Volume 263, number 2, 237–240 FEBS 08337 April 1990

Identification of the binding sites of benzyl penicilloyl, the allergenic metabolite of penicillin, on the serum albumin molecule

Mireille Yvon, Patricia Anglade* and Jean-Michel Wal

Laboratoire des Sciences de la Consommation and **Station de Recherches Laitières, INRA-CRJ, 78350 Jouy-en-Josas, France*

Received 14 February 1990

Tryptic digests of fragment $A_{299-585}$ of penicilloylated serum albumin obtained from two penicillin-treated patients or prepared by in vitro conjugation, were analyzed by a tandem immunoaffinity reversed-phase HLPC. 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 were located on lysines 432, 541 and 545. Six major BPO binding sites were thus identified on the whole albumin molecule. All of them are lysine residues and correspond to a limited number of definite structures in which lysine and serine residues appear to be closely associated.

Albumin; Penicillin; Benzyl penicilloyl: Lysine residue; Serine residue

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 three BPO-binding sites have been located on the fragment $C_{124-298}$ of albumin, on lysines 190, 195 and 199 [6]. The purpose of this work was firstly to identify the other BPO-binding sites on the albumin molecule, and secondly to determine if similar sequences are involved in these fixations, in order to confirm the possible mechanism of penicilloylation suggested by the previous results.

2. MATERIALS AND METHODS

2.1. *Reagents and penicilloylated albumins*

For high-performance liquid chromatography (HPLC), all the chemicals used were of HPLC grade.

Correspondence address: J.M. Wal, Laboratoire des Sciences de la Consommation, INRA-CRJ, 78350 Jouy-en-Josas, France

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

Control HSA was obtained from Centre National de Transfusion Sanguine. Free fatty acids were removed according to Chen [7].

Sera were obtained from two patients in the Institut Pasteur Hospital, who received intravenously 50×10^6 IU penicillin G per day during 3–4 weeks and the fast serum albumin was separated from each serum by ion exchange chromatography on DEAE Sephadex [5,8].

A BPO-human serum albumin (BPO-HSA) conjugate was prepared in vitro as previously described [8].

2.2. *Preparation, reduction and carboxymethylation of the CNBr fragment $A_{299-585}$*

Control and penicilloylated fragments A were prepared from control HSA and from patients' fast serum albumin fractions or from in vitro BPO/HSA conjugate as described; i.e. CNBr digestion followed by an HPLC separation of fragments A, B and C [6].

Denaturation, disulfide cleavage and alkylation were performed as described by Swenson et al. [9]: the A containing fractions were dried and dissolved in 1 M Tris-HCl buffer, pH 8.0, containing 6 M urea and a 10-fold molar excess of dithiothreitol over the –SH concentration and kept overnight at 40°C under nitrogen. Iodoacetic acid in 1 N NaOH (1.3-fold molar excess over total thiols) was added and the mixture was maintained in the dark for 4 h, before the reaction was stopped by addition of β-mercaptoethanol.

2.3. *Obtainment and tryptic digestion of the four peptides $A_{1\ 447-549}$, $A_{2\ 330-446}$, $A_{3\ 299-329}$, $A_{4\ 549-585}$*

The mixture obtained after reduction was loaded onto an Aquapore RP-300 column equilibrated in trifluoroacetic acid (TFA) 0.05% and the peptides were eluted with a 30 min linear gradient from 0% to 100% of acetonitrile/2-propanol/water 2:1:2, v/v, 0.05% TFA at a flow rate of 1 ml/min. The HPLC fractions collected every 30 s were evaporated to dryness and BPO was detected by Enzyme Immuno Assay (EIA) as previously described [6].

Tryptic digestion was performed on penicilloylated fragments A_1 and A_2 in 0.1 M phosphate buffer, pH 7.8, overnight at room temperature using TPCK-trypsin (Sigma) (molar ratio E/S = 1/50). The enzymatic reaction was stopped by 10 min heating at 100°C.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

2.4. *Isolation and separation of the penicilloylated fragments from A_1 and A_2 tryptic digests*

2.4.1. Preparation of immunoaffinity column by covalent immobilization

13 mg of affinity pure rabbit anti-BPO antibodies obtained from the immune sera already prepared [10] were dissolved in 18 ml of 1 M phosphate buffer, pH 7, and recycled through an Ultraffinity-EP column (5 cm × 4.6 mm i.d., Beckman) overnight at a 0.2 ml/min flow rate. 8 mg of IgG were thus immobilized. The column was washed in 1 M phosphate buffer, pH 7, and stored in the same buffer. It was equilibrated with 10 mM phosphate buffer, pH 7, 0.15 M NaCl (PBS) before use.

