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Relationship between endo- and exopeptidases in a processing enzyme system: Activation of an endoprotease by the aminopeptidase B-like activity in somatostatin-28 convertase

(brain cortex/basic amino acid pairs/peptide substrates/protease inhibitors/prohormone maturation)

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ABSTRACT **The somatostatin-28 convertase activity involved *in vitro* in the processing of somatostatin-28 into the neuropeptides somatostatin-28-(1–12) and somatostatin-14 is composed of an endoprotease and a basic aminopeptidase. We report herein on the purification to apparent homogeneity of these two constituents and on their functional interrelationship. In particular we observed that after various physicochemical treatments, the 90-kDa endoprotease activity was recovered both at this molecular mass and as a 45-kDa entity. Moreover, the production of [Arg^{-2},Lys^{-1}]somatostatin-14 from somatostatin-28 by the action of the endoprotease was activated in a cooperative manner by the aminopeptidase B-like enzyme. A 10-fold activation occurred when the exopeptidase was inhibited by 6.5 mM diisopropyl fluorophosphate and allowed the determination of a half-maximal activation constant ($K_{1/2}$) of ≈13 nM. These observations strongly suggest that both enzymes act in a concerted manner *in vitro* and that they may form a complex *in vivo*.**

Peptide hormones and neuropeptides are released from higher molecular weight precursors by enzymatic cleavage. Examination of prohormone primary structures indicate that the most common cleavage sites minimally consist of pairs of basic amino acids (1, 2) that may reside in β-turns in the precursor (3). Enzyme activities that cleave synthetic peptides containing partial sequences of the prohormone either at a pair of basic residues (4–6) or at a single (7) basic residue have been described. When the cleavage site is a pair of basic amino acids, cleavage has been observed after, between, or before the pair (3). In most cases the peptide fragments released after processing do not contain the original arginine or lysine residue(s) of the cleavage site. Thus, the release of these final forms, from which extra basic amino acids have been removed, has to involve either an endopeptidase activity alone or both endopeptidase and exopeptidase B-like activities (1, 2, 8). The processing enzymes that have been described appear to possess only one of the two required activities, suggesting that two enzymes are needed to completely excise the bioactive fragments (9). Only a few of these enzymes have been purified to homogeneity (10, 11).

The somatostatin precursor is a particularly suitable model because of its relative simplicity. In mammals a single, 92-amino acid prohormone appears to be encoded by a single gene whose sequence is highly conserved between rodents and humans (12–14). During processing, the carboxyl-terminal domain of prosomatostatin (somatostatin-28) (15) is cleaved at an Arg-Lys pair to yield two fragments of known biological activity: the carboxyl-terminal tetradecapeptide somatostatin-14 and the amino-terminal dodecapeptide somatostatin-28-(1–12) (16).

We have described an endoprotease that cleaves the peptide bond on the amino side of the Arg-Lys doublet in the somatostatin-28 sequence (17, 18), releasing the somatostatin-28-(1–12) fragment and [Arg^{-2},Lys^{-1}]somatostatin-14. The released [Arg^{-2},Lys^{-1}]somatostatin-14 is further processed by an aminopeptidase B-like activity (18, 19) that is also present in the preparation. We report herein the purification to apparent homogeneity of these two activities. The study of their functional relationship led to observations on their mutual interactions.

MATERIALS AND METHODS

Somatostatin-28 convertase was isolated from 70 male Wistar rats (100–120 g). After rapid decapitation, cerebral cortices were immediately removed and the freshly dissected tissue was homogenized in a Potter–Elvejhem homogenizer in 50 mM potassium phosphate, pH 7.4/100 mM KCl [10% (wt/vol)]. The extract was then centrifuged for 15 min at 1000 × *g* in a refrigerated Beckman TJ-6 centrifuge, and the supernatant was precipitated at pH 4.7 with 1 M ammonium acetate/5 mM 2-mercaptoethanol, pH 4.5, and centrifuged at 1000 × *g* for 30 min. The recovered supernatant was concentrated to 10% of its initial volume in a Speed-Vac system (Savant) and chromatographed on a Sephadex G-150 column in 0.25 M Tris·HCl, pH 7.5/5 mM 2-mercaptoethanol. The resulting active fractions were diluted 1:1.5 with distilled water, applied to a DEAE-Trisacryl ion-exchange column (IBF Biotechnics, France) preequilibrated in 50 mM Tris·HCl (pH 7.7) containing 50 mM NaCl and 5 mM 2-mercaptoethanol, and eluted stepwise by a discontinuous NaCl gradient (from 50 to 400 mM).

