FURTHER STUDIES ON SUBUNIT III OF BOVINE PROCARBOXYPEPTIDASE A

Structure and reactivity of the weakly functional active site

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1. Introduction

The activation of pancreatic zymogens results from the tryptic cleavage of the first basic bond in the N-terminal part of the molecules [1]. As indicated by crystallographic data [2,3], the major event induced by this cleavage is the appearance at the newly formed N-terminus of the chain of a positively charged hydrophobic residue (Ile 16 in bovine chymotrypsinogen A) which moves into the interior of the molecule and forms an ion pair with the buried carboxylate of Asp 194. The concomitant repositioning of other residues, has been proposed to improve the structure of the primary substrate binding site and that of the oxyanion hole [4]. However, the charge relay system between Asp 102, His 57 and Ser 195 which plays a key role in catalysis is largely if not fully developed in the zymogens. The resulting latent active sites are able to react at a measurable rate with titrants and substrates [5].

Bovine procarboxypeptidase A is composed of 2 or 3 subunits [6,7] which can be dissociated under non-denaturing conditions after dimethylmaleylation of the complex [8]. Subunit I is the immediate precursor of carboxypeptidase A whereas subunit II is a chymotrypsinogen of the C type [9]. In contrast, no activity has ever been found in subunit III prior to or following tryptic treatment. This subunit has been assumed to be a new type of a non-activatable zymogen for the following 2 reasons:

(i) Partial information about its sequence suggests a substantial homology with several zymogens of serine proteases but its chain is shorter by several residues located in the N-terminal region including the hydrophobic residues essential for activation [8].

(ii) The subunit contains a weakly functional active site reacting slowly with diisopropylfluorophosphate and chymotrypsin or elastase substrates such as *N*-acetyl-L-tyrosine ethylester and *N*-acetyl-L-trialanine methylester [8,10].

Evidence presented below will show further structural homologies between subunit III and serine protease zymogens, and strengthen the hypothesis that the subunit contains a weakly functional active site.

Abbreviations: Boc-Ala-ONp, tertiary butyloxycarbonyl alanine *p*-nitrophenyl ester; MUGB, 4-methylumbelliferyl-*p*-guanidinobenzoate; MUTMAC, 4-methylumbelliferyl-*p*-trimethylammonium cinnamate; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; Pipes, piperazine-*N*,*N'*-bis (2-ethane sulfonic acid)

2. Materials and methods

Purified bovine procarboxypeptidase A-S6 [11] was dissociated by dimethylmaleylation [8]. Subunits II and III were purified to homogeneity as in [8]. Bovine trypsinogen and trypsin (once and twice crystallized), as well as 3X crystallized chymotrypsinogen A and α-chymotrypsin were Worthington products. NPGB, MUGB and MUTMAC hydrochlorides were from Sigma, while Boc-Ala-ONp and 4-methylumbelliferone were from Bachem and Fluka, respectively.

The two peptides resulting from CNBr cleavage of *S*-carboxymethylated subunit III (100 mg) were separated by gel filtration in 7 M urea [10]. The sequence of ~100 nmol C-terminal peptide was determined in a 890 C Beckman Sequencer using the 0.1 M Quadrol program. Phenylthiohydantoins were identified by thin-layer chromatography or gas chromatography in a GC 45 Beckman equipment. The manual microse-

quencing method in [12] was also used because of its high sensitivity.

Hydrolysis of Boc-Ala-ONp by zymogens and enzymes [5] was monitored in a Beckman Model 25 double-beam spectrophotometer using A_{400} = 12 200 for released *p*-nitrophenol at pH 7.2. The kinetic parameters K_m and k_{cat} of the reaction were computed using a least squares program. If necessary, zymogens (1 mg/ml of a 50 mM Tris–HCl buffer (pH 7.8)) were activated at 0°C for 1 h with 1/100 (w/w) of trypsin.

Specific acylation of Ser 195 in enzymes and zymogens (10–50 nmol/ml in a 100 mM veronal buffer (pH 8.3)) was performed at 25°C with 50 μl 20 mM NPGB in dimethylformamide (molar excess, 20–100-fold). Acylation was followed as above using A_{400} = 16 595. Values were corrected for unspecific hydrolysis of NPGB. For titration with MUGB and MUTMAC stock enzyme solutions (10 mM) in *N*-methyl pyrrolid-2-one were diluted and treated as in [13]. Zymogens at the highest possible concentration were first incubated with the undiluted titrant for 6–8 h at 25°C to allow maximal acylation, then diluted to the same concentrations as above. The fluorescence due to released 4-methylumbelliferone was measured in a Fica Model 55 double-beam spectrofluorimeter [13].

