

Crystallographic Analysis Reveals Common Modes of Binding of Medium and Long-chain Fatty Acids to Human Serum Albumin

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Human serum albumin (HSA) is an abundant plasma protein that is responsible for the transport of fatty acids. HSA also binds and perturbs the pharmacokinetics of a wide range of drug compounds. Binding studies have revealed significant interactions between fatty acid and drug-binding sites on albumin but high-resolution structural information on ligand binding to the protein has been lacking. We report here a crystallographic study of five HSA-fatty acid complexes formed using saturated medium-chain and long-chain fatty acids (C10:0, C12:0, C14:0, C16:0 and C18:0). A total of seven binding sites that are occupied by all medium-chain and long-chain fatty acids have been identified, although medium-chain fatty acids are found to bind at additional sites on the protein, yielding a total of 11 distinct binding locations. Comparison of the different complexes reveals key similarities and significant differences in the modes of binding, and serves to rationalise much of the biochemical data on fatty acid interactions with albumin. The two principal drug-binding sites, in sub-domains IIA and IIIA, are observed to be occupied by fatty acids and one of them (in IIIA) appears to coincide with a high-affinity long-chain fatty acid binding site.

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Introduction

Long-chain fatty acids are required for the synthesis of membrane lipids, hormones and second messengers, and serve as an important source of metabolic energy. Fatty acids are stored as triacylglycerols in adipose tissue and released into the circulation where their low aqueous solubilities (typically < 1 μ M) are overcome by serum albumin, an abundant plasma protein. Human serum albumin (HSA) greatly enhances the transport capacity of plasma, since it is present at around 0.6 mM and can carry at least six molecules of fatty acid. Under normal physiological conditions, HSA carries around 0.1–2 mol of fatty acid per mol protein (Fredrickson *et al.*, 1958).

HSA is capable of binding an extraordinarily broad range of drugs, and much of the clinical and pharmaceutical interest in the protein derives from its effects on drug pharmacokinetics (Robertson & Brodersen, 1991; Dubois *et al.*, 1993; Jakoby *et al.*, 1995; Vorum & Honoré, 1996; Itoh *et al.*, 1997; Demant & Friche, 1998; Molla *et al.*, 1998). Pioneering work by Sudlow found two primary drug-binding sites on the protein, named sites I and II (Sudlow *et al.*, 1975, 1976). Many subsequent studies have shown that the presence of fatty acids has unpredictable effects on drug binding, and both co-operative and competitive interactions have been observed (Vallner, 1977; Birkett *et al.*, 1978; Wanwimolruk *et al.*, 1983; Ivarsen & Brodersen, 1989; Brodersen *et al.*, 1990; Vorum & Honoré, 1996; Curry *et al.*, 1998).

The binding of fatty acids to serum albumin has been studied for over 40 years (Carter & Ho, 1994; Peters, 1995) but our understanding of these interactions is far from complete. Although it is now well established that the protein has multiple fatty acid binding sites of varying affinities (Kragh-Hansen, 1990; Carter & Ho, 1994; Peters, 1995), the

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Abbreviations used: HSA, human serum albumin;
BSA, bovine serum albumin.

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precise number of binding sites is not known; a current consensus seems to be that there are two or three high-affinity sites on the protein and at least three further sites of lower affinity (Carter & Ho, 1994; Peters, 1995). A variety of biochemical and biophysical methods have been used to investigate the nature and locations of fatty acid binding sites and many significant insights have accrued. The mixed electrostatic and hydrophobic nature of the binding interaction has been characterised (Reynolds *et al.*, 1968; Parks *et al.*, 1983; Cistola *et al.*, 1987b) and it has been shown that at several binding sites the carboxylate head-group of the bound fatty acid is more rigidly anchored than the methylene tail (Hamilton *et al.*, 1984). Moreover, the domain locations of some sites have been mapped (Reed *et al.*, 1975; Hamilton *et al.*, 1991), and there are clear indications that the primary sites for medium-chain and long-chain fatty acids are distinct (Means & Bender, 1975; Soltys & Hsia, 1978). However, progress in understanding fatty acid binding has been hampered, due largely to the complexity of the multiple binding interactions but also to the absence of structural information. Almost all of the methods used to identify binding sites have resorted to some form of modification of the protein (Reed *et al.*, 1975; Shaklai *et al.*, 1984; Hamilton *et al.*, 1991) or the ligand (Sklar *et al.*, 1977; Berde *et al.*, 1979; Reed, 1986), which inevitably complicated interpretation of the results. Only recently have structural and mutational approaches been applied to the analysis of ligand binding to HSA.

The first crystallographic analyses of HSA revealed that the protein, a 585 amino acid residue monomer, contains three homologous α -helical domains (I-III) (He & Carter, 1992; Carter & Ho, 1994). The domains each contain ten helices and are divided into six-helix and four-helix sub-domains (A and B); the first four helices of A and B form similar anti-parallel α -helix bundles. These investigations found that drug-binding sites I and II on HSA are located in sub-domains IIA and IIIA, respectively (He & Carter, 1992; Ho *et al.*, 1993). We subsequently reported the crystal structure of HSA complexed with a medium-chain fatty acid (myristic acid, C14:0) (Curry *et al.*, 1998, 1999), which brought to light the precise locations of six fatty acid binding sites. This study also uncovered a previously undetected binding site for drug compounds, immediately adjacent to a bound fatty

acid molecule in sub-domain IA (Curry *et al.*, 1998).

However, C14:0 is not found in appreciable quantities circulating in plasma (Saifer & Goldman, 1961) except under certain conditions of disease or clinical treatment (Bach & Babayan, 1982; Babayan, 1987; Bougnères *et al.*, 1989). Therefore, to assess the generality of our findings from the structure of the HSA-C14:0 complex, we have extended our analysis and present the crystal structures of four new HSA-fatty acid complexes. These include complexes with the saturated fatty acids C10:0, C12:0, C16:0 and C18:0 (capric, lauric, palmitic and stearic acid, respectively; in the *Cm:n* nomenclature adopted for this work, *m* gives the number of carbon atoms in the methylene tail and *n* is the number of double bonds). We have re-refined the structure of the HSA-C14:0 complex using a more complete high-resolution data set than that available previously.

