Bovine Serum Albumin: Characterization of a Fatty Acid Binding Site on the N-Terminal Peptic Fragment Using a New Spin-Label†

J. C. Hsia,* L. T. Wong, C. T. Tan, S. S. Er, S. Kharouba, E. Balaskas, D. O. Tinker, and R. C. Feldhoff

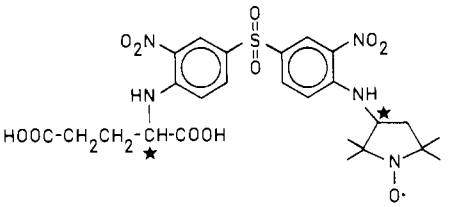
ABSTRACT: A new spin-label, 4-(L-glutamo)-4′-[(1-oxy-2,2,5,5-tetramethyl-3L-pyrrolidinyl)amino]-3,3′-dinitrodiphenyl sulfone, is shown to bind to one high-affinity binding site on bovine serum albumin ($K = 5 \times 10^4$ M^{-1}, $n = 1$). Analysis of the binding of the spin-label to the amino-terminal half (peptic fragment PB) and the carboxy-terminal half (peptic fragment PA) of BSA, and their complex (PA–PB), indicates that the spin-label binds to a long-chain fatty acid binding site located on PB. The usefulness of the novel specificity of the spin-label in characterizing this binding site is discussed.

Albumin is the most abundant plasma protein. It binds and transports bilirubin, fatty acids, L-tryptophan, and many drugs. Albumin consists of a single polypeptide chain of 581 residues and exists in a multidomain structure with complex ligand-binding specificities. A striking feature of the structure of albumin is its repeating double-loop motif, formed by cross-linking of adjacent cysteine residues. These double loops define the three domains or homogeneous regions of the molecule. This, together with the folding restrictions imposed by 17 disulfide bridges, led Brown (1976) to postulate a domain structure for albumin resulting from gene duplication. In this model, Brown proposes that the small ancestral albumin gene has undergone a series of duplications and deletions to give the present three-domain structure. Studies of the DNA sequence of the murine α-fetoprotein gene, which has the same ancestral gene as albumin, have lent strong support to Brown's gene duplication model (Eiferman et al., 1981).

Recently we have prepared a series of molecular probes consisting of mono- and dicarboxylic acids linked to nitroxide spin-labels either directly (Wood & Hsia, 1977) or indirectly through a dinitrophenyl (DNP)[1] cross-linker (Soltys & Hsia, 1978a). We have shown that these spin-labels bind to albumin with specificities similar to that of bilirubin. It is interesting that these spin-labels also bind to medium- and long-chain fatty acid and steroid binding sites (Soltys & Hsia, 1978b,c). Results of these studies have led to the proposal of an allosteric domain model based on Brown's original concept (Hsia, 1978). The stereospecificity of albumin's ligand binding sites has also been explored (Hsia et al., 1982).

More recently Hsia (1981) proposed that the multiple physiological ligand binding sites arise through the conservation of a primordial ligand binding site in accordance with Brown's model. However, Hsia's hypothesis and the allosteric domain model have not been rigorously tested, due to the complexity of albumin's binding properties.

In the present paper we describe a new spin-label, 4-(L-glutamo)-4′-[(1-oxy-2,2,5,5-tetramethyl-3L-pyrrolidinyl)-amino]-3,3′-dinitrodiphenyl sulfone [or L-Glu-DNDPS-L-SL-(5)] (I), which binds to a single saturated long-chain fatty acid binding site on bovine serum albumin (BSA). By studying the binding of this spin-label to the carboxy- and amino-terminal halves of BSA, using the peptic fragments PA and PB and the BSA molecule reconstituted from them (Reed et al., 1976), we show that this single binding site is located on the amino-terminal half of albumin and that it is independent of the primary bilirubin binding site. This new spin-label should be extremely useful in further characterizing this ligand binding site.



