Crystal structure analysis of warfarin binding to human serum albumin: anatomy of drug site I

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Running title: Crystal structure of HSA-myristate-warfarin

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Summary

Human serum albumin (HSA) is an abundant transport protein found in plasma that binds a wide variety of drugs in two primary binding sites (I and II) and can have a significant impact on their pharmacokinetics. We have determined the crystal structures at 2.5 Å resolution of HSA-myristate complexed with the R–(+) and S–(-) enantiomers of warfarin, a widely used anticoagulant that binds to the protein with high affinity. The structures confirm that warfarin binds to drug site I (in subdomain IIA) in the presence of fatty acids and reveal the molecular details of the protein-drug interaction. The two enantiomers of warfarin adopt very similar conformations when bound to the protein and make many of the same specific contacts with amino-acid side chains at the binding site, thus accounting for the relative lack of stereospecificity of the HSA-warfarin interaction. The conformation of the warfarin binding pocket is significantly altered upon binding of fatty acids and this can explain the observed enhancement of warfarin binding to HSA at low levels of fatty acid.

Introduction

Human serum albumin (HSA) is the major protein component of blood plasma but is also distributed to the interstitial fluid of the body tissues. The protein binds a number of relatively insoluble endogenous compounds such as unesterified fatty acids, bilirubin and bile acids and thus facilitates their transport throughout the circulation (1,2). HSA is also capable of binding a wide variety of drugs (1-3) and much of the interest in this abundant protein derives from its effects on drug delivery. Drug binding to plasma proteins such as HSA can be an
important determinant of pharmacokinetics, restricting the unbound concentration and affecting distribution and elimination. In some cases the major fraction of the administered drug is sequestered by HSA; this is particularly true of warfarin, a widely-used anticoagulant, which is 99% bound to the protein under normal therapeutic conditions and consequently has a small volume of distribution and low clearance (4).

HSA has a limited number of binding sites for endogenous and exogenous ligands so that drug binding to the protein may be affected by a variety of factors. Although the effects on pharmacokinetics of drug-drug competition for the same sites on HSA are generally held to be of little clinical import (4,5), physiological or diseased states which cause variations in the plasma levels of albumin or its primary endogenous ligands can influence drug binding and may require dosages to be closely monitored. Added to this, genetic polymorphisms in HSA can also alter drug binding and may further complicate the clinical picture (6,7). In order to understand the molecular basis of these effects, structural information is required to fully delineate the binding sites for drugs and endogenous ligands. Such information will also be invaluable to efforts to exploit the carrier properties of HSA in the development of novel therapeutic reagents for drug targeting (8) or oxygen transport (9).

HSA, a 585-residue protein is monomeric but contains three structurally similar α-helical domains (I-III); each domain can be divided into sub-domains A and B which contain six and four α-helices respectively (3,10). Structural studies have mapped the locations of the fatty acid binding sites (11,12) and the primary drug binding sites on the protein (10). The fatty acid binding sites are distributed throughout the protein and involve all six sub-domains; by contrast many drugs
bind to one of the two primary binding sites on the protein, known as Sudlow’s sites I and II (13). Although examples of drugs binding elsewhere on the protein have been documented (13-15), most work has focused on the primary drug sites. These investigations have largely employed competition binding methods to investigate the selectivity of the primary drug binding sites. Drug site I, where warfarin binds, has been characterised as a conformationally adaptable region with up to three sub-compartments (16-18).

Crystallographic studies located drug sites I and II in subdomains IIA and IIIA respectively and provided the first detailed description of the geometry and chemistry of the binding environment (10). Several reports have analysed structural aspects of the binding of an aspirin analogue, 2,3,5-tri-iodobenzoic acid (TIB) to defatted HSA (10), to the HSA-myristate complex (11) or to equine serum albumin (19). However these studies were done at fairly modest resolutions (3.0-4.0 Å) and in any case it is not clear whether TIB, which carries three iodine atoms, is properly representative of typical HSA drug ligands.

The binding of warfarin to HSA, and its interactions with other HSA ligands have been investigated for many years. Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] binds to drug site I with high affinity \( (K_d \approx 3 \mu M) \) (13,20,21). A crystallographic study in 1992 confirmed subdomain IIA as the binding locus for single molecule of warfarin but was unable to give specific details on the binding interactions because of the limited resolution of the data (10). Warfarin shares this binding site with a range of other drugs (including phenylbutazone, tolbutamide, indomethacin) and thus competes with them for binding to HSA (13,16,17). Other studies have shown that low levels of fatty acids (22,23) or
elevated pH (24,25) may enhance the affinity of HSA for warfarin by up to three-fold but the molecular mechanisms of these effects are not well understood.

