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Identification of lysine residue 199 of human serum albumin as a binding site for benzylpenicilloyl groups

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Cyanogen bromide and tryptic fragments of penicilloylated serum albumin from penicillin-treated patients were separated by HPLC. Determinations of benzylpenicilloyl (BPO) were performed on the different fractions. A BPO containing peptide was identified by its amino acid sequence and the bound BPO group was located on the lysine residue 199.

Albumin; Penicillin; Benzylpenicilloyl; Lysine

1. INTRODUCTION

Benzylpenicilloyl groups (BPO) result from the cleavage of the β lactam ring of penicillin G. They form covalent binding between their carbonyl group and ϵ-amino groups of albumin molecules. They thus give rise to penicilloyl-albumin conjugates which have lost all antibacterial activity but possess an immunogenic potential; BPO are thus considered to be the major antigenic determinant in penicillin allergy [1–3].

In patients treated with large amounts of penicillin, a transient hypoalbuminemia was observed [4], and it has been demonstrated that the covalent fixation of BPO was responsible for the presence of the fast electrophoretic band of albumin [5]. Recently BPO binding sites were located on cyanogen bromide (CNBr) fragments of serum albumin from penicillin-treated patients, the fixation occurring mainly on fragment $C_{124-298}$ and on the C-terminal fragment $A_{299-585}$ [6].

The purpose of this work was to identify the sites of BPO fixation on fragment C of fast serum albumin molecules.

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2. MATERIALS AND METHODS

2.1. *Reagents and serum*

For high-performance liquid chromatography (HPLC), all the chemicals used were of HPLC grade, the water was filtered through an Elgastat UHQ system and the solvents were degassed before use.

For amino acid analysis, as for recurring Edman degradation, the chemicals used were of sequanol grade. The other reagents were of analytical grade.

Serum was obtained from a patient in the Institut Pasteur Hospital, who received intravenously 50×10^6 IU penicillin G per day for 22 days. The collect was achieved 2 days after cessation of the treatment. The fast serum albumin was separated as described by Lapresle and Wal [5].

2.2. *CNBr digestion of fast albumin and HPLC separation of fragments*

CNBr cleavage was carried out on 10 mg of fast albumin as described [7]. Separation of fragments was performed by reversed phase HPLC using a Waters Ass. chromatographic system. The CNBr digest was dissolved in 1 ml of solvent A (0.05% aqueous solution of trifluoroacetic acid (TFA)). 50 μl were injected onto an Aquapore RP-300 column (7 μm, 30 × 4.6 mm i.d.) (Brownlee), equilibrated in a mixture of 50% solvent A and 50% solvent B (acetonitrile/2-propanol/water (2:1:2, v/v), 0.05% TFA).

Elution of peptides was achieved with a 30 min linear gradient from 50 to 100% of solvent B at a flow rate of 1 ml/min. All runs were performed at room temperature and peptides were detected at 215 nm. Fractions were collected every 30 s using a TDC 80 microcol (Gilson). 20 runs were performed, the fractions were pooled and the solvent was removed using a Speed Vac evaporator.

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2.3. *Tryptic digestion of fragment C, carboxymethylation and separation of peptides*

Fragment $C_{124-298}$ obtained from the CNBr digest was dissolved in 0.1 M phosphate buffer, pH 7.8, and digested overnight with TPCK-trypsin (Sigma) (enzyme/substrate, 1:20) at room temperature.

Reduction and carboxymethylation were performed directly on the tryptic digest, as described [8]. The mixture was then fractionated on a Nucleosil 5-C18 column (5 μm, 25 cm × 4.6 mm i.d.) (Société Française Chromato Colonne) equilibrated with solvent A. A linear gradient was operated, over 30 min, at 1 ml/min from 0 to 100% solvent B. Fractions, collected every 30 s, were evaporated to dryness.

The BPO containing fractions were then purified on the same column, using the same linear gradient at the same flow rate but with another elution system: solvent A′: 0.01 M phosphate buffer, pH 7, and solvent B′: solvent A′/acetonitrile (40:60, v/v). Fractions were collected every 0.1 min.

