Efficient identification of photolabelled amino acid residues by combining immunoaffinity purification with MS: revealing the semotiadil-binding site and its relevance to binding sites for myristates in domain III of human serum albumin

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To identify photoaffinity-labelled amino acid residue(s), we devised an effective method utilizing immunoaffinity purification of photolabelled fragments, followed by matrix-assisted laser-desorption ionization-time of flight (MALDI-TOF) MS and nanoelectrospray ionization tandem MS (nano-ESI-MS/MS) analysis. Human serum albumin (HSA) was photolabelled with an azidophenyl derivative of semotiadil, FNAK {(+)-(R)-3,4-dihydro-2-[5-methoxy-2-[3-[N-methyl-N-[2-(3-azidophenoxy)ethyl]amino]propoxyl]phenyl]-4-methyl-2H-1,4-benzothiazin-3-(4H)-one}, since HSA is a major binding protein for semotiadil in serum. After lysyl endopeptidase digestion, photolabelled HSA fragments were adsorbed selectively on to Sepharose beads on which an anti-semotiadil antibody was immobilized, and fractions were eluted quantitatively by 50% acetonitrile/10 mM HCl. MALDI-TOF MS analysis of the eluted fraction showed that it contained two photolabelled fragments of m/z 2557.54 (major) and 1322.44 (minor), corresponding to Lys-414–Lys-432 and Ala-539–Lys-545, respectively. Further nano-ESI-MS/MS analysis revealed that Lys-414 was the photolabelled amino acid

residue in fragment 414–432 and Lys-541 was a likely candidate in fragment 539–545. Based on the photolabelling results, we constructed a three-dimensional model of the FNAK–HSA complex, revealing that FNAK resides in a pocket that overlaps considerably with myristate (Myr)-binding sites, Myr-3 and -4, by comparison with crystallographic data of HSA–Myr complexes described in Curry, Mandelkow, Brick and Franks (1998) *Nat. Struct. Biol.* **5**, 827–835. Moreover, addition of Myr increased photo-incorporation into Lys-414, whereas incorporation into Lys-541 decreased under conditions of $[Myr]/[HSA] < 1$. Further addition of Myr, however, uniformly decreased photo-incorporation into both Lys residues. These results indicate that FNAK labelling can also be used to monitor Myr binding in domain III. An interpretation for the concomitant local conformational change of HSA is provided.

Key words: drug-binding site, fatty acid binding, mass spectrometry, photoaffinity labelling.

INTRODUCTION

Photoaffinity labelling is widely used to locate ligand-binding sites in biomolecules (for reviews, see [1–4]). Since the introduction of this technique, investigators have used radioactive photoaffinity reagents and purified photolabelled fragments by conventional chromatographic techniques, such as HPLC, followed by amino acid sequence analysis. Although these procedures are tedious and labour-intensive, successful investigations have been reported ([2] and references cited therein) even with membrane proteins, in which photo-incorporation efficiency was high and the photoproducts resistant to Edman degradation [3]. However, photolabelling efficiency varies and only a small percentage of labelling is achieved in many cases (reviewed in [1,3]).

To overcome difficulties in isolating labelled peptide fragments produced in small quantities, an alternative method has been employed, using anti-peptide antibodies to isolate and identify

radioactive photolabelled fragments. Several reports describe the application of this method to locate successfully the site of action of drugs or toxins in ion channels (see [5–8] for examples) at sub-picomolar levels. The method, however, requires preparation of a large number of antibodies to efficiently identify only a limited number of labelled fragments. In addition, resolution of the labelled sites by this method is limited to the size of the digested peptides (in the order of several kDa in most cases) because fragments are generated by selective cleavage with enzymic and chemical methods.

Recent progress in MS enables us to detect peptides and determine their sequences in sub-picomolar quantities (for reviews, see [9–11]). Matrix-assisted laser-desorption ionization-time of flight MS (MALDI-TOF MS) provides accurate information about peptide sizes, even when the sample is a peptide mixture, and mass-fit analysis gives a possible amino acid sequence when the primary structure is known. To analyse the sequence of a peptide fragment precisely, several methods,

Abbreviations used: nano-ESI, nanoelectrospray ionization; FNAK, (+)-(R)-3,4-dihydro-2-[5-methoxy-2-[3-[N-methyl-N-[2-(3-azidophenoxy)ethyl]amino]propoxyl]phenyl]-4-methyl-2H-1,4-benzothiazin-3-(4H)-one; HSA, human serum albumin; Lys-C, lysyl endopeptidase; MALDI-TOF, matrix-assisted laser-desorption ionization-time of flight; MS/MS, tandem MS; Myr, myristate; SIAB, N-succinimidyl (4-iodoacetyl)-aminobenzoate; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase HPLC.

