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The activation pathway of procarboxypeptidase B from porcine pancreas: Participation of the active enzyme in the proteolytic processing

VIRTUDES VILLEGAS, JOSEP VENDRELL, AND FRANCESC X. AVILÉS

Departament de Bioquímica i Biologia Molecular, Unitat de Ciències and Institut de Biologia Fonamental, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

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Abstract

The activation process of porcine pancreatic procarboxypeptidase B (pro-CPB) has been studied in detail by a number of complementary methodologies, and a description of the molecular events that lead to the generation of active carboxypeptidase B (CPB) has been deduced. The generated CPB participates in the degradation of its own activation segment by excising C-terminal residues from fragments produced by tryptic proteolysis. The trimming action of CPB is, however, not essential for the release of a fully functional enzyme, in contrast to what was previously reported for porcine procarboxypeptidase A (pro-CPA). In the model presented here, the activation process is solely dependent on the first tryptic cleavage, at the limit between the activation segment and the enzyme region, and the former piece loses all of its inhibitory capacity once severed from the proenzyme. The use of heterologous inhibitors of CPB activity during the study of the tryptic activation process of pro-CPB has been required for the capture of short-lived, otherwise nondetectable, intermediates. This has allowed a complete description of the process and shown that the first proteolytic action of trypsin can also take place on a second target bond. Structural considerations that take into account the three-dimensional structures of the A and B forms of the proenzymes lead us to propose that the differences in conformation at the region that connects the globular activation domain to the enzyme are the main responsible elements for the differences observed in the activation processes of both proenzymes.

Keywords: activation domain; activation segment; inhibition mechanism; limited proteolysis; procarboxypeptidase; zymogen

Porcine pancreatic secretion contains considerable amounts of the precursor forms of the two major pancreatic carboxypeptidases: A1 and B. Procarboxypeptidase A1 (pro-CPA1) is found in the monomeric state and as a member of a binary complex with proproteinase E, whereas procarboxypeptidase B (pro-CPB) is found only in the monomeric state (Avilés et al., 1993). The oligomeric association is the most common occurrence of pro-CPAs (Yamasaki et al., 1963; Uren & Neurath, 1972; Kobayashi et al., 1978; Oppezzo et al., 1994), and it has been observed that the quaternary structure affects their activation behavior (Puigserver & Desnuelle, 1977; Kobayashi et al., 1981; Puigserver et al., 1986; Chapus et al., 1987). No complex with proteinase precursors has yet been described for the B form. The presence of the two monomeric zymogens in porcine pancreas makes this system suitable for comparison studies on their activation processes.

The activation process of pancreatic pro-CPs stands out among those of other pancreatic zymogens because of the length of the N-terminal pro-pieces (also called activation segments): 94 residues for the A1 form and 95 residues for the B form; other important digestive zymogens (e.g., trypsinogens or proelastases) have much shorter activation pro-pieces. This differential characteristic suggests that the activation peptides of pro-CPs might provide the conformational elements that modulate the activation process of these proenzymes.

Sequential data are available for a number of pro-CPs from different species (for a review, see Avilés et al., 1993). On average, the percentage of sequence identity between pro-CPA1 (403 residues) and pro-CPB (402 residues) is about 45%. However, this degree of homology decreases to only 31% when the sequences of the activation segments are compared (Burgos et al., 1991). The 3D structures of the A1 and B porcine proenzymes have been deduced from X-ray diffraction studies on sin-

Reprint requests to: Francesc X. Avilés, Departament de Bioquímica i Biologia Molecular, Unitat de Ciències and Institut de Biologia Fonamental, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

Abbreviations: BZS, benzylsuccinic acid; CP, carboxypeptidase; PCI, potato carboxypeptidase inhibitor; MMGETP, 2-mercaptomethyl-3-guanidinethylthiopropanoic acid.

gle crystals (Coll et al., 1991; Guasch et al., 1992), and the solution structure of the globular domain of the pro-CPB activation segment has been derived from NMR spectroscopy (Vendrell et al., 1991). Comparison of the 3D structures shows that the structural divergence in the activation segment regions is higher than in the enzyme regions and suggests that the former are independent folding units.

The activation processes of porcine pancreatic pro-CPA1 and pro-CPB have been studied previously in some detail (Vendrell et al., 1990; Burgos et al., 1991). Both proenzymes are readily activated by trypsin through a proteolytic cleavage that initially gives rise to the separated enzyme and activation segment moieties. However, the kinetics of activity generation are very different, the activation of the B form being much faster than that of the A1 form. On the other hand, pro-CPB is absolutely devoid of intrinsic enzymatic activity, whereas pro-CPA1 is able to catalyze the hydrolysis of small synthetic peptides (Lacko & Neurath, 1970; Reeck & Neurath, 1972).

We have undertaken a detailed study of the proteolytic activation process of procarboxypeptidase B in the presence and in the absence of inhibitors with the double aim of understanding the sequence of molecular events that occur during the process and that of demonstrating the hypothesized involvement of the active enzyme on the degradation of its own activation propiece. The results are compared with the activation process of pro-CPA1 and discussed with reference to the 3D structure of the proenzymes.

The work presented here completes a description of the differential behavior shown by porcine pancreatic pro-CPA and pro-CPB after activation by limited proteolysis. Both models should serve as a reference for understanding similar processes that affect other pro-CPs present in the pancreas of different species or in different tissues.