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Materials and Methods

Bovine serum albumin and its pepsin digestion fragments PA and PB were prepared as described by Feldhoff & Peters (1975). The proteins were defatted by charcoal treatment (Chen, 1967), and their concentration was determined spectroscopically with $E^{1\%}_{279nm} = 6.8$ for BSA and $E^{1\%}_{280nm} = 5.3$ and 7.4 for PA and PB, respectively. The system used was 50 mM barbital buffer, pH 8.6; the fragments were shown by cellulose acetate chromatography to quantitatively associate to form the PA–PB complex in this system.

Bilirubin was purchased from Sigma and further purified by the method of Fog (1964). All procedures involving bilirubin were carried out under minimum lighting conditions. To make bilirubin–protein complexes, bilirubin was dissolved in methanol in the sodium form, and the concentration was determined spectroscopically with $E_{453nm} = 6.22 \times 10^4$ in Me_2SO (Kuenzle, 1970). The required amount of bilirubin was transferred into a small vial and dried under nitrogen. The protein solution was then added immediately and blended on

† From the Department of Biochemistry, Faculty of Medicine, University of Toronto, Toronto, Canada M5S 1A8 (J.C.H., L.T.W., C.T.T., S.K., E.B., and D.O.T.), the Biosciences Division, Defence and Civil Institute of Environmental Medicine, Downsview, Ontario, Canada M3M 3B9 (J.C.H. and S.S.E.), and the Department of Biochemistry, School of Medicine, University of Louisville, Louisville, Kentucky 40292 (R.C.F.). *Received April 30, 1984.* Publication No. 84-P-00. Supported by a research grant from the Medical Research Council of Canada (MA-4129) and the Department of National Defence, Canada.

* Address correspondence to this author at the Department of Biochemistry, Faculty of Medicine, University of Toronto.

[1] Abbreviations: BSA, bovine serum albumin; PA, peptic fragment P-(307–581) of BSA; PB, peptic fragment P-(1–306) of BSA; DNP, dinitrophenyl; AcCN, acetonitrile; Me_2SO, dimethyl sulfoxide; ESR, electron spin resonance; DNDPS, 3,3′-dinitrodiphenyl sulfone; DFDNDPS, 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone; FDNDPS-L-SL(5), 4-fluoro-4′-[(1-oxy-2,2,5,5-tetramethyl-3L-pyrrolidinyl)amino]-3,3′-dinitrodiphenyl sulfone; spin-label I or L-Glu-DNDPS-L-SL(5), 4-(L-glutamo)-4′-[(1-oxy-2,2,5,5-tetramethyl-3L-pyrrolidinyl)amino]-3,3′-dinitrodiphenyl sulfone.

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a vortex mixer. Solutions were used within 1 h of preparation.

Palmitic acid was also purchased from Sigma and was used without further purification. Preparation of palmitate–protein complexes was essentially as previously described (Soltys & Hsia, 1977). Briefly, the sodium salt of palmitate was dried under vacuum to form a thin film in a small glass vial. The protein solution was added to the vial and gently blended on a vortex mixer until optically clear. Molar ratios of fatty acid to protein were quantitated with a trace of [^{14}C]palmitate (New England Nuclear).

The spin-label probe 4-(L-glutamo)-4′-[(1-oxy-2,2,5,5-tetramethyl-3L-pyrrolidinyl)amino]-3,3′-dinitrodiphenyl sulfone [L-Glu-DNDPS-L-SL(5)] was synthesized as follows: 1-Oxy-2,2,5,5-tetramethyl-3L-aminopyrrolidine was resolved from the DL mixture by use of benzoyl-L-leucine as described previously (Wong et al., 1974). Forty milligrams of the amine spin-label salt was dissolved in 2 mL of a dry $CHCl_3$–acetonitrile (AcCN) (1:1) mixture containing 0.5 mL of triethylamine. This was added dropwise to 70 mg of 4,4′-difluoro-3,3′-dinitrodiphenyl sulfone (DFDNDPS) dissolved in 1 mL of AcCN at 0 °C. The reaction mixture was stirred at room temperature for 15 h and then chromatographed on a small silica gel column using methylene chloride followed by diethyl ether. The ether-eluted product was concentrated to give 26 mg (53%) of 4-fluoro-4′-[(1-oxy-2,2,5,5-tetramethyl-3L-pyrrolidinyl)amino]-3,3′-dinitrodiphenyl sulfone [FDNDP-S-L-SL(5)].

