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FURTHER CHARACTERIZATION OF SUBUNIT III OF BOVINE PROCARBOXYPEPTIDASE A-S6 AS A NON ACTIVATABLE ZYMOGEN

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Summary

The homology of Subunit III of bovine procarboxypeptidase A-S6 (EC 3.4.12.-) with Subunit II (bovine chymotrypsinogen C) of the same complex, already reported in a previous publication (Puigserver, A. and Desnuelle, P. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2442—2445) has been extended to the position of the single methionine of the chains. The sequences linked by 4 disulfide bridges out of 5 are also probably homologous in the 2 proteins. The last bridge is displaced in Subunit III as a consequence of the deletion of the N-terminal half-cystine.

Subunit III, which is not activatable by trypsin, due to the loss of essential residues in the N-terminal region, has conserved a weakly functional active site reacting with concentrated diisopropylfluorophosphate at exactly the same rate as that of Subunit II.

Introduction

The 3 subunits (I, II and III) of bovine procarboxypeptidase A-S6 (EC 3.4.12.-) have recently been obtained in an apparently pure and native form after partial acylation of the amino groups of the trimer by 2,3-dimethylmaleyl anhydride followed by deacylation at pH 6.0—7.0 [1]. Data obtained by amino acid analysis and limited sequencing were consistent with the idea that Subunit III is homologous with Subunit II (bovine chymotrypsinogen C) but is unable to be activated by trypsin due to a deletion in the N-terminal region including the 2 hydrophobic residues normally pushing the chain after activation into the interior of the molecule [2].

Abbreviation: iPr_2P-F, diisopropylfluorophosphate.

The purpose of the present work is to present new data confirming the homology of Subunits II and III, and showing that, like Subunit II and other zymogens, Subunit III possesses a weakly functional active site reacting with concentrated iPr_2P-F.

Materials and methods

Enzymes and reagents. Pure bovine procarboxypeptidase A-S6 [3,4] was dissociated by reversible dimethylmaleylation and the resulting Subunits were purified by chromatography [1]. An extinction coefficient ($E^{1\%}_{1cm}$ at 280 nm) of 19 was adopted for the calculation of all protein concentrations. Pepsin and trypsin were from Worthington (twice crystallized). Chymotrypsin activity was determined at pH 7.5 and 25°C using 10 mM *N*-acetyl-L-tyrosine ethylester in 3% methanol as substrate. Triated water and [^{32}P]iPr_2P-F (specific radioactivities 0.2 and 2.0 mCi/mmol, respectively) were obtained from the Commissariat à l'Energie Atomique (France) and the Radiochemical Centre, Amersham (Great Britain).

Cyanogen bromide cleavages. Solutions of 30 mg of Subunit II or III in 3 ml of 70% formic acid were incubated at 5°C for 30 h under nitrogen with 20 mg of BrCN [5]. After a second incubation with the same amount of reagent for 20 h, the mixure was diluted with 5 vols. of water and lyophilized.

Terminal residues and sequences. N and C terminal residues were identified, respectively, by dansylation [6,7] and hydrogen-tritium exchange in alkaline medium [8]. *S*-carboxymethylated peptides (about 300 nmol) were sequenced, either manually by the Dansyl-Edman technique [9], or automatically with the aid of a Socosi Sequenator Model PS-100 using the dimethylbenzyl amine system. In this latter case, the thiohydantoins and their trimethylsilyl derivatives were identified by gas-liquid chromatography in a Beckman apparatus Model GC 45 [10] and by thin layer chromatography on Merck 60 F 254 fluorescent silicagel plates eluted with Edman's solvent E.

Peptide maps. Peptides from Subunits II and III were separated on Whatman 3 MM paper by electrophoresis (35 V/cm for 110 min at pH 6.5 in pyridine/ acetic acid/water (25 : 1 : 475 v/v)) and chromatography (18 h in *n*-butanol/ pyridine/ acetic acid/water (15 : 10 : 3 : 12 v/v)). Peptides containing cystine [11], histidine, tryptophan, tyrosine [12], or arginine [13] were specifically revealed on paper by suitable reagents.