Results

Generation of CPB activity

The action of trypsin on pro-CPB at 37 °C and pH 7.5 is very quick given that 100% activation of pro-CPB is achieved in 2 min at a 40/1 pro-CPB/trypsin ratio (w/w), in agreement with previous studies (Burgos et al., 1991). To facilitate the characterization of the activation course, as well as the identification and isolation of intermediates, the present study was carried out at 0 °C and at a 400/1 ratio. Under these conditions, the activation course is monotonic and the step pro-CPB → CPB can be fitted to pseudo-first-order kinetics. However, the expected activation intermediates containing an Arg residue at their C-terminus cannot be detected, presumably because of the trimming action of CPB on the generated peptides. The relative lengths of the peptides detected are presented in Figure 1, together with the sequence around the activation site.

In order to evaluate the participation of the generated CPB in the shortening of the activation fragments, and to identify and isolate a complete set of activation intermediates, specific inhibitors of CPs were added to pro-CPB before its tryptic activation. Organic inhibitors, such as MMGETP (K_I = 2 nM) (Plummer & Ryan, 1981) and BZS (K_I = 1.2 μM) (Zisapel & Sokolovsky, 1974), and the small protein inhibitor PCI (K_I = 5 nM) (Hass & Ryan, 1981), were used for this purpose.

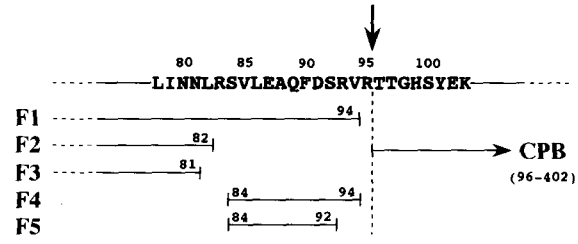

Fig. 1. Scheme representing the relative lengths of the peptides (F1–F5) isolated in the activation of pro-CPB at 400/1 proenzyme/trypsin ratio (w/w). F1–F3 share the N-terminal residue with the proenzyme; the position of their C-terminal residue is indicated. Positions of the two terminal residues are indicated for the shorter fragments F4 and F5. At the top of the figure, the sequence around the activation site is presented to serve as a guide for the interpretation of the results. The vertical arrow indicates the point of the primary tryptic activation. The CPB generated from this cleavage corresponds to residues 96–402 of the proenzyme.

A comparison of the activation processes of pro-CPB in the absence (control) and in the presence of CP inhibitors is provided in Figure 2. The degree of inhibition reached varied with the inhibitor, the protein inhibitor PCI at 0.2 mM being the most

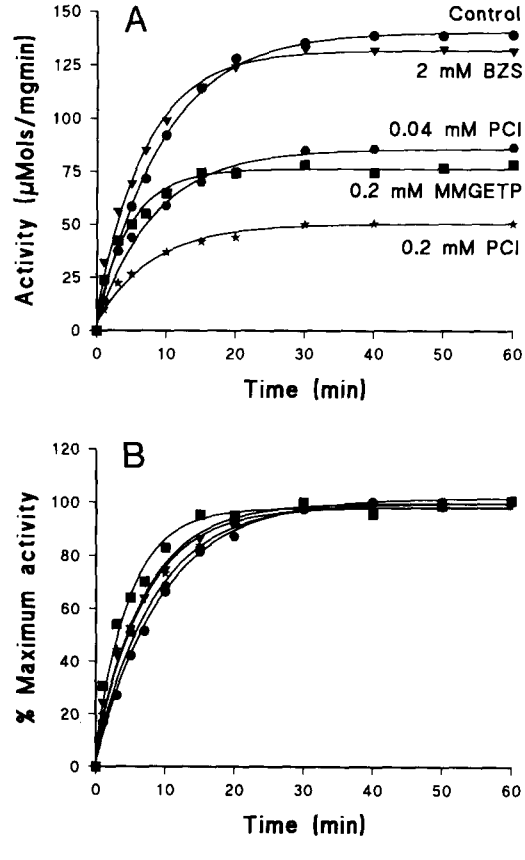

Fig. 2. Time-course generation of CPB activity after tryptic activation in the absence (control) or in the presence of inhibitors. The type of inhibitor used and its concentration is shown at each activity curve. **A:** Experimental activity values versus time after trypsin addition. **B:** Deduced percentages of activity measured relative to the maximum reached in every experiment are represented. Symbols used are the same as in A.

efficient, followed by 0.2 mM MMGETP, 0.04 mM PCI, and 2 mM BZS. There is no evident relationship between the degree of inhibition observed at different concentrations and the inhibition constants because the K_M values for the different substrates generated (i.e., the activation fragments) remain unknown. A complete inhibition of CPB was not reached under the experimental conditions tested because of the dilution required to measure CPB activity, which promotes dissociation of the complex of CPB with the competitive inhibitors used. Part B of Figure 2 shows the relative slopes of the "control" and "CPB-inhibited" activation courses. It can be observed that at initial times there is an acceleration of the generation of relative CPB activity in the presence of inhibitors. This acceleration can be estimated from the differences in the rate constants calculated for the individual curves. Thus, if the rate of the control activation is given a reference value of 1, the relative rate constant values are: 0.04 mM PCI, 1.16; 2 mM BZS, 1.45; 0.2 mM PCI, 1.47; 0.2 mM MMGETP, 2.1.

Electrophoretic analysis of the activation products

The electrophoretic analysis of the activation products in the absence and in the presence of CPB inhibitors gave similar results for the different inhibitors used. Shown in Figure 3 are a control experiment and an activation in the presence of 0.2 mM PCI. The addition of CPB inhibitor to the activation solution promotes an about twofold faster conversion of pro-CPB into CPB. This result is coincident with the steeper slope observed for the activations performed in the presence of inhibitors that have been described in the preceding section.

