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Determining molecular binding sites on human serum albumin by displacement of oleic acid

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Abstract

An NMR method was developed for determining binding sites of small molecules on human serum albumin (HSA) by competitive displacement of ^{13}C-labeled oleic acid. This method is based on the observation that in the crystal structure of HSA complexed with oleic acid, two principal drug-binding sites, Sudlow's sites I (warfarin) and II (ibuprofen), are also occupied by fatty acids. In two-dimensional [^{1}H,^{13}C]heteronuclear single quantum coherence NMR spectra, seven distinct resonances were observed for the ^{13}C-methyl-labeled oleic acid as a result of its binding to HSA. Resonances corresponding to the major drug-binding sites were identified through competitive displacement of molecules that bind specifically to each site. Thus, binding of molecules to these sites can be followed by their displacement of oleic acids. Furthermore, the amount of bound ligand at each site can be determined from changes in resonance intensities. For molecules containing fluorine, binding results were further validated by direct observations of the bound ligands using ^{19}F NMR. Identifying the binding sites for drug molecules on HSA can aid in determining the structure–activity relationship of albumin binding and assist in the design of molecules with altered albumin binding.
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Human serum albumin (HSA)[1] is a major transporter of fatty acids in plasma. HSA can also bind an extraordinarily diverse range of drugs, metabolites, and other organic compounds, especially small anionic aromatic compounds [1,2]. Because HSA is present in blood at high levels (~40 mg ml^{-1}, 0.6 mM), compounds with even medium affinity are mainly in HSA-bound form. HSA binding can extend the metabolic half-life of a compound for several days, providing a convenient way in which to extend the duration of activity for pharmacologically active molecules. But HSA binding also reduces the free concentration of compound in solution that can significantly alter activity in vivo. Therefore, albumin binding can have dramatic effects on potency and pharmacokinetics, making it an important factor to consider in drug design and delivery. Albumin has several sites that can potentially interact with molecules. Small molecule binding sites on HSA have been studied extensively by various biochemical and physical methods [3–11]. Two primary drug-binding sites on HSA, Sudlow's sites I (warfarin) and II (ibuprofen), are known [12]. Competitive displacement of fluorescent probes is often used to determine the site and affinity of compounds for albumin [13–20]. This often requires several experiments with different probes. There is a need for additional analytical methods that can, in a single experiment, determine whether, at which site, and the extent to which a compound binds to albumin. Here we describe a novel NMR method for locating the ligand's binding site(s) on HSA and for assessing affinity at the site(s), providing the first step in detailed structural characterizations of HSA–ligand

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[1] *Abbreviations used:* HSA, human serum albumin; 2D, two-dimensional; HSQC, heteronuclear single quantum coherence; DMSO, dimethyl sulfoxide; 1D, one-dimensional; SEC, size exclusion chromatography.

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complexes to assist in the design of molecules with altered albumin affinity.

HSA is a plasma protein with 585 residues containing three structurally similar α-helical domains I to III [21]. The primary ligands for HSA in vivo are nonesterified fatty acids. Under normal physiological condition, HSA carries approximately 0.1–2 mol of fatty acids/mol protein. The most common type associated with the protein is oleic acid (*cis*-9-octadecenoic acid), representing roughly 33% of the total fatty acids bound to albumin in normal serum. The crystal structure of HSA complexed with monounsaturated oleic acids has been solved [22]. Seven oleic acids were seen in the complex. Their locations, which are also occupied by medium- and long-chain saturated fatty acids [23,24], are distributed asymmetrically across the protein. One interesting observation from the structure is that the oleic acid-binding sites overlap with primary drug-binding sites on HSA. In two-dimensional (2D) [^{1}H,^{13}C]heteronuclear single quantum coherence (HSQC) experiments of HSA complexed with ^{13}C-methyl-labeled oleic acid at a molar ratio of 1:4, seven distinct resonances were observed. This suggests that oleic acids bind at seven distinguishable sites, consistent with the crystal structure. Resonances corresponding to oleic acids bound at Sudlow's sites I and II were identified through competitive displacement of molecules that bind specifically to each site. Therefore, with the atomic resolution provided by NMR, we demonstrate that oleic acid can be used as a probe molecule to follow the small molecule's binding to the primary drug-binding sites on HSA.