The endoprotease and the aminopeptidases B-like (I and II) activities were eluted from this column (19) in separate fractions, pooled, and concentrated in a Speed-Vac apparatus. Each activity was further purified by HPLC using a TSK G 3000 SW column (7.5 × 600 mm) (LKB) with a buffer containing 50 mM Tris·HCl (pH 7.0), 0.1 M NaCl, and 5 mM 2-mercaptoethanol. The effluent proteins were monitored by UV absorbance at 215 nm.

Enzyme activities in 20-μl aliquots of each fraction were assayed as described (19). Peptide I was used as the substrate to detect endoprotease, and peptide V was the substrate to detect the aminopeptidase B-like activity. The reaction products (i.e., peptides V, VI, and II) were identified by HPLC using the corresponding synthetic standards (19). When somatostatin-28 was used as substrate, the reaction products {i.e., somatostatin-28-(1–12), [Arg^{-2},Lys^{-1}]somatostatin-14, [Lys^{-1}]somatostatin-14, and somatostatin-14} were

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Abbreviation: aufs, absorbance units full scale.

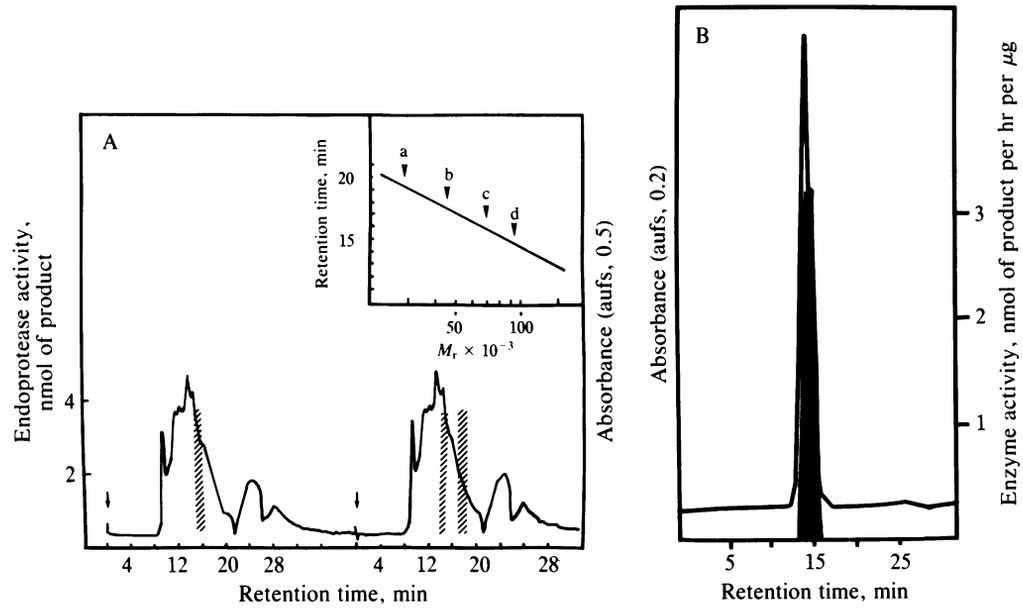

FIG. 1. HPLC elution profile of the endoprotease. (*A*) Fractions 140–160 from the DEAE-Trisacryl column (19) were pooled and concentrated to 1 ml. The left side presents the elution profile of 500 μl applied to a TSK G 3000 SW column and eluted in 0.1 NaCl/5 mM 2-mercaptoethanol/50 mM Tris·HCl, pH 7, at a flow rate of 1 ml/min. The right side presents the elution profile under the same conditions of 500 μl of enzyme, but after salt exposure for 24 hr. An aliquot (20 μl, 250 ng) of each fraction was assayed for enzymatic activity (hatched bars) by incubation with 5 μg of peptide I at 37°C for 5 hr in 200 μl of Tris·HCl (pH 7). The reaction products were analyzed as described (19). —, Absorbance. (*Inset*) Molecular weight calibration of the column. Arrows indicate the retention time of carbonic anhydrase, M_r 29,000 (arrowhead a); ovalbumin, M_r 45,000 (arrowhead b); bovine albumin, M_r 68,000 (arrowhead c), and phosphorylase *b*, M_r 94,000 (arrowhead d). (*B*) Activity recovered from fraction 14 of similar experiments (a total of 15 μg of protein) was applied to the same column and eluted as in *A*. Absorbance at 215 nm and endoprotease activity were monitored (—). Endoprotease activity was expressed as nmol of peptide V produced per hr per μg of protein (solid peak). aufs, Absorbance units full scale.

