Locations of the three primary binding sites for long-chain fatty acids on bovine serum albumin

(fatty acid binding/13C NMR)

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ABSTRACT Binding of 13C-enriched oleic acid to bovine serum albumin and to three large proteolytic fragments of albumin—two complementary fragments corresponding to the two halves of albumin and one fragment corresponding to the carboxyl-terminal domain—yielded unique patterns of NMR resonances (chemical shifts and relative intensities) that were used to identify the locations of binding of the first 5 mol of oleic acid to the multidomain albumin molecule. The first 3 mol of oleic acid added to intact albumin generated three distinct NMR resonances as a result of simultaneous binding of oleic acid to three heterogeneous sites (primary sites). Two of these resonances were seen upon addition of 1 or 2 mol of oleic acid to fragments representing either the carboxyl-terminal half (residues 307-582) or the carboxyl-terminal domain (residues 377-582); the third resonance was seen upon addition of 1 mol of oleic acid to the fragment representing the amino-terminal half (residues 1-306). The resonance patterns for the fourth and fifth moles of oleic acid added to albumin (secondary sites) could not be duplicated by addition of more oleic acid to individual fragments. These resonance patterns were generated, however, when the two complementary fragments were mixed in equimolar proportions to form an albumin-like complex with a reconstituted middle domain. Thus, two primary fatty acid binding sites are assigned to the carboxyl-terminal domain, one primary site is assigned to the amino-terminal half, and the secondary sites are assigned to the middle domain. This distribution suggests albumin to be a less symmetrical binding molecule than theoretical models predict. This work also demonstrates the power of NMR for the study of microenvironments of individual fatty acid binding sites in specific domains.

Serum albumin, the major transport protein for unesterified fatty acids in plasma, is able to bind several moles of fatty acid, although the exact locations and detailed structures of fatty acid binding sites on this multidomain protein are not yet known (1, 2). The homology of the three domains of albumin has invoked speculation that the domains are structurally similar and share similar ligand binding capabilities. One of the most elegant models was proposed by Brown and Shockey (3), who drew upon the triple-domain primary structure and amphipathic helical secondary structure to present albumin as a series of cylinders, each with the capacity to bind 2 mol of fatty acids inserted into their hydrophobic centers. They further suggested that the three primary binding sites for long-chain fatty acids were distributed with one site per domain.

Although a variety of chemical and spectroscopic studies have been undertaken to define and characterize the locations of the heterogeneous binding sites for long-chain fatty acids, to date none to our knowledge has successfully mapped the locations of the heterogeneous sites for fatty acids on albumin.

Chemical approaches, including studies of the binding of fatty acids to chemically modified albumins (4) and to proteolytic fragments of albumin (5, 6) and affinity labeling of fatty acid binding sites (7), have identified specific regions of albumin and in some cases specific amino acid residues that appear to contribute to binding sites. However, the use of nonnative fatty acid analogues or the modification of specific residues in the protein may perturb the native structure, resulting in loss of native sites or in production of new, nonnative sites. When the results of such studies are in disagreement with predictions of various models, it is impossible to tell whether the disagreement reflects a limitation of the model or the technique.

Spectroscopic approaches, particularly studies of fluorescence of bound parinaric acids (8) and electron spin resonance studies using spin-labeled fatty acids (9), have determined distances between binding sites and have suggested features of binding geometries. Yet spectroscopic studies may be of limited utility in characterizing individual sites because multiple binding sites on albumin can fill simultaneously rather than sequentially; therefore, spectroscopic changes may reflect composites of the contributions of heterogeneous populations of albumin–fatty acid complexes rather than characteristics of individual sites. Like the chemical studies described above, these studies also involve nonnative fatty acids, and results may reflect nonnative behaviors.

In contrast, NMR spectra of 13C-enriched fatty acids bound to albumin have unique resonances reflecting the different microenvironments of the heterogeneous binding sites (10-13). For example, the carboxyl carbon of oleic acid bound to albumin gives rise to NMR resonances encompassing a broad range (>3 ppm) of chemical shifts and thus acts as a sensitive and specific probe of the local environments of unique binding sites. Since NMR studies can monitor native fatty acids bound to native proteins, they do not induce artefactual perturbations of the tertiary structure of the protein. Therefore, the NMR results should provide a complete reflection of native binding behavior. Previous NMR studies have reported evidence of different microenvironments for different binding sites, but the locations of the individual sites for fatty acids corresponding to the resonances were not identifiable from these earlier studies (11, 12).

