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Binding of benzyl penicilloyl to human serum albumin

Evidence for a highly reactive region at the junction of domains 1 and 2 of the albumin molecule

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Tryptic digests of fragment $C_{124-298}$ of penicilloylated serum albumin, obtained from a penicillin-treated patient or prepared by in vitro conjugation, were analyzed by HPLC. Determinations of benzyl penicilloyl groups (BPO) were performed on the different fractions. Three BPO-containing peptides were identified by their amino acid sequence and the bound BPO was located on lysines 190, 195 and 199 and serine 193. These four main BPO-binding sites are all located on a very short region (10 amino acid residues) of the albumin molecule at the junction of domains 1 and 2.

Albumin; Penicillin; Benzyl penicilloyl; Lysine; Serine

1. INTRODUCTION

Benzyl penicilloyl 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 bisalbuminemia 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 have been located on the fragment $C_{124-298}$ of albumin, on histidine 146 [6] or on lysine 199 [7]. Since then other fragments have been isolated and the purpose of this work was to complete this analysis and identify the main BPO-binding sites located on the fragment C of albumin molecule which are involved in both in vivo and in vitro conjugation of BPO with albumin.

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2. MATERIALS AND METHODS

2.1. *Reagents and penicilloylated albumins*

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 sequanal grade.

The other reagents were of analytical grade.

Serum was obtained from a patient in the Institut Pasteur Hospital, who had 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 by ion-exchange chromatography on DEAE Sephadex and the elution performed with 0.2 M sodium phosphate buffer, pH 5.75, according to the methodology previously described [5] and slightly modified.

A BPO-human serum albumin (BPO-HSA) conjugate was prepared in vitro by incubating 100 mg HSA (obtained from Centre National de Transfusion Sanguine), from which free fatty acids have been removed according to Chen [8], with 30 mg

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benzyl penicillin (Specillin G, Specia) in 10 ml of 0.01 M phosphate buffer, pH 7.5, overnight at 37°C. Free penicillin was removed by ultrafiltration (micropartition system MPS-1, Amicon) and dialysis. The conjugate was then freeze dried.

2.2. *Analysis of the tryptic digests and separation of the penicilloylated peptides*

The preparation and purification of the two penicilloylated fragments C obtained by CNBr cleavage of patient's serum fast albumin and of BPO-HSA conjugate; the tryptic digestion of these fragments and the reduction and carboxymethylation of the digests were performed as previously described [7].

The penicilloylated peptides were then isolated by 3 steps of HPLC using a Waters Ass. Chromatographic System. The first step, carried out on the whole tryptic digest after reduction and carboxymethylation, was performed on a Nucleosil 5-C18 column (5 μm, 25 cm × 4.6 mm i.d.) (Société Française Chromato Colonne) equilibrated with solvent A_1 (0.01 M phosphate buffer, pH 7). Elution was achieved by a 30 min linear gradient from 0 to 100% of solvent B_1 [solvent A_1/acetonitrile (40:60, v/v)] at a flow rate of 1 ml/min.

Fractions were collected every 0.2 min using a TDC 80 microcol (Gilson). All the runs were performed at room temperature. Peptides were detected at 215 nm and BPO was detected in the different fractions by enzyme immunoassay as previously described [7].

The BPO-containing fractions were then purified on a cation-exchange column (Polysulfoethyl Aspartamide, Poly LC) (5 μm, 20 cm × 4.6 mm i.d.) equilibrated with solvent A_2 [0.05 M phosphate buffer, pH 3/acetonitrile (50:50, v/v)]. The elution system consists of a 5 min isocratic step with solvent A_2 followed by a 40 min linear gradient from 0 to 100% of solvent B_2 (solvent A_2 + KCl at a final concentration of 0.25 M). Fractions were collected and analyzed as before.

A final third step of purification and desalting was operated on the BPO-containing fractions by using a Nucleosil 5-C18 column equilibrated with solvent A_3 (0.05% aqueous solution of trifluoroacetic acid (TFA)). Elution was achieved with a 30 min linear gradient of solvent B_3 (acetonitrile/2-propanol/water (2:1:2, v/v), 0.05% TFA) and the fractions were collected every 0.1 min.

2.3. *Identification of peptides and location of BPO-binding sites*

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

A recurring Edman degradation using Tarr's procedure [9] was performed on each peptide and the EIA of BPO was realized at each cycle on the phenylthiohydantoin (PTH) derivative.

3. RESULTS

3.1. *Obtainment of the penicilloylated peptides from tryptic digests of fragments C*

The HPLC patterns of the two tryptic digests of in vivo and in vitro penicilloylated albumin fragments C were identical. Fig.1 shows that obtained with the in vitro conjugate. In both cases 3 BPO-containing peaks were collected e.g. Pep 12, Pep 15 and Pep 16 at retention times (RT) of 12, 15 and 16.7 min, respectively.