The molecular species generated during pro-CPB activation are CPB, the 95-residue-long primary activation segment, and the shorter fragments derived from it. The length of those fragments ranges from 81 to 94 amino acid residues for the longer ones and from 9 to 12 residues for the shorter ones, as was subsequently shown by MALDI-TOF mass spectrometry. It is not possible to resolve all the species generated by SDS-electrophoresis in the presence of urea. However, the fragments that will from now on be referred to as "F1" (92–95 residues in length), and those referred to as "F2" or "F3" (ranging from 81 to 83 amino acid residues) can be distinguished by electrophoresis and are identified as such in Figure 3. It is apparent from the figure that the relative mobility of F1 to F3 is reversed compared to what would be expected from their relative molecular masses. It is also evident that the transformation of fragments F1 into fragments F2–F3 is about twofold faster in the presence of inhibitors. The joint results of this and the preceding section seem to suggest that the addition of inhibitors might shift the position of the activation segment within the proenzyme, thus yielding an improved accessibility of target bonds to trypsin and accelerating the activation process.

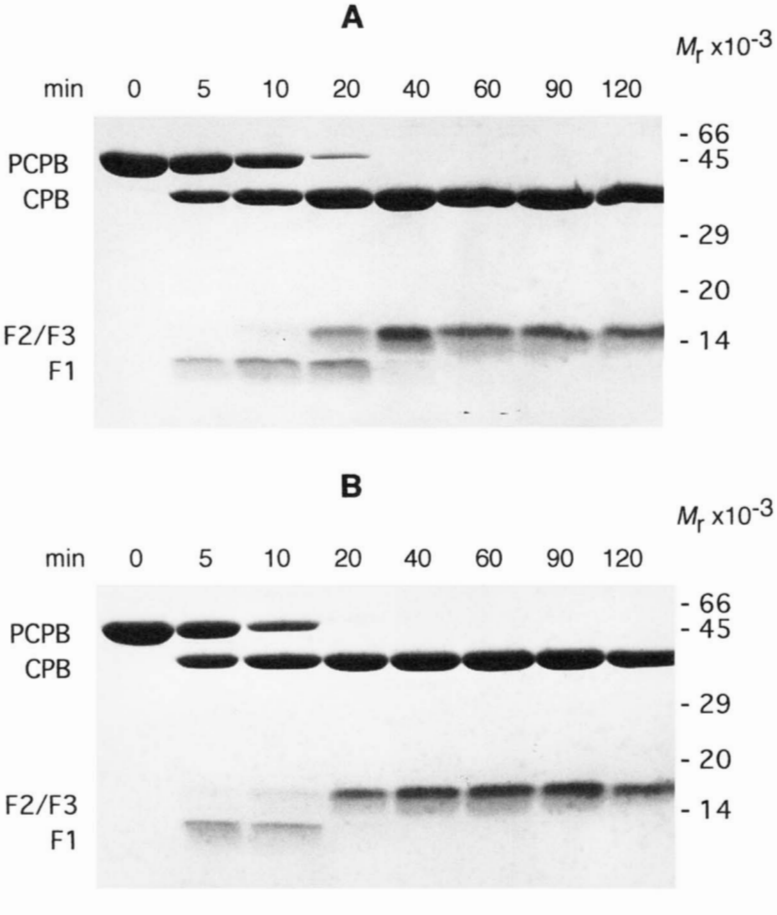

Fig. 3. Electrophoretic analysis of the activation process of pro-CPB by trypsin. **A:** Control activation. **B:** Activation in the presence of PCI at 0.2 mM. Samples were analyzed in SDS/7 M urea polyacrylamide gels. Species present in the activation mixtures are identified on the left side of the gel. Because F2 and F3 have the same electrophoretic migration, the band corresponding to these species is labeled F2/F3 in both experiments; however, no F3 is detected in the presence of inhibitors (see text for details). On the right site, the relative masses of molecular weight markers run in parallel are indicated.

Detection and purification of the activation products

Analysis of the products generated during the activation in the absence or in the presence of different inhibitors was performed by reversed-phase HPLC. Semipreparative reversed-phase HPLC was also employed for the isolation of the individual species.

Figure 4 shows the activation segment fragments detected in an activation in the absence (A) or in the presence (B) of inhibitors. None of the five activation fragments that may be observed and isolated during an activation in the absence of inhibitors (see Fig. 1) contains an Arg at its C-terminus, a fact presumably due to the rapid action of CPB on the newly generated fragments. The number of species detected in the presence of inhibitors is considerably increased, and one of these subfractions, F1α, was found to be the primary activation segment (residues 1-95). Our previous observations on the activation process of pro-CPA (Vendrell et al., 1990) showed that the release of the C-terminal Arg 94 from the primary activation segment was an important step in the activation. This is in contrast with the results of the present study because the generated full-length activation segment, F1α, does not behave as an inhibitor of CPB activity nor does it remain bound to the enzyme once it is released from the proenzyme by tryptic cleavage, as confirmed by electrophoretic analysis of activation mixtures in acrylamide gels with a transverse urea gradient (results not shown). This demonstrates that the activation process is solely dependent on the first tryptic cleavage.

