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The disulfide bridges of the immunoreactive forms of human pancreatic stone protein isolated from pancreatic juice

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Following the complete sequence elucidation of human pancreatic stone protein (immunoreactive form PSP S1 isolated from pancreatic juice) [(1987) Eur. J. Biochem. 168, 201–207], the location of the three S-S bridges of the protein was investigated. The cystine-containing peptides, detected after the separation of the peptic or chymotryptic digests on SP-Sephadex or Sephadex G-50, were submitted to Edman degradation and/or to oxidation. The cysteic peptides after separation on SP-Sephadex or Sephadex G-50 were characterized by their amino acid compositions. The pairing of the half-cystines: Cys 3-Cys 14, Cys 31-Cys 129 and Cys 104-Cys 121 was determined. The same experiments carried out with PSP S2–5 (other immunoreactive forms) gave an identical characterization.

Disulfide bridge; Pancreatic stone protein; (Pancreatic juice, Human)

1. INTRODUCTION

Various forms of human pancreatic stone protein deriving from the same native protein, synthesized in pancreatic acinar cells [1], have been revealed: PSP, the form prepared from calculi [2]; PSP S1 one of the immunoreactive forms (M_r 15000) [3,4]; and PSP S2–5, other immunoreactive forms (M_r 16000–19000) detected in pancreatic juice but not separated from each other [3,4]. Both PSP and PSP S1 have the same M_r as well as N-terminal sequence and C-terminal residues [4,5]. PSP S1 derives from PSP S2–5 through the tryptic cleavage of an Arg–Ile bond [5]. In a recent publication the total sequence of PSP S1 was elucidated [6]. The 6 half-cystines of the molecule were located at positions 3, 14, 31, 104, 121 and 129 in the alignment of the 133 amino acid residues of PSP-S1. Since no free thiol was detected in PSP S1 [4], the presence of three disulfide bridges was assumed. In a preceding paper [5] the first S-S bridge connecting Cys 3 to Cys 14 was demonstrated in the course of an investigation performed in view of revealing the nature of the bond cleaved in the conversion of PSP S2–5 to PSP S1. The present report confirms the cystine linkage between Cys 3 and Cys 14 and deals with the assignment of the two remaining bridges.

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2. EXPERIMENTAL

Most of the methods have been described: preparation of PSP S1 and PSP S2–5 [4], dansylation [7], succinylation [6], amino acid analysis [7,8], automated Edman degradation [6], 5,5′-dithiobis(2-nitrobenzoic acid) reaction in the presence of 10 mM EDTA and 1% SDS [9], location of the half-cystine-containing peptides eluted from column [10] and pepsin digestion [5]. The chymotryptic digestion was performed on 15 mg succinylated PSP S1 or PSP S2–5 in 1 ml of 60 mM NH_4HCO_3 buffer (pH 7.8) for 5 h at 37°C (enzyme/substrate, 1:50, w/w). The reaction was stopped by adding phenylmethylsulfonyl fluoride (1 mM). Thin-layer chromatography on silica gel (TIC plates, silica gel 60, Merck) was performed with development in a solvent system of acetonitrile/water/pyridine (75:20:5, v/v) adjusted to pH 6.0 with a few drops of acetic acid. Three successive runs in the same direction were carried out, the plate be-

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ing dried after each run. The peptides were located using ninhydrin reagent (0.05% in acetone) and were extracted with water followed by acetonitrile.

3. RESULTS AND DISCUSSION

The absence of free thiol in PSP-S1 and PSP-S2–5 [4] was confirmed by titration with 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of EDTA and SDS. Moreover, a mild reduction of the proteins with sodium arsenite was performed. In the case of SH groups having been partially oxidized into sulfenic or sulfinic radicals, this treatment would have reconverted them into their primitive forms [11]. No SH group was detected after this experiment.

