcine PCPB by trypsin is quick and apparently simple: the activation course is monotonic and is completed within a few minutes, even at much lower trypsin/PCPB ratios than those found in the digestive tract. The process is very much dependent upon the relative concentration of trypsin and follows pseudo-first-order kinetics both for the molecular conversion of PCPB in CPB and for the appearance of CPB activity. These processes, however, do not lead to the random destruction of the activation region. Thus, the first two proteolytic cleavages specifically occur at Val–Arg–Thr bonds, at the boundary between the whole activation region and CPB, and are clearly responsible for the generation of activity. Subsequently, a definite sequence of cleavages occurs at the C-terminal end of the released F1 (residues 1–94), giving rise to characteristic intermediates, F2, peptide F4, peptide F5, and to a single high molecular weight end product from the activation region, the F3 fragment (residues 1–81).

The participation of the generated CPB in the shortening of the activation fragments (whole activation segment, and fragments F2 and F4) is an interesting observation. This agrees with the previous report of Cox et al. (1963) on bovine PCPB in which it was deduced that the cleavage of an arginyl–threonyl bond is essential in the activation by trypsin and that free arginine, probably from the new C-terminal, is also released in this process. In our case, evaluation of the influence that the release of this arginine could have on the generation of activity from porcine PCPB is hindered by the extreme quickness of this step. However, preliminary kinetic analysis of PCPB tryptic activation in the presence of inhibitors of carboxypeptidases, carried out in our laboratory (Villegas et al., unpublished results), seems to favor the hypothesis that the progress of the activation process is much more dependent on the first tryptic cleavage of PCPB than on the immediate trimming action of CPB. This would be in contrast with our observations on the tryptic activation process of porcine PCPA (Vendrell et al., 1990a) in which the release of Arg-94 by CPA was found to be an important step in the activation.

The good fit between the experimental measurements and the theoretical model for the kinetics of transformation of activation segment fragments also allows us to deduce that the activation process follows a definite sequence of cleavages and not mixed activation pathways. After the definition of the cleavage sites, the former theoretical model (Scheme I) becomes more complete (Scheme II).

In Scheme II, afBx denotes each one of the different fragments from the activation region ending in a given C-terminal residue (x). Since $k_\alpha \gg k_a$ and $k_\beta \gg k_b$, from the

Scheme II



and, in parallel, F4 → F5 + Val + Arg

overall kinetic point of view this activation model is equivalent to the one previously shown in Scheme I, in which the activation rate is dependent of k_a, k_b, and k_c, and when analyzed by the former mathematical simplified approach, both should give similar values for the latter kinetic constants (k_a = 0.110 min^{-1}, k_b = 0.123 min^{-1}, and k_c = 0.013 min^{-1}).

These values indicate that the transformation of afB82 to afB81 is the slowest step in the degradation of large activation fragments. It can be considered as the terminal step in the proteolytic transformation of PCPB in vitro (at 0 °C and at low trypsin/PCPB ratios) because afB81 is resistant to further proteolysis from CPB, trypsin, and many other proteinases. The stability of afB81 against proteolysis indicates that it constitutes a structural globular domain, an assumption which has recently been experimentally shown by 2D NMR (Vendrell et al., 1990b) and by differential scanning microcalorimetry (Conejero, Mateo, Avilés, et al., unpublished results).

The production of a single species of CPB during the tryptic activation of porcine PCPB is another interesting observation. This is in agreement with previous reports (Cox et al., 1962; Reeck & Neurath, 1972) on bovine and lungfish homologous proenzymes. Besides, in the present work no inactive intermediates containing the carboxypeptidase region have been detected in the generation of CPB from porcine PCPB. This is in contrast with that previously shown for lungfish PCPB in which the loss of an N-terminal pentapeptide was found to precede the transformation of PCPB in CPB (Reeck & Neurath, 1972). Therefore, the first cleavage on porcine PCPB releases mature and homogeneous CPB, and further cleavages only act on the C-terminal part of the fragment(s) from the activation region.

On the whole, the results presented here give a detailed and quite complete insight on the molecular events which occur in the limited proteolysis of a pancreatic PCPB by trypsin. The deduced view on the activation of porcine PCPB complements that provided by Winsterberger et al. (1962) on bovine PCPB and by Reeck and Neurath (1972) on lungfish PCPB and allows several general conclusions on the PCPB systems to be drawn. It has also been shown that trypsin is the most probable, and perhaps the only, proteinase which can act as an activator of porcine PCPB in vivo, in the intestine.

The amino acid sequence of the activation region of porcine PCPB determined in this work is the first deduced for a PCPB directly from the protein, and supplies interesting comparative information with that recently derived from the cDNA of rat PCPB (Clauser et al., 1988). In both cases, the activation region contains 95 residues with a high percentage of acidic residues (22% for the porcine), with a single tryptophan and tyrosine residues, and lacking cysteines and methionines. This region in porcine PCPB also lacks side-chain posttranslational chemical modifications.

In the comparison between the activation region sequences of rat and pig PCBP, as shown in Figure 5, an overall percentage of 60% identity is found. However, when the activation region of porcine PCPB and those of porcine PCPA (Vendrell et al., 1986) or rat PCPA (Quinto et al., 1982) are compared, the percentage of identities decreases, respectively, to 31% and to 29%. The divergence of the activation regions of procarboxypeptidases A and B probably occurred before the radiation of mammals, a suggestion which is in agreement with the previous hypothesis of Gardell et al. (1988) about the evolutionary pathway followed by pancreatic procarboxypeptidases. It is also interesting to note that the segments located at positions 21–27 and 82–95 show the lowest percentage of residue identities between the activation regions of porcine and rat PCPB. The latter segment corresponds to the target region for proteolytic activation. This is a surprising fact taking into account the importance of this region in activation.

The trypsin activation pathway of porcine PCPB derived here and its molecular basis present important differences from those recently proposed by us for porcine PCPA (Vendrell et al., 1990a), and both explain several properties observed in the activation of these proenzymes from porcine and from other species. According to our results, the much quicker activation of PCPB with respect to PCPA—the most outstanding differentiating property—would be related to the inability of the released activation peptides to inhibit the CPB generated, an inhibition already demonstrated in the PCPA/CPA system (San Segundo et al., 1982; Vendrell et al., 1990a). Because of this, a more extensive proteolysis of the activation region is required in PCPA than in PCPB to generate carboxypeptidase activity. In both cases, the degradation of the activation segment occurs at its C-terminal end, and progresses toward the N-terminal. However, in PCPB, the proteolytic action stops at residue 81 due to the compact fold of the remaining piece F3. Also, in both cases, the generated carboxypeptidase takes part in the limited proteolysis of the activation peptides. This behavior is more understandable in PCPB because the severed activation peptides do not inhibit CPB and they possess some residues (arginines) at their C-terminal end which are easily released by this protease.

The dependency of PCPB and the relative insensitivity of PCPA activation processes to the concentration of added trypsin are, in each case, probably related to the degree and dynamics of binding of the activation peptides to carboxypeptidases. The primary cleavage point appears as highly accessible in both proenzymes, but the access of trypsin to the subsequent cleavage points in PCPA is probably highly restricted, and the kinetics of activation are more related to the dynamics of the activation segment/CPA association equilibrium than to the local concentration of trypsin molecules. In contrast, in PCPB, the quick release of the primary activation segment (due to its lack of inhibitory power) gives rise to its trimming and to the immediate appearance of CPB activity. Confirmation of these hypotheses would require further research on both systems.

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