Volume 299, number 2, 146–148 FEBS 10799 March 1992

Identification of hydrophobic fragments of α_1-antitrypsin and C1 protease inhibitor in human bile, plasma and spleen

Jan Johansson[a], Staffan Gröndal[c], Jan Sjövall[a], Hans Jörnvall[a,b] and Tore Curstedt[c]

[a]*Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden,* [b]*Center for Biotechnology, Huddinge Hospital S-141 86 Huddinge, Sweden* and [c]*Department of Clinical Chemistry, Karolinska Institutet at Danderyd Hospital, S-182 88 Danderyd, Sweden*

Received 7 January 1992; revised version received 22 January 1992

Hydrophobic peptides were isolated from the phospholipid fraction of human bile, plasma and spleen by exclusion chromatography in organic solvents. From plasma, the activation peptide of C1 protease inhibitor was recovered, from spleen the activation peptide of α_1-antitrypsin, and from bile, both these peptides, as well as a fragment generated by proteolytic cleavage of α_1-antitrypsin six residues N-terminal of the P1–P1′ peptide bond. Cleavages in this region inactivate antiproteases but have previously not been reported to occur in vivo. These peptides in human bile may reflect physiological actions in regulation of antiproteolytic activity or bile secretion processes, and/or be of importance for the physicochemical state of cholesterol, phospholipids and bile acids in bile.

Serpin fragment; Activation peptide; Inactivation peptide; Bile secretion; Plasma peptide

1. INTRODUCTION

During the complex formation between a serpin (serine protease inhibitor) and its target protease, the protease cleaves a specific peptide bond, the P1–P1′ bond, of the serpin active site. This cleavage liberates the C-terminal activation peptide, consisting of about 35 residues, from the antiprotease. The 10-residue segment of the serpin preceding the P1–P1′ bond is exposed in the tertiary structure [1,2] and susceptible to proteolytic cleavage (cf. [3]). Cleavage within this segment instead of at the P1–P1′ bond inactivates the antiprotease and liberates a correspondingly longer inactivation peptide. Such cleavages can be mediated by endogenous serpin target proteases [4–8], and by non-target proteases from both venom and bacterial toxins [7–9]. This mechanism has therefore been proposed to be involved in bacterial pathogenicity [7], and in inflammation [3,4], but an in vivo physiological production of these fragments has, to our knowledge, not been reported. We now identify such an inactivation peptide from α_1-antitrypsin, recovered in the phospholipid fraction of human bile.

2. MATERIALS AND METHODS

2.1. *Phospholipid isolation*

Spleen tissue was homogenized in chloroform/methanol (2:1, v/v). After filtration, 0.2 vols. of 0.1 M NaCl in water was added [10]. The lower phase was evaporated to dryness and the phospholipid fraction was isolated by liquid-gel chromatography on Lipidex-5000 in the solvent system methanol/ethylene chloride (4:1, v/v) [11].

Plasma and bile were mixed with 4 vols. of chloroform/methanol (2:1 v/v). The lower phase was filtered and the phospholipids were isolated as described above.

Correspondence address: J. Johansson, Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden. Fax: (45) 8337462.

2.2. *Protein purification*

Peptides in the phospholipid fraction of spleen, plasma and bile were isolated by chromatography on Sephadex LH-60 in chloroform/methanol (1:1, v/v), containing 5% 0.1 M HCl [11] and pooled into two fractions (corresponding to positions of elution of the lipopeptides SP-B and SP-C previously isolated from pulmonary phospholipids [11]). Aliquots of both pools from each source were reduced, treated with iodo[^{14}C]acetate and separated from excess reagents [12]. Analysis of the peptides from the three sources showed different sequences, but in each case the two pools gave identical results except for separate recoveries (more in the second pool, cf. section 3.1.).

2.3. *Structural analysis*

Acid hydrolysis for amino acid composition was performed in 6 M HCl/0.5% phenol at 110°C for 24 h, or at 150°C for 72 h. Sequencer analyses were carried out with an ABI 470A gas-phase instrument, equipped with an on-line 120A Analyzer for detection of liberated phenylthiohydantoin derivatives. The amino acid sequences obtained were identified by searching the Protein Identification Resource data base using the FASTA program. Plasma desorption mass spectrometry was performed as described [13].

3. RESULTS AND DISCUSSION

3.1. *Identification of antiprotease fragments*

Peptides in the phospholipid fraction of bile, plasma and spleen were submitted to Sephadex LH-60 chromatography, and the eluates were divided into two pools (cf. section 2.2.). In each case, the major part of the peptide material was recovered in the second pool, but the two pools gave identical sequence results. The as-

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signments, based on both sequence analysis and total compositions after hydrolysis, are summarized in Table I. The sequence of the peptide from plasma was identical to that of C1 protease inhibitor activation peptide ([14]; monitored for 27 residues), and that of the peptide from spleen was identical to α_1-antitrypsin activation peptide ([15]; monitored for 13 residues). In bile, both the C1 protease inhibitor activation peptide (monitored for 18 residues) and the α_1-antitrypsin activation peptide (monitored for 20 residues) were recovered. In addition, a third peptide starting six residues N-terminal of the P1–P1′ bond of α_1-antitrypsin was found in bile. This sequence was monitored for 17 residues and gave an extensive overlap beyond the P1–P1′ bond, which was therefore established to remain intact in this peptide.

