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Pancreatic Procarboxypeptidases: Their Activation Processes Related to the Structural Features of the Zymogens and Activation Segments

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Summary

The molecular events leading to the complete activation of pancreatic procarboxypeptidases A and B have been investigated. For both proteins the activation process follows a similar general scheme: trypsin is responsible for the first cleavage that separates the active enzyme from the activation segment, the degradation of the activation segment proceeds only from its C-terminal end, and activity release can be correlated with the disappearance of the long forms of the activation segment. In both systems, trypsin and the released carboxypeptidase participate in the trimming of the severed activation regions. However, the rate of enzymatic activation is much faster in the case of procarboxypeptidase B. This phenomenon may be explained by some structural differences in the connecting region which acts as a linker between the globular domain of the activation segment and the N-terminal end of carboxypeptidases and also by the higher efficiency of carboxypeptidase B for the digestion of its own activation segment. It is not due to unfolding of the activation domain, since the isolated activation domain retains its globular conformation in solution.

Introduction

Pancreatic carboxypeptidases, like other proteolytic enzymes, are secreted as inactive procursors or zymogens, a mechanism which ensures control of their activity (1). In contrast to other secretory digestive zymogens, the pro-peptide lost upon activation is remarkably long: 94 residues for procarboxypeptidase A (PCPA) (2-4) and 95 residues for procarboxypeptidase B (PCPB) (5). Some preliminary experiments showed that the activation segment from PCPA assumed a compact globular fold in the isolated state (6) and acted as a strong inhibitor of the active enzyme (7). This suggested a role of the activation segment in the inhibitory mechanism. The structural basis of the inactivity of the zymogens of serine proteinases such as trypsinogen (8) or aspartic proteinases such as pepsinogen (9) is very well defined. Procarboxypeptidases represent a third inhibition mechanism where the activation segment shields the active site, as recent X-ray diffraction studies have shown (10, 11).

Enzymes: Carboxypeptidase A (EC 3.4.17.1); carboxypeptidase B (EC 3.4.17.2)
Abbreviations: (P)CPA , (pro)carboxypeptidase A; (P)CPB, (pro)carboxypeptidase B; ADB, activation domain isolated from procarboxypeptidase B; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid

Procarboxypeptidase A occurs in the form of stable oligomeric complexes with zymogens of endoproteinases (12-14) and/or in monomeric forms. The *in vitro* tryptic activation of the bovine ternary complex form of the proenzyme had been shown to be a very slow process (15, 16) and the quaternary structure considered to be partially responsible for the slowness of activation. However, a similar phenomenon is observed in the monomeric forms of PCPA found in porcine (17) or human (18) pancreas. On the other hand, the *in vitro* activation of monomeric PCPB is very fast compared to the A form of the zymogen isolated from the same species (19, 20). We have started a project of rationalizing the different rates of activity release from examination of the three-dimensional structures of the zymogens and the corresponding isolated moities: activation segments and active enzymes.

In this work we present a comparison of the activation processes of procarboxypeptidases A and B at the molecular level. The differences in the activation rates may be explained as the result of different phenomena: (i) greater structural stability of the PCPA activation segment upon activation, (ii) a number of primary and tertiary structure differences between the two zymogens that leads to a tighter binding of the severed activation segment to the enzyme in the case of the A form and (iii) higher efficiency of CPB in the digestion of its own activation segment.

Materials and Methods

Tryptic activation studies

Monomeric procarboxypeptidases at 1 mg/mL in Tris 50 mM, $ZnCl_2$ 10 μM (pH 7.5) were treated with trypsin (TPCK) at final ratios ranging from 4/1 to 400/1 (w/w), at 0° or 25°C. For activity measurements, 10 μL aliquots were withdrawn from the activation mixture and analyzed for carboxypeptidase activity against Bz-Gly-L-Phe (CPA) or Bz-Gly-L-Arg (CPB) as described elsewhere (19, 20).

For HPLC analyses, 50 μL or 500 μL aliquots were withdrawn from the activation mixtures, made 0.05% in TFA and analyzed in Vydac C-4 analytical or semipreparative reverse phase columns. Chromatography was developed using a gradient between H_2O and acetonitrile in the presence of 0.05% TFA, at a flow rate of 0.5 mL/min, and detection was performed at 280 and 214 nm.