Results

Structure determination

HSA-fatty acid complexes were prepared by incubating the protein with a large molar excess of fatty acid (see Materials and Methods; Table 1). Five different complexes were prepared in the course of the present work using C10:0, C12:0, C14:0, C16:0 and C18:0 fatty acids. The complexes all crystallized isomorphously with HSA-C14:0 (Curry *et al.*, 1998) and the structures were solved by molecular replacement. Difference electron density maps showed clear density for multiple bound fatty acids in each case. At most sites (exceptions are noted below) the shape of the density, which had a significant broadening at one end, clearly indicated the position of the carboxylate moiety and thus defined the orientation of the bound lipid. This usually placed the carboxylate group of the fatty acid adjacent to an amino acid side-chain with which it could make a salt-bridge or hydrogen bond. Models for each HSA-fatty acid complex were constructed and refined to resolutions of 2.44–2.70 Å; the models have R_{free} values in the range 25.8–28.8% and reasonable stereochemistry (Table 2). While it was not possible at these resolutions to assign the dihedral angles within the methylene tails of the bound fatty acids precisely, the overall shapes of the tails were well-defined. Average *B*-factors for the different models are relatively high, ranging from 52–61 Å²; sub-domain

Table 1. Summary of co-crystallisation conditions

	Process	C10:0	C12:0	C14:0	C16:0	C18:0
Fatty acid (mM)	Complexing	10	5	2.4	2.5	0.8
HSA:FA molar ratio	Complexing	>40	30	12	20	>20
Fatty acid (mM)	Washing	5.0	0.5	0.1	0.1	0.05
PEG 3350 (% w/v)	Crystallisation	28	28	28	28	31
PEG 3350 (% w/v)	Harvest	35	35	35	35	34

Table 2. Data collection and model refinement statistics

HSA-fatty acid complex	C10:0	C12:0	C14:0	C16:0	C18:0
A. Data collection					
Unit cell dimensions					
<i>a</i> (Å)	186.78	188.50	191.84	190.09	189.60
<i>b</i> (Å)	39.19	38.90	39.02	38.75	38.84
<i>c</i> (Å)	95.34	95.77	95.88	95.87	95.98
β (deg.)	105.15	104.63	105.12	104.96	105.49
Source ^a	D-9.6	H-X31	D-9.5	H-X31	D-9.6
Resolution range (Å)	37.5-2.5	12.0-2.45	13.0-2.5	12.0-2.44	40.0-2.7
Independent reflections	22984	23657	23210	24122	17689
Multiplicity	3.2	1.5	1.9	2.7	2.5
Completeness (%) ^b	97.5 (100)	92.7(83.6)	96.2(92.4)	92.9(87.8)	93.7(96.8)
<i>I</i> / σ <i>I</i>	5.3(2.5)	9.5(3.1)	3.7(3.0)	9.4(4.0)	4.5(2.4)
<i>R</i> _{merge} (%) ^c	7.0(27.3)	3.8(22.6)	5.7(22.4)	4.6(17.8)	9.0(24.8)
B. Model refinement					
RCSB PDB ID	1e7e	1e7f	1e7g	1e7h	1e7i
Number of non-hydrogen atoms	4561	4478	4598	4554	4639
Number of water molecules	31	26	16	29	26
<i>R</i> _{model} (%) ^d	22.0	22.5	22.5	21.4	20.4
<i>R</i> _{free} (%) ^e	27.1	27.6	28.8	27.2	25.9
r.m.s deviation from ideal bond lengths (Å)	0.006	0.007	0.006	0.006	0.007
r.m.s deviation from ideal angles (deg.)	1.16	1.23	1.10	1.21	1.16
r.m.s. deviation in <i>B</i> -factors main/side-chain (Å ²)	1.7/2.6	1.7/2.6	1.7/2.7	1.7/2.7	1.7/2.6

^a D, Synchrotron Radiation Source, Daresbury, UK; H, DESY, Hamburg, Germany.

^b Values for the outermost resolution shell are given in parentheses.

^c $R_{\text{merge}} = 100 \sum_i \sum_j |I_{ij} - \bar{I}_i| / \sum_i \sum_j I_{ij}$, where $\bar{I}_i$ is the weighted mean intensity of the symmetry-related reflections I_{ij} .

^d $R_{\text{model}} = 100 \sum_{hkl} |F_{\text{obs}} - F_{\text{calc}}| / \sum_{hkl} F_{\text{obs}}$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively.

^e R_{free} is the R_{model} calculated using a randomly selected 5% sample of reflection data omitted from the refinement.

IIIB consistently exhibits higher than average *B*-factors (65–80 Å²).

Overview of fatty acid binding

For the four new HSA-fatty acid complexes in this study, the protein conformations are very similar to the structure of HSA-C14:0, which has been described in detail elsewhere (Curry *et al.*, 1998, 1999). The HSA-fatty acid complexes all display the conformational change from the defatted HSA structure (He & Carter, 1992; Sugio *et al.*, 1999) that was observed previously for HSA-C14:0 (Curry *et al.*, 1998). This conformational change involves substantial rotations of domains I and III relative to the central domain II, and appears to be primarily driven by fatty acid binding to the site located at the juncture of sub-domains IA and IIA.

Our original study on the HSA-C14:0 complex positively identified five binding sites for the fatty acid. These consist of a single site in sub-domain IB, one at the interface between sub-domains IA and IIA, two sites in IIIA (Sudlow's drug-binding site II) and a fifth in IIIB (Figure 1). A sixth C14:0-binding site was suggested, on the basis of fragmentary electron density, to lie at the interface between sub-domains IIA and IIB. The present results show that all six sites identified for C14:0 are occupied by all the fatty acids in our study (C10:0, C12:0, C16:0 and C18:0) (Figure 1). In addition, a seventh site has been identified within the drug-binding cavity of sub-domain IIA (Sudlow's drug-binding site I). Moreover, for med-

ium-chain fatty acids (C10:0 to C14:0), the data indicate the presence of further sites (see below).

Common binding sites for medium and long-chain fatty acids

In the discussion of fatty acid binding sites, we have adopted and extended the site-numbering scheme that was established for HSA-C14:0 (Curry *et al.*, 1998).