2.4. *Detection of the penicilloyl groups*

All the dried fractions collected from the analysis of CNBr and tryptic digests were dissolved in 0.1 ml water and the BPO detection was performed using an enzymoimmunoassay directly derived from the radioimmunoassay as described [9].

2.5. *Identification of peptides*

Amino acid analyses were performed with a Biotronik LC 5000 Analyzer after acid hydrolysis (5.7 N HCl, in vacuo, 110°C, 24 h). The C-terminal amino acids were identified by amino acid analysis after reaction with carboxypeptidase A (Boehringer); the N-terminal amino acids were identified by the recurring Edman degradation using Tarr's procedure [10].

Sequencing was achieved using a 470A sequenator connected to a 120A PTH HPLC analyzer (Applied Biosystems).

3. RESULTS

3.1. *Obtainment and identification of the penicilloylated fragment C after CNBr cleavage of the fast albumin*

Under the HPLC conditions described for the CNBr digest, two penicilloyl containing peaks were observed.

The first peptide, eluted at a retention time (RT) of 8.9 min was identified to the fragment $C_{124-298}$ by its amino acid analysis and by the determination of the C-terminal and N-terminal amino acids.

3.2. *Obtainment of the penicilloylated peptides after tryptic digestion of fragment C*

Fig.1 shows the chromatographic pattern of the tryptic digest, using the trifluoroacetic acid elution system (solvents A and B). Two BPO containing peaks are collected, e.g. RT 17 and 21 min. The HPLC of these two peaks using the second elution system (solvents A′ and B′) demonstrated that

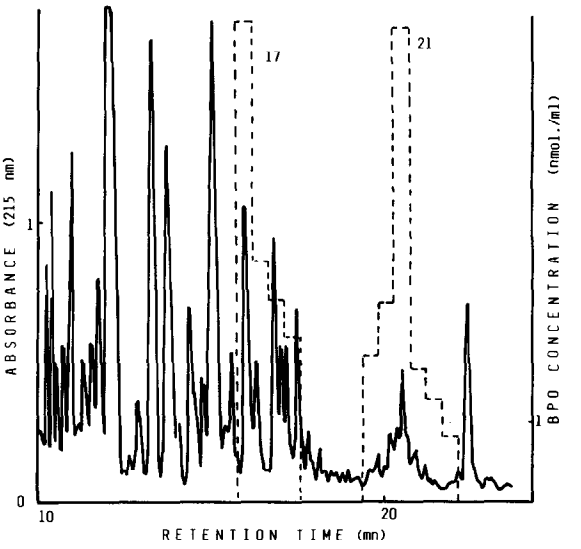

Fig.1. HPLC separation (TFA/acetonitrile/propanol) of the tryptic fragments from the fragment $C_{124-298}$ of the penicilloylated HSA. (——) Peptide determination at 215 nm; (– – –) EIA determination of BPO on the corresponding fractions.

peak 17 actually was a mixture of two penicilloylated fragments while peak 21 appeared to contain only a single BPO-peptide separated and collected at a RT of 16.8 min. Fig.2 shows the HPLC separation of this 'peptide 16'.

3.3. *Identification of peptide 16*

The amino acid sequencing of this peptide gave the following pattern:

Leu– –CM Cys–Ala–Ser–Leu–Gln–Lys

→ ⟶ ⟶ → → → → →

No amino acid could have been identified at the second step of the sequencing. The sequence of the peptide 198–205 of the 'normal' serum albumin (e.g. Leu–Lys–Cys–Ala–Ser–Leu–Gln–Lys) suggests that binding of the BPO group occurs on Lys-199.

