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Identification of two fixation sites for penicilloyl groups on the albumin molecule from penicillin-treated patients

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Fixation of penicilloyl groups to albumin in penicillin-treated patients was shown to involve histidine residues 146 and 338.

Albumin; Penicillin; Penicilloyl group; Histidine; (Human)

1. INTRODUCTION

Transient bisalbuminemia due to the presence of a fast albumin component in the serum of patients given large doses of penicillin was first described by Arvan et al. [1]. Fast albumin was subsequently isolated by ion-exchange chromatography and the presence of penicilloyl groups (BPO groups) on it was ascertained using RIA [2] and ELISA [3]. The kinetics of BPO group fixation to albumin of penicillin-treated patients as well as the immunological significance of this process have recently been reported [4].

Two fixation sites for BPO groups have been demonstrated on the albumin molecule and found to be located on CNBr fragments $C_{124-298}$ and $A_{299-585}$ [5]. In the present study, identification of these sites was carried out on purified tryptic peptides derived from CNBr fragments of fast albumin from penicillin-treated patients. It was shown that fixation of BPO groups involved histidine residues 146 and 338.

2. MATERIALS AND METHODS

Benzyl-penicillin sodium salt was obtained from Serva (France). Human serum albumin (HSA) 100% pure on electrophoresis was obtained from Mann. Trypsin (TPCK-treated) from bovine pancreas was from Serva. Polyamide plates (2.5 × 2.5 cm) were from Schleicher & Schüll. Phenylisothiocyanate (PITC) was purchased from Pierce. 4-*N*,*N*-Dimethylazobenzene (DABITC) was obtained from Fluka. *N*-Bromoacetyl-L-tryptophan was synthesized as specified by Gambhir and McMenamy [6].

BPO groups were determined by the enzyme-linked-immunosorbent assay described by Lapresle and Lafaye [3]. This ELISA consists of inhibiting by penicilloylated proteins the fixation of penicilloylated alkaline phosphatase (PAP) to antipenicilloyl antibodies.

Sera were taken from four patients (FAU, GER, LEV and LOI) whose clinical picture was reported by Lafaye and Lapresle [4]. These patients were given intravenously 50×10^6 IU of penicillin G over a 20–40-day period. Sera were collected at the end of each treatment and mixed. Fast albumin was prepared as described by Lapresle and Wal [2] using chromatography on DEAE-Sephadex. CNBr fragments of fast albumin were obtained according to Lapresle and Doyen [7]. Reduction and carboxymethylation of CNBr-fragments were carried out according to Crestfield et al. [8].

Immunoadsorbents were prepared by reacting 1 g of CNBr-activted Sepharose 4B with 10 mg of purified antipenicilloyl antibodies as recommended by the manufacturer (Pharmacia). The fragments were digested with TPCK-trypsin (1%, w/w) at 37°C for 24 h in 50 mM ammonium bicarbonate (1 mg/ml) and lyophilized. The trypsin hydrolysates were filtered on immunoadsorbents containing antipenicilloyl antibodies. A fraction not adsorbed under these conditions was eluted with 0.15 M NaCl and the immunoadsorbent was washed with 0.15 M ammonium acetate, pH 7.1, to eliminate NaCl; the material remaining adsorbed to the immunoadsorbent was eluted with 5% acetic acid and lyophilized.

Amino acid analysis was performed on a Biotronik amino acid analyzer LC 5001 using the single column procedure.

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Samples containing 5 nmol of peptides were hydrolyzed in vacuo with 0.1 ml of 6 N HCl for 15 h at 110°C.

The amino acid sequence of the N-terminal portions of the isolated tryptic peptides was determined on about 5–10 nmol of material by the manual 4-(dimethylamino)azobenzene 4′-isothiocyanate/phenylisothiocyanate double coupling technique. The resulting 4-(dimethylamino)azobenzene 4′-isothiocyanate derivatives were identified on polyamide plate as described by Chang et al. [9].

Human serum albumin was labeled with *N*-bromoacetyl-L-tryptophan as indicated by Gambhir et al. [10]. Two derivatives were synthesized: one containing per molecule of HSA one molecule of *N*-bromoacetyl-L-tryptophan and the other 2 molecules. 20 mg of each derivative were added with 10^3 IU of penicillin G in 1 ml of 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and allowed to stand at 37°C for 24 h. After dialysis against water the mixtures were lyophilized.