Pep 12 and Pep 15 were isolated and analyzed in the second step of purification. Fig.2 shows this ion-exchange HPLC pattern. For each of these two peptides a single BPO-containing peak was obtained and collected at RT 6.2 and 14.2 min for Pep 12 and Pep 15, respectively, while numerous non-penicilloylated contaminating peptides were discarded. This second step of purification appeared to be unnecessary for Pep 16.

In order to obtain a complete purification of the BPO peptides and to prepare them free of salts for sequencing, a third step of purification was performed on the 3 peptides. Fig.3 shows the patterns of the reversed-phase HPLC using the TFA elution system.

For the 3 steps of the purification procedure, identical profiles were obtained with the in vivo conjugate, the 3 purified BPO peptides obtained at the different steps of the analytical procedure being separated and collected at exactly the same retention times as those presented for the in vitro conjugate.

In both cases, the proportion of the total BPO present in fragment C was ~10% in Pep 12, 30% in Pep 15 and 40% in Pep 16. These 3 peptides thus account for ~80% of the total BPO present in the fragment C.

3.2. *Identification of peptides*

Each of the purified BPO peptides prepared from the in vivo and in vitro penicilloylated albumins was analyzed for its amino acid sequence. In both cases, the patterns were identical (e.g. separated at the same RT).

Pep(s) 12:

Asp - Glu - Gly - - Ala - Ser - Ser - Ala - Lys

Pep(s) 15:

Ala - Ser - Ser - Ala - - Gln - Arg

Pep(s) 16:

Leu - - CMCys - Ala - Ser - Leu - Gln - Lys

No amino acid could be identified in the fourth cycle of the sequencing of Pep(s) 12. This sequence corresponds to that of peptide 187–195 of the

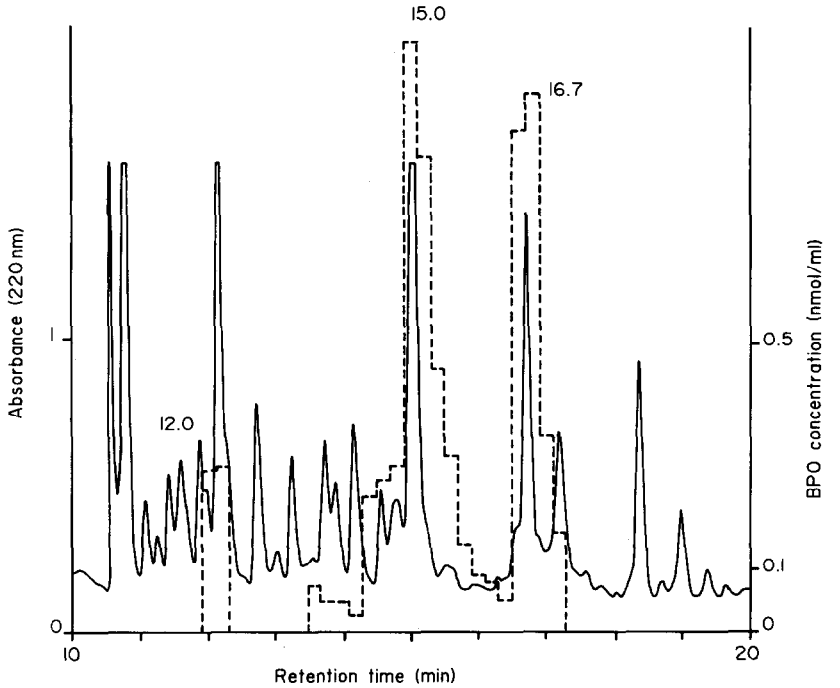

Fig.1. Reversed-phase HPLC analysis (phosphate buffer/acetonitrile, pH 7) of the tryptic digests of the whole fragment $C_{124-298}$ from penicilloylated albumin. Pep 12 (RT = 12 min), Pep 15 (RT = 15 min) and Pep 16 (RT = 16.7 min) were isolated for further purifications. (——) Peptide detection at 215 nm; (– – –) EIA determination of BPO on the corresponding fractions.