Ten milligrams of FDNDPS-L-SL(5) was dissolved in 1.25 mL of AcCN–acetone (3:2) and added dropwise to 8 mg of L-glutamic acid dissolved in 1 mL of water containing 12 mg of Na_2CO_3. The mixture was stirred at room temperature overnight and concentrated to 1 mL by bubbling N_2 gas through it to remove the organic solvents. The aqueous layer was extracted three times with 2-mL portions of ether and then carefully acidified to pH 3 with 0.1 N HCl. The yellow precipitate was filtered, washed with water, and dried to give 8 mg (63%) of L-Glu-DNDPS-L-SL(5) (I), mp 155–160 °C dec. Thin-layer chromatography of this product showed a single spot in all solvent systems tested.

Electron spin resonance spectra (ESR) were recorded with a Varian E-109S X-band spectrometer equipped with a variable temperature controller. The ESR system was interfaced with the Hewlett-Packard 9835B desktop computer system with signal-averaging capability. The temperature of the cavity was maintained at 37 ± 0.5 °C and monitored with a thermocouple. Samples were contained in disposable 50-μL glass pipets and used for ESR measurements.

For dilute samples an average of five scans per sample was taken to enhance the ESR signal. The magnetic field was calibrated with Fremy's salt. Free spin-label concentrations were determined by measurement of the peak-to-peak height of the high-field line of the sharp three-line spectrum (Hsia et al., 1973).

Results

Binding of the Spin-Label to BSA. Figure 1 shows the resonance spectra of the spin-label in the presence and absence of an equimolar concentration of BSA. The decrease in the intensity of the free spin-label peak in the presence of BSA indicates binding of the spin-label to albumin. Analysis of the binding isotherm using a Scatchard plot (Figure 2) indicates the presence of a single, high-affinity binding site for the spin-label on albumin with an association constant of 5×10^4 M^{-1}.

Specificity of the Spin-Label Binding Site. BSA is known to have at least one high-affinity binding site for bilirubin (Jacobsen, 1969) and at least four for long-chain fatty acids (Ashbrook et al., 1975). A Scatchard plot of the spin-label's binding to BSA in the presence of 1 molar equiv of bilirubin or 4 molar equiv of palmitate is also shown in Figure 2. This shows the spin-label to be displaced from its binding site by palmitate, while the presence of bilirubin does not greatly affect spin-label binding. These results suggest that the spin-label is binding not to the primary bilirubin binding site but to one of the long-chain fatty acid binding sites, a site that is separate from the primary bilirubin site.

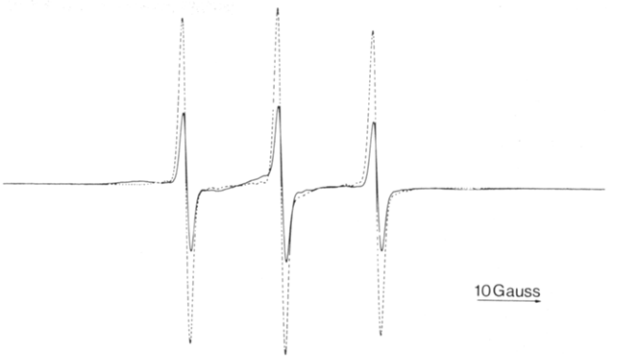

FIGURE 1: Electron spin resonance spectra of the spin-label in the absence (dashed line) and in the presence (solid line) of equimolar defatted BSA monomer. BSA and spin-label concentrations are 5×10^{-5} M in 50 mM barbital buffer, pH 8.6 at 37 °C. The magnetic field strength increases from left to right.