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including post-source-decay MALDI-TOF MS and tandem MS (MS/MS), have been used. In studies on photoaffinity labelling, several investigators have introduced MS analysis to identify photolabelled peptides and their labelled amino acid residues. However, most reports are still limited to mapping or identification of labelled peptides [12–19]. Recently, two groups reported successful identification of labelled amino acids by post-source-decay MALDI-TOF MS, either by using perfluoroarylazide as a photosensitive group to achieve high labelling efficiency [20] or by undertaking a combinatorial analysis with NMR [21]. Therefore, in order to locate the ligand-binding site precisely when photolabelling efficiency is low, a general method is required to identify not only photolabelled peptides but also their labelled amino acid residues. In the present study, we describe a method using a simple purification step followed by MS analysis to characterize semotiadiol-binding sites in HSA efficiently.

Semotiadiol is a new antagonist for L-type calcium channels. We previously reported the location of the semotiadiol-binding site in calcium channels from skeletal and cardiac muscle by photoaffinity labelling with (+)-(R)-3,4-dihydro-2-[5-methoxy-2-[3-[N-methyl-N-[2-(3-azidophenoxy)-ethyl]amino]propoxy]phenyl]-4-methyl-2H-1,4-benzothiazin-3-(4H)-one (FNAK), an azidophenyl derivative of semotiadiol [7,8]. It has also been reported that semotiadiol binds strongly to human serum albumin (HSA) in serum [22]. HSA is a major binding protein for many drugs and fatty acids. Binding sites for several drugs have been defined ([23] and reviewed in [24]). More recently, binding sites for some medium- and long-chain fatty acids [25,26] and those for fatty acids and general anaesthetics [27] have been analysed by crystallography. They provide us with valuable information to understand the structural basis of fatty acid binding and the interaction of fatty acids with drugs on HSA. However, most of the crystals of HSA complexes analysed have been obtained in the presence of the fatty acids and/or drugs at close to saturating concentrations. Therefore, high-affinity sites for drugs and fatty acids have been difficult to identify, and analysis of their interactions has been limited. In addition, no data are available on the semotiadiol-binding site in HSA and its relation to fatty acid binding.

Here we employ photoaffinity labelling and identify labelled site(s) using a semotiadiol analogue at the amino acid level. First we report an efficient method to identify photolabelled amino acid residues with FNAK in HSA by a combination of immunoaffinity purification and MS analysis. Secondly we show that photo-incorporation efficiency into the amino acid residues is regulated allosterically by the amount of myristate (Myr) added. Finally we discuss the FNAK-binding site in a three-dimensional HSA structure and correlate it with Myr-binding sites by constructing models of complexes, since the three-dimensional co-ordinates of HSA [28] and the HSA–Myr complex [25] are currently available from crystallographic analyses.

EXPERIMENTAL

Materials

[³H]FNAK (77.6 Ci/mmol) was synthesized as described in [29]. Semotiadiol and norsemotiadiol {(±)-2-[5-Methoxy-2-[3-[2-[3,4-(methylenedioxy)phenoxy]-ethylamino]propoxy]phenyl]-4-methyl-2H-1,4-benzothiazin-3-(4H)-one} were obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan). HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was used after defatting with activated charcoal by the described method [30]. Horseradish peroxidase-labelled goat anti-rabbit IgG was purchased from Zymed Laboratories. Lysyl

endopeptidase (Lys-C) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Conjugate preparation and antibody production

Norsemotiadiol was obtained by extraction with ethyl acetate after treatment of hydrogen oxalate salt of norsemotiadiol with saturated sodium bicarbonate. Norsemotiadiol (8.4 mg, 16.4 mmol) was reacted with equimolar *N*-succinimidyl (4-idoacetyl)-aminobenzoate (SIAB) and triethylamine in dimethylformamide for 24 h at room temperature and the residual mixture was purified on a silica-gel column with chloroform/acetone (3:1) as a solvent. The purified material (11.4 mg) in dimethylformamide (1.5 ml) was incubated with thyroglobulin in 3 ml of PBS, pH 8.0, for 1.5 h at room temperature. After dialysis against PBS, a norsemotiadiol–thyroglobulin conjugate was obtained. Spectral analysis of the conjugate indicated 112 mol of norsemotiadiol/mol of thyroglobulin. Japanese white rabbits were immunized with the conjugate (1.6 mg/rabbit) emulsified in Freund's complete adjuvant with a biweekly schedule of injection. After 4 weeks, the immunization was repeated an additional four times with the conjugate (0.8 mg/rabbit per immunization) in Freund's incomplete adjuvant. Titres were monitored by ELISA using norsemotiadiol–HSA conjugate and horseradish peroxidase-conjugated anti-rabbit IgG. The norsemotiadiol–HSA conjugate (49 mol of norsemotiadiol/mol of HSA) was prepared in a manner similar to the norsemotiadiol–thyroglobulin.