3. RESULTS

5 mg of either CNBr-fragment $C_{124-298}$ or $A_{299-585}$ derived from fast albumin were reduced, carboxymethylated and digested by trypsin. The hydrolysates were subjected to affinity chromatography on immobilized antipenicilloyl antibodies. The results obtained are illustrated in fig.1. This figure shows in both cases the emergence of a large peak of non-adsorbed material in 0.15 M NaCl. Successive washing with 5% acetic acid eluted a small peak of adsorbed material from the gel loaded with either of the fragments. BPO groups were assayed in the collected fractions by PAP inhibition reaction using immobilized antipenicilloyl antibodies. Relative to absorbance the second peak contained significantly higher activity than the first one. The elution pattern of the first peak on rechromatography on the same column was similar to that obtained in the first chromatography run.

The amino acid content of the material of the second peak derived from CNBr fragment $C_{124-298}$ corresponded to that of a tetrapeptide containing 1 Arg, 1 His, 1 Pro and 1 Tyr, whereas its partial N-terminal sequence was Arg-His-Pro.

The amino acid content of the material of the second peak derived from CNBr fragment $A_{299-585}$ corresponded to that of a pentadecapeptide containing 2 Arg, 1 His, 1 Pro, 1 Asp, 1 Tyr, 1 Ser, 2 Val, 4 Leu, 1 Ala and 1 Lys. Its partial N-terminal sequence was determined as Arg-His-Pro-Asp.

HSA was derivatized by treatment with *N*-bromoacetyl-L-tryptophan because this reaction was found to occur at histidine 146 [10]. Binding

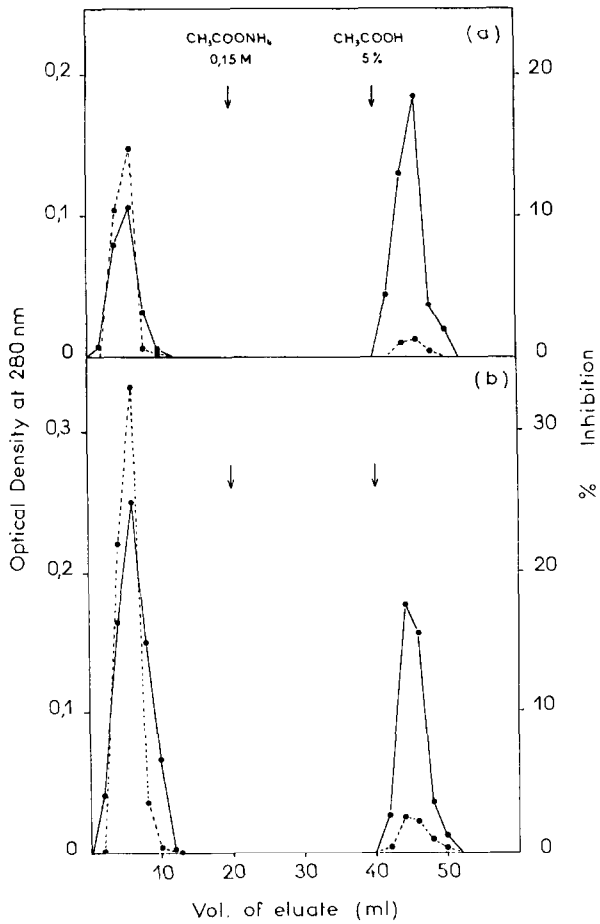

Fig.1. Affinity chromatography on immobilized antipenicilloyl antibodies of a tryptic hydrolysate of (a) fragment $C_{124-298}$ and (b) fragment $A_{299-585}$; (– – –) *A* at 280 nm; (——) % inhibition of the fixation of penicilloylated alkaline phosphatase to coated antipenicilloyl antibodies.

capacity of untreated HSA was 2.8×10^{-8} BPO groups per mg of protein. HSA when substituted with 1 mol of *N*-bromoacetyl-L-tryptophan per mol of protein fixed only 2.1×10^{-8} BPO groups and when substituted with 2 mol of reagent fixed 1.7×10^{-8} BPO groups per mg of protein.

4. DISCUSSION

In a previous study most BPO groups were found to be located preferentially on at least two sites of albumin isolated from the sera of penicillin-treated patients. Using CNBr-peptides derived from albumin, one of these sites could be

located between Met-123 and Met-298 and the other between Met-298 and the C-terminal residue of albumin [5].

In an attempt to locate more precisely the fixation sites for BPO groups, the albumin-derived CNBr-fragments $C_{124-298}$ and $A_{299-585}$ were degraded by trypsin and the peptides carrying BPO groups were isolated by immunoaffinity on immobilized antipenicilloyl antibodies. From each of the above fragments only one peptide was isolated. The amino acid composition and the partial N-terminal sequence of each of these peptides allowed us to allocate them to well-defined places in the albumin molecule by taking advantage of the sequence established by Behrens et al. [11] and Meloun et al. [12]. The peptide derived from fragment $C_{124-298}$ was identified to the tetrapeptide involving residues 145–148 and the other peptide derived from fragment $A_{299-585}$ was identified as the pentadecapeptide involving residues 337–351.