In the present work, we have combined the ability of NMR to distinguish microenvironments of individual fatty acid binding sites with the advantages of using isolated fragments to limit the regions and number of sites available for binding. The three fragments of bovine albumin chosen for this study are especially suitable for mapping native binding sites because they retain native properties and native structures when

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cleaved from the parent protein (5). For example, the CD spectrum of each fragment, when presented as mean residue ellipticity, is indistinguishable from that for albumin. This result reflects the symmetry of the highly helical secondary structure of the three domains of albumin and shows that it is retained by the isolated fragments. Each fragment also has at least one long-chain fatty acid binding site with a binding affinity comparable to that demonstrated by native albumin (5). The fragments even show the ability to refold from a completely reduced, denatured state to form correct disulfide binding patterns and regain antigenicity and fatty acid binding properties (14). Two of the fragments represent complementary halves of albumin. When mixed together in stoichiometric quantities, they form a noncovalent complex that migrates upon gel filtration with an apparent molecular weight of albumin. This complex exhibits some properties of native albumin not displayed by the individual isolated fragments (6).

By comparing NMR spectra of \(^{13}\text{C}\)oleic acid bound to these fragments and to intact albumin, we are able to assign NMR resonances to specific locations in the albumin molecule and to identify the order of filling of the primary and secondary sites on albumin.

**MATERIALS AND METHODS**

Albumin was Armour crystallized bovine serum albumin. Fragments representing residues 1–306 (designated PB) and 307–582 (designated PA) of bovine albumin were obtained by limited peptic digestion and were purified by ion-exchange and gel filtration chromatography (15). The fragment representing residues 361–582 (designated TA) was obtained by tryptic digestion of albumin bound to a palmitoyl-aminoethylamino-agarose column and was purified by ion-exchange and gel filtration chromatography (16). The sequence numbers for the fragments are based on the sequence reported by Brown and Shockley (3).

Isotopically enriched [1,\(^{13}\text{C}\)]oleic acid (99% enriched, Kor Isotopes, Cambridge, MA) was added as the potassium soap to aqueous solutions of albumin at 50 mg/ml or albumin fragments at 10–30 mg/ml in 0.05 M phosphate buffer, pH 7.4/0.1 M NaCl (11). \(^{13}\text{C}\) NMR spectra were obtained at 50.3 MHz on a Bruker WP200 NMR instrument as described (10–12); chemical shifts were measured relative to external tetramethylsilane in C\(_2\)H\(_2\)Cl\(_2\) with a reliability equal to or greater than ±0.5 ppm. In spectra of albumin fragments in the absence of added oleic acid, no resonances were detected downfield from the broad protein envelope at 172–178 ppm. All NMR spectra were collected at least twice with each of two independent preparations of each fragment.

**RESULTS**

The \(^{13}\text{C}\) NMR spectra of oleic acid bound to fragments TA and PA are strikingly different from the spectrum for PB, yet each spectrum shows specific \(^{13}\text{C}\)carbonyl resonances associated with the binding of oleic acid to intact albumin (Fig. 1). Addition of 0.5 or 1 mol of oleic acid per mol of TA or PA produces two narrow, partially resolved resonances [182 ppm, Fig. 1, spectra A (182.05 and 182.37 ppm) and B (182.08 and 182.33 ppm)]. Addition of a second mole of oleic acid produces an increase in intensity in resonances already seen and a broadening of the downfield side of the resonance envelope (shown for PA in Fig. 1, spectrum C) but no additional identifiable peaks, suggesting simultaneous filling of two sites shared by TA and PA. Addition of 1 mol of oleic acid to PB produces a single sharp peak at 180.70 ppm (Fig. 1, spectrum D). These three resonances are indistinguishable in chemical shift and linewidth to those for 3 mol of oleic acid bound to intact albumin (Fig. 1, spectrum E).