The BPO determinations were done again on each of the four first steps of the recurring Edman degradation of peptide 16. Most of the BPO was detected on the second step (corresponding to Lys-199). Only traces corresponding to a ≈10% overlap were detected on the third step while no BPO could have been detected on the first and fourth step, nor on the residual tetrapeptide. No

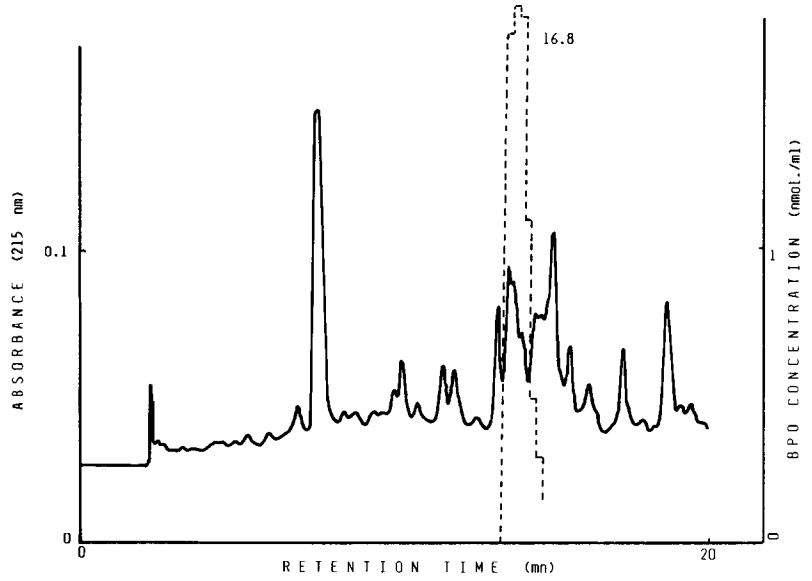

Fig.2. HPLC purification (phosphate buffer/acetonitrile) of the peak 21. (——) Peptide determination at 215 nm; (– – –) EIA determination of BPO on the corresponding fractions.

false positive result was observed in the EIA of a blank phenylthiohydantoin lysine derivative.

4. DISCUSSION

This work confirms our first observations on bisalbuminemia induced by penicillin therapy showing the presence of covalently bound BPO on the fast albumin molecules [5]. It also confirms the recent result from Lafaye and Lapresle [6] demonstrating that one major site of BPO fixation was fragment $C_{124\text{-}298}$, as well as the previous classical assumption that ϵ-amino groups of lysine were implicated in the formation of the allergenic metabolite of penicillin, e.g. BPO-protein conjugates [1,2].

The identification of Lys-199 of HSA as a binding site for BPO is to be compared to results obtained with other ligands. Walker [11] demonstrated that, under physiological conditions, lysine residue 199 reacts specifically with acetylsalicylic acid which thus transfers its acetyl group to HSA. Lysine 199 appears to be also a major site of the non-enzymatic glycosylation in vitro, this glycosylation being inhibited by aspirin [12]. In vivo, glucose becomes attached to Lys-199 too although it is not then the principal site of glycosylation [13,14]. The discrepancy between the rates of glycosylation of Lys-199 observed in vivo and in vitro could possibly result from the fact that in vivo the ϵ-amino group of Lys-199 is already covered by a ligand which would affect the availability of this site for glucose as suggested by Garlick et al. [13]. It could be hypothesized that in some cases, drugs such as BPO could be such a ligand.

Lys-199 has also been identified as one of the rapidly reacting lysines of HSA with 2,6-dinitro-4-trifluoromethyl benzene sulfonate [15].

In all these studies authors emphasize the role of the low pK_a of the ϵ-amino group of Lys-199, e.g. 7.9 according to Iberg and Fluckiger [14], whereas the other lysine ϵ-amino groups are likely to have pK values near 11. The lower pK should facilitate reaction with ligands since the reactive amino group must be uncharged for the chemical modification to occur. Similar observations were made concerning the in vitro aminolysis of penicillin in the presence of various amines, the reaction being more rapid and extensive as the pK of the amine acceptor was close to the pH of the reaction [16–18].

Lysine residue 199 thus appears to be an important binding site, for several ligands, in albumin

molecules. Concerning BPO fixation, it is largely implicated even if it is not the only site, as can be assumed from the HPLC patterns obtained as well as from previous observations on the electrophoretic heterogeneity of fast albumin [5] and from the results of Lafaye and Lapresle [6].

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