2.4.2. Apparatus

The immunoaffinity column was connected to a reversed-phase column (Nucleosil $5C_{18}$, 25 cm × 4.6 mm i.d., Société Française Chromato Colonne) via a 6-way valve (Rheodyne) as described by Rybacek et al. [11]. The switching valve contained the affinity column in place of a sample loop. The position of the switching valve determined whether the immunoaffinity and analytical columns were used in series or separately.

2.4.3. Obtainment and separation of the penicilloylated fragments

The tryptic digests of peptides A_1 and A_2 were loaded onto the affinity column in PBS at a flow rate of 1 ml/min. The column was then washed with 10–15 ml of PBS at the same flow rate. Non-retained peptides were collected to check the absence of BPO. The valve was then switched to place both columns in line. The bound antigens were desorbed from the immunoaffinity column into the analytical column with a 0.1% TFA solution at a flow rate of 1 ml/min. After 10 min, the valve was switched, placing the immunoaffinity column off-line to permit the analysis of the antigens by gradient elution on the analytical column. The reversed phase chromatographic separations were performed with a 30 min linear gradient from 0% to 100% of acetonitrile/isopropanol/water (2:1:2, v/v), and 0.1% TFA at a flow rate of 1 ml/min. All runs were performed at room temperature and peptides were detected at 220 nm. Fractions were collected every 0.25 min and analyzed for BPO concentration. Simultaneously, the affinity column was re-equilibrated with loading buffer.

2.5. *Identification of peptides and location of the BPO binding sites*

Amino acid analyses were performed with a Biotronik LC 5000 analyzer after acid hydrolysis (5.7 N HCl, in vacuo, 110°C, 24 h).

On each penicilloylated fraction obtained the amino acid sequencing was achieved using a 477 sequenator connected to a 120A PTH HPLC analyzer (Applied Biosystems). Moreover, a recurring Edman degradation using Tarr's procedure [12] was performed on each peptide and the EIA for BPO detection was realized at each cycle on the phenylthiohydantoin (PTH) derivative.

The C-terminal amino acids were identified by amino acid analysis after reaction with carboxypeptidase A (Boehringer).

3. RESULTS

3.1. *Obtainment and identification of the penicilloylated fragment A from the CNBr digests of the albumins*

Two penicilloyl containing peaks were observed from the CNBr digests of each of the 3 penicilloylated albumins, i.e. the two fast albumin fractions isolated from patients' sera and the BPO/HSA conjugate prepared in vitro. The first one eluted at a retention time (RT) of 8.9 min was previously identified with fragment $C_{124-298}$ [6], the second one (RT: 12 min) was identified with fragment $A_{299-585}$ by its amino acid analysis and by the determination of the C-terminal and N-terminal amino acids. No BPO group was detected in the fragment B_{1-123} of the different penicilloylated albumins.

3.2. *Separation of peptides from fragment A*

Fragment $A_{299-585}$ consists of 4 chains held together by S–S bonds. Actually 4 peptides were obtained after reduction, *S*-carboxymethylation and HPLC separation. The HPLC patterns of the reduced fragments A obtained from in vivo and in vitro penicilloylated albumins were identical. For each fragment A, two penicilloyl containing peaks were obtained, i.e. A_1 and A_2 at retention times of 20.8 and 23.2 min, respectively (Fig. 1). The peptides eluted in these fractions were identified with sequence 447–547 for A_1 and 330–446 for A_2 by their amino acid analysis and by the determination of the C- and N-terminal amino acids.