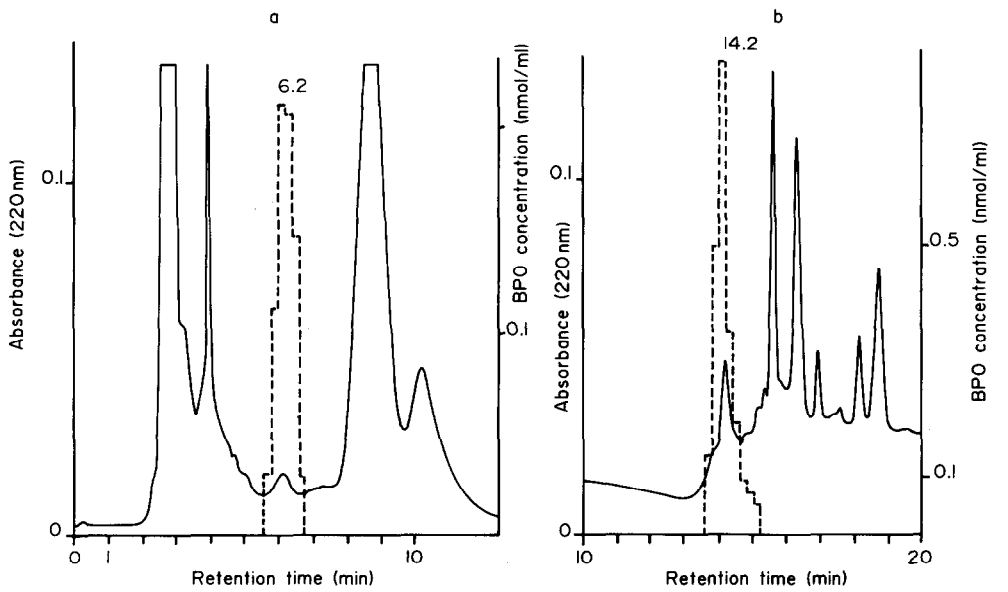

Fig.2. Cation-exchange HPLC analysis (phosphate buffer/acetonitrile, pH 3) of fragments isolated in fig.1. (a) Pep 12, (b) Pep 15. (——) Peptide detection at 215 nm; (– – –) EIA determination of BPO on the corresponding fractions.

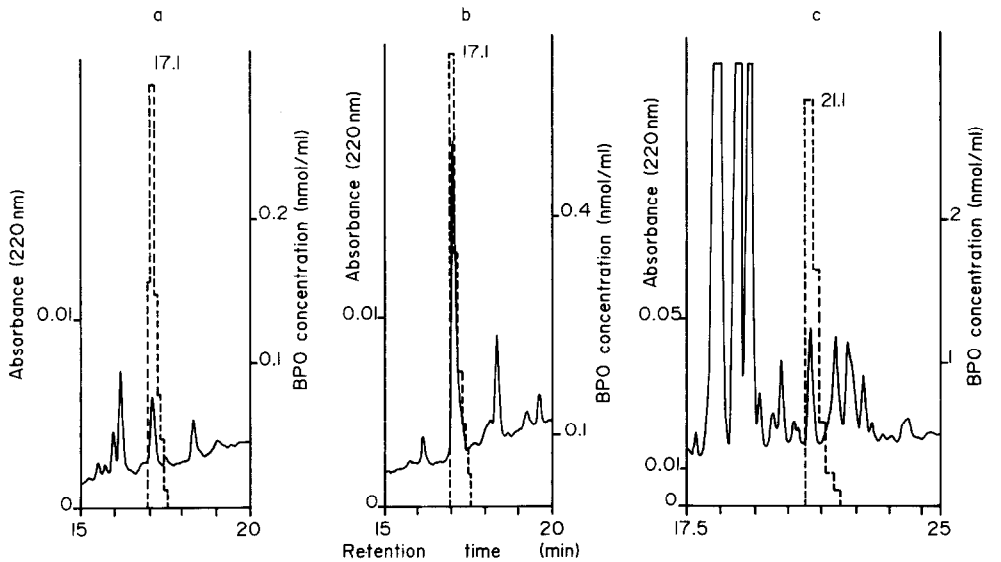

Fig.3. HPLC purification (TFA/acetonitrile/2-propanol/water) of fragments isolated in figs 1 and 2. (a) Pep 12, (b) Pep 15, (c) Pep 16. (——) Peptide detection at 215 nm; (– – –) EIA determination of BPO on the corresponding fractions.

'normal' serum albumin [10] suggesting that binding of BPO occurs at lysine 190.

Concerning Pep(s) 15, the fifth cycle of the sequencing corresponding to lysine 195 of peptide 191–197 gave no PTH derivative and can be supposed to correspond to the BPO-binding site of this peptide. It should also be noticed that less PTH serine was measured in the third cycle than in the second one. This result is unexpected since a ~10–15% overlap is usually observed and suggests that a part of Ser-193 could be undetected during sequencing.

Concerning Pep(s) 16 the sequencing pattern suggests it corresponds to segment 198–205, BPO being fixed on lysine 199.