Tryptic activation in the presence of carboxypeptidase inhibitors was carried out only in the case of PCPB. The inhibitors used were benzylsuccinic acid 2 mM, 2-mercaptomethyl-3-guanidinoethylpropanoic acid 2 mM or potato carboxypeptidase inhibitor (PCI) at 0.04 mM. The inhibitors were added individually to the procarboxypeptidase B activation mixture 2h prior to trypsin addition and activation was subsequently carried out as described above.

Compositional and sequential analysis of the fragments generated

The fragments generated during the activation were submitted to amino acid and N-terminal sequence analysis following established procedures (19, 21). The longer peptides were treated with trypsin and their tryptic peptide maps were obtained in either μ-Bondapak or Novapak C-18 reverse phase columns (Waters Ass.). The collected peptides were also analyzed for their amino acid composition and sequenced.

NMR spectroscopy and solution structure calculations

2D ^1H NMR spectra (in H_2O and D_2O) were recorded on a Bruker AM600 spectrometer. Sequence specific assignments were obtained by standard procedures (22). The input for the structure calculation was derived from two *NOESY* spectra in H_2O and D_2O recorded with a mixing time of 60 ms. Out of a total of 1136 cross-peaks, 757 were found to represent effective conformational constraints and were used for the structure calculations. In the final step, the programs DIANA (23) and X-PLOR (24) were used to collect the final 20 conformers which define the solution structure. Further details about the procedures used can be found in refs. 25 & 26.

X-ray diffraction studies

Details on X-ray data collection and crystal structure determination are found in the references 11 & 12.

Results and Discussion

Activation process of procarboxypeptidases

Different preliminary trials not shown here indicated that trypsin is the endoprotease of choice for the activation of procarboxypeptidases. In particular, activation tests of PCPB with other endoproteinases such as chymotrypsin, proteinase E, elastase, thermolysin, lysine endoproteinase and V8 proteinase did not result in any significat CPB activity release. Figure 1 shows a comparison of the tryptic activation time-course for both procarboxypeptidases. It may be observed that even at lower temperature and lower protein/trypsin ratio, the activation of the B form is much faster than that observed for the A form. This is also observed for the activation of PCPA included in a binary complex with proproteinase E (not shown). The biphasic curve obtained for PCPA was later demonstrated to be the result of the time-dependent interaction of CPA with a number of fragments derived from the activation segment which differ in C-terminal sequence and binding affinity.

This difference in activation rate does not seem to correlate with a differential degradation of the released activation segments from the two zymogens, since the electrophoretic follow-up of the process clearly shows that in both cases a high molecular weight species derived from the primary (complete) activation segment remains in solution at long activation times (i.e. 4 hours) (19, 20).

The chromatographic follow-up of the activation in the absence of inhibitors is shown if Figure 2. The analysis of the products arising from the proteolytic cleavage of the activation segments showed that the expected intermediates from a tryptic cleavage were only observed for PCPA. In that case the only exception is a C-terminal cleavage performed by CPA on the 94-residue activation segment. From that, the sequence of molecular events in PCPA activation could be formulated. On the contrary, in the case of PCPB none of the C-terminal sequences observed for the released fragments could correspond to a tryptic cleavage.

To complete the information regarding PCPB activation, a series of experiments in the presence of CPB inhibitors were carried out (an example is shown in Fig. 2c). As previously suspected from the rapid