To assess antibody specificity, competitive ELISA was performed by addition of a preincubated mixture of antiserum and test drugs at various concentrations to 96-well plates coated with the norsemotiadiol–HSA conjugate.

Antibody purification and preparation of antibody-immobilized gel

Pooled antiserum (7 ml) was loaded on to a thyroglobulin-immobilized agarose column (10 ml). The flow-through fractions were then loaded on to a Protein A–Sepharose column (2 ml) and IgG was eluted with 0.1 M glycine/HCl (pH 3.0). The eluted IgG was dialysed against 0.1 M sodium bicarbonate containing 0.5 M NaCl, concentrated to 5 mg/ml, and coupled with an equal volume of Affi-Gel 10 overnight at 4 °C. The IgG-immobilized gel was treated with 1 M ethanolamine containing 0.5 M NaCl (pH 8.3), washed with 0.1 M acetic acid containing 0.5 M NaCl (pH 4.0) and stored in TBS containing 0.05 % NaN₃ at 4 °C until use. Ethanolamine-treated Affi-Gel 10 was prepared in a similar manner as a control gel.

Photolabelling

HSA (0.67 mg, 10 nmol) in 1 ml of 50 mM Tris/HCl (pH 7.4) was incubated with 10–100 μM [³H]FNAK at 30 °C for 1 h in the presence and absence of 1 mM semotiadiol in 3 % DMSO. The incubation mixture was kept on ice and irradiated for 20 min with a 100 W black light/blue lamp (Ultra-Violet Products, San Gabriel, CA, U.S.A.) at a distance of 10 cm. After photolysis, the sample was concentrated to 0.1 ml in a SpeedVac concentrator (Savant) and cold acetone (0.8 ml) was added. The sample was allowed to stand overnight at –30 °C and then centrifuged in a microfuge (18000 g) at 4 °C for 10 min to obtain photolabelled HSA in the pellet. Photolabelled HSA was separated by SDS/PAGE (10 % gels). Lanes of the gel were cut manually into 3 mm slices and radioactivity was counted in ACSII scintillation

Figure 1 Binding specificity of anti-semotiadil antibody for semotiadil derivatives

Cross-reactivity was determined by the competitive ELISA method as described in the Experimental section. Before the antibody was added to the 96-well plates that were coated by norsemotiadil-HSA conjugates, it was preincubated with or without test compounds at various concentrations. Semotiadil (○), FNAK (●), diltiazem (△), verapamil (□) and SIAB (×) were used as test drugs. Results are shown as means $\pm$ S.D. ($n = 4$).

Figure 2 Saturable photo-incorporation of $[^3\text{H}]$ FNAK into HSA

Fatty acid-free HSA (10 μM) was photolabelled with $[^3\text{H}]$ FNAK at various $[^3\text{H}]$ FNAK/[HSA] ratios in the presence (△) or absence (■) of 1 mM semotiadil. Radioactivity photo-incorporated into HSA was determined after SDS/PAGE as described in the Experimental section. Results are shown as means $\pm$ S.D. ($n = 3$).

cocktail (Amersham Bioscience) with 3% (v/v) H_2O_2 . Photolabelling efficiency was determined by calculation of the incorporated radioactivity (mol) compared with HSA (mol). The K_d value of $[^3\text{H}]$ FNAK with respect to HSA was obtained as 4.1 μM (Hill's coefficient, 0.98; K. Kawahara and H. Nakayama, unpublished work) by using the equilibrium-dialysis method, and will be reported elsewhere in combination with the value of the enantiomeric isomer. The stoichiometry of photo-incorporation was measured by nanoelectrospray ionization (nano-ESI) MS using a Q-TOF instrument (Micromass, Manchester, U.K.) and MassLynx data acquisition. All HSA samples photolabelled with a 1:10 molar ratio of unlabelled FNAK/HSA

showed FNAK-modified HSA bearing only 1 mol of FNAK (m/z 66945.0, 67062.0 and 67158.0) and unmodified HSA (m/z 66439.0, 66557.0 and 66653.0). Therefore, photolabelled samples that were prepared using unlabelled FNAK (20 μM) and 20 μM HSA were used for enzymic digestion and the subsequent MS analysis. Protein amounts were determined by the Bradford method [31].