To elucidate the molecular details of the interaction of warfarin with HSA, we report here the determination of the crystal structures of HSA-myristate complexed with both the R–(+) and S–(-) enantiomers of the drug.

**Experimental procedures**

**Purification, crystallisation and drug soaking.** Purified recombinant HSA, produced in yeast and supplied by Delta Biotechnology Ltd. (Nottingham, UK), was purified, complexed with excess myristic acid and crystallised as described previously (11,12). Racemic warfarin, purchased from Sigma-Aldrich (A-2250) was separated into R–(+) and S–(-) enantiomers using established protocols (26). Crystals of HSA-myristate were harvested into solutions containing 37% (w/v) polyethylene glycol 3350, 50 mM potassium phosphate. Warfarin was dissolved at 20 mM in harvest buffer at pH 12.0 with continuous stirring for 24 hours; the pH was then restored to 7.0 with concentrated HCl. Crystals were soaked first in harvest buffer containing 10 mM of the R–(+) or S–(-) warfarin enantiomer for 15 hours and then in buffer containing 20 mM of the same enantiomer for at least 24 hours.

**Data collection and structure determination.** X-ray diffraction data were collected at room temperature at station X-11 of the EMBL Outstation at DESY, Hamburg Germany (Table 1). The data were indexed and measured with MOSFLM (A.
Leslie, personal communication). For both enantiomers, crystals of the HSA-myristate-warfarin complex were isomorphous with the HSA-myristate complex (11,12). The protein model for this structure, stripped of its ligands, was used for initial phasing of the x-ray data. The model, split into its six sub-domains, was initially refined using a rigid body protocol in CNS (27) 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 the positioning of the fatty-acid and warfarin ligands and bound waters and to make manual adjustments to the protein prior to further cycles of refinement. Details of the refinement statistics are summarised in Table 1. Co-ordinates have been deposited with the Protein Data Bank (ID codes are given in Table 1). Figures depicting the structure were prepared using BOBSCRIPT (28) and RASTER3D (29).

**Results**

**Structure determination**

HSA-myristate complexes were prepared and crystallised as described previously (11,12,30). Over a period of at least 48 hours the crystals were soaked in increasing concentrations of the warfarin R–(+) and S–(–) enantiomers (Figure 1) up to a final concentration of 20 mM (see Materials and Methods). The soaked crystals were isomorphous to crystals of HSA-myristate and the structures were solved by molecular replacement. Difference electron density maps showed clear density for a single warfarin molecule in subdomain IIA in each case (Figure 2). The
density was consistent with the open form of the warfarin molecule (31); features corresponding to the coumarin, benzyl and acetonyl moieties of the drug were evident and gave a clear indication of the overall orientation of the drug in the pocket. For both R–(+)
and S–(−) warfarin there nevertheless seemed to be a possible ambiguity in the orientation of the coumarin moiety. However, although the density appeared to allow the possibility of flipping the coumarin group by 180° about the C2-C13 bond (Figures 1 and 2), attempts to refine the drug in the alternative orientation resulted in higher B-factors for the ligand, a poorer fit to 3F_o -2F_c electron density maps and significantly more residual difference density (data not shown). The density is thus consistent with a single dominant conformation of the bound drug for each of the enantiomers. Models for R–(+)
and S–(−) warfarin bound to HSA-myristate were refined to 2.5 Å and have R_free values of 25.4 % and 24.8 % respectively and good stereochemistry (Table 1).

Structure of the complex

The overall structures of the HSA-myristate-warfarin complexes are very similar to those reported for HSA-myristate and other HSA-fatty acid complexes (11,12) — there are only minor conformational changes in a number of side chains located at the drug binding site. As expected, warfarin binds within subdomain IIA in Sudlow’s drug site I (Figure 3a) (10,13,32) and displaces the myristate molecule that binds to this domain (in fatty acid site 7, FA7) with relatively low affinity (12). In addition to the drug bound in IIA, there is a fragment of difference density adjacent to the myristate bound to subdomain IB at the same location observed for tri-iodobenzoic acid (11). The density would be large
enough to encompass the coumarin moiety of warfarin and may represent a secondary binding site for the drug but there is no density for the other portions of the drug and a second ligand molecule has therefore not been incorporated into the model.