identified by RIA after HPLC separation as described (19), with antibodies kindly provided by C. Rougeot and F. Dray (Unité de Radioimmunologie Analytique, Pasteur Institute), and R. Benoit (Montreal, Canada). Protein content was determined by the procedure of Bradford (20). The fractions containing the respective activities were pooled, divided into aliquots, and stored at −80°C for further use as purified enzymes.

For gel electrophoresis an aliquot of endoprotease or aminopeptidase B-like activity was diluted in Laemmli buffer (21) or in 1 M Tris·HCl, pH 8.0/10% (vol/vol) glycerol. A Mini-Protean II system (Bio-Rad) was used, with 9% polyacrylamide gels for the $NaDodSO_4$ electrophoresis and with 7% polyacrylamide gels for nondenaturing electrophoresis. Bands were identified by silver nitrate (22) or Coomassie blue (21) staining.

After electrophoresis, the nondenaturing gel was cut into 0.2-cm slices and protein was eluted overnight at 4°C in 0.2 ml of 0.1 M ammonium formate (pH 7.4). The enzyme activities were then assayed on each eluate as described (19).

Somatostatin-28, somatostatin-28-(1–12), and somatostatin-14 were provided by Bachem (Bubendorf, Switzerland). The following synthetic peptides were prepared and analyzed in the laboratory, as described (19), by TLC, HPLC, and fast atom bombardment mass spectrometry, amino acid composition, and amino-terminal sequencing: peptide I, [Ala^{17},Tyr^{20}]somatostatin-28-(10–20)-NH_2 (i.e., Pro-Arg-Glu-*Arg-Lys*-Ala-Gly-Ala-Lys-Asn-Tyr-NH_2); peptide II, [Ala^{17},Tyr^{20}]somatostatin-28-(15–20)-NH_2 (i.e., Ala-Gly-Ala-Lys-Asn-Tyr-NH_2); peptide V, [Ala^{17},Tyr^{20}]somatostatin-28-(13–20)-NH_2 (i.e., *Arg-Lys*-Ala-Gly-Ala-Lys-Asn-Tyr-NH_2); peptide VI, [Ala^{17},Tyr^{20}]somatostatin-28-(14–20)-NH_2 (i.e., *Lys*-Ala-Gly-Ala-Lys-Asn-Tyr-NH_2).

RESULTS

Endoprotease and aminopeptidase B-like activities were purified by differential precipitation, molecular sieve (Sephadex G-150) filtration, and ion-exchange (DEAE-Trisacryl) chromatography (19). The ion-exchange step separated the endoprotease and aminopeptidase activities. The endoprotease activity, eluted from the ion-exchange column with 400 mM NaCl, and further purified on a TSK G 3000 SW HPLC column, had a molecular mass corresponding to 90 kDa (Fig. 1*A Inset*). When the activity was chromatographed again on the TSK column, the enzyme activity eluted with the sharp protein absorbance peak (Fig. 1*B*) at a position corresponding to a molecular mass of 90 kDa. Interestingly, when the endoprotease was exposed to salt for 24 hr [i.e., activity that was collected after ion-exchange chromatography in 400 mM NaCl, concentrated by a factor of 5 (to 2 M NaCl)] and then chromatographed on a TSK HPLC column, the endoprotease

