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THE ACTIVATION PEPTIDE OF PANCREATIC PROCARBOXYPEPTIDASE A IS THE KEYSTONE OF THE BOVINE PROCARBOXYPEPTIDASE A-S6 TERNARY COMPLEX

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In some ruminant species, pancreatic procarboxypeptidase A is the central element of a ternary complex involving two other components, a C-type chymotrypsinogen and an inactive protease E. Although the complex is devoted to protein digestion, the fate of this system upon activation of its constituent subunits has, as yet, not been clearly established. In this paper, the activation peptide of procarboxypeptidase A is shown to play a key role in the association of the three subunits and a model is proposed for the *in vivo* function of the complex.

In ruminant species belonging to the bovidae subfamily, pancreatic procarboxypeptidase A (subunit I, 45 kDa) is secreted in a non covalent ternary complex (procarboxypeptidase A-S6) (1, 2) involving two other proteins, a C-type chymotrypsinogen (subunit II, 28 kDa) (3) and a truncated protease E (subunit III, 26 kDa) (4-6). The nature and strength of the interactions between the subunits as well as the macromolecular organization of the complex have been investigated using various approaches including sequential dissociation (7), small angle X-ray scattering (8), spectrofluorimetry (9), and microcalorimetry (10). Procarboxypeptidase A, the central element of the complex, possesses two topologically different binding sites for the other two subunits (11). No interaction between subunits II and III has ever been detected. The binding between the subunits involves ion pairing and procarboxypeptidase A has a higher affinity for subunit II than for subunit III. However, the binding sites have not been located on the various partners.

Although the complex is devoted to protein digestion, its physiological function remains hypothetical. One possible role of the association could be to prevent denaturation of procarboxypeptidase A by the acidic pHs in the duodenum of ruminants (6). The knowledge of the fate of the complex upon activation of its constituent subunits is an absolute prerequisite for

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Abbreviations

Enzymes: (pro)carboxypeptidase A, EC 3.4.17.1; protease E, elastase, EC 3.4.21.36; chymotrypsin(ogen); EC 3.4.21.1; trypsin EC 3.4.21.4.

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the understanding of the *in vivo* function of this complex.*In vitro*, the fast conversion of subunit II into active chymotrypsin does not result in any dissociation of the complex (12). Moreover the catalytic efficiency of chymotrypsin C is increased upon association with the other subunits (13). By contrast, the fate of the complex upon activation of procarboxypeptidase A is not obvious since the bulky activation peptide (10 kDa) generated by trypsin attack remains tightly bound to the enzyme and is a powerful inhibitor of the newly formed carboxypeptidase A (14). The inhibitory effect of the peptide can be abolished upon chemical treatment, without its removal. In this case no dissociation of the complex is observed (14).

Since Brown *et al.* (1) reported that the appearance of the carboxypeptidase A activity is concomitant to the disaggregation of the ternary complex, we decided to clarify the role of the activation peptide of procarboxypeptidase A in the association of the subunits.

EXPERIMENTAL PROCEDURES

Purification of the various molecular species. The bovine procarboxypeptidase A-S6 ternary complex was purified and dissociated as previously reported (7). The three subunits were further purified according to Kerfelec *et al.* (6). The full-length activation peptide from procarboxypeptidase A was isolated according to Chapus *et al.* (14).

Recovery of fully active carboxypeptidase A. Procarboxypeptidase A was incubated first with trypsin as previously reported (14) and then with elastase [enzyme/substrate 1/25 (w/w)] for 3 hours at 20°C in 10 mM Tris/HCl pH 8.0, containing 5 mM NaCl and 20mM $CaCl_2$. After addition of 0.2 mM PMSF, the incubation mixture was chromatographed on Mono Q using a Fast Protein Liquid Chromatography apparatus (Pharmacia). Elution was performed using a linear NaCl gradient from 0 to 0.5 M (total volume of the gradient, 30 ml; flow rate, 1ml/min). In these conditions, fully active carboxypeptidase A devoid of activation fragments can be obtained.

Activity measurements. The esterase activity of carboxypeptidase A was measured using 1mM hippuryl-L-phenyllactic acid according to McClure *et al.* (15). The peptidase activity of the enzyme was measured using 1mM benzyloxycarbonylglycyl-L-phenylalanine as described by Whitaker *et al.* (16).The specific esterase and peptidase activities were 700 and 27 µmol/min/mg respectively. Chymotrypsin activity was measured using N-acetyl-L-tyrosine ethyl ester as substrate as reported (2). The kinetic parameters were obtained by fitting the experimental data to the Michaelis Menten velocity equation using the Newton-Gauss method.