Site 1

This site lies in a D-shaped cavity in the centre of the four-helix bundle of sub-domain IB (Figure 2). In comparison to the other major fatty acid binding pockets on HSA, this site is relatively open and accessible to solvent. There is clear electron density for all the medium-chain fatty acids (C10:0–C14:0) in their respective complexes but the density becomes progressively weaker and more broken for longer fatty acids. In the HSA-C18:0 complex, the density for the bound fatty acid is especially weak at this site and the side-chain of Tyr138, which lines the binding cavity and contacts the ligand, also appears disordered. In the absence of fatty acid, Tyr138 stacks with Tyr161 and occludes the binding pocket but upon fatty acid binding at this site, the tyrosine side-chains rotate through about 90° so that their phenyl rings hold the lipid in a hydrophobic clamp (Curry *et al.*, 1999) (Figure 2). The disordering of Tyr138 in the HSA-C18:0 structure is an indication that this site

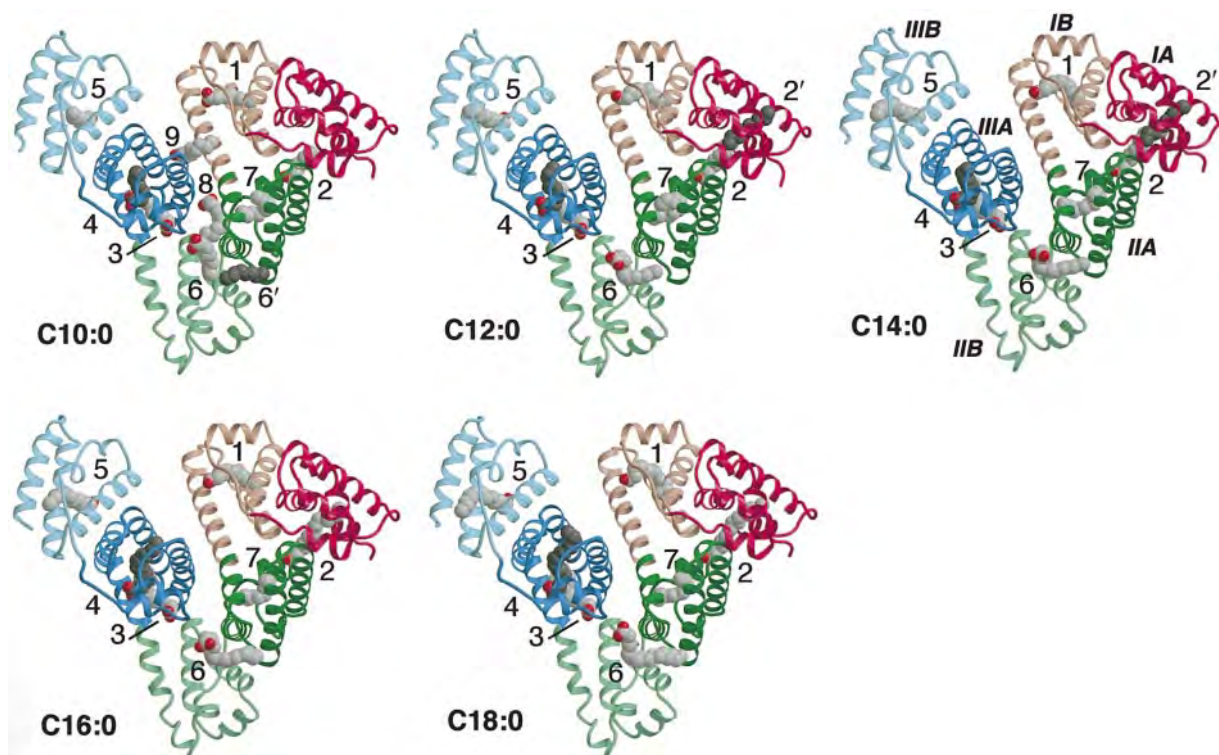


Figure 1. Structures of HSA complexed with five different fatty acids. The protein secondary structure is shown schematically and the domains are colour-coded as follows: I, red; II, green, III, blue. The A and B sub-domains are depicted in dark and light shades, respectively. This colour scheme is maintained throughout. Bound fatty acids are shown in a space-filling representation and coloured by atom type (carbon, grey; oxygen, red). Where two fatty acid molecules bind in close proximity, one of them is shown in a darker shade of grey. All Figures were prepared using Bobscrip (Esnouf, 1997) and Raster3D (Merrit & Bacon, 1997).

is not well occupied. The observation that the weakening of the ligand density at site 1 is progressive with increasing chain length for saturated fatty acids suggests that the presumed increase in binding affinity at this site as the ligand becomes longer and more hydrophobic (Ashbrook *et al.*, 1975) does not fully compensate for their lower aqueous solubilities. Although C18:0 may bind to this site at least as tightly as C16:0, its solubility may be too low to allow full occupancy; such “cut-off” effects have been observed for the protein binding of other series of aliphatic compounds (Franks & Lieb, 1985; Curry *et al.*, 1990).

All the fatty acids bind at site 1 in the same orientation with the carboxylate group hydrogen-bonded to Arg117 and to a water molecule that is also coordinated by the side-chain hydroxyl group of Tyr161 and the carbonyl oxygen atom of Leu182. For the longer-chain saturated fatty acids, the tail curls around the inside surface of the cavity so that the tip of the hydrophobic tail gradually approaches His146 at the lower end of the cavity opening (Figure 2). This suggests an explanation for the finding that C16:0 and C18:0 but not C8:0 can block the covalent modification of the equivalent His in bovine serum albumin (BSA) by *N*-dansylaziridine (Brown & Shockley, 1982).

Site 2

Located between sub-domains IA and IIA, site 2 is one of the most enclosed fatty acid binding sites on HSA, since even the carboxylate moiety of the ligand is largely shielded from solvent (Figure 3(a)). Binding of fatty acids at this site is implicated in driving the conformational change observed with the uptake of ligand because this site is dislocated into two half-sites in defatted HSA (Curry *et al.*, 1998, 1999); the formation of a contiguous pocket to accommodate the fatty acid requires rotation of domain I relative to domain II and appears to be stabilized by ligand binding. In all cases, the carboxylate head-groups of fatty acids bound to site 2 are anchored in sub-domain IIA by hydrogen bond interactions to the side-chains of Tyr150, Arg257 and Ser287 (Figure 3(a)). The methylene tails extend linearly within the narrow hydrophobic cavity formed by the realignment of IA and IIA; the longest fatty acid tails extend furthest into this cavity. There is good electron density for all the fatty acids (C10:0-C18:0) at this site, indicating that even fatty acids with just ten carbon atoms in the methylene tail are long enough to pin the two half-sites together as a binding pocket. Curiously, we have been unable to co-crystallise HSA with C8:0 in a form that is isomorphous with crystals of the other HSA-fatty acid complexes. Simple modelling