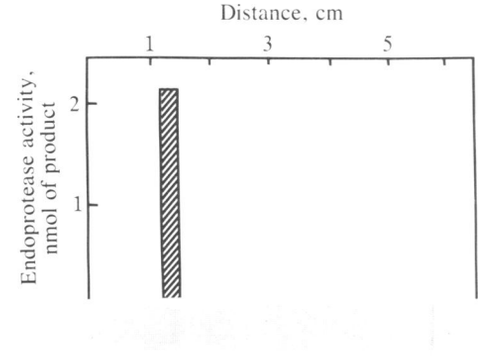

FIG. 2. Gel electrophoresis of the endoprotease. Fractions 14 and 15 obtained from the TSK column were pooled. An aliquot (30 μl, 370 ng) was electrophoresed in a 7% polyacrylamide gel with the Tris/glycine buffer system (27). (*Upper*) The gel was sliced and material was eluted from each 2-mm slice with 0.2 ml of 0.1 M amonium formate (pH 7.4) overnight at 4°C. Each eluate was assayed for endoprotease by incubation at 37°C with 5 μg of somatostatin-28 for 12 hr. The reaction products were analyzed as described (19). (*Lower*) Proteins were detected by the silver stain method (21).

Table 1. Purification of the endoprotease and the aminopeptidase B-like activity from 70 rat brain cortices

Purification step	Volume, ml		Protein, mg		Total activity, nmol·hr^{-1}·mg^{-1}		Specific activity, nmol·hr^{-1}·mg^{-1}		Purification factor		Yield, %	
	Endo	AP	Endo	AP	Endo	AP	Endo	AP	Endo	AP	Endo	AP
Homogenate	350											
Centrifugation (3000 rpm)	295		1425		3920	159,000	2.75	111				
Ammonium acetate precipitation	315		157		1685	73,000	10.73	466.2	3.9	4.2	43	46
Gel filtration (G-150)	125		40		1620	35,000	41.32	875	15	7.0	41	22
Ion exchange (DEAE)	25	33	1.72	2.200	1123	17,900	650	8,160	236	73	20	11
HPLC (TSK)	4		0.05	0.055	170	4,400	3404	85,272	1258	768	4.5	2.7

Endo, endoprotease; AP, aminopeptidase B-like activity. Homogenate activities were not evaluated because the total quantities of peptide substrate could not be recovered. The endoprotease activity was determined by incubation of 5 μg of somatostatin-28 with an aliquot of the enzyme obtained at each purification step. The production of [Lys^{-1}]somatostatin-14 and somatostatin-14 was taken into account with that of [Arg^{-2},Lys^{-1}]somatostatin-14 until the gel filtration step for the evaluation of the endoprotease specific activity. The aminopeptidase activity was determined by incubation of 5 μg of peptide V with an aliquot of the enzymes obtained at the various steps. Peptide II and peptide VI were added to evaluate the aminopeptidase activity.

activity was eluted in two peaks with apparent molecular masses of 90 and 45 kDa (Fig. 1*A*). When endoprotease purified by HPLC was submitted to gel electrophoresis under denaturing conditions, a single band was observed corresponding to 90 kDa (data not shown). When electrophoresis was performed under nondenaturing conditions, a single sharp band was observed that corresponded to the endoprotease activity eluted from the gel (Fig. 2). When the eluted activity was electrophoresed in a NaDodSO$_4$/polyacrylamide gel, it had an apparent molecular mass of 90 kDa (data not shown). Table 1 summarizes the endoprotease purification. The activity was enriched by a factor of 1250, which is probably an underestimate because contaminating unspecific proteolytic activities that degraded both the substrate and the reaction products were present in the crude extract, making the total endoprotease activity difficult to evaluate. Indeed, the rate and extent of unspecific substrate degradation by those proteases were significantly higher than the rate of its selective cleavage by the endoprotease. Therefore, under these conditions, accurate evaluation of its specific activity was impossible.