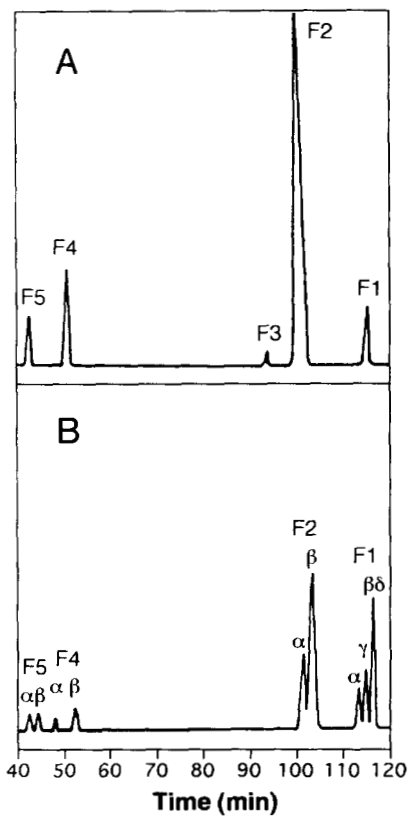

Fig. 4. Chromatographic profiles of the reverse-phase HPLC analysis of a control activation at 40 min (**A**) and an activation in the presence of 0.2 mM PCI at 20 min (**B**). Labeling of the different species and the chromatographic conditions are explained in the text.

The chromatographic follow-up of the activation time-course allowed us to define the kinetics of generation and transformation of the different activation fragments. A general discussion on the transformations observed depends on the individual characterization of all the species and we shall discuss this later on. However, some observations deserve a commentary at this point: (1) All the intermediates of the F1 group have been detected in all the activations carried out in the presence of inhibitors, although their relative quantities vary depending on the inhibitor and its concentration. Thus, the relative quantities of forms F1β (1-94) plus F1δ (1-92) observed in the activation carried out in the presence of 0.04 mM PCI were significantly greater than those observed when PCI concentration was 0.2 mM. These two fragments are a product of CPB action on F1α and F1γ, respectively (see below), and the relative quantities reflect the greater similarity between the less strongly inhibited experiment and the control. The commented differences in yield of the individual fragments were of help in their preparative isolation and further analysis. (2) The chromatographic analysis confirmed the acceleration in the generation of free enzyme observed using other methodologies. (3) As seen by chromatographic analysis, the transformation of fragments F1 into fragments F2 is also accelerated in the presence of inhibitors. (4) F3, the globular activation domain stable to further proteolysis (Vendrell et al., 1991), was not generated from F2 even in the poorly inhibited activations (0.04 mM PCI and 2 mM BZS), a demonstration that it is generated only through CPB action in the absence of inhibitors.

Characterization of the activation products

As stated in the preceding section, the use of inhibitors permitted the observation and isolation of a number of fragments differing in only one or two amino acid residues. Mass spectrometric analysis (MALDI-TOF) was used for the characterization of the molecular masses of the activation segment fragments, whereas capillary electrophoresis was used to establish the number of CPBs generated, which were also characterized by N-terminal sequence analysis.

Table 1 shows the results obtained in the mass spectrometric analysis of the isolated activation fragments. Two expected relative masses are given for each species, corresponding to a simple and a double protonization. The reason for labeling α to δ of F1 fragments and α and β of F2 fragments is now apparent: they describe molecular species that are shorter by a single amino acid residue than the species preceding them alphabetically. Fragments that could not be resolved individually by HPLC could be observed and distinguished by MS. An example is shown in Figure 5A, in which trace amounts of F1δ are detected in a control activation, and in Figure 5B, where fragments F1β, F1γ, and F1δ, which sequentially differ in only one amino acid residue, are clearly identified. The comparison of the HPLC and MS results was determinant in the unambiguous assignment of the molecular species to a chromatographic elution time.

Concerning the small peptides not shown in the table (F4 and F5), only the relative mass of F4α was determined and the exact theoretical value was obtained (1,406). All attempts to purify F5α failed because of the small amounts accumulated during the

Table 1. *Relative molecular masses derived by MALDI-TOF mass spectrometry analysis of the purified fragments isolated by HPLC from activation mixtures*[a]

Fragment analyzed	M_r expected	M_r obtained	Fragment identified as
F1α	11,148	**11,148**	Primary activation segment
	5,574	**5,575**	(residues 1-95)
F1γ	10,893	**10,891**	Fragment 1-93
	5,447	*5,455*	
		11,147	Slight contamination with
		5,575	F1α, due to close elution
F1b-δ	10,992	**10,984**	Fragment 1-94
	5,496	**10,728**	Fragment 1-92
	10,737	**10,889**	Slight contamination with
	5,369	*5,468*	F1γ, due to close elution
F1 (control)	10,992	**10,984**	Fragment 1-94
			(also referred to as F1β)
F2α	9,760	*9,746*	Fragment 1-83
	4,880	*4,886*	
F2β	9,604	**9,601**	Fragment 1-82
		9,765	Slight contamination with
			F2α, due to close elution
F2 (control)	9,604	**9,600**	Fragment 1-82
			(also referred to as F2β)
F3 (control)	9,490	*9,508*	Fragment 1-81
			(globular activation domain)
F4α	1,406	**1,406**	Fragment 84-95

[a] Fragments were obtained from either an activation in the presence of 0.2 mM MMGETP stopped at 10 and 30 min, or a control activation stopped at 30 min. F3 was individually obtained as described in the Materials and methods. Two relative masses are presented, corresponding to the mono- and biprotonated species. Values are omitted in those cases where the biprotonated species was not detected. Numbers are shown in italics when the value obtained deviates more than 0.1% from the theoretical value expected.