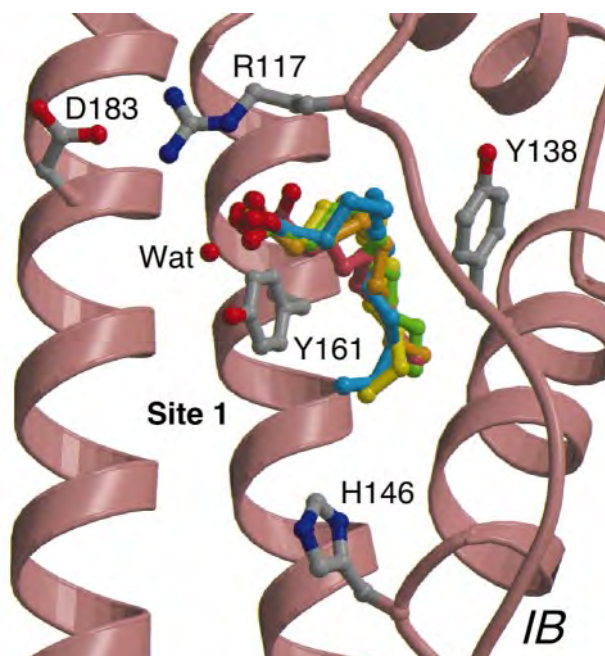


Figure 2. Fatty acid binding to site 1 in sub-domain IB. The protein is coloured as in Figure 1. The fatty acids are coloured using a rainbow scheme: C10:0, red; C12:0, orange; C14:0, yellow; C16:0, green; C18:0, blue (this colour scheme is maintained in subsequent Figures). Selected amino acid side-chains and a bound water molecule are shown coloured by atom type.

experiments suggest that this may be because C8:0 is too short for its methylene tail to make the stabilising contacts with sub-domain IA that are required for the conformational change.

In the case of C12:0, the electron density map showed clear evidence for the presence of a second fatty acid molecule binding in the upper part of the pocket within IA (designated 2'), its tail over-

lapping in an anti-parallel fashion with the first lipid molecule in site 2 (Figure 3(b)). Examination of a map for the HSA-C14:0 complex calculated from a new high-resolution data set (resolution 2.5 Å, Table 2) confirmed that a second C14:0 molecule was binding at this site, in a configuration essentially identical with that observed for the shorter C12:0 fatty acid. The carboxylate head group for the second molecule, which is not visible in either the HSA-C12:0 or HSA-C14:0 electron density maps, presumably extends into solvent from the upper surface of sub-domain IA and is disordered. In the HSA-C10:0 map, there is only very weak, broken density to suggest the presence of a second fatty acid molecule in site 2. The binding of a second molecule of C10:0 may be much weaker because the methylene tails are too short to allow the overlapping hydrophobic contacts observed for C12:0 and C14:0. For fatty acids longer than C14:0, there is no density at all for a second molecule, perhaps because binding of the first long-chain fatty acid at this site leaves too little room for a second molecule to bind with appreciable affinity.

Sites 3 and 4

Sub-domain IIIA was previously observed to contain two molecules of C14:0, bound at sites 3 and 4 (Curry *et al.*, 1998). The molecules bound approximately at right-angles to one another with their methylene tails in contact (Figures 1 and 4(a)). All the fatty acids included in this study exhibited the same pattern of binding within sub-domain IIIA, sites 3 and 4 both being occupied.

Within site 3, the head-group is fixed in the same position for all fatty acids, by hydrogen bonding to Ser342 and Arg348 from IIB, and Arg485 from IIIA. As the fatty acid tail length increases, the tail is forced into a U-bend configuration, since the longest dimension of the pocket is able to accommodate only about 12-14 methylene

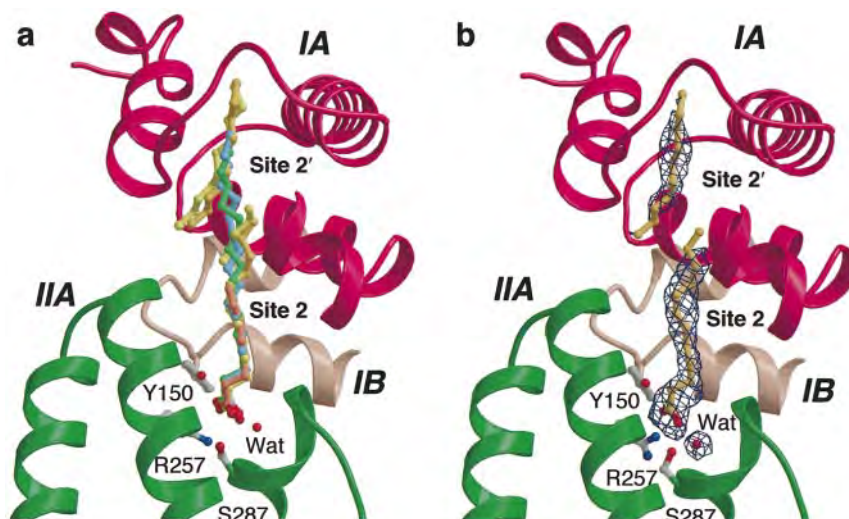


Figure 3. Fatty acid binding to site 2 in sub-domains IA and IIA. (a) Superposition of medium-chain and long-chain fatty acids. (b) Simulated annealing $F_o - F_c$ omit map contoured at 2.75σ , showing difference electron density for C12:0 in site 2. A second molecule is bound in the upper part of the site, designated 2'.

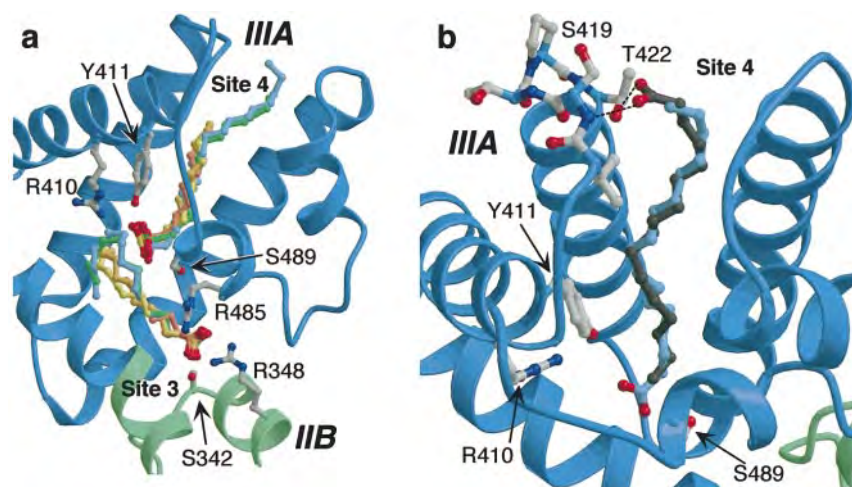


Figure 4. Fatty acid binding to sites 3 and 4 in sub-domains IIIA. (a) Superposition of medium-chain and long-chain fatty acids. (b) Model of C18:0 bound in two configurations in site 4. The configuration that is inverted relative to that observed for shorter fatty acids is depicted in with dark-grey carbon atoms. This orientation of the ligand allows hydrogen bonding of the carboxylate group to the amide nitrogen atom of S419 and the side-chain hydroxyl group of T422.

groups. The breadth of the pocket allows variation in the positions of the hydrophobic tails (Figure 4(a)).