Enzyme digestion and immunoaffinity purification

Photolabelled and acetone-precipitated HSA (1 mg/0.1 ml) was reduced with 25 mM dithiothreitol in 0.2 M Tris/HCl (pH 8.6) containing 10 M urea and 1 mM EDTA at 37 $^\circ\text{C}$ for 2 h and carboxyamidated with 0.1 M iodoacetoamide for an additional hour. After gel filtration on a Sephadex G-50 column, labelled HSA was digested with endoprotease Lys-C ([substrate]/[enzyme] = 20) in 50 mM Tris/HCl (pH 9.0) at 37 $^\circ\text{C}$ for 12 h. The digest ($\approx$ 30 pmol equivalent) was diluted with 50 mM Tris/HCl (pH 7.4) containing 0.5 M NaCl (0.1 ml) and incubated with the antibody-immobilized gel (30 μl) at 4 $^\circ\text{C}$ for 12 h. The gel was washed twice with 1 ml of 50 mM Tris/HCl (pH 7.4) containing 0.5 M NaCl, once with 1 ml of 50 mM Tris/HCl (pH 7.4) and twice with 1 ml of distilled water. The adsorbed materials were eluted three times with 0.1 ml of 50% acetonitrile/10 mM HCl and the combined eluates were concentrated to 10 μl using a SpeedVac concentrator. Yields of the adsorbed and eluted products were determined using the Lys-C digest of $[^3\text{H}]$ FNAK-labelled HSA. A control sample was prepared similarly using ethanolamine-treated Affi-Gel 10.

MALDI-TOF MS measurement

The eluted and concentrated sample from the antibody-immobilized gel was mixed (1:1) with an α -cyano-4-hydroxycinnamic acid solution [40% acetonitrile/60% water containing 0.06% trifluoroacetic acid (TFA)]. An aliquot ($\approx$ 100 fmol) of the mixture was put on a probe tip, air-dried and MALDI-TOF MS measurements taken using a Bruker's Reflex MALDI-TOF mass spectrometer.

Nano-ESI MS/MS analysis

Before analysis, the eluted samples (10 pmol) from the antibody-immobilized gel were subjected to a micro-HPLC system (PerSeptive, model 173A; Perkin Elmer) with a narrow-bore C_{18} column (1.5 mm $\times$ 150 mm, 300 $\text{\AA}$). Solvent A (0.1% TFA in water) and solvent B (0.085% TFA in acetonitrile) were used, and elution was performed with a 5–45% linear gradient of solvent B for 140 min at a flow rate of 5 $\mu\text{l}/\text{min}$, and monitored at 210 nm. Two fractions that were absent in the control (the eluted sample from control IgG-immobilized gel) were obtained at around 80 and 110 min. Recovery from the HPLC system was 70%, based on a separate run of the $[^3\text{H}]$ FNAK-labelled sample. Each peptide fraction was lyophilized and dissolved in 20 μl of 50% aqueous methanol containing 1% acetic acid. Then, 2 μl were loaded into a nanoflow tip and nano-ESI-MS/MS was acquired using a Q-TOF instrument and MassLynx data acquisition. A high voltage (1–1.2 kV) was applied to the nanoflow tip of the capillary. The collision energy was increased to an optimum value of 25–50 eV using argon collision gas.

In the case of fragment 539–545, photolabelled amino acids were not identified exclusively by nano-ESI-MS/MS but determined supplementally by a microsequencer (model Procise 492; Perkin Elmer Applied Biosystems).

Figure 3 MALDI-TOF MS analysis of the photolabelled peptide mixture

(a) After photolabelled HSA was digested with Lys-C ([substrate]/[enzyme] = 20) at 37 °C for 12 h, photolabelled peptide fragments were immunoprecipitated with Sepharose beads on which either anti-semotiadil antibody or control IgG (inset) was immobilized and analysed by MALDI-TOF MS as indicated in the Experimental section. Ion peaks marked with an asterisk were candidates for FNAK-labelled fragments. Ion peaks at m/z 1344.39 and 2579.61 are sodium ion adducts to the corresponding molecular ions and the ion peak at m/z 1279.55 is assigned as a doubly charged ion of the peak at m/z 2557.54. (b) The partial amino acid sequence of HSA (residues 401–585) is shown and putative photolabelled peptide sequences are underlined. (c) Photolabelled peptides that were detected by MALDI-TOF MS are summarized.