3. Results

3.1. *Conservation of Asp 194 and Ser 195 in subunit III*

As shown in table 1, the first 20 residues of the C-terminal cyanogen bromide peptide from subunit III starting from Met 180 show a high degree of homology with the corresponding regions of elastase, trypsin and chymotrypsin [14]. The characterization of Ser 195 and Asp 194 in a fully conserved sequence of seven residues is noteworthy. Residues Cys 182 and Cys 191 are also conserved, thus confirming the homology of subunit III with these proteases. The additional residues 184 A present in bovine trypsin and 188 A in bovine trypsin and porcine elastase are lacking in both bovine subunit III and chymotrypsinogen A.

3.2. *Intrinsic activity of subunit III on Boc-Ala-ONp*

Boc-Ala-ONp known to be a good substrate for chymotrypsinogen [5] was also hydrolyzed by the subunit at approximately the same rate (table 2). By contrast, the intrinsic activity of subunit II on the same substrate was 3–4-times higher while that of trypsinogen was lower by a factor of 4–12. Boc-Ala-ONp has already been reported to be a poor substrate for trypsinogen and trypsin [5]. Subunit III activity was completely abolished by petreatment with 80 mM phenylmethylsulfonylfluoride or diisopropylfluorophosphate, thus proving that Ser 195 in the subunit is actually involved in catalysis as it is in other zymogens.

Another point of interest revealed by table 2 and fig.1 was that the activity of subunit III towards Boc-Ala-ONp was enhanced at high ionic strength like that of authentic zymogens [15], eventhough, the effect was definitely smaller (3-fold in 4 M NaCl compared to 7-fold for chymotrypsinogen and 10-fold for trypsinogen). The NaCl effect was due to an increase of k_{cat} for both subunit III (from 0.011–0.029 s^{-1}) and chymotrypsinogen A (from 0.012–0.063 s^{-1}). The K_m value for both proteins was unchanged (0.04 and

Table 1
Amino acid sequence surrounding the active serine residue in several pancreatic proteases[a]

	180			(A) 185		(A) 190			195	
Elastase[b] :	Met	Val	Cys.Ala.Gly	---.Gly.Asn.Gly.Val.	Arg.Ser.Gly	Cys	Gln	Gly.Asp.Ser.Gly.Gly.Pro	Leu.	
Trypsin :	Met	Phe	Cys.Ala.Gly	Tyr.Leu.Glu.Gly.Gly.Lys.Asp.Ser		Cys	Gln	Gly.Asp.Ser.Gly.Gly.Pro	Val.	
α-Chymotrypsin:	Met	Ile	Cys.Ala.Gly	---.Ala.Ser.Gly.Val.	---.Ser.Ser	Cys	Met	Gly.Asp.Ser.Gly.Gly.Pro	Leu.	
Subunit III[c] :	Met	Val	Cys.Ala.Gly	---.Gly.Asp.Thr.Ser.	---.Ser.Gly	Cys	Asn	Gly.Asp.Ser.Gly.Gly.Pro	Leu.	

[a] The residue numbering is that of bovine chymotrypsinogen A; insertions in the sequence of other proteins are numbered 184 A and 188 A while deletions are indicated by (---); boxes enclose identical residues in the 4 proteins

[b] From porcine pancreas while trypsin and α-chymotrypsin are from bovine origin [14]

[c] Determined automatically with the aid of a sequenator; the sequence extending from Val 181–Gly 190 has also been confirmed by the manual method as in section 2

Table 2
Enzyme or zymogen catalyzed hydrolysis of Boc-Ala-ONp in the absence or presence of high salt concentration[a]

Protein	(μmol substrate hydrolyzed . min^{-1} . μmol $protein^{-1}$)[b]		
	No added salt	NaCl (3.0 M)	$CaCl_2$ (1.0 M)
Trypsinogen	0.1	0.7	0.2
Trypsin	13.2	52.8	17.2
Chymotrypsinogen A	0.8	3.2	2.0
α-Chymotrypsin	84.0	226.8	184.8
Subunit II	4.1	9.0	7.0
Activated subunit II	73.8	162.4	125.5
Subunit III	1.0	2.3	2.4

[a] Conditions were 1 ml of a 0.1 M Pipes buffer (pH 7.2) containing 10 μl of a 10 mM solution of Boc-Ala-ONp in dimethylformamide and 10 μl of a 10 mg/ml solution of zymogens or subunit III, or 0.2 mg/ml solutions of the enzymes
[b] Determined as the amount of *p*-nitrophenol released (A_{400})

0.03 mM). By contrast, a K_m decrease from 3.0–0.3 mM was observed for trypsinogen when NaCl was raised to 3.0 M while k_{cat} was only slightly modified (from 0.013–0.007 s^{-1}).