In site 4, which is longer and narrower than site 3, the conformations of the bound fatty acids are similar to one another because of the constriction of the hydrophobic tunnel that accommodates the fatty acid methylene tails at this site. The carboxylate head-groups all lie in more or less the same positions and are hydrogen-bonded by Arg410, Tyr411 and Ser489, which lie on the exterior surface on one side of sub-domain IIIA. As the chain length of the bound fatty acid increases, the carboxylate head-group remains fixed and the methylene tail protrudes further into the hydrophobic tunnel that runs through sub-domain IIIA. The longer fatty acids bind in an extended configuration that follows the shape of the tunnel. For C18:0 fatty acid, the methylene tail traverses the entire width of the sub-domain, the methyl tips emerging into solvent from the opposite surface. The electron density for this tip is not fully accounted for by the methyl group, suggesting that C18 fatty acids may also bind to site 4 in an inverted configuration. A second configuration for the C18:0 in site 4 has been incorporated into the model, which places the fatty acid carboxyl group within hydrogen bonding distance of the amide nitrogen atom of Ser419 and the side-chain hydroxyl group of Thr422 (Figure 4(b)). Intriguingly, the possibility that C18 fatty acids may bind in two opposing orientations in site 4 is supported by the finding that octadecanedioic acid, which has a carboxylate group at either end, binds to sub-domain IIIA of BSA (Tonsgard & Meredith, 1991). Our results suggest that only fatty acid species with at least 18 carbon atoms may span the distance between the polar ends of the pocket.

Site 5

This site is formed by a hydrophobic channel that spans the width of sub-domain IIIB. In all

cases, a single fatty acid molecule binds in an extended linear conformation (Figure 5). The carboxylate head-group of the bound fatty acid invariably interacts with the side-chain of Lys525, aided in most cases by Tyr401, while the methylene tail extends into the tunnel. As with site 4, the largest fatty acid used here (C18:0) is long enough to fill the channel completely; indeed, the tip of the tail of this fatty acid projects through the far side of the channel and is exposed to solvent. However, unlike site 4, there is no indication of inversion of the fatty acid ligand. Superposition of the HSA-fatty acid complex structures for different fatty acids suggests that the carboxylate head-groups are not as uniformly anchored as for other sites (especially sites 2-4); in part, this may be due to the inherent flexibility of Lys525.

Site 6

On the basis of fragmentary electron density, our original analysis of HSA-C14:0 complexes tentatively identified a sixth fatty acid binding site in a

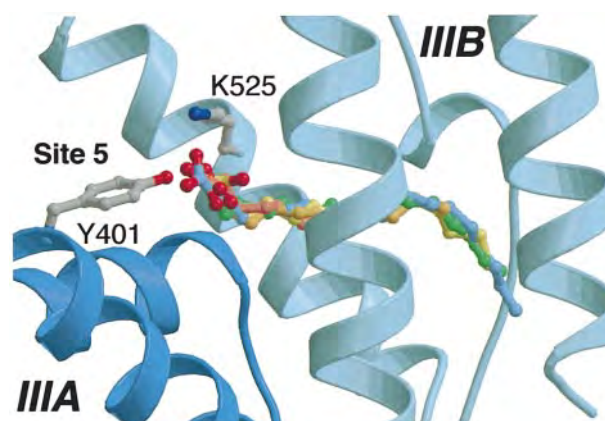


Figure 5. Superposition of fatty acids bound to site 5 in sub-domains IIIB. Amino acid side-chains for Y401 and K525 are shown coloured by atom type.

shallow trench on the surface of the protein at the interface between sub-domains IIA and IIB (Curry *et al.*, 1998, 1999). The results of the present study with fatty acids of varying chain lengths indicates that this site is occupied by both medium and long-chain fatty acids. The site differs most significantly from sites 1-5 in that there does not appear to be a cluster of well-placed amino acid side-chains that might co-ordinate the binding of the fatty acid carboxylate moiety (Figure 6). The ligand density is generally stronger on the IIB side of the binding site and, in some cases, shows the broadening associated with a carboxylate group, while on the IIA side there is no indication in any map of such broadening. The fatty acids modelled at this site have thus been built with their carboxylate groups interacting with IIB. This is consistent with evidence that C16:0 activated with Woodward's reagent K can interact with Lys349 in sub-domain IIIB of BSA, which corresponds to Lys351 in HSA (Reed, 1986). Nonetheless, it should be stressed that the carboxylate groups of the different fatty acids adopt a variety of positions and there is no conserved electrostatic interaction. Side-chains that may be involved, at least transiently, include Arg209, Lys351 and Ser480. In contrast, the middle portion of the methylene tail appears to be well anchored, probably because salt-bridges from Arg209 to both Asp324 and Glu354 forms a side-chain "strap" that helps to hold it in position (Figure 6).

The binding of C10:0 differs from that of the other fatty acids in that two molecules are observed to bind in site 6 in what appears to be a linear tail-to-tail configuration. The first C10:0 molecule binds in a conformation similar to that of the

other fatty acids, with its carboxylate head-group towards IIB; the second binds in the opposite orientation in the portion of the pocket (designated 6') that is occupied by the methylene tails of the longer fatty acids. This arrangement places the tips of the methylene tails of the two C10:0 molecules in the centre of the site where the middle portions of the tails of the longer fatty acids are well anchored. There is no electron density for the carboxylate moiety for the second C10:0 molecule bound in site 6, and the head-group has been omitted from the model. Additional unexplained density is associated with the side-chain of Lys212 nearby, a feature not observed for complexes with long-chain fatty acids; this suggests a possible interaction between this residue and the ligand carboxylate group but there is a break between the density adjacent to Lys212 and that for the methylene tail for the bound C10:0 and at present it is not clear how the two might convincingly be united.

The general absence of clear ligands for carboxylate groups of the fatty acids at this rather open site suggests that the binding affinity may be relatively low. Consistent with this notion, in crystals of HSA-C14:0 soaked with the general anaesthetic halothane, the fatty acid in site 6 was displaced by the drug, whereas sites 1-5 retained their bound lipids (Bhattacharya *et al.*, 2000).