Effects of Myr on photolabelling

HSA (3.35 mg, 50 nmol) in 5 ml of 50 mM Tris/HCl (pH 7.4) was preincubated with FNAK (10 μ M, molar equivalent to HSA) for 1 h. Sodium myristate (0–100 μ M, 0–10-times molar equivalent to HSA) was added to the mixture and incubated at 30 °C for 1 h. Photolabelling was carried out as described above. Subsequent procedures of reductive alkylation, Lys-C digestion and immunoaffinity purification were also performed as described above. Two photolabelled fragments (corresponding to Lys-414–Lys-432 and Ala-539–Lys-545) were separated by narrow-bore reversed-phase HPLC (RP-HPLC) as described above and quantified by comparison with standard samples that were separately isolated and calibrated by the microsequencer.

Construction of the three-dimensional complex models

The crystal structures of HSA (Protein Data Bank code 1bj5, and code 1ao6 for Lys-541 co-ordinates) were used for the modelling study of complexes with semotiadil at the Myr-binding pockets. Semotiadil was manually docked in the binding site by examining possible binding modes and then structures of complexes consisting of semotiadil and residues within 8 Å of the ligand were optimized with molecular dynamics. The molecular-dynamics calculation was performed at 298 K with an 8 Å cutoff, a dielectric constant of 1 and a time step of 1 fs for 100 ps by sampling a

conformation every 1 ps using Discover 3 (version 98.5; Molecular Simulations). Structure minimization was carried out until the final root-mean-square deviation was less than 0.1 kcal/mol per Å. Two most energetically favoured models of complexes were selected from the 100 structures generated. One of the models has a fairly large pocket in subdomain IIIA, and the other one has rather a small hydrophobic hole in subdomain IIIB.

A model complex of FNAK at the large pocket was also generated by replacing the methylenedioxy moiety of semotiadil with the azide group. For the structural minimization of the FNAK complex, a linear moiety, 1-propyne, was used as a substitute for the azide group, since parameters of the azide group for the molecular mechanics calculated were not available in Discover 3.

RESULTS

Generation and specificity of anti-ligand antibody

The antibody selectively recognized semotiadil and its phenyl-azido derivative FNAK (Figure 1). SIAB, the crosslinker used to prepare the antigenic conjugate of norsemotiadil with thyroglobulin, was not recognized by the antibody. Semotiadil is a hybrid compound of diltiazem and verapamil, since not only is the benzothiazine ring of semotiadil homologous to the benzothiazepine ring of diltiazem, but also has a structural component,

Figure 4 Nano-ESI-MS/MS spectra of the photolabelled peptide Lys-414–Lys-432

The peptide was obtained by immunoprecipitation followed by fractionation by capillary RP-HPLC as described in the Experimental section. (a) Nano-ESI-MS/MS of the triply charged ion (m/z 853.46) using a collision energy of 45 eV and argon as collision gas. y and b ion series of the peptide fragments corresponding to the internal cleavages in Lys-414–Lys-432 are indicated. Fragment ion series are defined using standard nomenclature [36]. (b) and (c) show expanded spectra of (a).

Ar-C(R₁R₂)-CH₂CH₂CH₂-N(Me)-CH₂CH₂-Ar (where Ar is an aryl group), similar to verapamil [29]. However, the antibody showed no cross-reactivity with diltiazem or verapamil. The antibody recognized FNAK-labelled HSA in a Western blot, whereas unmodified HSA was not recognized, indicating that the antibody recognizes the semotiadil moiety (results not shown).

Photolabelling of HSA

To determine photolabelling efficiency, labelling was performed at various [³H]FNAK/[HSA] ratios, and the photo-incorporated radioactivity into HSA was measured after SDS/PAGE. Figure 2 shows that the photolabelling efficiency was enhanced progressively by increasing the [³H]FNAK/[HSA] ratios and reached a plateau (20%) at a ratio of 3. In the presence of excess semotiadil, a parent compound of FNAK, labelling was effec-

tively inhibited. These results suggest that labelling was specific to the binding site. Alternatively, in nano-ESI-MS analysis, all HSA samples photolabelled with a 1 : 10 molar ratio of unlabelled FNAK/HSA showed FNAK-modified HSA bearing only 1 mol of FNAK (m/z 66945.0, 67062.0 and 67158.0) and unmodified HSA (m/z 66439.0, 66557.0 and 66653.0, which correspond to HSA, HSA modified with 1 mol of cysteine and HSA modified with 1 mol of glutathione, respectively; results not shown).