Incubation of proteins with [^{32}P]iPr_2P-F. Zymogens and the enzymes resulting from their activation by trypsin (1 : 100 by weight; 90 min at 0°C) were treated with iPr_2P-F. The enzymes were incubated as usual with 1 mM iPr_2P-F for 10 h at 0°C in a 0.1 M KH_2PO_4 buffer pH 7.3. For the much less reactive zymogens, the iPr_2P-F concentration was raised to 80 mM and the temperature to 25°C. The pH was maintained at 7.3 throughout these latter assays with the aid of a Radiometer pH-stat containing 1 M NaOH. At the end of each incubation, low molecular weight radioactive contaminants were carefully removed by a 24 h dialysis against a 50 mM Tris · HCl buffer pH 8.0 followed by a filtration through a Sephadex G 100 column (1 × 100 cm) equilibrated with the Tris · HCl buffer containing 200 mM NaCl. Then, the radioactivity incorporated into the proteins was counted in a Packard Tri-Carb Scintillation Spectrometer

Model 3380 after dissolution of the samples in Bray's mixture. The specific radioactivity of the iPr_2P-F solution serving for the incubations was derived from an assay with chymotrypsin α whose complete inhibition was assumed to correspond to the incorporation of exactly 1 phosphorus atom per molecule.

Results

Cyanogen bromide peptides from Subunits II and III

Subunits II and III are known to contain a single methionine [1] instead of 2 and 4 in chymotrypsinogens A and B. The position of this methionine in the chain can be expected to be easily ascertained after cyanogen bromide degradation of the proteins.

Gel filtration of the cyanogen bromide peptides resulting from degradation of both subunits gave very similar diagrams. A typical example related to Subunit III is reproduced in Fig. 1. The fasted peak (designated III in Fig. 1) was found by terminal residues determination to contain undegraded molecules.