Site 7

A seventh binding site was observed for all fatty acids within the hydrophobic cavity of sub-domain IIA (Figure 7). Although the position of the site is analogous to the binding cavity in IIIA (which contains sites 3 and 4), the site itself is considerably smaller. Previously we did not report C14:0 bind-

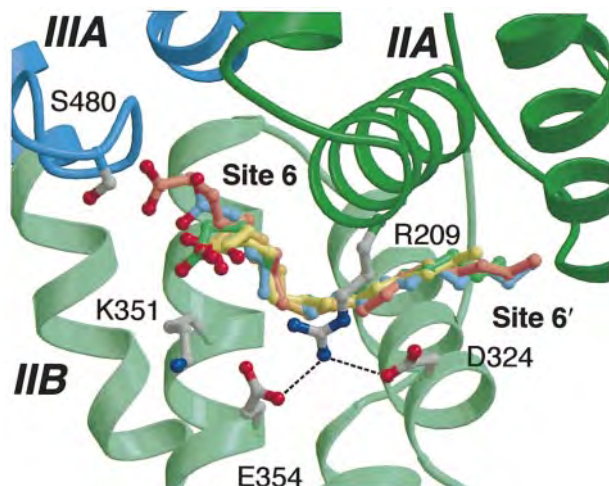


Figure 6. Superposition of fatty acids bound to site 6 in domain II. Observe the variability in the positions of the carboxylate head-groups of the fatty acids. The salt-bridges from R209 to D324 and E354 are shown as dotted lines. The portion of the pocket that binds a second molecule of C10:0 (shown with carbon atoms coloured red) is labelled Site 6'.

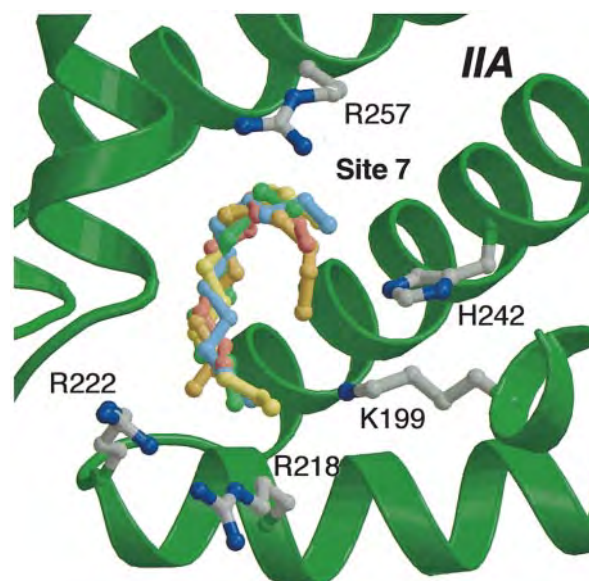


Figure 7. Superposition of fatty acids bound to site 7 in sub-domain IIA. The carboxylate groups of the fatty acids were not modelled due to lack of electron density.

ing within IIA, since the original map for this ligand contained only a very short crescent of density (Curry *et al.*, 1998). However, density of a very similar shape was observed for all other fatty acids in the same position and was not observed in maps of defatted HSA (data not shown). In all cases, it is truncated and lacking the characteristic broadening at one end that was used elsewhere to locate the carboxylate head-group of the ligand. Accordingly, the bound fatty acids have been modelled conservatively with just their methylene tails. Although we cannot be definitive about side-chain interactions with the carboxylate head-group, a number of basic residues, including Lys199, Arg218, Arg222, His242 and Arg257 (which makes a salt-bridge to the carboxylate group of the fatty acid bound in site 2), are close enough to be involved. The fatty acids bind in a curved configuration, the tail being co-planar with aromatic drugs that bind at this site (Curry *et al.*, 1998). Previous data show that C14:0 may be displaced from site 7 in the presence of 0.5 mM tri-iodobenzoic acid and suggest that the site has relatively low affinity for fatty acids (Curry *et al.*, 1998), which is consistent with the lack of a fixed carboxylate interaction. However, other evidence (see below) suggests that site 7 in sub-domain IIA may be a primary binding site for shorter-chain fatty acids.

Additional binding sites for medium-chain fatty acids

The shortest fatty acid used in this study, C10:0, binds at two additional sites on the protein that were not initially observed for any of the longer fatty acids (sites 8 and 9). Both sites occur within the large crevice between domains I and III.

Sites 8 and 9

A single molecule of C10:0 is observed to bind close to the base of the inter-domain crevice and is actually tucked into the juncture between domains II and III (site 8; Figures 1 and 8(a)). The methylene tail of the C10:0 molecule bound to site 6 helps to form the hydrophobic end of the site 8 pocket, indicating a possible cooperative interaction. At the other end of the cavity, there is an opening ringed by polar residues (Lys195, Lys199, Arg218, Asp451 and Ser454). However, the carboxylate group of the bound fatty acid does not appear to have a basic amino acid ligand, though there is a hydrogen bond to the side-chain of Ser454.

Located further up the inter-domain crevice, site 9 provides a rather open binding environment so that only one flank of the bound C10:0, which faces towards the bottom of the crevice, is in full contact with the protein (Figure 8(b)). On the top flank of the ligand (towards the solvent) there are contacts from the side-chain of Lys190 and from a bridging interaction between Glu187 of domain I and Lys432 of domain III, which is similar to the side-chain strap that occurs in site 6 (see above).

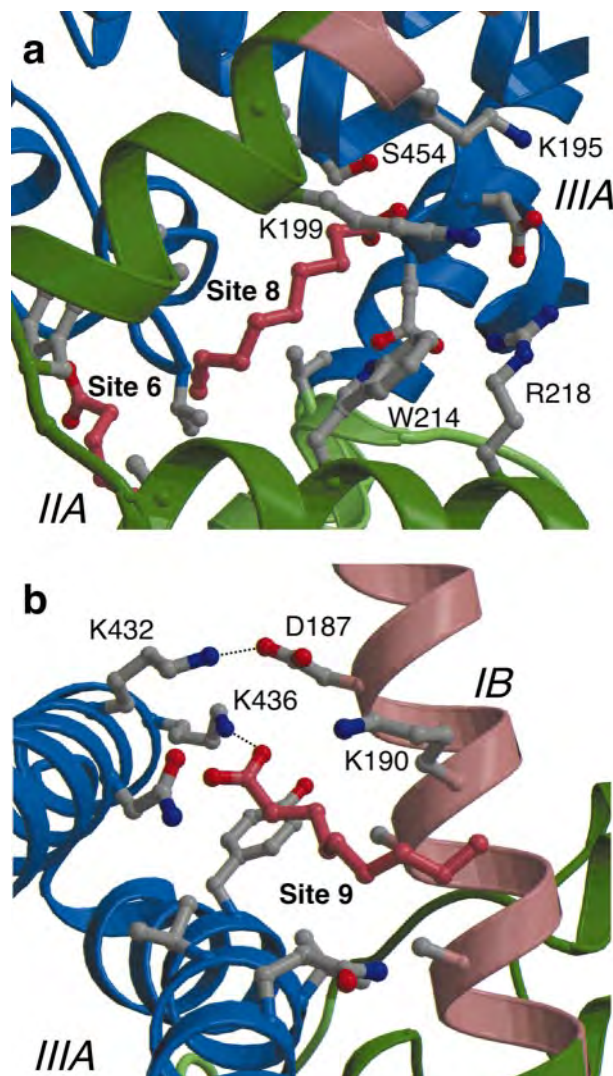


Figure 8. Additional binding sites for C10:0 fatty acid. (a) Site 8, which occurs at the base of the crevice between sub-domains IA-IB-IIA on one side and IIB-IIIA-IIIB on the other (see Figure 1). (b) Site 9, which lies in an upper region of the crevice. A salt-bridge between D187 and K432 across the top of the crevice helps to hold the ligand in place.