MALDI-TOF MS analysis of the photolabelled peptide

The MALDI-TOF MS spectrum showed three ion peaks at m/z 2557.54, 1322.44 and 1279.55 in the eluate from the antibody-immobilized column, whereas no apparent peaks were observed in the eluate from the control column (Figure 3a). Among the three ion peaks described above, the m/z 1279.55 ion peak was

Figure 5 Nano-ESI-MS/MS spectra of the photolabelled peptide Ala-539-Lys-545

(a) Nano-ESI-MS/MS of doubly charged ion (m/z 661.88) using a collision energy of 25 eV and argon as collision gas. (b) and (c) show expanded spectra of (a). b^* ion indicates b -399.

assigned as a doubly charged ion of the m/z 2557.54 peak. Therefore, the eluted fraction contained two photolabelled fragments at m/z 2557.54 (major) and 1322.44 (minor). As shown in Figures 3(b) and 3(c), mass-fit analysis of the two fragments in the HSA sequence revealed that m/z 2557.54 and 1322.44 were in complete agreement with the calculated mass of the peptides Lys-414-Lys-432 and Ala-539-Lys-545, respectively, when 505 was taken as the mass number of the photo-incorporated phenylnitrene generated from FNAK during photolysis.

Nano-ESI-MS/MS analysis of the photolabelled amino acid

To identify photolabelled amino acid residues in the two fragments, nano-ESI-MS/MS analysis was performed after micro-RP-HPLC separation. Figure 4 shows the MS/MS spectrum of the triply charged ion (m/z 853.46) for the labelled peptide of m/z 2557.54. Two significant peaks were observed at m/z 399.22

and 1080.64. The m/z 399.22 ion peak was determined to be the fragment derived from cleavage at the C—O bond adjacent to the substituted aniline moiety of the labelled peptide (the structure is shown in Figure 4a), and the m/z 1080.64 was the doubly charged ion for the resultant labelled peptide fragment. In the expanded spectra 400–1075 (Figure 4b) and 1100–2100 (Figure 4c), we assigned the sequence of the second to the fifteenth amino acids as VPQVSTPTLVEVSR, based on the observation of contiguous y ion series. In addition, $b2$ and $b4$ ions were also detected at m/z 733.46 and 958.57, respectively. Taken together with the observed y ion series, we conclude that the N-terminal Lys was the photolabelled amino acid residue in the peptide. This observation is consistent with the fact that the Lys residue remained at the N-terminus of the labelled peptide after Lys-C digestion.

In the second labelled peptide of m/z 1322.44, the MS/MS spectrum of the doubly charged ion (m/z 661.85) showed two strong peaks of m/z 399.2 and 924.5 (Figure 5). Although the C-

Figure 6 Effect of Myr on FNAK photolabelling of HSA

Fatty acid-free HSA (10 μ M) was photolabelled with 10 μ M FNAK in the presence of Myr at the indicated [Myr]/[HSA] ratio. Photolabelled HSA was digested with Lys-C and mixed with anti-semotiadil antibody-immobilized gel. After elution, the sample was fractionated by capillary RP-HPLC and the amount of the photolabelled peptides (in pmol) were determined by microsequence analysis. Results are shown as means $\pm$ S.D. ($n = 3$).

terminal short sequence QLK was assigned, based on the part of a contiguous b and b^* ($= b-399$) ion series, the entire sequence and the labelled amino acid residue were not determined. This may be due to the sensitivity of the labelled peptide to ionization at or near the photolabelled residue. Nevertheless, the Lys residue that lies in the third position of the peptide is a strong candidate for the labelled site, since it was resistant to Lys-C digestion. The photolabelled amino acid was not identified by nano-ESI-MS/MS but could be determined by amino acid sequence analysis, in which six amino acid residues of the heptapeptide were determined, except for the third Lys residue (results not shown).

Different regulation of photolabelling on Lys-414 and Lys-541 by addition of Myr

The effect of Myr on FNAK photolabelling of HSA was investigated by isolating and quantifying two photolabelled fragments by HPLC. Photo-incorporation into Lys-414 increased on the condition of [Myr]/[HSA] < 1 but decreased when the ratio was greater than 1 (Figure 6). In the case of photo-incorporation into the Lys-541-containing peptide, however, a mild decrease was observed up to a ratio of 1, followed by a steep decrease with further addition of Myr. The results indicate that Myr binding affected FNAK photolabelling in two phases: Myr addition up to [Myr]/[HSA] = 1 increased labelling of Lys-414, while it decreased that of Lys-541, but further addition of Myr uniformly decreased labelling of both lysine residues. Therefore, FNAK labelling is closely associated with Myr binding. We interpret changes in FNAK labelling as being indicative of Myr binding to HSA.