3.3. *Obtaining of the penicilloylated fragments from tryptic digests of peptides A_1 and A_2*

Isolation and separation of penicilloylated fragments from A_1 and A_2 tryptic digests were achieved using the above-described dual-column chromatographic system. The HPLC patterns of the 3 tryptic digests of peptides A_1 obtained from the two in vivo BPO/HSA and the in vitro conjugate were quite similar. Fig. 2 shows those obtained with one of the in vivo conjugates. In the 3 cases, two BPO-containing peaks were collected, i.e. Pep $19A_1$ (RT = 19.3 min) and Pep $21A_1$ (RT = 21.4 min). The retention times of the two peaks observed with the different digests of peptides A_1 obtained from the 3 penicilloylated albumin samples were identical but their respective height may vary from one sample to another. In the same way, the patterns of the 3 tryptic digests of peptides A_2 were similar. Fig. 3 shows one obtained from an in vivo BPO/HSA. Two BPO-containing peaks were obtained i.e. Pep $20A_2$ (RT = 20.1 min) and Pep $21A_2$ (RT = 21.2 min). A few other peaks were detected at 220 nm in all the digests, but they did not contain BPO. These peptides may have been non-specifically bound to the affinity column. Furthermore, non-retained peptides on the affinity column were collected for BPO detection: no penicilloylated peptides were detected.

3.4. *Identification of penicilloylated fragments and location of BPO-binding sites*

Each of the penicilloylated fragments isolated from the in vivo and in vitro penicilloylated albumins was analyzed for its amino acid sequence. In order to confirm the BPO-binding site, BPO determinations were done again at each cycle of the Edman degradation.

The same results were obtained whatever the origin of the peptides.

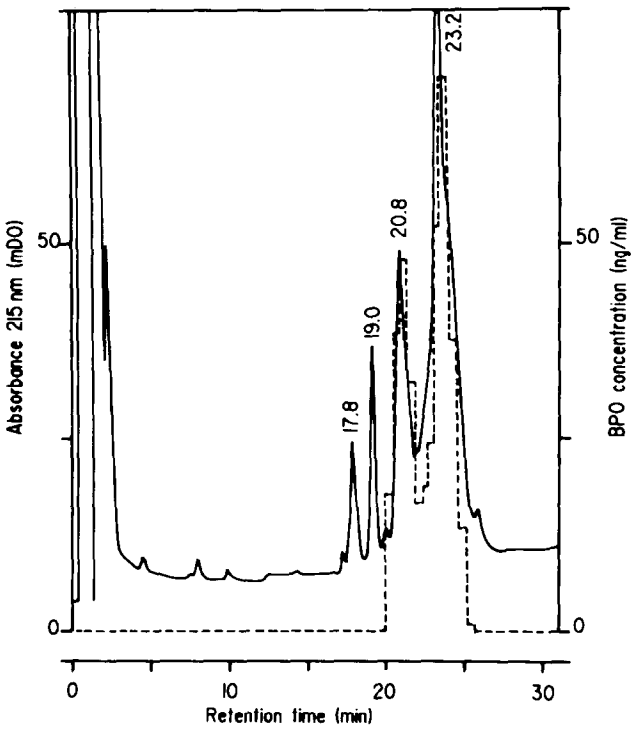

Fig. 1. HPLC separation of the 4 peptidic chains from the CNBr fragment $A_{299-585}$ of the fast albumin fraction isolated from a patient' serum. (——) Peptide determination at 220 nm; (- - -) EIA determination of BPO on the corresponding fractions.