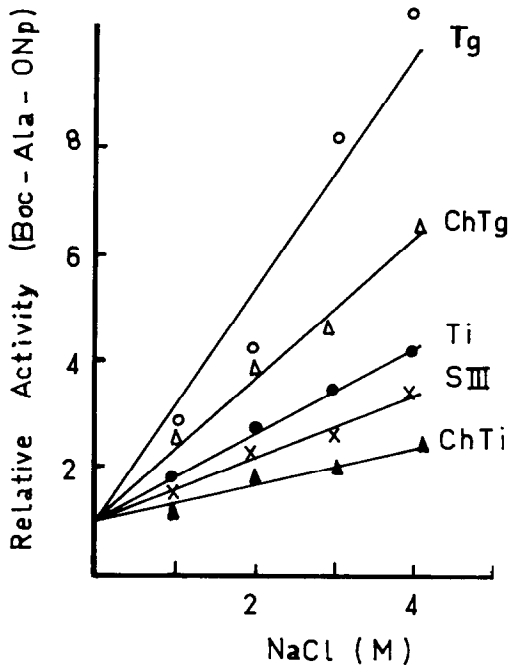

Fig.1. Effect of NaCl concentration on the activity of subunit III (S III), trypsinogen (Tg), trypsin (Ti), chymotrypsinogen (ChTg) and chymotrypsin (ChTi) towards Boc-Ala-ONp. The assay conditions were as in table 2. Rates measured in the absence of added salt were taken as unity.

3.3. *Acylation of subunit III with specific titrants for serine proteases*

Subunit III was found to react with NPGB at about the same rate as chymotrypsinogen [16]. Although slow, this reaction suggests a specific acylation of Ser 195 which is conserved in the subunit.

The properties of the subunit latent active site were further explored using MUGB and MUTMAC known to be specific titrants, respectively, for trypsin and chymotrypsin [13]. As expected, these compounds were not hydrolyzed by the subunit under conditions used for enzyme titration (0.1 μM protein incubated with 1.0 μM titrant). When higher concentrations of both protein (4.0 μM) and titrant (40 μM) were used as for zymogens, the subunit reacted quite well with MUGB but not with MUTMAC, thus suggesting in this case an analogy with trypsinogen.

Although no burst of methylumbelliferone or *p*-nitrophenol was observed during incubation of subunit III with MUGB and NPGB, the acylation of subunit III was estimated after a 3 h incubation to be, respectively, 25% and 65%. The acyl derivatives were stabilized by lowering the pH to 3.0 and isolated by gel filtration. Some properties of acylated subunit III will be reported shortly.

4. Discussion

Our data confirm that subunit III of bovine procarboxypeptidase A is a non-activatable zymogen of a pancreatic serine protease [8,10]. The sequence fol-

lowing Met 180 and including the two essential residues Asp 194 and Ser 195 presents a very high degree of homology with chymotrypsinogen, trypsinogen and elastase. Like these zymogens or unactivated subunit II of the same complex, subunit III promotes slow hydrolysis of the substrate Boc-Ala-ONp and most importanly this activity is modulated by high salt concentration. The fact that the reaction of subunit III with specific serine proteases inhibitors and substrates were mutually exclusive confirmed that residue Ser 195 in the subunit is really involved in catalysis. Finally, subunit III was slowly acylated by active site titrants for chymotrypsinogen and trypsinogen, leading to acyl derivatives shown to be stable at low pH.

The zymogen of pancreatic serine proteases presenting the highest homology with subunit III has not yet been characterized. Structural problems will only be settled when the sequence of subunits II and III are elucidated. As for the active sites, the data show striking similarities with chymotrypsinogen in most cases but also with trypsinogen. It must be stressed here that, as shown by the example of trypsin [17] and chymotrypsin [18], the specificity of an enzyme (or zymogen) towards substrates and titrants may be entirely modified by the substitution of a single residue in an important region.

The most intriguing problem that remains to be solved is why subunit III is not active or activatable. The absence in the subunit of the N-terminal sequence [8] containing the activating bond and the 2 hydrophobic residues normally present in zymogens suggest that inactivability is due to the loss or modification of essential residues. Alternatively, the presumably flexible region of the molecule in which activating transconformations should occur may be assumed to be partly rigidified by an alteration of the sequence and/or of the disulfide bridges. In this latter respect, the bridge Cys 1–Cys 122 present in zymogens is already known to be displaced in subunit III because of the loss of Cys 1 [10]. Work is in progress to clear up these points.

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