DISCUSSION

An efficient method to identify photolabelled amino acid residues

We have developed a sensitive method to identify photolabelled sites at the level of amino acid residues by a combination of immunoaffinity purification and MS analysis. It is noteworthy that our trials by MALDI-TOF MS did not detect FNAK-

labelled fragments in Lys-C digests before immunoaffinity purification. Photo-incorporation efficiency of [3 H]FNAK into HSA was $\approx 20\%$ where $\approx 70\%$ (calculated using a K_d value of 4.1 μ M) of HSA formed a 1:1 complex. Theoretically, HSA should yield ≈ 56 peptide fragments after complete digestion by Lys-C and, therefore, labelled fragments were calculated to be less than 0.4% of the total number of fragments produced. Based on the above results, we conclude that MALDI-TOF MS detects only a fraction of labelled fragments in a peptide mixture. By contrast, after immunoaffinity purification with anti-ligand antibody two labelled fragments were easily detected by MALDI-TOF MS.

Subsequent nano-ESI-MS/MS analysis of purified samples revealed most of the amino acid sequences in the two peptides by observing y and b (or b^*) ion series, which allowed determination of the photolabelled amino acid in the peptide of m/z 2557.54 in a quantity of 500 fmol. Observation of the photoproduct-derived fragment ion (m/z 399.2) confirmed that the affinity-purified peptides were photolabelled. On the other hand, observation of a strong peak of the fragment ion suggests that the photoproducts from this reagent are sensitive to the ionizing process and cleave readily at photolabelled amino acid residues. We were hampered in our ability to identify the exact photolabelled site exclusively in the case of the peptide of m/z 1322.44 by the instability of the photoproducts. As the instability would depend mostly on the chemical nature of the photoproducts against ionizing energy, this point must be considered generally in the design or selection of photoprobes to be used.

It is significant that both labelled amino acid residues were lysine. Photosensitive phenylazides in FNAK react not via short-lived singlet nitrenes, as assumed originally, but rather through either a seven-membered ring of a didehydroazepine intermediate formed by intramolecular rearrangement of the singlet nitrene, or triplet nitrenes formed by intersystem crossing [2,3]. Although determination of the photoproduct structure remains to be solved, it is worth noting that the present method identified photolabelled amino acids with high sensitivity. Phenylazides are still the most commonly used reagents since they are easily synthesized, and therefore the novel method presented here is widely applicable for identifying labelled sites.

With respect to efficient purification of photolabelled fragments, the avidin/biotin system may be a promising alternative [32,33] when biotin-containing photoprobes are used. As the avidin-biotin interaction is highly specific and of high affinity, this system would be useful unless additional biotin decreases the binding affinity of ligands to receptors. Our present method is particularly advantageous because it employs normal photo-affinity ligands without a biotin pendant and uses only a single anti-ligand antibody, enabling us to identify labelled fragments containing ligand structures.

FNAK-binding site in HSA and its relevance to those for Myr

We identified two photolabelled sites by FNAK on Lys-414 and Lys-541 of HSA. Under photolabelling conditions, HSA binds 1 mol of FNAK. A binding stoichiometry of 1:1 was also reported for semotiadil binding to HSA [34]. Therefore, the FNAK-binding site is located near Lys-414 and Lys-541. When we constructed the model of the semotiadil-HSA complex, there were two plausible pockets for semotiadil binding. At a fairly large pocket that accommodates two Myrs (Myr-3 and -4) in subdomain IIIA, the 2-methoxyphenyl-1,4-benzothiazine-3-one moiety of semotiadil replaced the two Myrs. The other binding pocket, however, allowed semotiadil to bind only the 1,4-

Figure 7 Models of FNAK-HSA complexes in the presence and absence of Myr

Top panel: FNAK bound to subdomain IIIA of HSA. A local main-chain structure is shown by a ribbon. Particular residues (side chains only) and helices (h) that are noted in the text are shown for clarity. For amino acid residues of HSA, carbon atoms are coloured grey, and heteroatoms are black. For FNAK, carbon atoms are coloured black, nitrogen atoms are white and oxygen and sulphur atoms are grey. Bottom panel: a proposed complex model of FNAK and Myr at their binding sites. The cage shows the binding hole for FNAK or Myr-3 and -4. Myristates and amino acid residues are shown as stick structures. Colours are used in a manner similar to that described in the top panel.

benzothiazine-3-one moiety at the hydrophobic hole in subdomain IIIB, whereas the remaining moiety was exposed at the cleft formed by subdomains IIIA and IIIB. A definite conclusion was not arrived at concerning which model is more favoured. However, based on the photolabelled results described above, we propose a model of the FNAK-HSA complex, as shown in Figure 7 (top panel).