The aminopeptidase was recovered after elution from the DEAE-Trisacryl column by 150 mM NaCl and further purified by HPLC on a molecular sieve (TSK) column. A single peak was obtained with an apparent molecular mass of 60 kDa (Fig. 3*A*). When the aminopeptidase activity was rechromatographed on the same column, protein absorbance and enzyme activity were coeluted (Fig. 3*B*). The second type of aminopeptidase B-like species, which was eluted with 50 mM NaCl, was unstable during this step. A final purification factor of 768 (Table 1) was achieved, but it is also probably an underestimate because of the presence of nonspecific proteases. Under nondenaturing electrophoretic conditions, the position of the enzyme activity corresponded

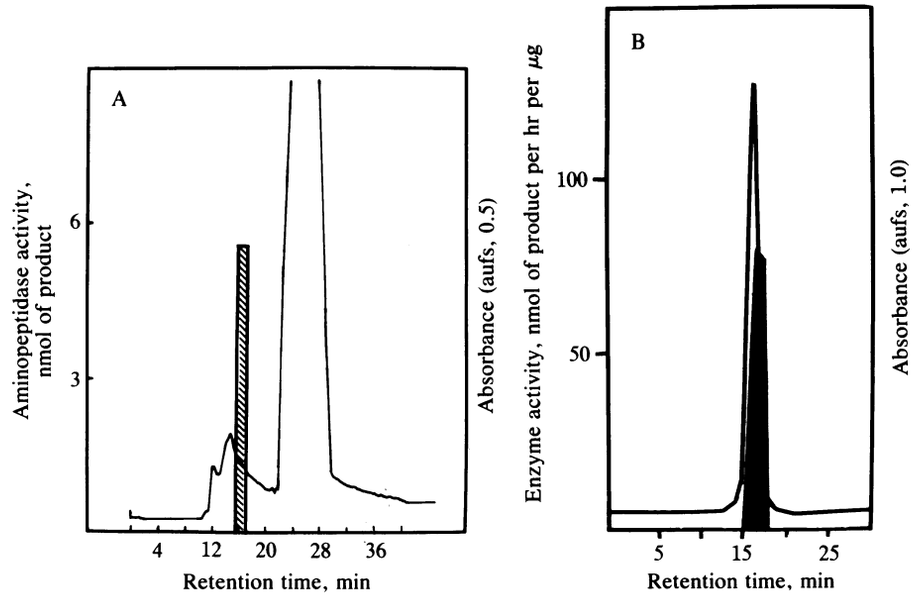

FIG. 3. HPLC elution profile of the aminopeptidase B-like activity. (*A*) Fractions 60–80 obtained from the DEAE-Trisacryl column were pooled and concentrated to 1 ml. Then 500 μl was applied to a TSK G 3000 SW column and eluted in 0.1 M NaCl/5 mM 2-mercaptoethanol/50 mM Tris·HCl, pH 7, at a flow rate of 1 ml/min. The effluent was monitored by absorbance at 215 nm (—). An aliquot (20 μl, 260 ng) of each fraction was assayed for aminopeptidase B activity (hatched bar) by incubation with 5 μg of peptide V, and the reaction products were analyzed as described (19). (*B*) Activity recovered from fraction 16 of similar experiments (a total of 125 μg of protein) (see *A*) was rerun on the same column, and the absorbance profile (—) and aminopeptidase activity (solid peak) were monitored. Aminopeptidase B activity is expressed as nmol of peptide II produced per hr per μg of protein.

