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LOCALIZATION OF THE TWO FREE THIOL GROUPS IN THE PORCINE PANCREATIC α-AMYLASE I SEQUENCE

Lyda PASERO, Yvette MAZZEI, Brigitte ABADIE, Danielle MOINIER*
Michel FOUGEREAU* and Guy MARCHIS-MOUREN+

Institut de Chimie Biologique, Place Victor-Hugo, 13331 MARSEILLE Cedex 3. *Centre d'Immunologie, 70, route Léon-Lachamp, 13288 MARSEILLE Cedex 9, France

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Porcine pancreatic α-amylase I, a single 496 residue long polypeptide chain, contains 5 disulfide bridges and 2 free -SH groups. The conditions for specific blocking of native amylase either with radioactive N-ethyl maleimide or with labeled iodoacetic acid were determined. Under these conditions 2 moles of blocking reagent are incorporated per mole of amylase. [^{14}C]-S-succinimido amylase was cleaved by CNBr and the resulting peptides were purified. Only one of them the CNBr 2+3 peptide (178 residues) was found labeled. Tsl a 33-residue peptide containing the whole radioactivity was purified from the tryptic digest of this large fragment. After reduction and carboxymethylation TslA, (22 residues) was obtained which contains 2 moles of succinyl-Cys and one mole of CM-Cys per mole of peptide. Chymotryptic digestion of TslA yielded 2 equally labeled peptides: C1 (16 residues) and C2 (6 residues). Automated sequencing of both peptides and counting of the PTH-amino acids shows that the free cysteines are only 15 residues apart in the sequence :

C(SH) G S G A A A G T G T T C G S Y C(SH) N P G N R

Porcine pancreatic α-amylase (α-1,4 glucan-4-glucano hydrolase EC 3.2.1.1) is a single chain polypeptide (496 residues) (1). The major part of its amino acid sequence has been reported (2-5). It contains 5 disulfide bridges and 2 -SH groups (4,6) which are masked to bulky thiol reagents but can be unmasked by removal of the tightly bound calcium (7). It is now clear that the -SH groups are not necessary to amylase activity, although they might be part of the substrate binding site (8,9). However the chelation of calcium makes the enzyme more susceptible to proteolytic attack with subsequent loss of activity. In the present work conditions for specific blocking of native amylase either with [^{14}C]-NEM or with [^{14}C]-iodoacetic acid were first determined. Then for structural studies, [^{14}C]-S-succinimido amylase was used because it allows a better characterization of

+ To whom all correspondence should be addressed.

ABBREVIATIONS : PTH : Phenylthiohydantoine, CNBr : Cyanogen bromide, DTNB : 5,5'-dithiobis-(2-nitrobenzoic acid), NEM : N-ethyl maleimide.

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-SH groups as succinyl-Cys. Two radioactive peptides were obtained from CNBr2+3 peptide digested subsequently with trypsin and chymotrypsin. Both peptides were finally sequenced and counting of the radioactivity in the PTH-amino acid derivatives allowed the localization of the free -SH groups.

MATERIAL AND METHODS

The techniques used for α-amylase I purification, chain fragmentation by CNBr, trypsin digestion, peptide separation and automated sequence determination are reported in our previous publications (2,3,4,10). The conditions used for amino acid analysis allowed to separate succinyl-Cys and CM-Cys. Analysis were carried out in a LC 2000 Biotronik autoanalyzer using citrate buffers pH 3.03, 3.90, 4.30, 4.51 with sodium concentration respectively : 0.18 M, 0.2 M, 0.2 M, 1.6 M. Chymotrypsin digestion was carried out at 37°C in 0.06 M NH_4Cl-NH_4OH pH 8 for 3 hr, E/S (w/w) : 1/10. The reaction of 5,5'-dithio-bis (2-nitrobenzoate) (DTNB) with -SH groups was carried out according to the method of Ellman (11).

Chymotrypsin was purchased from Boehringer and the TPCK trypsin from Worthington. Radioactive N-ethyl [2,3 ^{14}C] maleimide (10 mCi/mM) and iodo-[2-^{14}C] acetic acid (27.6 mCi/mM) were from Amersham. Counting of [^{14}C] radioactivity was performed in an Intertechnique SL 40 scintillation counter using the aqueous counting scintillant (ACS) from Amersham. Trisacryl GF-05 was from IBF (France) and Biogel P6 was from Biorad (U.S.A.).