In order to confirm that the missing residue in the sequencing is the real BPO-binding site, BPO determinations were done again at each cycle of the Edman degradation of purified peptides 12, 15 and 16. Concerning Pep 16, these determinations were performed on both peptides obtained from in vivo and in vitro penicilloylated albumins. For Pep 12 and Pep 15, they were only performed on the in vitro conjugate since too small quantities of purified peptides remained available after the analysis of the in vivo penicilloylated albumins.

3.2.1. Pep 16

Most of the BPO was detected in the second cycle (Lys-199). Only traces corresponding to a ~10% overlap were detected in the third cycle while no BPO was detected in either the first and fourth cycle, or the residual tetrapeptide.

3.2.2. Pep 12

BPO was detected in the fourth cycle (Lys-190) while a 10–15% overlap was still present in the fifth step. No BPO was present in any of the other cycles.

3.2.3. Pep 15

Most of the BPO (~50% of the total BPO present on this fragment) was detected in the fifth cycle (Lys-195) and a ~10% overlap in the sixth cycle. However, a significant amount of BPO (~30%) was detected in the third cycle corresponding to Ser-193 which was partly missing in the sequencing pattern. A ~10% BPO overlap was still present in the fourth cycle.

It has to be mentioned that the whole procedure was performed on a blank HSA sample and on blank lysine and serine phenylthiohydantoin derivatives. No false positive result occurs at any step of the analysis.

4. DISCUSSION

The location of BPO-binding sites on fragment

C of HSA is supported by the identification of penicilloylated lysines 190, 195 and 199. The absence of cleavage of the bonds 190–191, 195–196 and 199–200 in the peptides 12, 15 and 16 confirms that the lysine is substituted.

It is worth noticing that the same binding sites have been identified for both in vitro and in vivo serum albumin penicilloylation; they account for ~80% of the total BPO present on the albumin fragment C.

Lysine 199 (on Pep 16: 198–205), already identified as a binding site for in vivo fixation of BPO to albumin [7], is confirmed and appears to be involved in in vitro conjugation too.

We did not find any BPO on histidine 146 either on the in vivo or on the in vitro penicilloylated albumins. This residue was described as a BPO-binding site by Lafaye and Lapresles [6] working on penicilloylated albumin isolated and analyzed from a pool of penicillin-treated patients' sera by a methodology slightly different from ours. Although no definite and unequivocal identification of a penicilloylated histidine-146 was brought, this binding site could exist and account for a part of the non-identified bound BPO (~20%) in our study. However, one should notice that this lone putative histidine BPO-binding site cannot by itself explain the faster mobility of the penicilloylated albumin from which it has been isolated, since at pH 8.7 (at which the electrophoretic separation of the penicilloylated fast albumin is observed [5]), the histidine residues ($pK_a = 7$) are not protonated and an eventual fixation of BPO would not have changed the global electric charge of the albumin molecule.

On the opposite the fixation of BPO on various lysine residues, as previously hypothesized [1–4], can explain the faster mobility and the heterogeneity observed on penicilloylated albumins [5].

Moreover one must notice the respective positions of the reactive lysine and serine residues in the sequence of serum albumin. Each lysine is located near a serine from which it is separated by two amino acids e.g.

Pep 12: —— —— —— Lys_{190} —— —— Ser_{193}
Pep 15: Ser_{192} —— —— Lys_{195} —— —— ——
Pep 16: —— —— —— Lys_{199} —— —— Ser_{202}

These sequences represent the configuration in which α-helix Lys and Ser residues are the nearest. This supports the mechanism proposed by Yamana et al. [11] for the formation of penicilloylamide allergenic determinants. The penicilloylation of a hydroxyl group (e.g. of a serine residue) should occur rapidly with the assistance of general acid-base catalysis toward penicillin β-lactam to yield the corresponding penicilloyl esters, which are then rapidly converted to a stable penicilloylamide by nucleophilic attack by a proximate ϵ-amino group of lysine on the protein. What is needed is the combination of closely located functional groups on the protein.

This proposed mechanism is confirmed by the presence of the penicilloylated serine residue 193 on Pep 12. However Ser_{193} and Lys_{190} are never both penicilloylated on the same molecule; Ser_{193} could thus be considered as a first intermediate site which rapidly reacts with penicillin before the BPO is transferred to the proximate Lys_{190}.

According to the structural protein model of Brown [10] all the BPO-binding sites identified here are located on a very short fragment of the albumin molecule (10 amino acids residues: 190–200) located at the junction of domains 1 and 2. This fragment is part of a larger flexible region of human serum albumin possessing high-affinity binding sites for salicylate, glucose, warfarin and other ligands [12–15].

Concerning the binding of BPO to HSA the same reactive sites are involved whether the penicilloylation occurs in vivo in the penicillin-treated patient or in vitro under 'physiological' conditions.

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