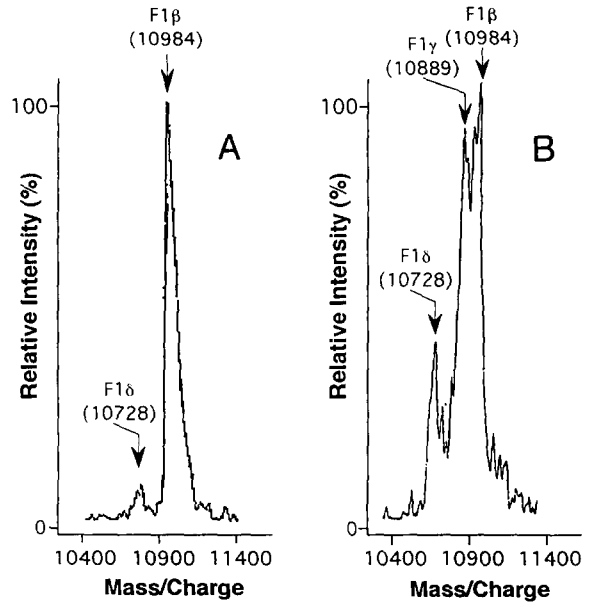

Fig. 5. MALDI-TOF mass spectrometric analysis of HPLC-isolated activation segment fragments. Samples of 0.5 μL in a mixture of water-synapinic acid (1:1), typically containing 5 pmol of peptide, were applied and analysis was performed in the reflectron mode. **A:** MS analysis of the chromatographic peak labeled F1 in an activation in the absence of inhibitors (see Fig. 4A). **B:** MS analysis of the chromatographic peak containing a mixture of F1β, F1γ, and F1δ, isolated from an activation in the presence of inhibitors (see Fig. 4B). Labels indicate the relative molecular masses of the fragments.

activation. However, given that F4β and F5β had been previously sequenced (Burgos et al., 1991), it became clear that the only possible precursor of F5β is F5α, as the kinetics of generation/transformation clearly indicates.

Relative masses for the CPB generated were also obtained from MS analyses of activation mixtures at different times, but these results are not shown in the table because their deviation from the theoretical value expected was greater than 0.1%. However, deviations in the mean relative mass value of the CPB generated in the presence of inhibitors suggest the presence of heterogeneity. To test this, capillary electrophoresis was performed on samples taken during activations in the presence or absence of inhibitors. A double and a single peak, respectively, corresponding to the elution time of CPB were detected. To check if the double peak was indicative of the generation of a new CPB species, N-terminal sequence analyses of the membrane-transferred electrophoretic band with migration characteristics of CPB were performed. The following double sequence was obtained:

Thr + Val/Thr + Arg/Gly + Thr/

His + Thr/Ser + Gly/Tyr + His,

which corresponded to the N-terminal sequences Thr 1-Thr 2-Gly 3-His 4-Ser 5-Tyr 6 and Val 94-Arg 95-Thr 1-Thr 2-Gly 3-His 4. Thus, a second CPB is detected in the presence of inhibitors, which is the product of the tryptic activation at a target bond shifted two amino acid residues N-terminally.

Analysis of the amino acids released during activation

The follow-up of released amino acid residues during activation has a key role in the demonstration of the step-by-step transformation of one species into another. Three Arg, one Leu, and one Val were released in a control activation at long activation times. In the presence of all the assayed inhibitors, only three Arg were detected and the release kinetics was slower (see Fig. 6). The null detection of Leu is in agreement with the lack of generation of F3 in the presence of inhibitors. On the other hand, the nonobservation of Val indicates that F1γ, as well as F5α, are generated by tryptic cleavage at Arg 93, and not by carboxypeptidasic transformation from F1β and F4β, respectively, because the enzyme is inhibited and unable to catalyze the release of an amino acid toward which it shows a low specificity.

Discussion

The analysis of the kinetics of generation/transformation of the different activation products, their isolation and characterization, and the quantitation of the free amino acids released

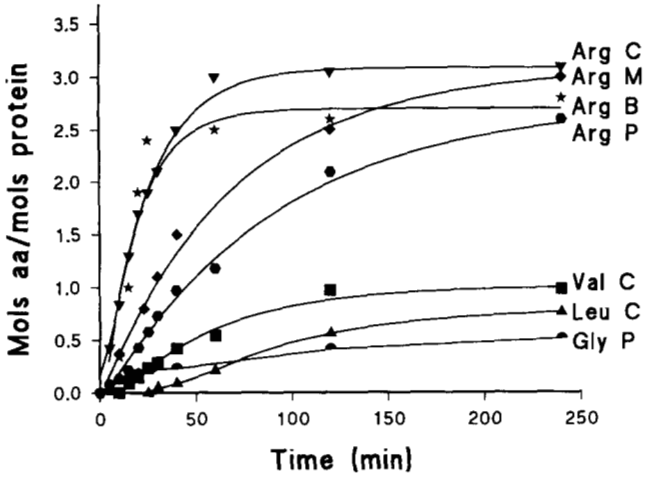

Fig. 6. Quantification of the free amino acids released during the tryptic activation of pro-CPB. The capital letter that follows the amino acid abbreviation refers to the type of activation followed: M, activation in the presence of 0.2 mM MMGETP; P, activation in the presence of 0.2 mM PCI; B, activation in the presence of 2 mM BZS; C, control activation. Note that a Gly is released in the activation carried out in the presence of PCI; this corresponds to the C-terminal residue of the inhibitor, which is cleaved by CPB.

during the tryptic activation process of pro-CPB, both in the absence or in the presence of inhibitors, leads us to propose a model for the molecular events that sequentially take place (Fig. 7).