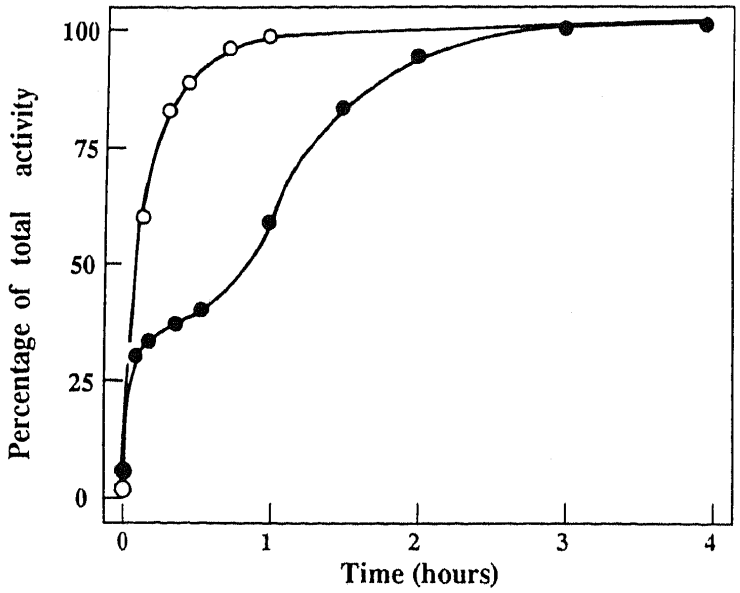

Figure 1. Generation of carboxypeptidase A (●) and carboxypeptidase B (○) activity by the action of trypsin. Protein/trypsin ratios were 40/1 for PCPA and 400/1 for PCPB and the activation temperature was 25 and 0 degrees Celsius, respectively . Other details are found in the text.

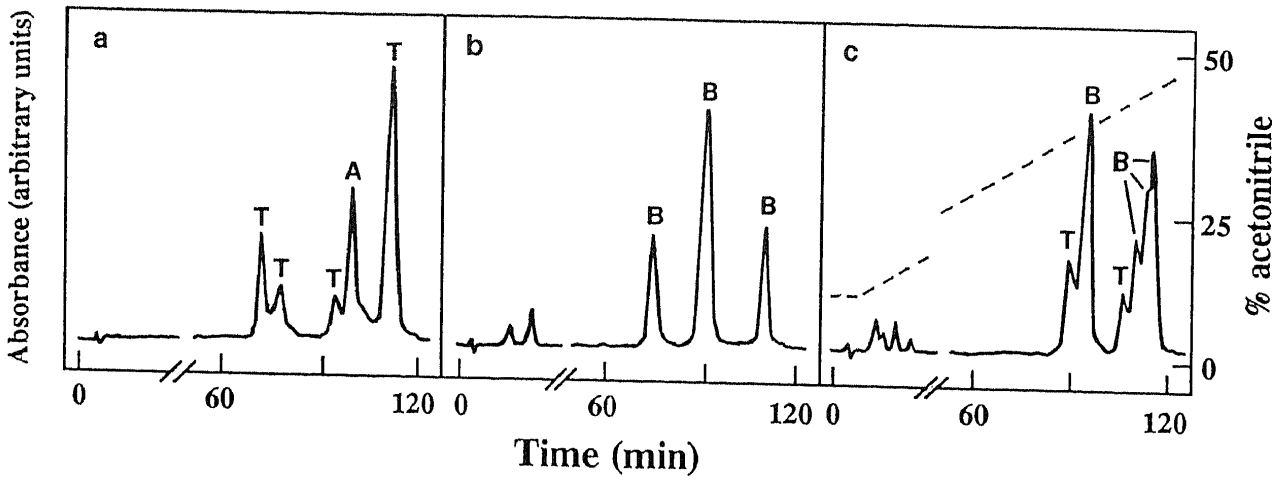

Figure 2. Analysis of the activation process by reverse-phase HPLC. a) PCPA; b) PCPB; c) PCPB in the presence of carboxypeptidase B inhibitors. In all cases the displayed chromatogram is a "sum" chromatogram of all fragments released and is not indicative of any particular activation time. The letters above the chromatographic peaks indicate the enzyme responsible for their appearance: T: trypsin; A: CPA; B: CPB. See M&M for other details.

activity release and from the specificity of CPB itself, the experiment corroborated that the active enzyme collaborates in the trimming of its own activation segment in a more extensive way than that performed by CPA on its corresponding pro-peptide. All fragments in Figure 2b are peptides obtained as a product of the joint action of trypsin and CPB, whereas those in 2c are "frozen" intermediates resulting from the much lower proteolytic action of CPB due to the presence of inhibitors.