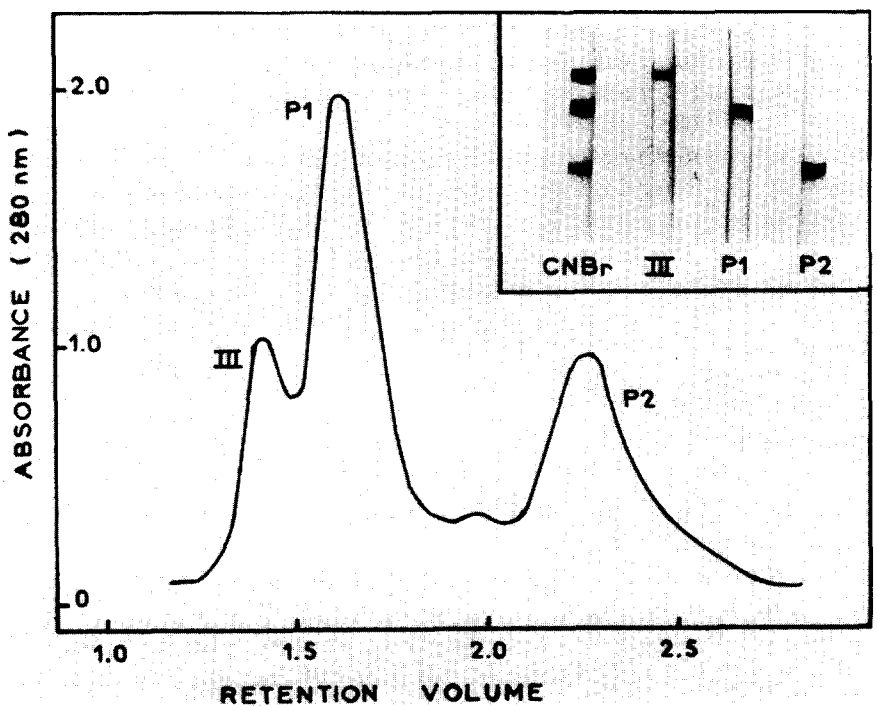

Fig. 1. Filtration through 3 successive Sephadex G 100 columns (1 cm × 100 cm) of the cyanogen bromide peptides of Subunit III. The columns were equilibrated with 0.5% ammonium bicarbonate (pH 8.5) containing 7 M urea and 5 mM mercaptoethanol. Fraction volume, 0.9 ml. Total retention volume of the columns, 67 ml. Flow rate, 1 ml/h. Symbols III, P_1 and P_2 stand for intact Subunit III and the 2 cyanogen bromide peptides. Inset: Gel electrophoresis after *S*-carboxymethylation of the unfractionated mixture (CNBr) and of the material under peaks III, P_1 and P_2, in presence of 1% sodium dodecyl sulfate [14]. Gels were calibrated with *S*-carboxymethyl bovine serum albumin (mol. w. 69 000), ovalbumin (43 000), pepsin (35 000), bovine chymotrypsinogen A (25 000) and the chains B and C (13 000 and 11 000, respectively) of bovine chymotrypsin α.

Each of the other two contained, as expected, a single peptide, P_1 or P_2, respectively, related to the N and C-terminal moities of the chains.

The amino acid composition of peptides P_1 and P_2 from the 2 Subunits is given in Table I. The composition of the C-terminal peptides P_2 shows a number of similarities (same number of Arg, Asx, Gly, His, Lys) as expected from the analogies already noted for the parent proteins [1]. The total number of residues in the two peptides does not differ by more than 7. Their molecular weight calculated by summation of the residues or determined by gel electrophoresis (Table II) are very close. A poorer agreement is noted for the N-terminal peptides P_1. In fact, the peptide originating from Subunit III contains 15 residues less than that from Subunit II and its molecular weight is lower. But, these differences are largely explained by the N-terminal deletion in Subunit III. Therefore, the above data are consistent with the view that the position of the methionine is about the same in Subunits II and III.

Another indication that the degree of homology between Subunits II and III may be high was obtained by sequencing the peptides P_2 from their amino end. Only short sequences could be identified, probably because of the use of urea for peptide purification (Fig. 1) and also probably of the existence of an un-

TABLE I

AMINO ACID COMPOSITION OF THE CYANOGEN BROMIDE PEPTIDES FROM SUBUNITS II AND III

Residues	Number of residues per mol of			
	C-terminal peptide P_2		N-terminal peptide P_1	
	Subunit II	Subunit III	Subunit II	Subunit III
Ala	7	10	11	10
Arg	3	3	6	4
Asx	13	13	17	15
Cys *	4	5	4	4
Glx	7	4	19	15
Gly	13	13	13	14
His	1	1	4	4
Ile	5	6	8	7
Leu	7	3	17	15
Lys	4	4	4	5
Met **	0	0	1	1
Phe	4	6	4	2
Pro	4	5	8	9
Ser ***	7	11	8	10
Thr ***	5	8	14	7
Trp †	5	3	3	5
Tyr	2	1	5	6
Val	8	10	14	13
Total	99	106	160	146
N-terminal residue	Val	Val	Cys	Asn
C-terminal residue	Leu ††	Tyr ††	Hse	Hse

* Uncorrected value of *S*-carboxymethyl cysteine.

** As homoserine deriving from homoserine lactone [15].

*** Extrapolation to zero time after 24, 48 and 72 h of hydrolysis.

† Colorimetric estimation.

†† Determination on the native Subunits (ref. 4 for Subunits II and this work for Subunit III).

TABLE II

MOLECULAR WEIGHT OF PEPTIDES P_1 AND P_2

Method	Peptide P_2		Peptide P_1	
	Subunit II	Subunit III	Subunit II	Subunit III
Amino acid composition	10 660	10 940	17 860	16 280
Gel electrophoresis *	8 900	9000	18 500	17 600

* 10% Polyacrylamide gel in presence of 1% sodium dodecyl sulfate [14].

favorable bond [16] if the identified Asx is an asparagine followed by a glycine in Subunit III as well as in Subunit II (see below). However, the 6 residues after the methionine were found by the Dansyl-Edman and automatic Sequenator techniques to be identical in the two subunits. These residues are:

Met ¦ -Val-Cys-Ala-Gly-Gly-Asx.