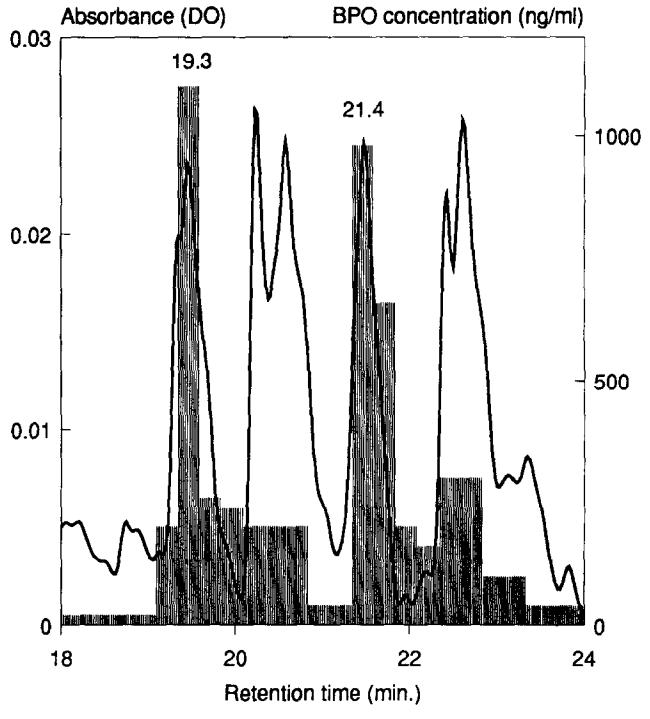

Fig. 2. HPLC separation of the penicilloylated fragments from the tryptic digest of peptide A_1 obtained from the same in vivo conjugate. (——) Peptide determination at 220 nm; (▒▒▒) EIA determination of BPO on the corresponding fractions.

3.4.1. Fragments from peptide A_1

Pep $19A_1$: Ala - Thr - - Glu - Gln - Leu - Lys

This sequence corresponds to that of fragment 539–545 of the human serum albumin. No amino acid could be identified in the third cycle of the sequencing while BPO was found in this fraction proving that binding of BPO occurs on lysine-541.

Pep $21A_1$: Glu - Gln - Leu - - Ala - Val

This peptide was identified with sequence 542–548 of HSA, although the last PTH amino acid could not be identified. In fact, since this peptide came from a CNBr cleavage, the methionine-548 has been changed into homoserine or homoserine-lactone which cannot be identified as PTH derivatives.

The fourth cycle of the sequencing corresponding to lysine-545 gave no PTH-derivative and was shown to be the BPO-binding site of this peptide since most of the BPO was detected at this cycle.

3.4.2. Fragments from peptide A_2

Pep $21A_2$: Asn - Leu - Gly - - Val - Gly - Ser - Lys

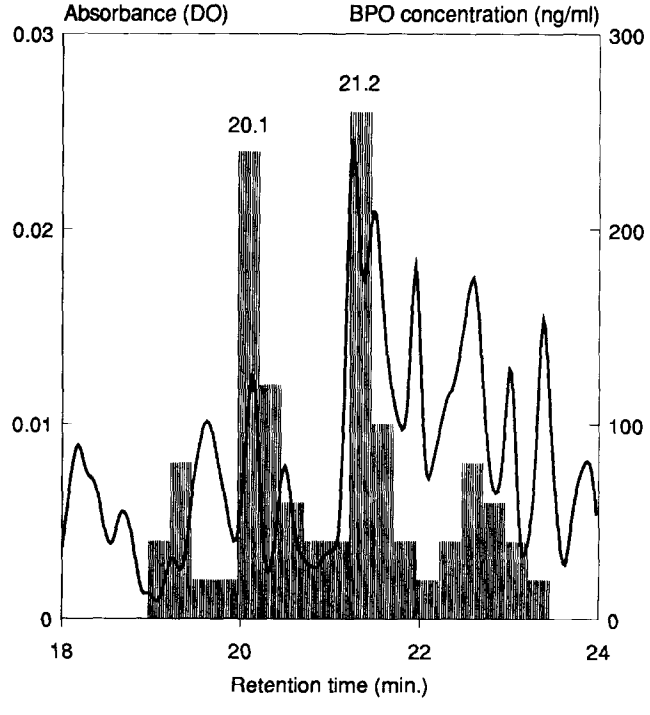

Fig. 3. HPLC separation of the penicilloylated fragments from the tryptic digest of peptide A_2 obtained from the same in vivo conjugate. (——) Peptide determination at 220 nm; (▒▒▒) EIA determination of BPO on the corresponding fractions.

The sequencing pattern shows that Pep $21A_2$ corresponds to sequence 429–436, BPO being fixed on lysine-432 since no PTH derivative could be identified in the Edman cycle corresponding to this residue and BPO was detected in the same fraction.

Pep $20A_2$: Asn - Leu - Gly - - Val - Gly - Ser - Lys

and Ala - Thr - - Glu - Gln - Leu - Lys

This fraction consisted of a mixture of two peptides corresponding to Pep $21A_2$ and Pep $19A_1$. A partial separation of these two linked peptides was obtained by reversed-phase HPLC in acidic conditions. After elution with acetonitrile, 0.1% TFA, only 20% of the mixture could be dissociated. Successive HPLC was necessary to achieve the separation of the two peptides.