Figure 4 summarizes the sequence of events in both activation processes deduced from the above experiments. The cleavages affecting the sequences shown are indicated by arrows. It may be observed

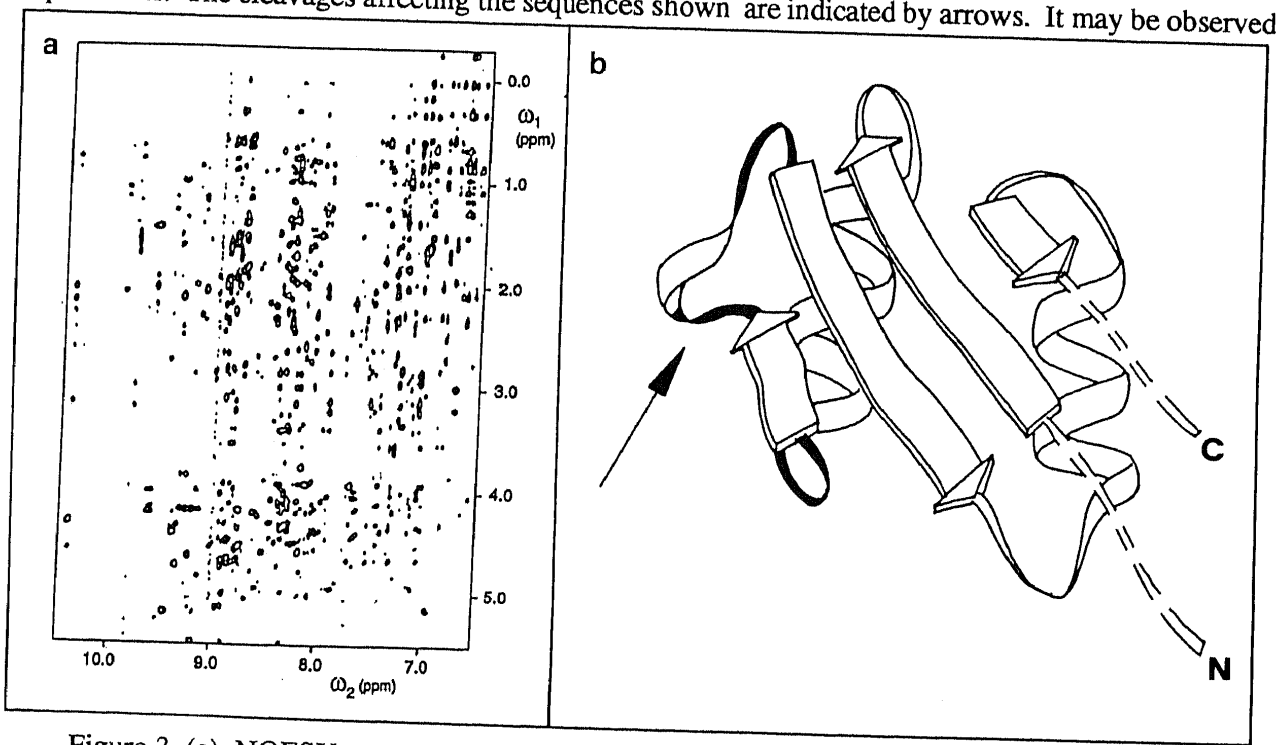

Figure 3. (a) NOESY spectrum of a 3.5 mM solution of activation domain B; the spectral region shown corresponds to the resonances from the amide protons and aromatic protons along ω_2 and to those from the side chain protons (aromatic protons excluded) along ω_1. (b) Schematic drawing of the solution structure of the activation domain B. The disordered ends are shown with dashed lines and the flexible loops with black lines. The flexible loop contacting the enzyme moiety is indicated by an arrow.

that the trimming of the activation segment is more extensive in PCPB than in PCPA. Degradation of the pro-pieces proceeds only from the C-terminal end, since all isolated fragments share the same N-terminal sequence, which coincides with that of the corresponding proenzyme.

NMR solution structure of the activation domain B

The activation domain B (residues 1-81 of the PCPB activation segment) is the limiting peptide which is resistant to further proteolysis released fom PCPB after tryptic activation (see Fig. 4, cleavage B*). A determination of the solution structure of activation domain B was started with the aim of observing how its globular fold was affected by the activation process. Figure 3 shows a part of a NOESY spectrum used for the structure calculations and a schematic view of the global fold. The peptide adopts a well defined backbone fold for residues 11-76, where three short segments are significantly less well defined than the rest of the molecule: 32-35, 39-43 and 56-61 The comparison of the solution structure of activation domain B and the X-ray crystal structure of the same fragment in the intact proenzyme shows that their fold is very similar, with a root-mean-square distance between the backbone atoms of both molecules of 1.0 Å.(27). This observation, together with the fact that only one of the three loops with increased disorder coincides with a region contacting the enzyme domain, indicates that activation domain B (ADB) is inefficient in inhibiting CPB because its interaction with the enzyme domain is not very tight, and that other reasons for a rate-difference in the activation of PCPA and PCPB must be found somewhere else than in the globular domains of the activation segments.