The automatic technique permitted to realise 2 additional productive cycles with Subunit II yielding, respectively, Gly and Val.

Disulfide bridges

Subunits II and III are devoid of any detectable free SH groups. Consequently, the 10 half-cystine residues found in both proteins [1,4] should correspond to 5 disulfide bridges *. The presence of 5 bridges is common to all known chymotrypsinogens. However, the deletion of the N-terminal half-cystine in Subunit III may be expected to have induced the displacement of one of the bridges.

A comparative study of the disulfide bridges in Subunits II and III was undertaken by submitting the proteins (20 mg in 3 ml of 10 mM HCl) to a 30 h digestion by pepsin (2.5 mg) at 37°C. The mixtures were fractionated by electrophoresis-chromatography on paper. The resulting maps stained for cystine peptides are reproduced in Fig. 2. Both show the expected 5 spots. Four spots are seen to occupy identical or very similar positions, suggesting a high degree of conservation in the corresponding regions of the proteins. By contrast, the last ones marked "2" in Fig. 2 are well separated. Diagonal electrophoresis confirmed that they really corresponded to cystine peptides, thus establishing the existence of a new bridge in Subunit III. Full identification of this bridge is in progress.

The maps were also spread with reagents specific for arginine, histidine, tyrosine and tryptophan. An interesting finding was that the cystine-containing spots marked "1" in Fig. 2 gave a positive response with the Pauly reagent, suggesting that the "histidine loop" found in chymotrypsinogens A and B is conserved in Subunit II representative of the chymotrypsinogen C line and also in Subunit III. Few significant correlations could be established between the spots

* The uncorrected results given in Table I for the cyanogen bromide peptides from Subunits II and III correspond to a somewhat lower half-cystine content, probably due to the known instability of *S*-carboxymethyl cysteine during acid hydrolysis.

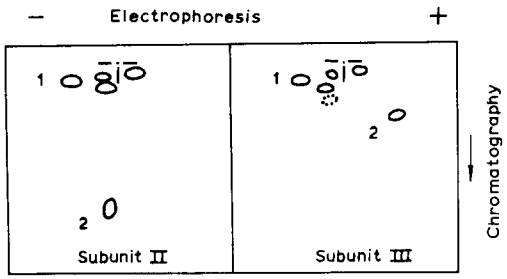

Fig. 2. Cystine peptides from Subunits II and III separated by electrophoresis-chromatography on paper. The very weak spot indicated in the right map by an interrupted line has not been investigated in detail.

revealed by other reagents. This last remark confirms that, in spite of a number of similarities, Subunit III does not directly derive from Subunit II.

Incorporation of iPr_2P-F into Subunits II and III.

Subunit III is not activatable by trypsin because of the already reported N-terminal deletion. But, its homology with Subunit II suggests that it may have conserved the weakly functional site characteristic of chymotrypsinogens and other zymogens and reacting with concentrated iPr_2P-F at temperatures higher than 0°C [17,18]. Curve A in Fig. 3 shows that Subunits II and III incorporate