**Fig. 1.** Carbonyl region of the \(^1\text{H}\)-decoupled Fourier transform 50.3-MHz \(^{13}\text{C}\)NMR spectrum at 35°C of the TA fragment with 1 mol of oleic acid (12,000 spectral accumulations; spectrum A), the PA fragment with 1 mol of oleic acid (7500 spectral accumulations; spectrum B), the PA fragment with 2 mol of oleic acid (10,000 spectral accumulations; spectrum C), the PB fragment with 1 mol of oleic acid (6000 spectral accumulations; spectrum D), and native bovine albumin with 3 mol of oleic acid (3000 spectral accumulations; spectrum E). This spectrum is similar to previously published spectra (9). All spectra were obtained with 10–30 mg of protein fragment or 50 mg of bovine albumin in 0.05 M phosphate buffer at pH 7.4 or 8.6 with 16,384 time-domain points, a 10-kHz spectral width, and a pulse interval of 2.0 sec. All spectra were processed with 3-Hz line-broadening except spectrum E.

Addition of 2 more mol of oleic acid to intact albumin (for a total of 5 mol of oleic acid per mol of albumin) introduces NMR resonances for three additional sites (compare spectrum D of Fig. 2 with spectrum E in Fig. 1). These are most noticeable as a weak, well-resolved peak at 183.55 ppm; a weak, partially resolved peak at 182.7 ppm; and an intense peak at 182.08 ppm, overlapping the resonances for the primary sites seen at 182.05 and 182.36 ppm. In contrast, addition of a second mole of oleic acid to PB (Fig. 2, spectrum A) or a third mole of oleic acid to PA (Fig. 2, spectrum B) produces strong resonances in the region of 182.6–182.7 ppm. The resonance for the secondary sites of PB is more apparent than for PA because of the larger difference in chemical shift between the primary and secondary sites for PB.

Since PA and PB form a complementary complex that regains some properties of albumin not observed for either of the fragments alone (6), we recorded the spectrum for \(^{13}\text{C}\)oleic acid bound to a stoichiometric mixture of PA and PB to determine if any secondary sites for oleic acid were regenerated in the complex. We compared this spectrum to that for albumin and a mathematical sum of the spectra for the two fragments alone.
and relative intensities to that for 5 mol of oleic acid bound to intact albumin. The similarity of spectrum E to spectrum D in Fig. 2 demonstrates that secondary binding sites of albumin, which were not present on the individual fragments, were restored when the fragments were mixed to form a complementary complex.

The association constants for oleic acid binding to the three primary sites must be at least 1 order of magnitude greater than those for the secondary sites, since the resonances for the secondary sites did not begin to appear until those for the primary sites had reached maximum intensity. For PB, this result is in agreement with equilibrium binding studies (5), which showed two binding sites for fatty acids with a 20-fold difference in affinity. For TA and PA, the simultaneous appearance of two resonance peaks that grew in intensity at approximately the same rate suggests the presence of two sites with similar affinities. However, the equilibrium binding data showed two sites with at least a 10-fold difference in affinity (5). Such seemingly different results might be explained if the binding of one fatty acid to either of two sites of nearly equivalent affinity induced a conformational change that lowered the binding affinity of the remaining unfilled site. Scatchard analysis, which assumes independent and noninteracting sites, is unable to determine binding affinities for individual sites when they are dependent, interacting sites. Instead, a more general model, determining empirical stepwise equilibrium constants that reflect net binding rather than binding at individual sites, would be applicable to such systems (17).

These NMR results permit us to assign each of the NMR resonances for oleic acid bound to albumin to specific regions (Fig. 3). The site reflected by the resonance at 180.60 ppm is seen in PB and therefore is assigned to the amino-terminal half of albumin (domain I and subdomain Ia). The two sites reflected by NMR resonances at 182.05 and 182.36 ppm are assigned to the carboxyl-terminal domain (domain II) because these are seen in PA and TA, the two fragments that

![Fig. 2. Carbonyl region of 1H-decoupled Fourier transform 50.3-MHz 13C spectra at 35°C: the PB fragment with 2 mol of oleic acid (1800 spectral accumulations; spectrum A), the PA fragment with 3 mol of oleic acid (5000 spectral accumulations; spectrum B), digital summation of spectra A and B (spectrum C), native bovine albumin with 5 mol of oleic acid (6000 spectral accumulations; spectrum D), and reassembled bovine albumin formed from a mixture of PA with 3 mol of oleic acid and PB with 2 mol of oleic acid (20,000 spectral accumulations; spectrum E). All spectra were processed with 3-Hz line-broadening. There is a small peak at 182.7 ppm in both the native and reassembled bovine albumin (spectra D and E), while there is a much larger relative intensity at 182.7 ppm in the spectral sum (spectrum C).]

