LYSINE RESIDUE 199 OF HUMAN SERUM ALBUMIN IS MODIFIED BY ACETYLSALICYLIC ACID

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1. Introduction

Acetylsalicylic acid has been shown previously to transfer its acetyl group under physiological conditions in vitro and in vivo to the ϵ-amino group of a specific lysine residue of human serum albumin, thereby increasing the ability of this protein to bind the anionic drug acetriozate [1,2]. A peptide in a tryptic digest of this acetylated human serum albumin has been identified by fingerprinting techniques as being that containing the modified lysine residue and was found to contain two lysine residues (one of which was acetylated) [3,4]. However, the reported amino acid composition of this peptide [3,4] does not correspond to that of any peptide which could be expected from this tryptic digest on the basis of either the amino acid sequence of this protein as described by Behrens et al. [5] or of that somewhat different version of the sequence of this protein as described by Meloun, Morávek and Kostka [6].

In view of this anomaly it was decided to reisolate this modified peptide and to determine its amino acid sequence and to thereby establish the identity of an amino acid residue in one of the binding sites of the albumin molecule.

2. Materials and methods

2.1. *Protein and chemicals*

Human serum albumin (Fraction V powder, fatty acid free) was from Miles Laboratories Inc., Elkhorn, Indiana, USA, and TLCK-treated trypsin was from Worthington Biochemical Corp., Freehold, N. J., USA [1-^{14}C]acetylsalicylic acid (10.8 mCi/mmol, > 98% pure) was purchased from the Radiochemical Centre, Amersham, UK. Iodoacetic acid was recrystallised from hexane. All other chemicals were of the highest available purity.

2.2. *Determination of radioactivity*

Radioactivities were measured in a Nuclear Chicago Unilux I scintillation counter in Bray's solution [7].

2.3. *Amino acid analysis*

Protein and peptides were hydrolysed in vacuo with 6 N HCl containing 0.1% phenol for 24 h. Hydrolysates were analysed with a Durrum D-500 analyser.

2.4. *Reaction of human serum albumin with [1-^{14}C] acetylsalicylic acid*

This was performed as described earlier [3]. Human serum albumin (70 mg, 1.1 μM) was dissolved in sodium phosphate buffer (6 ml, 0.1 M, pH 7.4) and [1-^{14}C]acetylsalicylic acid (5.8 μM, 60 μCi) dissolved in chloroform (25 μl) added. This solution was then incubated at 37°C for 24 h and then dialysed against three changes of a buffer (1 litre) containing 0.15 M NaCl and 0.01 M sodium salicylate and finally against 0.5% ammonium bicarbonate. The specific radioactivity of the modified protein was determined by measurements of the radioactivity of portions of the protein solution and by measurement of the protein concentration by amino acid analysis of hydrolysates of portions of this solution.

2.5. *Carboxymethylation*

After reaction with [1-^{14}C]acetylsalicylic acid,

albumin was reacted with iodoacetic acid as follows. The protein (70 mg) was dissolved under nitrogen in 50 mM Tris-HCl (pH 8.2, 6 ml) containing 6 M guanidinium hydrochloride. After 15 min dithiothreitol (28 mg, 180 μM) was added followed 1 h later by the addition of iodoacetic acid (134 mg, 70 μM). After 1 h this solution was dialysed against ammonium bicarbonate solution (10 mM) and freeze-dried.

2.6. *Digestion with trypsin*

This was performed in 1% ammonium bicarbonate solution, pH 8.4, containing 2 M guanidinium hydrochloride at 37°C using a trypsin–modified albumin ratio (w/w) of 1:50. After 4 h the reaction mixture was freeze-dried.

2.7. *Peptide fractionation*

The tryptic digest of modified human serum albumin was fractionated on a column of Sephadex G-50 (superfine) (100 × 2.5 cm i.d.) in 0.5% ammonium bicarbonate buffer. The absorbance at 225 nm of the effluent was monitored with a Cecil 272 ultraviolet spectrophotometer with a flow cell (5 mm path length). Fractions of 2.7 ml were collected. The radioactivities of portions of each fraction were determined. Certain fractions from this column were freeze dried and then fractionated further by high voltage paper electrophoresis at pH 6.5 and 2.1 at 60 V/cm on Whatman No. 1 paper [8]. Radioactive peptides were detected by overnight radioautography using Kodak Autoprocess AP-54 film. Peptides were eluted from paper with 1% ammonia.