Molecular sieving experiments. The protein samples were chromatographied on a Superose 12 HR 10/30 column using a Fast Protein Liquid Chromatography apparatus (Pharmacia). Elution was performed using a 10 mM Tris/HCl buffer pH 7.0 containing 1 M NaCl.

Sedimentation experiments. Sedimentation analyses were performed in a Spinco Model E ultracentrifuge equipped with a Schlieren optic. The experiments were carried out at 58083 rpm and 25°C. The protein samples were extensively dialysed at 4°C against a 10 mM Tris/HCl buffer pH 8.0 containing 1 M NaCl before sedimentation analyses. Because of the high salt concentration, the experimental data was corrected for water as the solvent according to Svedberg and Pedersen (17) in order to obtain the s_{w25} sedimentation constants.

Polyacrylamide gel electrophoresis. Electrophoresis in the presence of 0.1 % sodium dodecyl sulfate was carried out in 10-12 % polyacrylamide gels according to Laemmli (18). Proteins were stained with 0.25 % Coomassie brilliant blue and destained in acetic acid/ethanol/water mixture [1.5/1.0/17.5, (v/v/v)].

RESULTS

Influence of the procarboxypeptidase A activation peptide on the association between carboxypeptidase A and the other two subunits (II and III).

Molecular sieving experiments (Fig. 1) were performed using protein concentrations one order of magnitude higher than the dissociation constant values determined by Granon *et al.* (9) and Michon *et al.* (10). The chromatographies were performed in the presence of 1 M NaCl in order to stabilize carboxypeptidase A. As reported (10), this salt concentration does not modify the affinity between the native subunits of the complex. The column was calibrated (Fig. 1a) with a mixture of native ternary complex (10^{-5} M) and native subunit III (10^{-5} M). As expected, two well defined peaks were obtained, the first one containing the complex and the second one, the free subunit III. Chromatography on the same column of a mixture of carboxypeptidase A (10^{-5} M) devoid of any trace of activation peptide, subunit II (10^{-5} M) and a three fold molar excess of subunit III yielded a single large peak (Fig. 1b) which eluted at the same place as the monomeric subunit III. By contrast, when the same experiment was performed in the presence

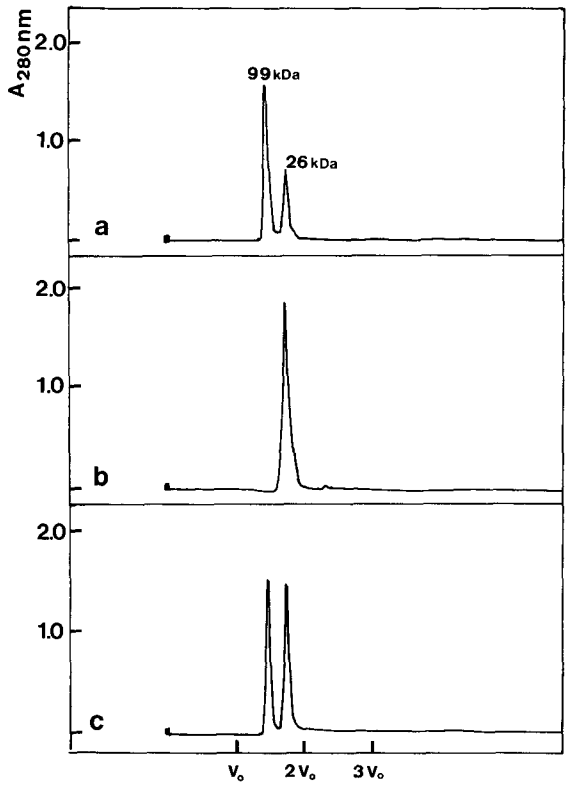

Figure 1. *Molecular sieving of mixtures of subunits II and III either with procarboxypeptidase A or carboxypeptidase A in the presence or absence of its activation peptide.* (a) native ternary complex (10^{-5}M) and native subunit III (10^{-5}M). (b) Mixture of carboxypeptidase A devoid of its activation peptide (10^{-5}M) with subunit II (10^{-5}M) and subunit III ($3x10^{-5}$M). (c) Same experiment as in (b) in the presence of the activation peptide of procarboxypeptidase A (10^{-5}M). For details, see experimental procedures and text.