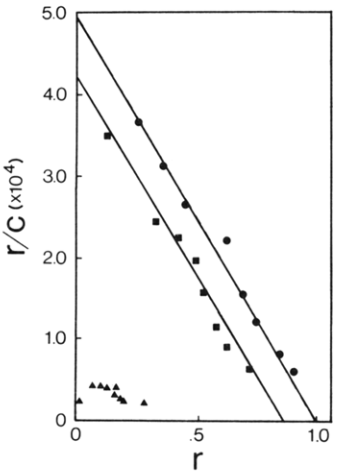

FIGURE 2: Scatchard plots of the binding of the spin-label to defatted BSA monomer in the absence (●) and in the presence of 1 molar equiv of bilirubin (■) and 4 molar equiv of palmitate (▲). BSA concentration is 5×10^{-5} M. r = moles of spin-label bound; c = free spin-label concentration. Other experimental conditions were the same as described in Figure 1.

Binding of the Spin-Label to PA, PB, and the PA–PB Complex. Figure 3 shows Scatchard plots of the binding of the spin-label to the peptic fragments PA and PB of BSA and to the PA–PB complex. In Figure 3A, the carboxy-terminal half of the protein, the PA fragment, shows no binding of spin-label. The amino-terminal fragment, PB, however, shows some affinity for the spin-label, with an r/c intercept of about 5×10^3 M^{-1} and an r intercept of 0.5 (the native BSA control shows intercepts of 5×10^4 M^{-1} and 1.0; see Figure 2). In Figure 3B, the PA–PB complex shows a 6-fold increase over PB in spin-label binding affinity ($K = 3 \times 10^4$ M^{-1}, $n = 1$), suggesting that reassociation of these fragments restores the spin-label binding site. The spin-label binding affinity of the PA–PB complex is about 60% of that of native BSA, in good agreement with the previous finding (Reed et al., 1976) that the complex binds palmitate 74 ± 6% as strongly as BSA.

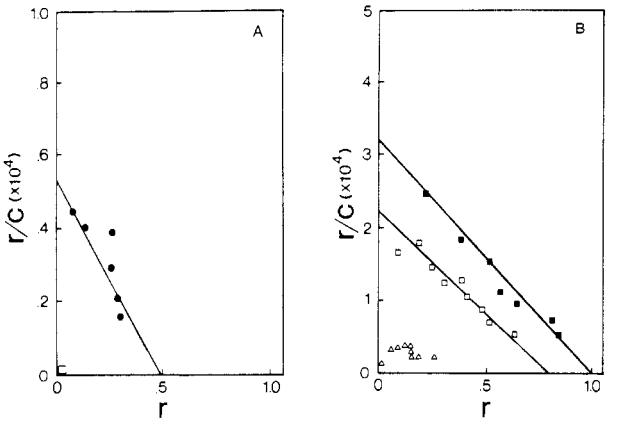

FIGURE 3: Scatchard plots of the binding of the spin-label to (A) the defatted peptic fragments PA (□) and PB (●) and (B) the defatted PA–PB complex in the absence (■) and presence of 1 molar equiv of bilirubin (□) and 4 molar equiv of palmitate (△). The concentrations of PA, PB, and the PA–PB complex are 5×10^{-5} M. The experimental conditions were the same as in Figure 2.

Specificity of the Reconstituted Spin-Label Binding Site on the PA–PB Complex. A Scatchard plot of the binding isotherms of spin-label binding to the PA–PB complex in the presence of 1 molar equiv of bilirubin or 4 molar equiv of palmitate is shown in Figure 3B. The effects of these ligands on spin-label binding are the same as those observed for BSA (Figure 2): palmitate completely displaces the spin-label and bilirubin does not. This indicates that the specificity of the spin-label site for long-chain fatty acids survives in the PA–PB complex.