Five activation segment fragments and one CPB can be detected during the course of an activation in the absence of inhibitors. At long activation times, however, only two species are accumulated: F5β, a nonapeptide, and F3, the only high molecular weight end product. F3 corresponds to residues 1–81 of the activation segment and is a fragment resistant to further proteolytic degradation, even in the presence of high trypsin/protein ratios (Burgos et al., 1991). In the isolated state, F3 folds as a globular stable domain as observed by 2D-NMR spectroscopy (Vendrell et al., 1991). From the scheme representing the control activation, it can be deduced that all the cleavages at the C-terminal ends of the generated fragments are the result of CPB action on them. This is also true for fragment F1δ (residues 1–92), which is not included in the scheme but was detected in small quantities in the MS analysis (see Fig. 5A). This fragment can be the product of CPB action on F1γ, after the secondary tryptic activation that releases F1γ and CPB′ has taken place (see Fig. 7C). In addition to its cleavage of C-terminal Arg residues, CPB is thus able to eliminate a C-terminal Val residue from an undecapeptide (F4β) and also a C-terminal Leu from F2β (82

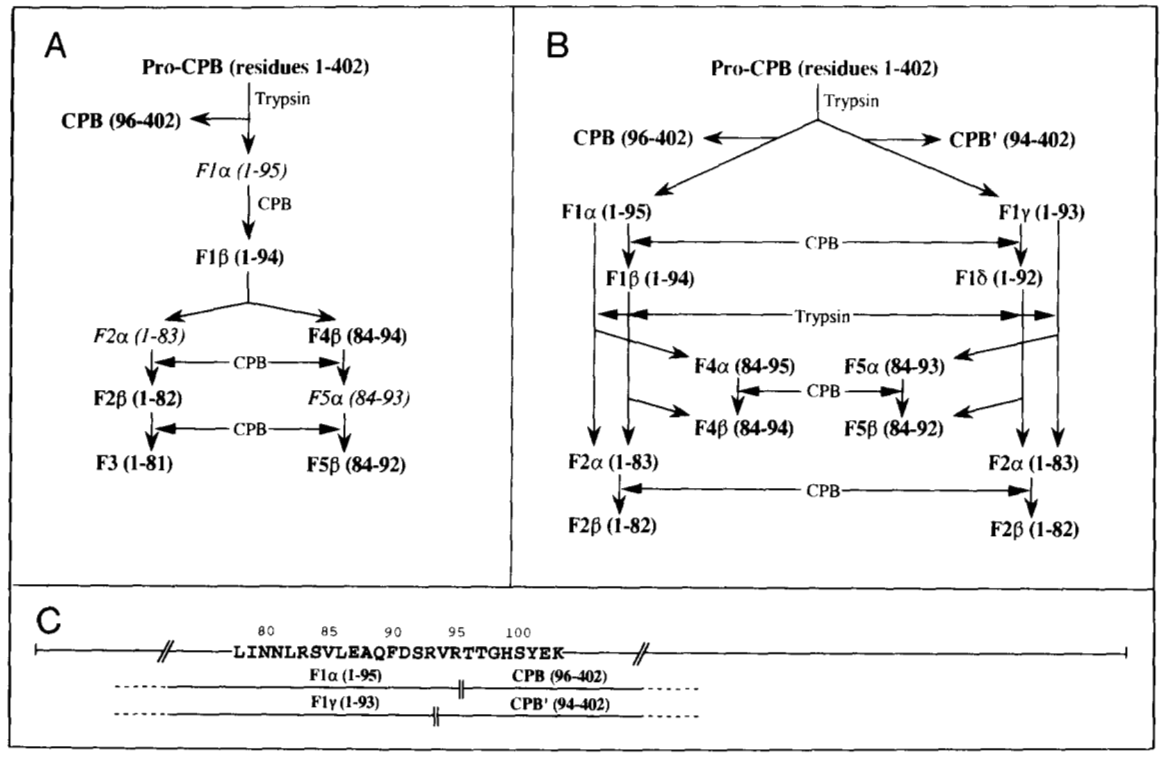

Fig. 7. Schematic view of the sequence of proteolytic cleavages observed during the activation process of pro-CPB. **A:** Control activation. **B:** Activation in the presence of inhibitors. The fragments detected and characterized are shown in bold type. Those shown in italics correspond to species whose presence as intermediates has been deduced but cannot be isolated individually because of their rapid clearance from the medium by CPB action. Fragment F1δ has not been included in the scheme corresponding to the control due to its minor occurrence and is only commented in the text. **C:** Localized view of pro-CPB showing the sequence that links the activation segment and the enzyme moieties and contains all the target bonds cleaved during activation. Below are shown the two sets of initial fragments generated by tryptic cleavage. Numbering is that of the whole proenzyme. Residue 96 of pro-CPB would be residue 1 of CPB in the normal numbering.

residues in length) to generate the two finally accumulated species in a control activation.

All the newly detected fragments in the activation carried out in the presence of inhibitors contained an Arg at their C-terminus. The detection of F1γ proves that there are two "primary" activation segments generated directly from the tryptic activation of pro-CPB, F1α (His 1–Arg 95) and F1γ (His 1–Arg 93). This is only clearly observable in the presence of inhibitors. It had been established that F1β, the only fragment of the F1 type detected in a control activation, was the substrate for trypsin to generate F2 and F4 and that this step was very fast (Burgos et al., 1991). Thus, the minor detection of F1δ in a control activation can only be due to the also minor generation of F1γ and CPB′ and not to transformation from F1β. This finally agrees with another observation: F1β did not disappear earlier than F1δ from the activation mixture, a fact incompatible with a hypothetical precursor character of F1β respective to F1δ. Both fragments F1β and F1δ are substrates for trypsin to generate F2α plus F4β and F2α plus F5β, respectively. The subsequent transformation into F2β, the only high molecular weight product that accumulates in an activation in the presence of inhibitors, is again in charge of CPB itself.