In a previous paper [5], the peptic digest of PSP S1 had been filtered on Sephadex G-50 and the cystine peptide (1–15) had been easily determined being included in a peak separated from the major compounds. Here, the peptic digest was chromatographed on SP-Sephadex. The half-cystine-positive fractions were selected (A–C). Only rectangles indicate their positions in the elution diagram (fig.1). The study of fraction B did not yield any interesting results given the low amount of half-cystine included. Fraction A and an aliquot of fraction C were performic acid-oxidized (AO and CO) and chromatographed on an SP-Sephadex column under the same conditions as the peptic digest. Only one peak (AO1) containing cysteic acid peptide was derived from fraction AO (fig.1a). Two peaks CO1 and CO2 containing cysteic acid peptides were obtained from fraction CO (fig.1b). Fractions X and Y contained no amino acid after hydrolysis. The amino acid compositions and N-terminal amino acid residue(s) of A, AO1, C, CO1 and CO2 are listed in table 1. Moreover, the N-terminal sequence of an aliquot of fraction C was performed using automated Edman degradation. Two chains were simultaneously degraded (fig.2). The non-characterization of the derivative at step 2 for the two chains was due to the presence of half-cystine residues. All these results demonstrated that cystine linkages exist between Cys 3 and Cys 14 (confirmation of the results in [5]) as well as between Cys 31 and Cys 129.

Having coupled 4 half-cystines out of 6, it is logical to assume that the two remaining half-

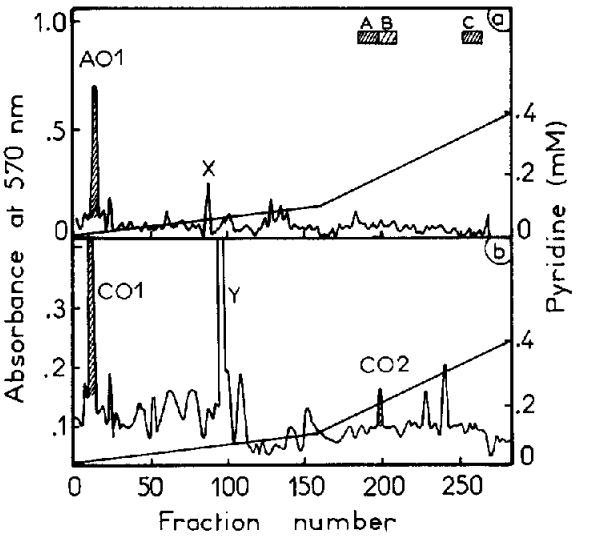

Fig.1. Chromatography of the oxidized peptic peptides A and C (AO, CO) on SP-Sephadex C-25. (a) Fraction AO, (b) fraction CO. Rectangles (more or less shaded according to the cystine concentration) indicates the place of cystine-containing fractions in the elution of the peptic hydrolysate of PSP S1 (400 nmol) chromatographed under the same conditions. The column (0.6 × 35 cm) was equilibrated with 5 mM pyridine-acetate buffer (pH 2.5) and eluted with 4 successive gradients of increasing pH and pyridine concentration. I (5 mM, pH 2.4 to 0.1 M, pH 3.75); II (0.1 M, pH 3.75 to 0.4 M, pH 4.8); III (0.4 M, pH 4.8 to 0.8 M, pH 5.0); IV (0.8 M, pH 5.0 to 4 M, pH 5.8). Only the first two gradients were necessary for these chromatography steps, the volume of each of the two gradient chambers was 80 ml, the flow rate 9.1 ml/h and 1.3-ml fractions were collected at room temperature. Eluants were monitored by ninhydrin assay after alkaline hydrolysis.

cystines Cys 104 and Cys 121 are linked. In the above experiment this cystine compound was not found. The peptide cleavages might not have been specific enough in this area to give a cystine peptide in a good yield. Therefore, chymotryptic hydrolysis of succinylated PSP S1 was carried out. Succinylation of PSP S1 was performed to ensure the solubility of the hydrolysate. The digest was filtered on Sephadex G-50 (fig.3). Fractions I–III after oxidation contained cysteic acid peptides. Since in fractions II and III it was possible to detect peptides of the S-S bridges 1–14 and 104–121 respectively, only fraction I was investigated.

As anticipated the latter was cystine-positive. 15 steps of automatic Edman degradation were performed on an aliquot of fraction I. Two chains

were sequenced (fig.2). Chain 1 contained more than 15 amino acid residues. It was sequenced from residue 92 to 106. Chain 2 was made up of 9 amino acid residues in position 117–125 of the PSP S1 sequence. The two non-identified derivatives correspond to Cys 104 and Cys 121.