Discussion

With the use of spin-label I, we demonstrate here the presence of a specific long-chain fatty acid binding site, independent of the primary bilirubin binding site, on the amino-terminal half (PB) of albumin. Both PA and PB of BSA were previously shown to bind palmitate (Reed et al., 1976). Our demonstration of a long-chain fatty acid binding site on PB supports the notion of multiple, similar ligand binding sites on albumin, which is implicit in Brown's gene-duplication hypothesis (Brown, 1979) and in the proposition that the multiple ligand binding sites of albumin arose through conservation of a binding site present on the primordial albumin (Hsia, 1981). It is interesting that the spin-label binds to the fatty acid binding site on PB but not on PA of the albumin molecule, which indicates divergence in binding specificity between these long-chain fatty acid binding sites on BSA.

To characterize the multiple ligand binding sites and specificities of albumin will require the development of spin-labels for each. Spin-labels binding specifically to the primary bilirubin binding site are known (Wood & Hsia, 1977; Soltys & Hsia, 1978a), and the present results indicate that spin-label I is a specific probe for the secondary long-chain fatty acid binding site. It is interesting that replacement of the DNP cross-linker by DNDPS changes the spin-label's specificity from one resembling that of bilirubin to one resembling that of a fatty acid. Unpublished results indicate that piperidine and pyrrolidine derivatives of DNDPS have the same binding specificity to albumin. Thus the change in the spin-label's binding specificity from bilirubin-like to fatty acid like is due to the change of cross-linker from DNP to DNDPS.

It is known that changing from the L to the D enantiomer of the DNP spin-label changes the latter to a spin-label specific for the primary bilirubin binding site of human serum albumin (Hsia et al., 1982). Spin-label I contains two asymmetric carbons. The four diastereoisomers of spin-label I, therefore, may well prove to have distinct and useful binding specificities. The unique binding specificity of the DNDPS spin-labels will be further exploited in the mapping of physiological ligand and drug binding sites of albumin and α-fetoprotein.

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Registry No. I, 92958-30-0; DFDNDPS, 312-30-1; FDNDPS-L-SL(5), 92958-31-1; 1-oxy-2,2,2,5-tetramethyl-3L-aminopyrrolidine, 34272-83-8; L-glutamic acid, 56-86-0.

References

Ashbrook, J. D., Spector, A. A., Santos, E. C., & Fletcher, J. (1975) *J. Biol. Chem. 250*, 2333–2338.

Brown, J. R. (1976) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 2141–2144.

Chen, R. F. (1967) *J. Biol. Chem. 242*, 173–181.

Eiferman, A. E., Young, P. R., Scott, R. W., & Tilghman, S. M. (1981) *Nature (London) 294*, 713–718.

Feldhoff, R. C., & Peters, T., Jr. (1975) *Biochemistry 14*, 4508–4514.

Fog, J. (1964) *Scand. J. Clin. Lab. Invest. 16*, 49–54.

Hsia, J. C. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1424.

Hsia, J. C. (1981) *Period. Biol. 83*, 99–104.

Hsia, J. C., Wong, L. T. L., & Kelow, W. (1973) *J. Immunol. Methods 3*, 17–24.

Hsia, J. C., Er, S. S., Tan, C. T., & Tinker, D. O. (1982) *J. Biol. Chem. 257*, 1724–1729.

Jacobsen, J. (1969) *FEBS Lett. 5*, 112–114.

Kuenzle, C. C. (1970) *Biochem. J. 119*, 395–409.

Reed, R. G., Feldhoff, R. C., & Peters, T., Jr. (1976) *Biochemistry 15*, 5394–5398.

Soltys, B. J., & Hsia, J. C. (1977) *J. Biol. Chem. 252*, 4043–4048.

Soltys, B. J., & Hsia, J. C. (1978a) *J. Biol. Chem. 253*, 3029–3034.

Soltys, B. J., & Hsia, J. C. (1978b) *J. Biol. Chem. 253*, 3023–3028.

Soltys, B. J., & Hsia, J. C. (1978c) *J. Biol. Chem. 253*, 4266–4269.

Wong, L. T. L., Piette, L. H., Little, J. R., & Hsia, J. C. (1974) *Immunochemistry 11*, 377–379.

Wood, D. J., & Hsia, J. C. (1977) *Biochem. Biophys. Res. Commun. 76*, 863–869.