This interaction is not observed in any other complex because the residues involved are too far apart; it occurs in HSA-C10:0 because of small but significant movements of domains I and III, which cause a 1-2 Å narrowing of the crevice between them. In the configuration modelled for the bound fatty acid, the carboxylate head-group of the C10:0 is salt-bridged to the side-chain of Lys436; however, both ends of the binding pocket have distributions of polar side-chains and the shape of the electron density does not exclude the possibility that the orientation of the fatty acid is the opposite of that shown in Figure 8(b).

Discussion

HSA serves to greatly amplify the capacity of plasma for transporting fatty acids. However, because the protein binds a wide variety of drugs, it can have a serious impact on drug pharmacokinetics. While previous studies have established that fatty acids and drugs may interact both cooperatively and competitively, the structural basis for these effects is not well understood. Here, we have focused on the interactions of the protein with its primary class of ligand and examined how HSA binds a variety of fatty acid molecules. The study includes one of the most physiologically important fatty acid ligands known to bind HSA *in vivo*: palmitic acid (C16:0) (Saifer & Goldman, 1961). The structures of the five HSA-fatty acid complexes permit an extensive and highly detailed survey of the interactions of medium and long-chain fatty acids with the protein. The results show that there is a common set of seven binding sites for fatty acids ranging in chain length from ten to 18 carbon atoms and reveal how the variable geometry of the pockets can accommodate fatty acids of different lengths. Further, our findings have identified four additional binding locations on the protein that can be accessed by medium chain-length fatty acids (C10:0-C14:0).

The binding sites are distributed across the three homologous domains of HSA, though each domain accommodates its complement of fatty acids in a different way, as noted previously (Hamilton *et al.*, 1991; Curry *et al.*, 1998) (Figure 1). Although there are clear variations in the modes of binding of fatty acids at different sites, some common themes emerge. For sites 1-5, the carboxylate moieties of the fatty acids are anchored by the same electrostatic interactions to basic and polar side-chains, irrespective of chain length (Figures 2-7). At these sites, the methylene tails are accommodated within hydrophobic cavities and extend further into these pockets as the chain length increases. This mode of binding occurs whether the pocket is short and wide (sites 1 and 3) or long and narrow (sites 2, 4 and 5); it allows both electrostatic and hydrophobic interactions to contribute to the binding energy and readily accounts for the progressive increase in binding affinity as the methylene tail lengthens (Reynolds *et al.*, 1968; Ashbrook *et al.*, 1972, 1975). A similarly bipartite interaction has been observed for other types of lipid-binding proteins (Lalonde *et al.*, 1994; Young *et al.*, 1994; Wu *et al.*, 1999).

In contrast, at site 6 in domain II of HSA, there is no clear indication of a basic or polar side-chain that anchors the carboxylate group of the bound fatty acid. At site 7, the fatty acid binds to the cavity within sub-domain IIA and, although there are several basic residues in the vicinity, the crystallographic data do not indicate a fixed interaction with the ligand carboxylate group. Drug-binding experiments indicate that it is possible to displace the fatty acid C14:0 from both sites 6 and 7 (Bhattacharya *et al.*, 2000), suggesting that they

may be of lower affinity, though other data indicate that site 7 in sub-domain IIA may be a primary site for shorter-chain fatty acids (see below). Our structural observations are consistent with NMR data, which showed that binding sites with no significant electrostatic interaction between the protein and fatty acid are low-affinity sites and are occupied only at high molar ratios of fatty acid to protein (Cistola *et al.*, 1987a,b). The additional sites that were identified for medium-chain fatty acids probably fall into this low-affinity category. These include the upper portion of site 2 in sub-domain IA (designated site 2'), which is occupied by a second ligand molecule in the HSA-C12:0 and HSA-C14:0 structures, the binding location of the second molecule of C10:0 in site 6', and sites 8 and 9, which show significant ligand density only for C10:0. It is notable that these additional sites were observed just for medium-chain fatty acids. They are unlikely to be physiologically relevant and were probably observed to be occupied in the crystal complexes only because of the relatively high concentrations of medium-chain fatty acids used in co-crystallisation.

Numerous reports provide evidence to suggest that the primary binding sites for medium and long-chain fatty acids may be distinct (Ashbrook *et al.*, 1972; Reed, 1977; Soltys & Hsia, 1978; Koh & Means, 1979; Brown & Shockley, 1982; Vorum & Honoré, 1996). Covalent modification by *N*-dansylaziridine of Lys220 in sub-domain IIA of BSA (which maps to Arg222 in HSA) is blocked at low fatty acid concentrations by C8:0 but not by the long-chain fatty acids C16:0, C18:0 or C18:1 (Brown & Shockley, 1982), indicating that IIA (site 7) is a primary site for short-chain but not long-chain fatty acids. That IIA is not a primary site for long-chain fatty acids is suggested also by findings that, at low concentrations, C16:0 actually enhances the binding of warfarin, a drug known to bind to sub-domain IIA (He & Carter, 1992); only at high molar ratios does the fatty acid start to displace the drug (Vorum & Honoré, 1996). Similarly, low molar ratios of long-chain fatty acids do not prevent the binding of bilirubin, another substance known to bind in IIA (Reed, 1977). Elsewhere on the protein, Koh and Means (1979) showed that the esterase activity associated with Tyr411 in sub-domain IIIA was effectively inhibited by medium-chain fatty acids up to C10:0; the relatively poor inhibition of this activity by longer-chain fatty acids was explained by reasoning that they bind primarily to other sites on the protein (Koh & Means, 1979). Collectively, these findings suggest that medium-chain fatty acids (at least up to C10:0) bind preferentially to sub-domains IIA and IIIA; that is, to the primary drug-binding sites. Our structural analyses of the different HSA-fatty acid complexes reveal seven sites that can bind both medium-chain and long-chain fatty acids in broadly similar configurations. At face value, this would appear to conflict with the biochemical data but, since the complexes for crystallisation were

invariably prepared using high molar ratios of fatty acid to protein, it is likely that both high and low-affinity sites are occupied in the conditions used for X-ray data collection.