RESULTS AND DISCUSSION

N-ethyl [2,3 ^{14}C] maleimide was first used for labeling the -SH groups of amylase in the presence of 10 mM EDTA. At the times indicated in Table I, the remaining -SH groups were titrated by DTNB : no -SH groups could be detected by DTNB after 20 min. Furthermore calculation of the radioactivity incorporated into the S-succinimido amylase showed that both -SH groups have been labeled at this time. Proper conditions were also worked out with iodoacetic acid. After a 40 min incubation period the two -SH groups were completely carboxymethylated (Table I).

The radioactive S-succinimido amylase was then cleaved by CNBr under the same conditions as for amylase (2) and CNBr-peptides were filtered through a Biogel P10 column as previously reported. The same absorbance pattern was obtained but only the (2+3) fraction was [^{14}C]-labeled (not shown). It thus appears that both -SH groups are located in the N-terminal part of the amylase chain (3). The radioactive CNBr-peptide was further purified. Trypsin hydrolysis was performed on the citraconylated 2+3 fragment and the pH 4 soluble peptides were purified by gel filtration through a Biogel P6 column. As shown from the elution pattern (Fig. 1) seven major peaks were obtained. Most of the [^{14}C]-radioactivity was found in the first one (Ts1) which elutes at 1.5 Vo. The Ts1 fraction was further purified by a new filtration on the same column. This chromatography allows a good separation of Ts1 from the neighbouring contaminating peptide (Ts2). Final purification of Ts1 was achieved by ion exchange-chromatography on a DEAE-Sepharose column equilibrated with 0.02 M ammonium bicarbonate : the labeled pep-

TABLE I : Number of -SH groups reacting with NEM or iodoacetic acid in α-amylase.

Blocking agent		Remaining SH (DTNB)***	Incorporated (^{14}C)radioactivity	
			Specific radioactivity (cpm/μmole)	Number of blocked -SH per mole
None		1.8-2.0	-	-
NEM*	10 min	0.19	7.6 10^5	1.51
	20 min	0.03	9.4 10^5	1.87
Iodoacetic acid**	30 min	0.02	1.150 10^6	1.87
	40 min	0.00	1.23 10^6	2

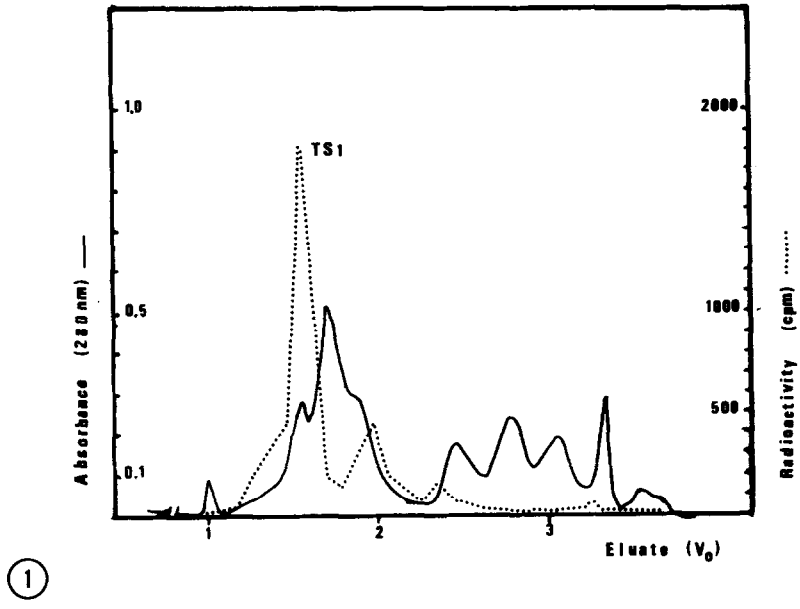
*Reaction of α-amylase (8-10 mg/ml) was performed at pH 7.0 in 0.02 M pH 7 phosphate buffer with 0.01 M EDTA to which a 10 fold molar excess of NEM over enzyme was added. The incubation was carried out at 30° for the indicated period of time. and stopped by adding concentrated HCl up to 1 M final concentration. The NEM-amylase was pelleted and washed twice at 0° with 1 M HCl. Specific radioactivity of (^{14}C) NEM :5.03 10^5 cpm/μmole.