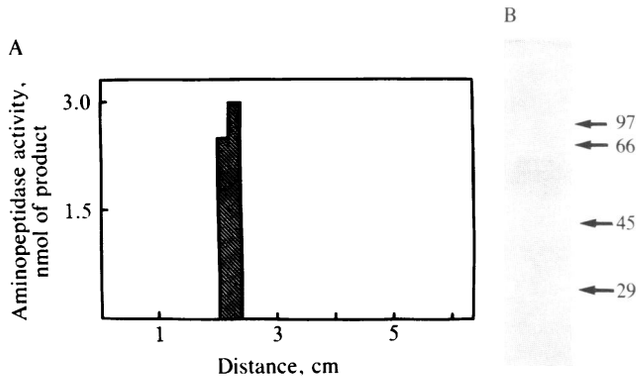

FIG. 4. (*A*) Recovery of the aminopeptidase B-like activity after gel electrophoresis. An aliquot of fraction 16 of the TSK column was electrophoresed on a nondenaturing 7% gel. The gel was then treated as described in Fig. 2. The eluates were assayed for aminopeptidase B by incubation with 5 μg of peptide V for 12 hr at 37°C, and the reaction products were analyzed as described (19). (*B*) Gel electrophoresis of the aminopeptidase activity. Protein with aminopeptidase B-like activity (1 μg) recovered from the rerun of TSK column (Fig. 3*B*) was electrophoresed on a $NaDodSO_4$/9% polyacrylamide gel containing 2-mercaptoethanol. The gel was stained with Coomassie blue. Molecular mass markers are in kDa.

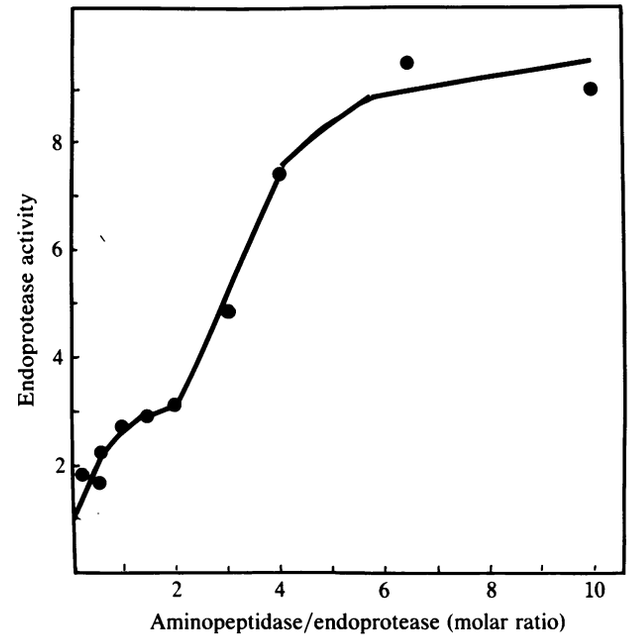

FIG. 5. Dependence of the endoprotease activity on aminopeptidase B-like activity concentration. Somatostatin-28 (5 μg) was incubated with 20 μl (250 ng) of the endoprotease obtained from the TSK G 3000 SW column for 5 hr at 37°C in 200 μl of Tris·HCl (pH 7.0). The reaction products were analyzed on a C_{18} μBondapak column as described (19). The HPLC analysis was followed by RIA (19). The level of production of [Arg^{-2},Lys^{-1}]somatostatin-14 is reported as 1. All the other incubations contained the same reaction mixture but with increasing quantities of aminopeptidase inhibited by diisopropyl fluorophosphate (from 260 ng to 2.6 μg) added. The level of production of [Arg^{-2},Lys^{-1}]somatostatin-14 is expressed relative to the basal level obtained with the endoprotease alone.

to a single sharp band (Fig. 4*A*). Under denaturing electrophoretic conditions, a single band of 60 kDa was found (Fig. 4*B*).

When both the endoprotease and aminopeptidase B-like activities obtained from ion-exchange chromatography, or from HPLC, were mixed together, greater amounts of the reaction products were generated (Table 2). To exclude the possibility that this effect might depend upon the relative rate of aminopeptidase B-like and endoprotease activities, similar experiments were done, but the aminopeptidase B-like activity was completely inhibited by diisopropyl fluorophosphate (Table 2). Under these conditions no further degradation of [Arg^{-2},Lys^{-1}]somatostatin-14 was observed. Moreover, endoprotease action, in the absence of aminopeptidase, was not sensitive to diisopropyl fluorophosphate (Table 2); therefore, the endoprotease activation that was obtained by addition of increasing amounts of the diisopropyl fluorophosphate-inhibited aminopeptidase B-like activity (Fig. 5) could be attributed only to protein–protein interactions. Moreover, this 10-fold activation was achieved at an aminopeptidase B-like activity/endopeptidase molar ratio of 1:6–7. The saturation curve (Fig. 5) yields a value for the half-maximal activation constant ($K_{1/2}$) of ≈13 nM (Fig. 5). Interestingly, bestatin significantly inhibited the endoprotease activity (Table 2), whereas its epi derivative produced 30% inhibition (Table 2).