Where are the highest-affinity long-chain fatty acid binding sites? Their locations have not been definitively established, though accumulated evidence suggests that there is at least one in domain I and one in domain III (Brown & Shockley, 1982; Shaklai *et al.*, 1984; Reed, 1986; Hamilton *et al.*, 1991; Kjelsden *et al.*, 1998). Titration studies in which fatty acid binding to BSA was examined using NMR have shown that the highest-affinity long-chain fatty acid binding sites are those that can make electrostatic interactions with the ligand carboxylate group (Cistola *et al.*, 1987a,b). From our crystallographic results, it is probable that the high-affinity pockets belong to the set of five sites (1-5) that have side-chains interacting with the carboxylate head-group of the bound ligand. Strikingly, these sites are found within domains I and III of the crystal structures, though there are contributions from domain II to sites 2 and 3. It is not yet possible to distinguish rigorously between these five primary sites because of the difficulty in matching biochemical information precisely to the more recently acquired crystallographic data, though some of the most convincing biochemical evidence indicates that site 5 in sub-domain IIIB may be the highest-affinity site (Reed *et al.*, 1975; Hamilton *et al.*, 1991), as we have discussed elsewhere (Curry *et al.*, 1999). It seems likely that site 2, which straddles sub-domains IA and IIA, may correspond to the high-affinity site assigned to domain I, since it provides a much more enclosed binding environment than the site 1 pocket in sub-domain IB. We attempted to identify the high-affinity binding sites experimentally by gradually reducing the concentration of free fatty acid in the crystal harvest solution, assuming that the weakest binding sites would be the first to release their ligands. However, an electron density map calculated using data collected from a crystal soaked in 5 μ M C14:0, the minimum concentration that did not induce crystal damage, showed no significant change in fatty acid occupancy at any of the binding sites (data not shown). A complete and reliable assignment of the highest-affinity fatty acid binding sites on HSA will require further work.

Our results indicate that the primary drug-binding sites on HSA (Sudlow's sites I and II) can both be occupied by fatty acids, though they may well be differentially affected by the presence of fatty acids. While drug site I (sub-domain IIA) appears to be a primary site for medium-chain fatty acids (see above), drug site II (sub-domain IIIA) is likely to have a high affinity for long-chain fatty acids. This follows from the observation that fatty acid binding sites 3 and 4 in sub-domain IIIA, unlike site 7 in sub-domain IIA, provide side-chain ligands for the fatty acid carboxylate group. Consistent with this view, in drug soaking experiments with crystals of HSA-C14:0 we have been able to

displace bound C14:0 from drug site I (IIA) but not from site II (IIIA) (Curry *et al.*, 1998; Bhattacharya *et al.*, 2000). Since long-chain fatty acids ($\geq$ C16) are normally most prevalent in the circulation, the binding of site II drugs may be more susceptible to variations in the plasma levels of fatty acid or HSA.

In summary, the structural data accord well with a large body of biochemical data on fatty acid binding to albumin and establish a reliable map of fatty acid binding locations. This structural information should facilitate more direct approaches, such as mutagenesis and high-resolution NMR, to questions of relative binding affinities of fatty acids. Moreover, detailed information on fatty acid binding will guide structural and biochemical investigation of the interactions of the panoply of other compounds, both endogenous and exogenous, which also bind to this versatile protein.

Materials and Methods

Protein purification, complex formation and crystallisation

Purified recombinant HSA, produced in yeast and supplied by Delta Biotechnology Ltd. (Nottingham, UK), was purified further by gel-filtration as described (Curry *et al.*, 1998). Fatty acids were purchased as the highest purity preparations available from Sigma-Aldrich Ltd. Details of the conditions used to prepare complexes of HSA with each of the fatty acids used in this study are summarised in Table 1. The protocol was based on the method developed previously for the formation of HSA-C14:0 complexes (Curry *et al.*, 1998). In brief, to prepare HSA-fatty acid complexes, the fatty acids were added to 20 mM potassium phosphate (pH 7.5), to achieve nominal concentrations in the range 0.8 to 10 mM (see Table 1). These solutions were heated to 50 °C to facilitate dispersal of the fatty acid, allowed to cool briefly and then mixed with solutions containing 100 mg/ml HSA in the same buffer to achieve fatty acid to protein molar ratios of between 12 and 40. After incubation for an hour at room temperature, excess undissolved fatty acid was removed by centrifugation. The HSA-fatty acid complex was then washed in a solution containing a known concentration of free fatty acid by repeated cycles of dilution and concentration using a 10,000 Da molecular mass cut-off centrifugal concentrator (Vivaspin, Millipore).

HSA-fatty acid complexes were typically concentrated to 100 mg/ml for use in crystallisation trials. Crystallisation was achieved by sitting-drop vapor diffusion using conditions essentially identical with those described (Carter *et al.*, 1994; Curry *et al.*, 1998). Variations in the polyethylene glycol (PEG) 3350 concentrations used in each case are summarised in Table 1. As before, the largest crystals were obtained by streak seeding into drops that had been allowed to equilibrate for five to seven days. Crystals nevertheless tended to grow in clusters; single crystals were detached for X-ray diffraction analysis using a cat-whisker tool. These were harvested into solutions containing slightly higher PEG 3350 concentrations than were used for crystallisation. The washing concentration of fatty acid was maintained in the harvest buffers.

Data collection and structure determination

X-ray diffraction data were collected at room temperature using CuK α and synchrotron radiation sources (Table 2). The data were indexed and measured with MOSFLM (A. Leslie, personal communication). In all cases, the HSA-fatty acid complex crystallized essentially isomorphously with HSA-C14:0 (Curry *et al.*, 1998). The protein model for this structure, stripped of its myristate, was used as a starting model for phasing of the X-ray data. The model, split into its six sub-domains, was first refined as a rigid body using X-PLOR (version 3.85) (Brünger *et al.*, 1987) and then subjected to cycles of positional and *B*-factor refinement before calculation of initial $F_o - F_c$ and $3F_o - 2F_c$ maps. These maps were used to guide positioning of the fatty acid ligands and bound water molecules and to make manual adjustments to the protein prior to further cycles of refinement. Details of the refinement statistics are summarised in Table 2. Co-ordinates have been deposited with the RCSB Protein Data Bank (ID codes are given in Table 2).

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