**The reaction conditions were as above except for the phosphate buffer (0.05 M pH 8) (^{14}C) Iodoacetic acid (specific radioactivity 0.622 10^6 cpm/μmole) was in 23 fold molar excess. The incubation was carried out for the indicated time at 37° and stopped as described above.

***Remaining -SH groups determined according to Ellman (11).

tide was not retained on the column but non radioactive impurities were adsorbed. At this stage of purification the amino acid composition of peptide Tsl was : Asn_2, Thr_4, Ser_3, Glu_1, Pro_2, Gly_6, Ala_3, Cys_4, Val_1, Leu_1, Tyr_3, Lys_1, Arg_2. Therefore this 33-residue peptide contains one disulfide bridge in addition to the two -SH groups. Reduction and carboxymethylation of Tsl yielded 2 smaller components TslA and TslB which eluted from the same Biogel P6 column at 1.75 Vo and 2.18 Vo, respectively. As indicated by the elution pattern, only TslA was [^{14}C]-labeled. The amino acid composition of both components was : Asp_2, Thr_3, Ser_2, Pro_1, Gly_6, Ala_3, CM-Cys_1, succinyl-Cys_2, Tyr_1, Arg_1 for the radioactive one (TslA) which thus contains 22 residues, and Thr_1, Ser_1, Glu_1, Pro_1, CM-Cys_1, Val_1, Leu_1, Tyr_2, Lys_1, Arg_1 for the other one (TslB) which contains 11 residues. Accordingly it can be concluded that peptide TslA and TslB are linked by a disulfide bridge and that the 2 -SH groups are located in the 22-residue peptide, named 3T1 as the 1st tryptic peptide of CNBr-3 (4) (Fig. 2).

The last question was to determine which of the three cysteines were either free or associated in the disulfide bridge. Peptide TslA was then digested with chymotrypsin and the digest analyzed by gel filtration through a Trisacryl GF-05 column in 0.02 M ammonium bicarbonate. Two radio-



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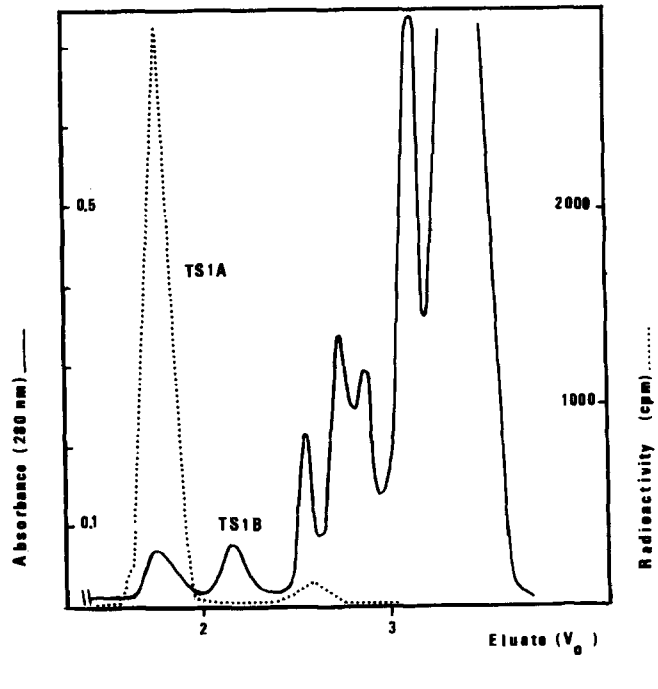

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Fig. 1 : Filtration pattern of the tryptic digest of the citraconylated 2+3 fragment. The Biogel P6 column (1.6 x 100 cm) was equilibrated with 30 per cent formic acid (w/w). Vo : 60.5 ml. Flow rate : 9.6 ml/hr. 100 µl of each fraction (2.5 ml) was taken for radioactivity counting.

Fig. 2 : Filtration pattern of peptides from reduced and carboxymethylated Ts1. The Biogel P6 column and the conditions were as in Fig. 1. The material on the right side of the pattern is due to the reduction and carboxymethylation reagents.