The shorter fragments ending with Arg, F4α (Ser 84–Arg 95) and F5α (Ser 84–Arg 93), are only observed when inhibitors are present, as expected. F4α is generated from F1α, through tryptic cleavage. On the other hand, F4β accumulates in the presence of inhibitors, indicating that it is not the precursor of F5α and F5β, as happens in the control activation. Thus, F5α is originated almost exclusively from F1γ, through tryptic cleavage. This consideration is supported by the fact that, although F4β is generated in larger amounts in the control activations, it disappears more quickly and is not accumulated.

The results presented here demonstrate the participation of CPB in the activation process of its own proenzyme: CPB acts readily on C-terminal Arg residues and also shows an exopeptidase activity on C-terminal residues of fragments from the proregion, toward which it has a low specificity. It is more efficient on short, unstructured peptides than on longer or structured ones. Thus, whereas CPB is very efficient in the elimination of the C-terminal Arg of any of its substrates during activation (F1α, 95 residues long; or F4α, 12 residues long), its ability to excise a C-terminal Val is much higher for F4β (11 residues) than for F1β (94 residues).

A second proteolytic target for tryptic activation has been detected in the experiments performed in the presence of inhibitors, which leads to the generation of two CPB forms: the one described in the literature (Thr 1–Tyr 307, or Thr 96–Tyr 402, if the numbering for the complete zymogen is used), and a second form that is two residues longer at the N-terminus (Val (−2)–Tyr 307, or Val 94–Tyr 402). Consequently, this also leads to the generation of two primary activation segments (F1α, His 1–Arg 95 and F1γ, His 1–Arg 93). The second proteolytic target becomes more accessible when an inhibitor molecule is present at the active site within the proenzyme. The presence of the inhibitor could produce a shift in the position of the activation segment within the proenzyme, originating the new target for trypsin activity. The minor detection of this secondary cleavage in a control activation leads us to propose that the presence of low molecular weight substrates could also originate the exposure of the Arg 93–Val 94 bond. It is known that pro-CPB has no intrinsic activity on small substrates due to a salt bridge formed between Arg 145 in the enzyme and an Asp residue in the activation segment, which renders Arg 145 unable to bind the C-terminal carboxylate of the substrate molecules (Coll et al., 1991). However, substrates could still bind to the rest of accessible free subsites and promote an action similar to that observed in the presence of inhibitors.

In the in vivo situation (digestion at the intestine), the pancreatic zymogens are activated in the presence of high concentrations of peptide substrates, some of them of low molecular weight. These substrates might act on pro-CPB by competing with the pro- segment for the binding to the enzyme moiety, thus distorting the proenzyme structure, as hypothesized for the inhibitors used in this work. Therefore, from our point of view, the activation pathway deduced from the experiments carried out in the presence of inhibitors does not greatly differ from the control and the "physiological" pathways. The use of inhibitors merely permits the detection of real intermediates in the activation process through the effect of slowing down the proteolytic degradation of the generated activation segment.

The tryptic activation of pro-CPB is in many terms different from that of pro-CPA. In the former, the primary nondegraded activation segment neither plays an inhibitory role on the free enzyme nor does it bind to it in a significant way, in contrast to that observed for pro-CPA activation (Vendrell et al., 1990). The release of the C-terminal Arg from the primary activation segment had also been shown to be of importance in the latter case. Now we have seen that the progress of the activation process of pro-CPB is solely dependent on the first tryptic cleavage and not on the trimming action of CPB on the released activation segment.

Under the conditions used in our experiments, and even under more drastic ones, a stable globular domain resistant to proteolysis is generated (residues 1–81). This globular domain, referred to as the activation domain, keeps the same conformation adopted within the proenzyme (Billeter et al., 1992). From our results, it seems clear that the activation domain of pro-CPB should not be considered to have any inhibitory power, because CPB reaches a total activity at times of activation shorter than those needed for the full generation of that domain. Binding studies carried out by electrophoresis in polyacrylamide gels with a transverse urea gradient show that no activation segment fragment is able to interact with CPB.

We conclude that the main differences observed between pro-CPB and pro-CPA proteolytic activation courses are not related with the structural characteristics of their activation domains, but only with the regions connecting them to the enzyme moiety. This connecting region, which folds partially as an α-helix, is longer and more structured in pro-CPA, with about two more helical turns in this case (Guasch et al., 1992). At the same time, the second tryptic target within the activation segment is less accessible in pro-CPA. These differences could explain why the release of the activation segment, or shorter fragments derived from it, is less efficient in pro-CPA, favoring a continued interaction with the enzyme and making the structural relaxation needed for full release (and for full activation) slower. The trimming action of the generated CPs on the connecting region is therefore much more important in the generation of activity from pro-CPA than from pro-CPB. These two models for the activation of pancreatic pro-CPs may help to understand the differential proteolytic maturation process of other monomeric and oligomeric pro-CPs.

Materials and methods

Preparative isolation of pro-CPB

Porcine pro-CPB was isolated from acetone pancreatic powder by anion-exchange chromatography on DEAE sepharose according to a previously reported procedure (Vilanova et al., 1985b; Burgos et al., 1991). As a last purification step, an additional FPLC chromatography on an Ultrapac TSK-DEAE column 5PW (150 × 21.5 mm, 10 μm particle size, 0.1 μm pore, from Toyoshoda) in 20 mM Tris-acetate, pH 8.0, with an elution gradient between 0 and 0.8 M ammonium acetate, was included.