**The warfarin binding pocket**

The binding pocket is formed by the packing of all six helices of subdomain IIA (Figure 3c). The R–(+)- and S–(–)-enantiomers bind in the pocket in almost identical conformations, both in the open configuration (31). The coumarin and benzyl moieties of the R–(+)- and S–(–)-forms are nearly perfectly superimposable, presumably because stabilisation of the open conformation allows these groups to rotate about the C2-C13 bond. The main difference in the drug conformations occurs in the acetonyl group which branches from the chiral carbon and is located at the mouth of the pocket. However, at 2.5 Å this difference is barely detectable in our electron density maps. The finding that the enantiomers bind in essentially the same way to HSA is consistent with the observation that they have similar binding affinities for the protein. At 25°C, close to the temperature of our diffraction data collection, the R–(+) and S–(–) enantiomers have dissociation constants of 3.8 and 2.9 µM respectively (21), though the difference is slightly more pronounced at lower temperatures (20,21).

Given the ability of HSA to accommodate a wide range of different drug compounds in site I, it is perhaps surprising to find that warfarin fits quite snugly into the binding pocket (Figure 3c). The binding site has two sub-chambers which accommodate different portions of the warfarin molecule. The interaction
between drug and protein appears to be dominated by hydrophobic contacts but there are also a number of specific electrostatic interactions. The benzyl moiety binds in a sub-pocket formed by Phe 211, Trp 214, Leu 219 and Leu 238 with additional aliphatic contacts from Arg 218 and His 242 (Figure 3b). Direct contact between the benzyl ring and the side chain of Trp 214 explains why modification of this residue reduces warfarin binding to HSA (33). The proximity of the benzyl ring to Arg 218 also accounts for the reduction in binding affinity associated with mutations at this position (6). The coumarin moiety fits into the main chamber furthest from the entrance which is the same portion of site I that is occupied by TIB (11). Figure 3b shows that this chamber has an additional side-pocket (delimited by Leu 219, Arg 222, Phe 223, Leu 234, Ile 264, Leu 257 and Ile 290) which is not occupied by warfarin but may accommodate hydrophobic portions of other site I drugs. The coumarin group makes primarily hydrophobic contacts with the surrounding side chains (Table 2 and Figure 3c). The roof and floor of the pocket are delimited by Ala 291 and Leu 238 respectively; the back end of the coumarin ring contacts Ile 260, Ile 264, Ile 290 and the aliphatic portions of Arg 257 and Ser 287 and lies close to but does not directly contact the fatty acid in the adjacent binding site (FA2). According to the view in Figure 3c, there are further hydrophobic contacts on the right flank from Val 241 and on the left from the aliphatic portion Arg 222. In addition, two of the three oxygen atoms contribute to electrostatic interactions. The O4 atom of the hydroxyl group makes hydrogen bonds to the side chain of His 242 (2.9 Å) and to a bound water molecule (2.8 Å). On the other side of the drug, although the O1 atom faces a gap in the wall of the binding pocket and has no specific interaction with the protein, the O2 atom is positioned 3.7 Å from Ne of Arg 222. Closer to the pocket entrance
the O3 of the acetonyl group lies 3.3 Å from NH2 of Arg 222. The combination of electrostatic interactions made by O2, O3 and O4 probably helps to fix the orientation of the bound warfarin but complementarity between the shape of the drug and the pocket is clearly also important for binding. There is no significant difference in the electrostatic contacts made by the two enantiomers.

Discussion

Drug binding to HSA has been extensively investigated over the past thirty years. Much of this work has been performed using binding and competition assays to map out the number and selectivity of binding sites on the protein (13,16-18,34), though more recently mutagenesis techniques have been applied to the study of drug interactions (6,35). While initial crystallographic studies at moderate resolution have focused mainly on the binding of TIB, a drug analogue, to HSA (10,11), there has been a lack of high resolution structural information with which to interpret the amassed biochemical and biophysical data on HSA-drug interactions.

The structures reported in this paper provide the first high-resolution view of what might reasonably be termed a “classic” site I drug bound to HSA. Although many different drugs bind to site I, they are generally bulky heterocyclic compounds with a negative charge located towards the centre of the molecule (34). Warfarin exemplifies this type of compound and the structure of the HSA-myristate-warfarin complex shows how this broad selectivity is achieved. The coumarin and benzyl moieties are accommodated in separate but adjacent chambers of what has previously been described as a “sock-shaped” pocket (10).
The polar features of the drug are all found at the basic mouth of the pocket with
the exception of the hydroxyl oxygen (O4) which interacts specifically with the
side chain of His 242 on one wall of the pocket (Figure 3c).