Materials and methods

Human serum albumin (fatty acid free, A-1887), phenylbutazone, and ibuprofen were purchased from Sigma and used without further purification. ^{13}C-methyl-labeled oleic acid was purchased from Cambridge Isotope Laboratories. Oleic acid, phenylbutazone, and ibuprofen were prepared as 100-mM solutions with deuterated dimethyl sulfoxide (DMSO). The HSA protein samples were prepared at 0.5 mM in 50 mM sodium phosphate D_2O buffer (pH 7.0). Aliquots of the 100-mM oleic acid solution were added to the HSA solution for the desired ligand/protein molar ratios. The mixture was equilibrated at room temperature for about 2 h before NMR experiments. This equilibration period provided adequate time for oleic acid to interact with HSA and provided consistent results.

2D (^{1}H/^{13}C) HSQC NMR spectra were collected on a Varian INOVA 600-MHz spectrometer equipped with a triple-resonance probe. Gradient-selected, sensitivity-enhanced HSQC pulse sequence was employed for recording the data. The spectral widths in the ^{1}H and ^{13}C dimensions were 8000 and 3200 Hz, respectively. Typically, the spectra were acquired with 256 complex points in t_1 and 2048 complex points in t_2, 4 scans per increment, 2 s recycle delay at 30 °C. ^{19}F NMR spectra were obtained on a Varian INOVA 500-MHz spectrometer with a double-resonance ^{1}H/^{19}F probe. No proton decoupling was employed during data acquisition.

Results

Ligand environments of HSA-bound oleic acid molecules

In previous studies, one-dimensional (1D) NMR ^{13}C direct observation experiments were used to examine the binding of oleic acid to bovine serum albumin [25–28], but resonances were in general poorly resolved. In the current work, this problem was addressed by using ^{13}C-methyl-labeled oleic acid as well as a 2D NMR experiment. The chemical shifts of a methyl group are extremely sensitive to the local environment. Spectroscopic advantages for detecting the methyl groups are well known, including three times more sensitivity from the degenerate protons and the increased spectral resolution from free methyl rotations. A 2D (^{1}H/^{13}C) correlation experiment (2D [^{1}H,^{13}C]HSQC) was used to further improve the spectral resolution. Fig. 1A shows an NMR spectrum for a 4:1 molar ratio of oleic acid/HSA preparation. Seven distinct resonances are evident (numbered arbitrarily) and are reasonably well resolved. These resonances are from the ^{13}C-methyl-labeled groups given that the spectrum from an HSA-only solution (carbons at natural abundance) does not have any peaks in this chemical shift region (data not shown). Furthermore, these resonances do not represent the unbound oleic acid given that their chemical shifts do not coincide with those of oleic acid in aqueous solution. Also, the amount of free oleic acid in our experimental conditions is too low to be detected ($<$50 nM) [29]. Therefore, the multiple resonances observed in Fig. 1A indicate that there are at least seven distinguishable microenvironments for oleic acids on HSA and that ligands are in slow exchange among them. They likely correspond to the seven oleic acid-binding sites identified in the crystal structure [22].

Oleic acid resonance assignments to HSA drug-binding sites

Assignment of all resonances in Fig. 1A to the binding locations observed in the crystal structure is a nontrivial task, but for the current application identification of those resonances from oleic acids bound at the primary drug-binding sites is sufficient. This was achieved through competitive displacement of molecules that bound specifically to each site. Phenylbutazone, a known ligand for the warfarin site, was added to the HSA/oleic acid solution. Resonance 4 completely disappeared at a 1:1 molar ratio of phenylbutazone/HSA (Fig. 1B). Thus, phenylbutazone was able to completely displace the oleic acid bound at the warfarin site. Some small chemical shift perturbations on resonance 7 were also seen, but this was likely due to allosteric interactions instead of direct displacement given that the resonance's intensity remains about the same. Ibuprofen, a known

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