Interpretation of the structural data.

Given the close coincidence of the ADB structures free in solution and in PCPB in crystals, the structural clues for the different activation rates must be searched in the connecting segment which links the ADB with the enzyme moiety. Figure 4 shows a summary of the structural and activation data collected for this fragment. The number and types of contacts observed in the crystals between the connecting segment and

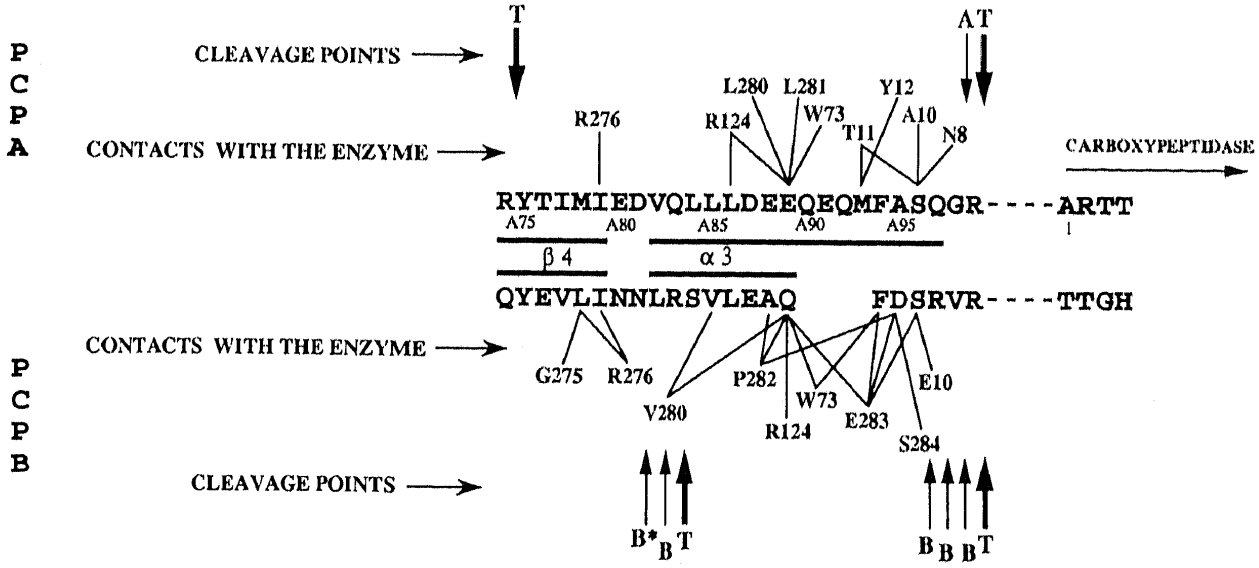

Figure 4. Summary of structural and activation data concerning the connecting region of PCPA and PCPB. The cleavage points observed during activation are indicated by arrows; T refers to trypsin and A and B to CPA and CPB actions, respectively. The observed contacts with residues in the enzyme moieties are indicated above and below the respective sequences. The numbering below PCPA sequence corresponds to that adopted for PCPA activation segment (11). The secondary structure elements identified are shown by bars between the two sequences.

the enzyme are very similar in both cases. However, important differences are observed in the local structure and in the number and localization of the cleaved bonds. Thus, helix 3 in PCPB is two turns shorter than in PCPA, and the number of cleavage points at the C-terminal of the activation segment is larger for PCPB, probably due to both the lack of secondary structure (residues SerA96-ArgA99) and to CPB action. We propose that, given the lack of local structure for the last residues, the first tryptic cleavage is able to promote a rapid unfolding of the connecting segment in PCPB . The cooperative action of CPB may help to accelerate the process and thus the global interaction is disrupted. In contrast, the PCPA activation segment is held in position by a stable local structure (observe in Fig 4 that the residues GluA89, MetA93 and SerA96, which contact the enzyme, are in a α helix), and this may prevent the nascent CPA molecules to extensively collaborate in its degradation. Finally, the second trypsin target in PCPA is located in a tight secondary structure that makes its access difficult. In summary, the connecting segment is required for both proper binding of the activation domain to the enzyme and the global inhibition of carboxypeptidases in the A and B proenzymes.

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