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

4. DISCUSSION

The penicilloylated tryptic peptides of albumin fragment A were isolated using a tandem immunoaffinity and reversed-phase HPLC. This method permitted one to obtain the purified penicilloylated peptides by a single step procedure. Three binding sites for penicilloyl residue were unequivocally established: Lys-432, Lys-541, Lys-545. They account for approximately 75% of the total BPO present on the albumin fragment A.

It is worth noting that the same sites have been identified for the two in vivo and for the in vitro penicilloylated albumins.

The 3 binding sites identified on fragment A must be added to the 3 others, already identified on fragment C [8]. BPO binding sites thus appear to be located on lysine residues. Moreover, only 6 different lysine residues out of a total of 59 can be penicilloylated. This result is in agreement with the theoretical model of Ahlstedt et al. [14]. In conditions of penicillin dosages similar to those of the present study, these authors calculate that a maximum of 6 different binding sites can be expected on the same albumin molecule.

As previously observed for the 3 binding sites located on fragment C, Lys-432 is located near a serine from which it is separated by two amino acids, this position represents in an α-helix structure Lys and Ser residues as being the nearest. This supports the hypothetical mechanism of penicilloylation proposed by Yamana et al. [14]: an intermediate ester of penicilloic acid is likely to be formed with a hydroxyl group of a serine residue. This unstable ester could then undergo an acyl transfer to the primary amino group of a lysine residue in a suitable steric position. In contrast, for Lys-541 and Lys-545 there is no evidence for proximity to hydroxylated residues in the sequence. However, another serine residue could be suitably located in the tertiary structure of the albumin molecule or in the environment of other HSA molecules. This hypothesis is supported by the obtaining of Pep $20A_2$. This fragment consists of two peptides: Pep $19A_1$ and Pep $21A_2$ held together by strong interactions, since they are only partially broken under acidic dissociating conditions. This could suggest that these two sequences are closely located. So, Ser-435 could also participate in penicilloylation of Lys-541 or of Lys-545.

BPO binding sites thus seem to correspond to a limited number of definite structures. Two other lysine residues, namely Lys-205 and Lys-276, are located in similar respective positions from serine residues. However, they do not appear to be penicilloylated. It should be noted that for Lys-205, Ser-202, which would be associated with its penicilloylation, is already involved in penicilloylation of Lys-199. Concerning the second lysine, one can suppose it to be located in a part of the albumin molecule which would not be in the α-helix structure.

REFERENCES

[1] Levine, B.B. and Ovary, Z. (1961) J. Exp. Med. 114, 875–904.
[2] Schneider, C.M. and De Weck, A.L. (1969) Int. Arch. Allergy 36, 129–139.
[3] Ahlstedt, S. and Kristofferson, A. (1982) Prog. Allergy 30, 67–134.
[4] Arvan, D.A., Blumberg, B.S. and Melartin, L. (1968) Clin. Chim. Acta 22, 211–218.
[5] Lapresle, C. and Wal, J.M. (1979) Biochim. Biophys. Acta 586, 106–111.
[6] Yvon, M. and Wal, J.M. (1988) FEBS Lett. 239, 237–240.
[7] Chen, R.F. (1967) J. Biol. Chem. 242, 173–181.
[8] Yvon, M., Anglade, P. and Wal, J.M. (1989) FEBS Lett. 247, 273–278.
[9] Swenson, R.P., Williams, M., Massey, V., Rouchi, S., Minchiotti Galliano, M. and Curti, B. (1982) J. Biol. Chem. 257, 8817–8823.
[10] Wal, J.M., Bories, G., Mamas, S. and Dray, F. (1985) FEBS Lett. 57, 9–13.
[11] Rybacek, L., D'Andrea, M. and Tarnowski, S.J. (1987) J. Chromatogr. 397, 355–364.
[12] Tarr, G.E. (1982) in: Methods in Protein Sequence Analysis (Elzinga, M. ed.) pp. 223–232, Humana, Clifton, NJ.
[13] Ahlstedt, S., Ekström, B., Svärd, P.O., Sjöberg, B., Kristofferson, A. and Ortengren, B. (1980) CRC Crit. Rev. Toxicol. 1, 219–277.
[14] Yamana, T., Tsuji, A., Miyamoto, E. and Kiya, E. (1975) J. Pharm. Pharmacol. 27, 771–774.