Proteolytic activations of pro-CPB

Pro-CPB at 1 mg/mL in 50 mM Tris-HCl/1 μM $ZnCl_2$, pH 7.5, was treated with trypsin (TPCK-treated from Worthington) at a 400/1 ratio (w/w) and at 0 °C. Several activation mixtures were prepared: (1) control (no inhibitors added); (2) in the presence of 0.2 mM MMGETP (Calbiochem) (Plummer & Ryan, 1981); (3) in the presence of 2 mM BZS (Sigma) (Zisapel & Sokolovsky, 1981); and (4) in the presence of PCI (Sigma) (Hass & Ryan, 1981) at concentrations of 0.04 or 0.2 mM. MMGETP and PCI were previously dissolved in water and BZS in ethanol. In order to correct for the changes on the dielectric constant between the several activation mixtures, equal amounts of pure ethanol or ethanol-dissolved inhibitor were added to all of them.

At given times after trypsin addition, aliquots were removed for activity measurements, for electrophoretic, HPLC, and mass spectrometry analysis, and for the quantitation of the released amino acids. For activity measurements, 10 μL of the activation mixture were added onto 190 μL of aprotinin (BPTI) at 0.1 mg/mL in 20 mM Tris/0.1 M NaCl, pH 7.5, and 10 μL of this new mixture were used to carry out spectrophotometric activity measurements with the substrate benzoyl-glycyl-L-arginine (BGA, Sigma) according to Wolf et al. (1962). The experimental data were fitted to a monoexponential curve and a correlation coefficient better than 0.99 was obtained in every case.

For electrophoretic analysis, 20 μL of the activation mixture were mixed with 2 μL of TLCK (Sigma) at 22 mM in water to reach a final trypsin inhibitor concentration of 2 mM. Each sample was immediately mixed with electrophoretic loading buffer (containing 1% SDS and 3% β-mercaptoethanol), heated at 100 °C for 1 min, and stored at −20 °C until its analysis. Electrophoresis was carried out in polyacrylamide gels in the presence of 0.1% SDS/7 M urea, according to Hashimoto et al. (1983).

For HPLC analyses, 60-μL samples (for analytical studies) or 1-mL 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.

For the quantitation of the amino acids released into the activation mixture, 90-μL samples were removed (2 nmol of initial pro-CPB) and mixed with trifluoroacetic acid up to a final concentration of 0.5%. 1.5 nmol of norleucine were added as quantitative reference before the addition of three volumes of ethanol in order to precipitate proteins and peptides. The supernatant was lyophilized and analyzed for amino acid composition.

Chromatographic analysis and separation of the activation products

Samples removed from the activation mixtures were analyzed by reverse-phase HPLC on Vydac C4 supports. A 214TP54 column (250 × 4.6 mm, 5 μm particle-size, 0.3 μm pore) was used in analytical studies, and chromatographies were performed in 0.1% trifluoroacetic acid with an eluting linear gradient between water (solvent A) and acetonitrile (solvent B), according to the following steps: 10% B from 0 to 10 min and 52% B at 130 min. A 214TP1010 column (250 × 10 mm, 10 μm particle size, 0.3 μm pore) was used in semipreparative runs. The chromatographic solvents and conditions were the same used with the analytical column. For the isolation of the individual activation fragments the activation in the presence of 0.2 mM MMGETP was selected. Fragments identified as F1 and F2 were isolated from a sample taken at 10 min activation, and fragments identified as F4 and F5 were purified from a 30-min activation sample. A control experiment was also performed and analyzed at 30 min of activation. Purified fragments were finally lyophilized and kept at −20 °C until needed.

Fragment F3 was obtained from pro-CPB by limited proteolysis carried out at 25 °C and at a 4:1 pro-CPB/trypsin ratio (w:w) as described previously (Burgos et al., 1991).

Binding studies

A control sample of 900 μL was trypsin-activated during 10 min and proteolytic action was stopped by the addition of 90 μL of TLCK (Sigma) to reach a final inhibitor concentration of 2 mM. The sample was immediately mixed with electrophoretic loading buffer (containing 7 M urea), heated at 100 °C for 1 min, and analyzed in a polyacrylamide gel with a transverse gradient of urea (0–9 M), as described previously (Vilanova et al., 1985a).

Mass spectrometry

HPLC-purified fragments were dissolved in water to reach different concentrations. One-tenth of each sample was chromatographed under the same conditions used for isolation to assess its purity and the rest was mixed with an equal volume of synapinic acid. Samples of 0.5 μL were analyzed on a MALDI-TOF-MS system (Karas & Hillenkamp, 1988; Hillenkamp et al., 1991).

Capillary electrophoresis and N-terminal sequence analysis of the generated CPB

CPB samples were obtained from a control activation and from an activation in the presence of 0.2 mM MMGETP. In order to analyze the generated CPB species by capillary electrophoresis (CE), 25-μL samples were removed at different times and tryptic action was stopped by addition of an equal volume of 100 mM sodium phosphate/4 M urea (pH 2.5). Samples containing 12 ng of protein were analyzed on a Beckman P/ACE System 2210. Capillary zone electrophoresis (500 mm × 75 μm) was used to perform the analysis at 15 kV during 35 min at 25 °C.

The generated CPB was also analyzed for its N-terminal sequence. Samples of 25 μL were taken from the activation mixture and TLCK was added to reach a final concentration

of 2 mM. Each sample was immediately mixed with electrophoretic loading buffer (containing 1% SDS and 3% β-mercaptoethanol), heated at 100 °C for 1 min, and loaded onto a polyacrylamide gel according to Laemmli (1970). Proteins were transferred onto a PVDF membrane at 100 V during 30 min as described by Towbin et al. (1979). N-terminal sequence analyses of CPB-containing bands were performed on a Beckman LF3000 Protein Sequencer.

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