A further confirmation of the existence of the bridge linking Cys 104 to Cys 121 was given by the following experiment: an aliquot of fraction I was oxidized (IO) and chromatographed on a thin layer of silica gel. Two spots IO1 and IO2 migrating as Lys and Val, respectively, were obtained (fig.3).

Table 1

Amino acid composition and characteristics of the cystine-containing peptides and their oxidation products

Amino acids	A	AO1	Sequence 1–15	C	CO1	Sequence 30–39	CO2	Sequence 128–133	I	IO1	Sequence 117–125	IO2	Sequence 92–107
Cys	1.5	1.6	2	1.5	1.0	1	1.1	1	2.0	0.6	1	0.9	1
Asx	1.0	0.9	1	3.8	3.0	3	1.0	1	3.1	2.2	2	1.1	1
Met (O2)				1.2	0.8	1							
Thr	0.8	0.6	1						0.9				
Ser	1.8	2.0	2	1.1	1.0	1			4.0			3.0	3
Glx	1.0	1.1	1	1.1	1.1	1			2.6	1.3	1		
Pro	1.1	1.2	1						1.9	0.8	1	1.9	2
Gly	1.1	1.0	1	1.0	1.0	1			3.1			3.0	3
Ala	1.1	1.0	1						2.2			1.0	1
Val				1.0			1.1	1	2.6	0.9	1	3.2	2
Ile	0.9	0.8	1						1.0			0.5	1
Leu				0.9	1.0	1			0.9			1.0	1
Tyr	2.1	2.1	3	1.0	0.9	1			0.5			ND	1
Phe				0.9			0.9	1	1.5	1.0	1		
His													
Lys				2.2			1.7	2	2.6	1.7	2		
Arg	1.0		1										
Total residue			15			10		6			9		16
Recovery (%)	78	15		43	9		14		12	6		2	
N-terminal residue(s)	Ile	Ile	Ile	Tyr Val	Tyr	Tyr	Val	Val	Gly Lys	ND	Lys	ND	Gly

All analyses were made after performic acid oxidation of the compounds. Cys and Met were titrated as cysteic acid and methionine sulfone. Oxidized tyrosine gave one or two peaks of low yield eluting between Phe and His. Experimental values are data of 24 h hydrolysis without corrections for losses

Steps	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
-Fraction C																
Chain 1	Tyr	---	Gln	Asn	Met	Asn	Ser	Gly	Asn	Leu	---	---				(30- 39)
Chain 2	Val	---	Lys	Phe	Lys	Asn	---	---	---	---	---	---				(128-133)
-Fraction I																
Chain 1	Gly	Ile	Gly	Ala	Pro	Ser	Ser	Val	Asn	Pro	Gly	Tyr	---	Val	Ser	(92-106)
Chain 2	Lys	Asp	Val	Pro	---	Glu	Asp	Lys	Phe	---	---	---	---	---	---	(117-125)

Fig.2. Automated Edman degradations of the half-cystine-containing peptides in fractions C and I. 12 and 15 steps were performed for fractions C and I, respectively. For fraction C, initial amount was 5 nmol and initial and repetitive yields 18 and 91%. For fraction I: initial amount was 3 nmol and initial and repetitive yields 34 and 84%.

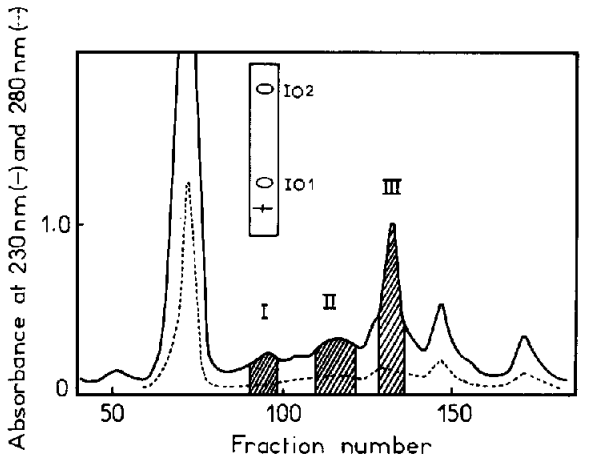

Fig.3. Gel filtration on Sephadex G-50 (superfine) of the chymotryptic digestion of the succinylated PSP S1. Two columns (1.5 × 100 cm) were connected in series and equilibrated and eluted at room temperature with 60 mM ammonium bicarbonate buffer (pH 7.8). Flow rate 12 ml/h. 2 ml fraction volume. Absorbances were measured at 230 nm (——) and 280 nm (- - -). Shaded areas: fractions containing cysteic acid after oxidation. Above peak I, TLC of the peak I fractions after oxidation is illustrated.