BPO groups have for a long time been considered to be the major antigenic determinants responsible for allergic reactions to penicillin [13–17]. These groups were supposed to react with proteins essentially through binding to ϵ-amino groups of lysine residues [13–16]. However, Bundgaard [18] and Yamana et al. [19] showed that reaction with lysine residues was very slow at neutral pH which is consistent with the pK_a of lysine (8.45–10.53). Since the pK_a of histidine is equal to 7, Bundgaard [20] and Yamana et al. [21] were prompted to assume that BPO groups should first react with histidine before reacting with lysine. The putative involvement of histidine in fixation of BPO groups could be inferred from the report of Wagner et al. [22] who showed that carboxymethylation of albumin resulted in reduction of BPO group fixation.

The involvement of lysine residues in fixation of BPO groups in our experiments can be excluded. In fact, lysine is lacking in the tetrapeptide. In the pentadecapeptide the single lysine is located at the C-terminal position. Substitution of this lysine by BPO groups would have prevented the adjacent peptide bond from being cleaved. In contrast, each of the two peptides contains one histidine (residues 146 and 338) which of all the constituent amino acids is the only one prone to reacting with BPO groups. In support of this it was verified that 25–39% less BPO groups were fixed in vitro by HSA labeled with *N*-bromoacetyl-L-tryptophan.

The ready reactivity with BPO groups of histidines 146 and 338 out of the 16 histidine residues present in the albumin molecule can be accounted for on the basis of their particular location on the polypeptide segments connecting helices of the large loops 1-C and 2-C (fig.2). In the structural model by Brown [23] these polypeptides are located in regions rich in reactive groups.

The two histidine residues 146 and 338 exhibit some structural common features. They are at near equidistance of disulfide bonds. On the N-terminal side, both are preceded by six identical amino acid residues (Leu-Tyr-Glu-X-Ala-Arg-Arg) and on the C-terminal side they are followed by a proline residue. In addition, the two large loops to which they belong exhibit 37% of homology in amino acid sequence.

Unmasking of BPO groups bound to the albumin molecule requires previous enzymatic degradation as was also the case with CNBr fragments $C_{124-298}$ and $A_{299-585}$. Moreover, in the

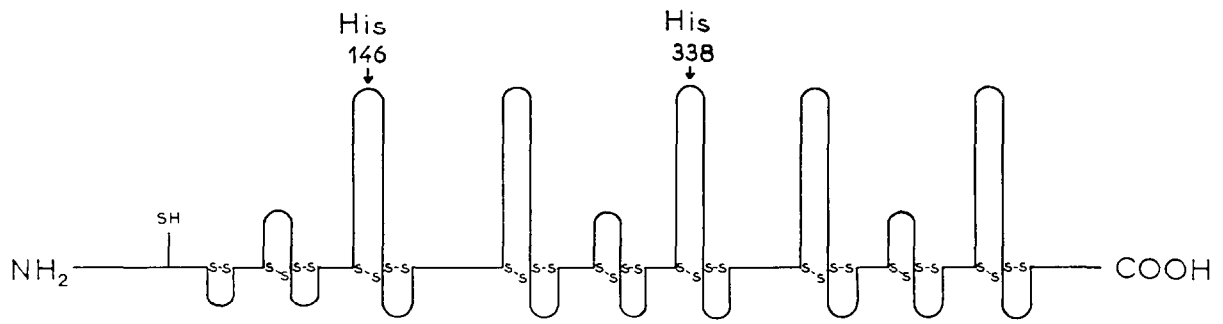

Fig.2. Schematic representation of structure of the loops of serum albumin.

latter instance BPO groups were more efficiently exposed after disulfide bond reduction than after enzyme digestion [5]. Thence, it appears that the unavailability of BPO groups should not be dependent upon the overall structure of the albumin molecule but rather upon the structure held by the disulfide bonds of the loop to which these sites belong. This suggests that BPO groups should be buried within each loop once fixed to histidine residues.

Fixation of BPO groups has extensively been studied in in vitro experiments. It is highly probable that a wide variety of free amino groups can be involved in fixation depending upon the experimental conditions. The experiments reported in the present paper have aimed at defining those sites which under physiological conditions are responsible for the fixation of BPO groups on the albumin of penicillin-treated patients.

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