DISCUSSION

In the purification procedure here described, a significant enrichment of both enzyme activities was obtained. The low final yield might be explained by inactivation during purification. The heterogeneous molecular masses observed for the endoprotease activity upon contact with high salt or under drastic storage conditions, or both, could be explained by assuming that the enzyme possesses active domains with a molecular mass of 45 kDa.

A significant enhancement of the endoprotease activity is produced by interaction with the aminopeptidase B-like

Table 2. Effect of the aminopeptidase on endoprotease activity

Addition(s) to incubation mixture	Peptide produced, nmol				
	Somatostatin-28	[Arg^{-2},Lys^{-1}]-Somatostatin-14	[Lys^{-1}]-Somatostatin-14	Somatostatin-14	Somatostatin-28-(1–12)
None	240	260	—	—	208
iPr_2P-F	228	279	—	—	220
AP (DEAE)	58	198	190	54	332
AP (DEAE) + iPr_2P-F	190	275	—	—	236
AP (TSK)	118	96	74	196	304
AP (TSK) + iPr_2P-F	166	320	—	—	262
Bestatin	432	50	—	—	ND
Epibestatin	299	210	—	—	ND

Somatostatin-28 (5 μg) was incubated with 20 μl (250 ng) of endoprotease (Endo) alone or with the aminopeptidase (AP) active or inactivated by diisopropyl fluorophosphate (iPr_2P-F) (6.5 mM) obtained either from the DEAE-Trisacryl (DEAE) or from the TSK column in 0.3 ml of Tris·HCl buffer (pH 7) for 2 hr at 37°C. The products of reaction were analyzed with a C_{18} μBondapak HPLC column as described (19). The eluted fractions were assayed for their content in somatostatin-28, [Arg^{-2},Lys^{-1}]somatostatin-14, [Lys^{-1}]somatostatin-14, somatostatin-14, and somatostatin-28-(1–12) by RIA.

enzyme. That this activation results from direct protein–protein interactions is inferred from the fact that the process was observed while the aminopeptidase B-like activity was completely inhibited by diisopropyl fluorophosphate. Moreover, a similar activation was observed with an endoprotease preparation from the ion-exchange chromatography or the HPLC step. This reinforces the original conclusion that there is a direct interaction between the enzyme proteins and makes unlikely the possibility that this effect might arise from contaminants copurifying with the aminopeptidase B-like enzyme. However, the mechanisms involved in the modification of endoprotease activity remain to be determined. Kinetic measurements may help to elucidate the molecular mechanism underlying these interactions.

Indeed, the results of these reconstitution experiments argue in favor of a concerted action of both enzymes. Endoprotease and aminopeptidase B-like activity should colocalize to the secretory apparatus, although this remains to be shown experimentally. Some weak interactions with the secretory granule membranes have been reported (23), but these interactions cannot be described precisely, as yet. Cloned cDNAs encoding these enzymes, or their zymogens, could be used to determine whether they possess domains involved in transmembrane traffic.

The $K_{1/2}$ here measured reflects a strong interaction, and allosteric modification of the endoprotease activity could result from such an interaction. Several examples indicate that enzymes can be regulated through selective interactions with protein effectors (24–26). The biological significance of this activation remains to be elucidated, although it is possible that somatostatin-28 convertase is part of a multienzyme complex *in vivo* in which its activity is regulated allosterically by its association with the aminopeptidase. Alternatively, this *in vitro* observation might simply reflect the reconstitution of an *in vivo* system in which neighboring interactions favor a thermodynamically and sterically more efficient process.

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