of the procarboxypeptidase A activation peptide (10^{-5} M) two peaks were obtained (Fig. 1c), one eluted at the same place as the native ternary complex, indicative of a reassociation, and the other one at the same place as monomeric subunit III. Electrophoresis on polyacrylamide gels in the presence of SDS (data not shown) confirmed that the peak corresponding to the reconstituted complex contained the four molecular species (carboxypeptidase A, activation peptide, subunit II, subunit III) whereas the second peak contained the excess of monomeric subunit III.

As shown in Fig. 2a, sedimentation experiments performed on an equimolar mixture of native procarboxypeptidase A, subunit II, and subunit III yielded a single symetrical bell shaped curve. The value of the sedimentation constant (s_{w25} = 5.97 S) is in fairly good agreement with that previously reported for the native ternary complex (9). The same experiment performed on an equimolar mixture of carboxypeptidase A devoid of any trace of its activation peptide and subunit II, and III, yielded an asymetrical bell shaped curve (Fig. 2b). A mean sedimentation constant of s_{w25} = 3.67 S was obtained indicating that no association between the subunits had occurred. Upon addition of an equimolar concentration of the activation peptide (Fig. 2c), a symetrical bell shaped sedimentation curve was again observed. The value of the sedimentation constant (s_{w25} = 5.8 S) corresponds to that of the ternary complex and clearly shows that in the presence of the activation peptide, the reassociation between the subunits had occurred. All the experiments were performed in 1M NaCl in order to stabilize carboxypeptidase A as above mentioned.

Influence of procarboxypeptidase A and carboxypeptidase A on the kinetic parameters of activated subunit II.

The kinetic parameters of the hydrolysis of N-acetyl-L-tyrosine ethyl ester by activated subunit II (C-type chymotrypsin) either in the absence or in the presence of procarboxypeptidase A or carboxypeptidase A are presented in Table I. Whereas carboxypeptidase A has no effect on the kinetic parameters, the presence of procarboxypeptidase A leads to both a slight increase of kcat and a two fold decrease of Km. As a consequence, the catalytic efficiency of activated subunit II is increased two to three fold upon association with procarboxypeptidase A.

DISCUSSION

Whereas the activation of the C-type chymotrypsinogen subunit of the bovine pancreatic ternary complex has been unambiguously shown not to induce any dissociation, the fate of the complex upon procarboxypeptidase A activation has not yet been clearly established. In order to clarify this point which is critical for the understanding of the *in vivo* function of the ternary complex, reassociation experiments between native subunits II and III and carboxypeptidase A either in the presence or in the absence of its activation peptide were carried out. Using either molecular sieving or sedimentation experiments, it was unambigously demonstrated that carboxypeptidase A alone is unable to reassociate with the other two subunits, in contrast to previous observations (11,19) and that its activation peptide plays a key role in the ternary complex formation. However, whether the activation peptide is directly involved in the binding with subunits II and III is still questionable. The discrepancy between our results and previous ones concerning the ability of carboxypeptidase A to reassociate with the other subunits of the complex might result from the difficulty to obtain carboxypeptidase A fully devoid of its activation peptide.