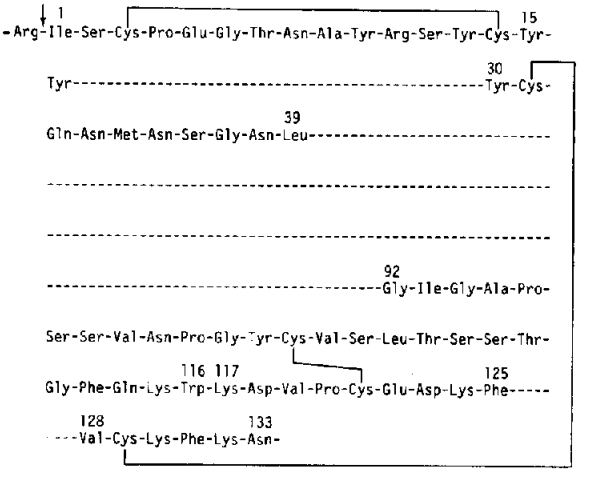

Fig.4. Schematic representation of the polypeptide chain of PSP S1 showing the location of its three disulfide bridges: Cys 3-Cys 14, Cys 31-Cys 129 and Cys 104-Cys 121. The arrow indicates the bond cleavage converting the polypeptide chain of PSP S2–5 into that of PSP S1.

The amino acid compositions of these peptides (table 1) enabled their identification with peptide sequences 117–125 and 92–107, respectively. The amino acid composition of IO2 was not as satisfactory as that of IO1 on account of the small quantity of this peptide available for the determination.

In fig.4, the three S-S bridges of PSP S1 are represented. The N- and C-terminal sequences are held together by the disulfide bond connecting Cys 31 to Cys 129. The two other disulfide bonds linking Cys 3 to Cys 14 and Cys 104 to Cys 121 only form small loops in the protein sequence. The same experiments as those described above for PSP S1 were performed on the forms PSP S2–5 and identical S-S bridges were found.

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REFERENCES

[1] Lechêne de la Porte, P., De Caro, A., Lafont, H. and Sarles, H. (1986) Pancreas 1, 301–308.
[2] De Caro, A., Lohse, J. and Sarles, H. (1979) Biochem. Biophys. Res. Commun. 87, 1176–1182.
[3] Montalto, G., Lusher, M., De Caro, A., Multigner, L., Sarles, H. and Delaage, M. (1985) CR Acad. Sci. Ser. III 300, 199–202.
[4] Montalto, G., Bonicel, J., Multigner, L., Rovery, M., Sarles, H. and De Caro, A. (1986) Biochem. J. 238, 227–232.
[5] Rouimi, P., Bonicel, J., Rovery, M. and De Caro, A. (1987) FEBS Lett. 216, 195–199.
[6] De Caro, A.M., Bonicel, J.J., Rouimi, P., De Caro, J.D., Sarles, H. and Rovery, M. (1987) Eur. J. Biochem. 168, 201–207.
[7] Bianchetta, J.D., Bidaud, J., Guidoni, A.A., Bonicel, J.J. and Rovery, M. (1979) Eur. J. Biochem. 97, 395–405.
[8] De Caro, J.D., Behnke, W.D., Bonicel, J.J., Desnuelle, P.A. and Rovery, M. (1983) Biochim. Biophys. Acta 747, 253–262.
[9] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[10] Erlanson, C., Charles, M., Astier, M. and Desnuelle, P. (1974) Biochim. Biophys. Acta 359, 198–203.
[11] Le Gaillard, F. and Dautrevaux, M. (1978) FEBS Lett. 94, 63–67.