From each of the three sources, the peptides in Table I were the only major fragments detected. Thus, the antiprotease fragments are the main peptides recovered from the phospholipid fractions of human plasma, spleen and bile. No radioactivity was incorporated into the protein fractions after [^{14}C]carboxymethylation, further indicating that the Cys-free activation and inactivation peptides are the only proteins present in appreciable amounts.

The α_1-antitrypsin activation peptide was subjected to plasma desorption mass spectrometry. Ions at m/z 4172, 2085 and 1390 were obtained, corresponding to molecular ions with one, two and three charges, respectively. These values are in good agreement with the calculated molecular mass (4130) of the intact activation peptide, provided that the ions observed are mainly sodium and potassium ion adducts, as for other peptides analyzed with plasma desorption mass spectrometry [13]. This shows that the α_1-antitrypsin activation peptide had not undergone proteolytic processing.

3.2. *Presence of antiprotease fragments in bile*

Protein constitutes a substantial part of the solutes in bile [16] but fragments of antiproteases have, to our knowledge, not previously been detected in bile. A partially characterized anionic protein fraction previously purified from bile [17] is unlikely to have contained antiprotease fragments since these have an excess of positive charges and amino acid compositions different from those then obtained from an anionic bile protein fraction [17].

3.3. *In vivo production of both inactivation and activation peptides*

The α_1-antitrypsin peptide with a starting point six residues N-terminal of the P1–P1′ bond is considered to be a true in vivo product for three reasons. First, it was found in about the same amount as the activation peptides. Second, its P1–P1′ bond is not cleaved, and third, no evidence of unspecific proteolytic processing within the activation peptides was obtained. The finding of this peptide in human bile may indicate that cleavage within the protease sensitive region constitutes a mode of regulation of serpins during normal physiology. Thus, α_1-antitrypsin has been found to be insensitive to treatment with elastase from human neutrophils [4], but is cleaved by proteases from *Staphylococcus aureus* [7] and elastase from *Pseudomonas aeruginosa* [18] and macrophages [6]. If the inactivation peptide in human bile is generated by its natural target protease, elastase, this would have a greater possibility to cleave within the exposed region of α_1-antitrypsin in vivo than previously detected in vitro. Irrespective of the mode of production, the presence of the inactivation peptide in vivo may reflect a physiological balance of the proteolytic/antiproteolytic activities in the system.

No α_1-antitrypsin fragments were found in plasma (Table I) although the normal plasma concentration of α_1-antitrypsin is about 5 times that of C1 protease inhibitor. This raises the possibility of local production of the fragments in the liver and/or bile ducts. Thus, the hydrophobic peptide fragments recovered in bile may be of importance for specific association with bile lipid components. This appears interesting for two reasons. First, cholesterol and phospholipids seem to be secreted as vesicles from the hepatocytes aided by the secretion of bile acids [19–21]. Although there is no established dependence on antiprotease activities in this process, patients with α_1-antitrypsin deficiency often suffer from intrahepatic cholestasis (see [22]). Thus, the excretion of the peptides in bile could reflect a function of antiproteases or antiprotease fragments in targetting or

Table I

Summary of peptides identified by sequence analysis of the hydrophobic peptide fractions from human plasma, spleen and bile

Source	Peptides identified	Recovery (mg/kg)
Plasma	C1 protease inhibitor activation peptide (Thr^{467}–Ala^{500})	0.2
Spleen	α_1-Antitrypsin activation peptide (Ser^{383}–Lys^{418})	30
Bile	C1 protease inhibitor activation peptide	0.2
	α_1-Antitrypsin activation peptide	
	α_1-Antitrypsin inactivation peptide (Leu^{377}–Lys^{418})	

Start and end points of the peptides identified are indicated by residues and positional numbers, referring to intact serpin amino acid sequences for C1 protease inhibitor [14] and α_1-antitrypsin [15]. Recoveries are given as mg of total hydrophobic peptides isolated per kg starting material. In bile, the three fragments were recovered in roughly equimolar amounts.

secretion of cholesterol/phospholipid vesicles into bile. Second, cholesterol and phospholipids occur in bile as vesicles and other aggregates or as mixed micelles with bile acids [23], and the form in which cholesterol occurs and interacts with biliary proteins influences the nucleation process in gallstone formation [24–26]. The hydrophobicity of the peptides identified and their association with phospholipids might indicate that they are of importance for the distribution of cholesterol between different physical forms. Thus, the peptides may influence the development of diseases such as intrahepatic cholestasis and gallstone formation.

Acknowledgements: We are grateful to Anne Peters and Elin Arvesen for excellent assistance. This work was supported by the Swedish Cancer Society (project 1806), the Oscar II:s jubileumsfond and the Axel Tielman Fund.

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