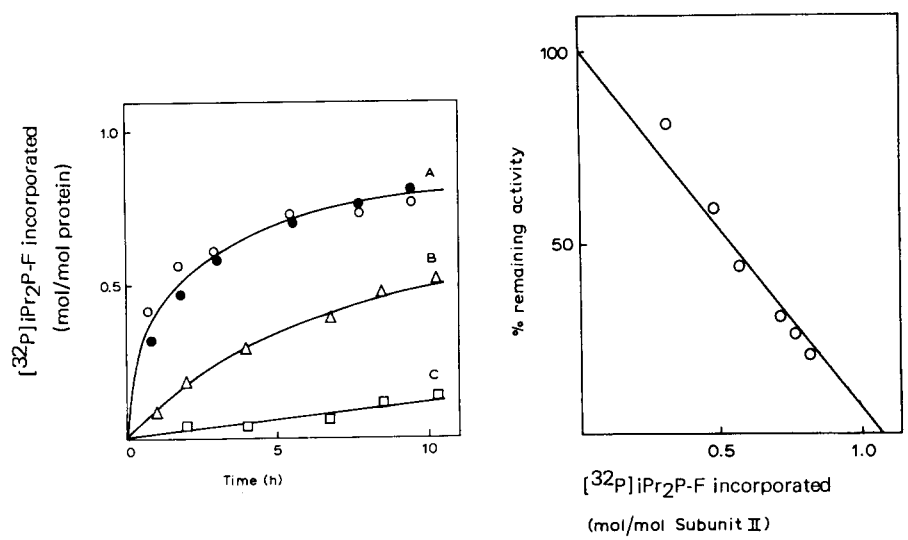

Fig. 3. Time course of iPr_2P-F incorporation into Subunits II and III, either free (curve A, • and ○, respectively), or integrated in procarboxypeptidase A-S6 (curves B and C, respectively). Incubations were performed under the conditions given for zymogens in the Materials and Methods section (80 mM iPr_2P-F at 25°C and pH 7.3).

Fig. 4. Relationship between iPr_2P-F incorporation and loss of potential activity of Subunit II. Same incubations as in Fig. 3.

radioactivity at exactly the same rate when treated with 80 mM [^{32}P]iPr_2P-F at 25°C. A point of great interest is that the stoichiometry of the reaction is approximately 1 : 1 for both subunits. Nonspecific incorporation due to the high iPr_2P-F concentration is ruled out in the case of Subunit II by the curve of Fig. 4. This curve shows that complete loss of potential activity is attained when not more than 1.05 mol of iPr_2P-F is incorporated per mol of protein.

Other assays realized with native procarboxypeptidase A-S6 and also reported in Fig. 3 (curves B and C) showed that the integration of the Subunits into the trimer distinctly slowed down iPr_2P-F incorporation.

Discussion

It had been reported in a previous publication [1] that Subunit II (bovine chymotrypsinogen C [19,20]), and Subunit III have a similar amino acid composition. Moreover, an identity or a very high homology was observed between the sequence of the 7 first residues of Subunit III and that extending from residue 18 to 24 in bovine chymotrypsinogen A (serving as reference for the numbering of residues), chymotrypsinogen B and Subunit II. Therefore, it could be assumed that Subunit III represents a new type of chymotrypsinogen, homologous with but distinct from the already known types A, B and C. The lack of potential activity in the Subunit was readily explained by the loss of 17 residues including Arg 15 and the 2 hydrophobic residues at position 16 and 17 which are essential for activation. In the course of the present work, the homology between Subunits II and III has been extended to the position of the single methionine of the chain and to the sequence of the next 6 residues. Furthermore, Subunit III, like all known chymotrypsinogens, contains 5 disulfide bridges. Four of them probably link homologous sequences in Subunits II and III. Therefore, the fact that Subunit III belongs to the chymotrypsinogen family is now well established. The fact that the Subunit does not merely derive from Subunit II by autolysis or any other degradation, but is representative of a new type of chymotrypsinogen, has also been confirmed. Maps of arginine, histidine, tyrosine and tryptophan peptides generated by pepsin from Subunits II and III show relatively few analogies. An interesting consequence of the deletion of the N-terminal half-cystine in Subunit III is that the bridge normally linking this residue to Cys 122 in chymotrypsinogen should have been suppressed or displaced. Displacement involving the creation of a new half-cystine somewhere in the chain is proved by the conservation of 5 bridges in the Subunit and by the fact that one of these bridges is quite different from the corresponding one in Subunit II.

A pre-existing, weakly functional active site has been characterized in bovine chymotrypsinogen and trypsinogen [17,18]. This site combines at 0°C with iPr_2P-F and suitable titrants at rates 10^4 to 10^7-fold lower than those observed with the related enzymes. But, the reaction is enhanced by using concentrated iPr_2P-F at 25°C. Data reported in the present work are consistent with the view that a weakly functional site also exists, not only in Subunit II, but also in Subunit III which, therefore, appears to be the first known example of a non activatable zymogen.

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