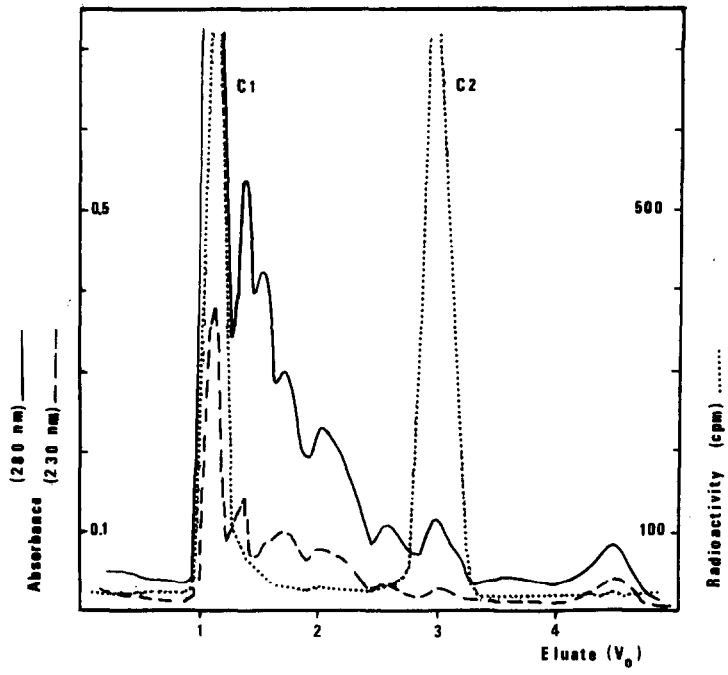

Fig. 3 : Filtration pattern of the chymotryptic peptides from Tsl A. The Tris acryl GF-05 column was equilibrated with 0.02 M ammonium bicarbonate, Vo : 42 ml. Flow rate : 14 ml/hr. 100 µl of each fraction (2.5 ml) was taken for the counting of radioactivity.

activity peaks were then separated : one is eluted at 1.1 Vo and the other at 3 Vo. Both were found equally radioactive containing 58 290 and 56 435 cpm, respectively (Fig. 3). The smaller peptide does not contain any aromatic residue since it has no 280 nm absorbance. Both peptides were analyzed for their amino acid content. The composition reveals a 16-residue fragment (C1) in which 1 succinyl-Cys and 1 CM-Cys are present and a 6-residue component (C2) containing only one mole of succinyl-Cys per mole of peptide. To determine the location of both cysteine derivatives in peptide C1, the peptidic chain was sequenced by automated Edman degradation and part of each collected PTH-amino acid was taken for amino acid characterization while the [^{14}C]radioactivity was counted in the remaining part. For comparison, peptide C2 was also sequenced. As shown in Table II, the first PTH-amino acid from peptide C1 is [^{14}C]-labeled (1891 cpm) in contrast to the PTH-amino acid n° 13 which contains no significant radioactivity. It is thus clear that Cys n° 1 (Cys n° 103 in the sequence) is one of the free cysteine whereas Cys n° 13 (Cys n° 115 in the amylase sequence) is engaged in a disulfide linkage with the 11-residue peptide (TslB) here above mentioned. Counting of the PTH-amino acids from peptide C2 (6 residues) showed that only the 1st PTH-amino acid is significantly labeled

TABLE II : (^{14}C) radioactivity incorporated into PTH-amino acid from the chymotryptic peptides C_1 and C_2.

Peptide C_1			Peptide C_2		
residue n°	PTH	radioactivity (cpm)	residue n°	PTH	radioactivity (cpm)
1	C*	1 891	1	C*	4 300
2	G	592	2	N	207
3	S	249	3	P	94
4	G	191	4	G	73
5	A	167	5	N	80
6	A	86	6	R	73
12	T**	70			
13	C	100			
14	G	60			
15	S	60			
16	Y	nd			

*PTH-derivative of succinyl-Cys. **PTH derivative of CM-Cys. Only a fraction of each PTH-amino acid was counted. Due to differences in the sequencing yields and the difficulty of PTH-Succ-Cys evaluation no quantitative determination could be done. The C-terminal Y was not determined by the sequenator.

(4300 cpm). It is thus confirmed that this cysteine, Cys n° 119 in the amylase molecule, is the other free Cys in porcine pancreatic α-amylase I.

Both -SH groups are thus only 15 residues apart in the amylase sequence and thus very likely quite close in the three dimensional arrangement of the molecule (12). This agrees with the properties of these groups previously reported (8, 9, 12).

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