When the spectra of 3 mol of oleic acid bound to PA and 2 mol of oleic acid bound to PB were added digitally (Fig. 2, spectrum C), the resultant spectrum was quite different from that for 5 mol of oleic acid bound to intact albumin (Fig. 2, spectrum D). Oleic acid bound to intact albumin has a small but significant peak at 183.55 ppm that is not seen in the composite spectrum for the fragments, and the composite spectrum for the fragments shows a large broad peak at 182.70 ppm that does not resemble the partially resolved peaks at 182.3 and 182.7 ppm seen with intact albumin. These differences in chemical shift and relative peak intensities suggest that the secondary sites on the individual fragments are different from the secondary sites on intact albumin.

The spectrum of 5 mol of oleic acid bound to a stoichiometric complex of PA and PB (Fig. 2, spectrum E) bore a striking similarity with respect to chemical shifts, linewidths,
contain domain III. The location of the secondary sites is less certain than those for the primary sites. We have assigned the secondary sites to the middle domain (domain II) because the resonance pattern for albumin is reproduced by the reassembled complex of PA and PB in which the middle domain is reconstituted but not by the sum of the individual fragments in which the middle domain is dissociated. Although reassembly could induce changes in other parts of the molecule and generate sites in domains I or III, chemical and other spectroscopic evidence is more consistent with the fourth and fifth moles of long-chain fatty acids binding near the middle of the molecule (4, 8).

DISCUSSION

Long chain fatty acid binding to serum albumin, as detected by the binding of \([^{13}C]\)oleic acid, shows an asymmetric distribution of primary and secondary sites, with two of three primary sites being in the carboxyl-terminal domain and the third being either in the first domain or the amino-terminal part of the second domain and with the weaker, secondary sites being in the middle domain.

These assignments are different from those of Brown and Shockley (3) who proposed that six fatty acids would be symmetrically distributed among three domains. In their model, each domain was envisioned as an open cylinder with two fatty acids inserted in antiparallel fashion with the hydrophobic tails in the interior and the hydrophilic heads extending out the ends; one strong site and one weak site were proposed for each domain.

The binding of \([^{13}C]\)oleic acid, as determined in this work, follows a distribution consistent with the x-ray crystallographic assignments of major binding sites based on the binding of heavy metal. We have assigned two oleic acids in domain III and one in the region encompassed by PB (residues 1-306). The crystallographic structure shows that subdomains IA, IB, and IIA form a particularly compact region as do subdomains IIB, IIIA, and IIIB (18, 19). Domain II is consequently a relatively open domain, consistent with its susceptibility to cleavage by pepsin to produce PA and PB. In making comparisons of behavior in solution to x-ray structure, it is well to keep in mind that albumin in solution is a flexible molecule that may adopt various conformations to accommodate binding of fatty acids.

The close correspondence of chemical shifts for high-affinity sites in the fragments and the native protein is striking, especially in the case of PB, whose single resonance is well separated from resonances for secondary binding sites as well as the primary binding sites on the carboxyl-terminal fragments. This suggests a preservation of tertiary structure for the primary sites in these large proteolytic fragments (in contrast to the secondary sites, which are different in fragments and native or reassembled albumin). The close correspondence of carboxyl linewidths and relative peak intensities in the spectra for oleic acid bound to the fragments and native albumin is additional evidence that these NMR results reflect native behavior on the part of the fragments rather than binding of oleic acid to artefactual binding sites on the fragments with chemical shifts that fortuitously overlap those seen with the native protein. The integrity of structure reflects a very stable multidomain structure of albumin. It is therefore most probable that the high-affinity site in PB is localized predominantly within domain I—the more intact domain of PB. This assignment is also consistent with an affinity-labeled site on Lys-116, within the first domain of PB (7).

The presence of two high-affinity sites for oleic acid in domain III but only one associated with domain I may be the result of differences in amino acid composition or in three-dimensional structure. Domain III has one more disulfide bond than domain I, which may provide a conformation critical to accommodating a second high-affinity site for long-chain fatty acids.

The assignment of unique NMR resonances for fatty acids bound to specific regions of albumin now makes NMR a more powerful tool for future investigations of perturbations of fatty acid binding and release at the specific-site level. The effects of (i) changes in pH or ionic strength, (ii) the presence of competing ligands, or (iii) the effects of chemical modifications of albumin can now be assessed in terms of their impact on individual fatty acid binding sites, overcoming limitations of other techniques that monitor only net effects.

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