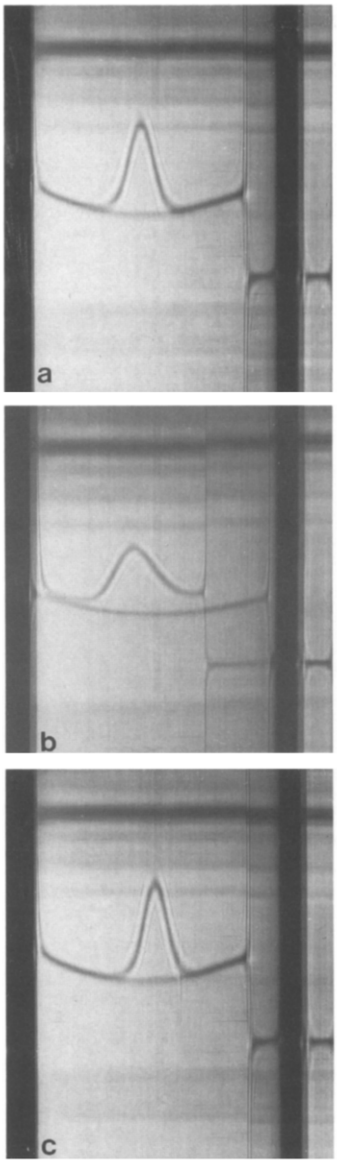

Figure 2. *Sedimentation patterns of the different mixtures after a 76 min run.* (a) Equimolar mixture of procarboxypeptidase A, subunit II and subunit III (10^{-4}M). (b) Equimolar mixture of carboxypeptidase A, subunit II and III (10^{-4}M). (c) Same experiment as in (b) in the presence of the procarboxypeptidase A activation peptide (10^{-4}M).For experimental details, see Experimental procedures.

These results are further supported by the finding that only the zymogen form of carboxypeptidase A has an influence on the enzymatic activity of activated subunit II. Moreover the determination of the kinetic parameters of the hydrolysis of a synthetic substrate clearly demonstrates that the enhanced catalytic efficiency of activated subunit II upon binding to procarboxypeptidase A mainly results from a decrease of the Km value. This is to be correlated

Table I

Kinetic parameters of the hydrolysis of N-acetyl-L-tyrosine ethyl ester by activated subunit II either in the absence or in the presence of carboxypeptidase A or procarboxypeptidase A

Molecular species	kcat (s^{-1})	Km (M^{-1})	kcat/Km (s^{-1} x M)
Activated subunit II alone	47.3 ± 1	(5.3 ± 0.4) x 10^{-3}	8.9 x 10^3
Activated subunit II + carboxypeptidase A	49.5 ± 2	(6.2 ± 0.6) x 10^{-3}	7.9 x 10^3
Activated subunit II + procarboxypeptidase A	53 ± 1	(2.6 ± 0.1) x 10^{-3}	20.4 x 10^3

with previous results showing a more rigid conformation of the active site of subunit II when it is bound to procarboxypeptidase A (9).

This work sheds a new light on the physiological function of the ternary complex. As illustrated in Fig. 3, a two-step process can be postulated. In a first step, trypsin attack of the native ternary complex results in the activation of C-type chymotrypsinogen. Simultaneously, the cleavage of the Arg_{94}-Ala_{95} bond of procarboxypeptidase A occurs without release of the activation peptide which is a powerful inhibitor of carboxypeptidase A. Consequently, only the endopeptidase activity of the complex is expressed. During this step, the subunits remain associated leading both to an increase of the endopeptidase activity and to the protection of

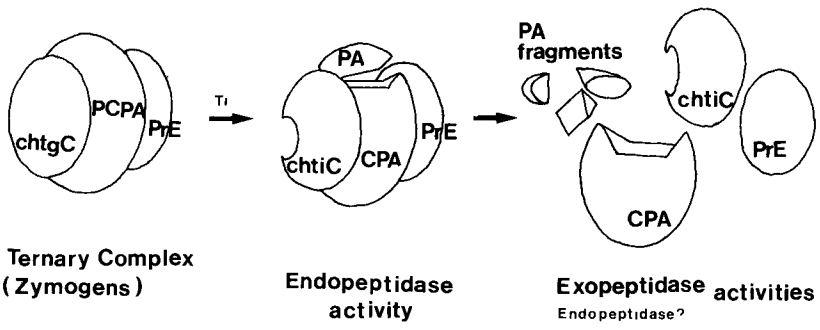

Figure 3. *Diagrammatic representation proposed for the hypothetical sequential activation of the procarboxypeptidase AS6 complex in the intestine of ruminants.* ChtgC, C-type chymotrypsinogen (subunit II); ChtiC, C-type chymotrypsin; PCPA, procarboxypeptidase A; PA, activation peptide; CPA, carboxypeptidase A; PrE, protease E (subunit III); Ti, trypsin. For explanations, see text.

carboxypeptidase A from the acidic pHs in the upper part of the intestine. Then, in a second step, extensive proteolytic cleavage of the activation peptide would result in the dissociation of the complex. Consequently, the released carboxypeptidase A can express its exopeptidase activity on the peptides generated by endopeptidases during the previous step. During this second step, the exopeptidase activity of subunit II is likely to be reduced.

In this respect, the physiological function of the ternary complex would be to modulate the activity of its constituent subunits and to transport the very unstable carboxypeptidase A in a native form to the place where it acts.

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