2.8. *The dansyl-Edman method*

This was carried out as described by Hartley [9]. Dansyl amino acids were identified by chromatography on polyamide sheets (Schleicher and Schüll) [9] following hydrolysis of dansylated peptides at 110°C for 4 h in vacuo.

3. Results

The radioactivity incorporated into human serum following transacetylation with [1-^{14}C]acetylsalicylic acid, 2.7 × 10^5 cpm/μmol of albumin corresponds to the reaction of 1.1 μM of acetylsalicylic acid/μM of albumin. The majority (85%) of this radioactivity was located in one peak of the elution profile resulting from the fractionation of the tryptic digest of the transacetylated *S*-carboxymethyl human serum albumin (fig.1). Only one radioactive peptide was detected in this fraction by autoradioautography following high voltage paper electrophoresis at pH 6.5 and 2.1. The amino acid analysis of the radioactive peptide showed that it included two lysine residues and 1 carboxymethylcysteine residue in its composition. Sequence analysis of this peptide demonstrated that radioactivity was associated with the lysine residue at position 2 of the peptide (table 1). This peptide corresponds to residues 198–205 of human serum albumin as determined by Meloun, Morávek and Kostka [6]. The corresponding residue in the sequence described by Behrens et al. was assigned as arginine [5].

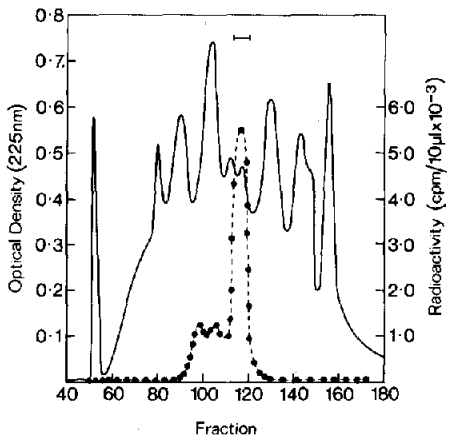

Fig.1. Gel filtration on Sephadex G-50 (superfine) of a tryptic digest of human serum albumin previously modified by reaction with [1-^{14}C]acetylsalicylic acid. The protein was reduced and *S*-carboxymethylated prior to digestion with trypsin as described in Materials and methods. (——) Absorbance at 225 nm; (- - - • - - -) radioactivity. For details see Materials and methods.

4. Discussion

The human serum albumin molecule appears to contain a number of binding sites which enable it to bind an extraordinarily wide range of chemical substances [10]. The acetylation of human serum

Table 1
Properties of the major radioactive tryptic peptide isolated from human serum albumin following reaction with [1-^{14}C]acetylsalicylic acid

Property determined	Result
Amino acid composition	$CMCys_{0.9}$, $Ser_{1.0}$, $Glu_{1.0}$, $Ala_{1.0}$, $Leu_{1.9}$, $Lys_{1.8}$
Mobility at pH 6.5	0
Charge	0
Amino acid sequence	Leu–$\overset{*}{\text{Lys}}$–CMCys–Ala–Ser–Leu–Gln–Lys

—→ denotes sequence determined by dansyl-Edman method. At each stage of the degradation a portion of the peptide was removed and its radioactivity subsequently measured. After removal of the second amino acid residue the peptide was no longer radioactive.

albumin with acetylsalicylic acid has been shown previously to profoundly affect the ability of this protein to bind various anionic compounds. Thus, the binding of acetriozate [1,2,4] and phenylbutazone [11] is enhanced and concomitantly with the latter observation flufenamic acid binding is decreased. Further, salicylate and acetriozate prevent the transacetylation reaction and moreover, acetriozate binding is inhibited by a variety of drugs [4]. The transfer of an acetyl group from acetylsalicylic acid to the ϵ-amino groups of a lysine residue of human serum albumin is thus a convenient method of chemically labelling a specific amino acid residue in one of the binding sites of this protein and has here enabled lysine residue 199 to be identified as being in this site. It appears that this site is distinct from the tryptophan binding site which contains histidine residue 146 [12], since tryptophan does not inhibit salicylate binding [4].

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