The binding site is located primarily in subdomain IIIA, which is composed of helices 1, 2, 3 and 6, and helix 9 is also in close apposition. In the model, the aromatic ring of the azidophenyl group interacts with the aromatic ring of Tyr-411 almost in parallel arrangement, and the hydroxy group of Tyr-411 forms a hydrogen-bond interaction with the carbonyl group of the 1,4-benzothiazine-3-one moiety. The methylamino group of FNAK is bonded by a salt bridge to the side chain of Glu-492, which is held in place by a second hydrogen-bond interaction with the side chain of Lys-414. The azidophenyl group of FNAK can take

two distinct conformations with respect to the orientation of the *m*-substituted azide moiety. In one conformation, the azide moiety is located 3 Å from the ϵ -amino group of Lys-414, as shown in Figure 7 (top panel). In the other conformation, the azide moiety orientates Lys-541 to reside within 3 Å from its ϵ -amino group when the side chain of Lys-541 forms an extended conformation. Alternatively, a more flexible conformation of the Lys-541 side chain is consistent with the results that Lys-541 was photolabelled less efficiently than Lys-414.

When the model complex is compared with the three-dimensional structure of binding sites for tri-iodobenzoate [28], Lys-414 is located at the limb of the tri-iodobenzoate-binding site in subdomain IIIA (site II) whereas Lys-541 is located at the outer surface in subdomain IIIB far from the tri-iodobenzoate-binding site. Therefore, the FNAK-binding site partly overlaps the tri-iodobenzoate site in subdomain IIIA. Our observation that semotiadil showed only a slight decrease ($\approx 5\%$) in dansyl-

sarcosine (site II drug) binding and no effect on warfarin (site I drug) binding (results not shown) suggests that the FNAK-binding site may partially overlap with that for site II drugs in subdomain IIIA. In contrast, FNAK labelling was altered markedly by addition of Myr. When the model of the FNAK-HSA complex was compared with X-ray crystallographic data of the HSA-Myr complex, the FNAK-binding site overlapped considerably with sites for Myr-3 and -4.

It is important to note that Myr affects the labelling efficiency of two Lys residues differently, depending upon the amount of Myr added (see Figure 6). A recent X-ray diffraction study by Curry et al. [25] clearly revealed five Myr-binding sites, indicating that two of the five Myr-binding sites on HSA are proximal to subdomain IIIA (Myr-3 and -4). However, our results that photo-incorporation into Lys-414 was not competitively inhibited but rather increased following addition of Myr on the condition of $[Myr]/[HSA] < 1$, suggests that the primary Myr-binding site in the presence of FNAK is located elsewhere.

Based on our results, we interpret the primary binding site of Myr, up to one stoichiometric amount, to be Myr-5 in subdomain IIIB during the photolabelling, in which FNAK occupies the Myr-3 and -4 sites (Figure 7, bottom panel). Myr binding can induce conformational changes in HSA, making its shape more compacted, as has been described for long-chain fatty acid binding of 1–2 mol [35]. The conformational change induced by Myr binding to Myr-5 may orient Lys-414 in subdomain IIIA closer to the azide moiety of FNAK, resulting in enhanced labelling in Lys-414. Such a change may also cause Lys-541 in domain IIIB to move away from the azide moiety, resulting in reduced labelling. It is also possible that Myr binding makes the Lys-541 side chain more flexible, as co-ordinate parameters for the side chain of Lys-541 are not described in the HSA-Myr complex [25], whereas they are described in unliganded HSA [28].

Curry et al. [25] suggested that Myr-5 might not be the primary binding site in subdomain III, based on observations that the carboxylate of Myr is usually liganded by $2 \times \text{Arg/Ser}$ and Arg/Tyr/Ser in Myr-3 and -4, respectively, while Myr-5 is held by Lys and Tyr. However, they also found that binding sites for Myr-3 and -4 are a large, branched hydrophobic cavity in which two Myrs may bind co-operatively [25]. The binding pocket for Myr-5, on the other hand, is a tunnel binding the hydrophobic part of only one Myr molecule near the bottom of subdomain IIIB [25]. Myr-induced enhancement of Lys-414 labelling that we observed follows addition of Myr up to 1 mol. Therefore, it is likely that Myr primarily binds to Myr-5 during photolabelling with FNAK.

In summary, our method utilizing anti-ligand antibody and modern MS has proven efficient in identifying photolabelled amino acids. The method also reveals that Myr binding to Myr-5 (subdomain IIIB), up to 1 stoichiometric amount, may induce a significant conformational change in the FNAK-binding site of subdomain IIIA of HSA, but further addition of Myr, which may bind to Myr-3 and -4, results in exclusion of FNAK by competition. The method presented here will be generally applicable to the identification of sites even in cases of low labelling efficiency and should expand the utility of photoaffinity labelling in structural biology studies.

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