Both enantiomers of warfarin bind in essentially the same way to the protein.
This accounts for the poor stereoselectivity of albumin and suggest the prospects
for developing it as a reagent for chiral separations of warfarin are unfavourable.
Conversely, the structure clearly reveals the details of the HSA-warfarin
interaction and provides a structural basis for the possible design of warfarin
derivatives with altered HSA binding properties. The binding of several warfarin
derivatives to HSA has already been characterised; acenocoumarin binds more
loosely than warfarin and phenprocoumon binds more tightly (16,22,34). The
weaker association of acenocoumarin is probably because the addition of an NO2
group to the benzyl ring sterically hinders its accommodation in the hydrophobic
compartment formed primarily by Phe 211, Trp 214, Leu 219 and Leu 238. By
contrast the tighter binding of phenprocoumon, in which the CH2-CO-CH3
acetonyl group found on warfarin is replaced by a propyl moiety (C3H7), may be
due to the formation of hydrophobic contacts between this propyl group and the
side chains of Trp 214 and Arg 218 (Figure 3c).

Comparison with the structure of HSA-myristate-TIB (11) reveals differences in
the ways that TIB and warfarin bind to site I. The heterocyclic coumarin moiety
of warfarin binds in the same location as the benzyl ring of TIB. Though the
planes of the aromatic rings are inclined at about 20° to each other, they make
many of the same interactions with the walls of this part of the pocket and both
drugs can also from specific hydrogen bonds with His 242. The presence of bulky
iodine atoms projecting from the TIB benzyl group make it too wide to fit into the sub-pocket that accommodates the benzyl ring of warfarin.

Effect of fatty acids on warfarin binding

Our structural results offer a plausible explanation for the observation that binding of fatty acids to HSA can enhance the affinity of the protein for warfarin and some other site I ligands (22,23,34). The presence of up to 3 moles of long-chain fatty acids per mole of HSA can increase the affinity for warfarin by almost a factor of three (22,23). Fatty acid binding to HSA induces a substantial conformational change in the protein involving rotations of domains I and III relative to domain II (11,12,36). One of the principal driving forces behind this conformational change is binding of a fatty acid molecule to the binding site (FA2) that spans the interface between subdomains IA and IIA and lies close to the warfarin binding site. In the absence of fatty acid the side chain of Tyr 150 from the linker connecting helices h2 and h3 of subdomain IB projects into the warfarin binding site (Figure 3d). However, occupation of site FA2 by myristate displaces domain IB, rotating helices h2 and h3 and pulling the side chain of Tyr 150 out of the drug pocket to allow it to hydrogen bond with the carboxylate group of the fatty acid (FA2). In concert with the movement of Tyr 150 the side chain of Arg 257 rotates to hydrogen bond to the same fatty acid carboxylate. The changes induced by fatty acid binding — removal of a hydroxyl group from the drug pocket and re-positioning of the aliphatic portion of Arg 257 — thus render the binding environment for the coumarin moiety more hydrophobic. This appears to be the most likely explanation for the increased binding affinity of
HSA for warfarin. Curiously, the binding of phenprocoumon is only marginally (≈20%) enhanced under similar conditions (22). This suggests that minor conformational changes induced by fatty acid binding in the vicinity of the mouth of the pocket may also affect drug binding.

The enhancement of warfarin binding by fatty acids is maximal with 3-4 moles of fatty acid bound per mole of HSA (22,23). At higher fatty acid concentrations the affinity for warfarin falls off, an observation that is probably attributable to direct competition between the drug and fatty acid for binding to the pocket in IIA. Consistent with this hypothesis we have detected fatty acid binding at this site (designated FA7), in a manner that is suggestive of a low affinity interaction (primarily because of the absence of specific interactions between the protein and the fatty acid carboxylate group) (12). Thus we would only expect to find displacement of the drug by high levels of fatty acid.

**Effect of pH on warfarin binding**

Over the pH range 6-9 HSA undergoes a transition from the neutral (N) to the basic (B) form that is linked to an increase in the affinity of the protein for a number of site I ligands, including warfarin (which binds about threefold tighter at alkaline pH) (24). The precise nature of the structural changes associated with the N-B transition are incompletely characterised. The transition appears largely to involve changes in domains I and II though there is some involvement of domain III (32). It remains unclear whether there is any link between the structural changes induced by shifts in pH and those induced by fatty acid binding, though the observation that fatty acid binding lowers the pH of the mid-
point of the N-B transition suggests a possible parallel between the two effects (25). In view of the paucity of data on this point, attempts to explain the pH effect on warfarin binding to HSA from the crystal structure are problematic. Our results show that the only likely titratable group in the binding site is the side chain of His 242 which makes a hydrogen bond to the drug. However, raising the pH would de-protonate the histidine which would be expected to reduce the strength of its interaction with warfarin, contrary to the results of binding experiments. It thus appears that other effects of pH upon the protein are responsible for tighter binding at alkaline pH and further work will be required to determine what these are.

Conclusions

Recently methods to study structural aspects of drug binding using engineered fragments of HSA have being developed in an effort to make the analysis more tractable (35,37). While such approaches are likely to be very valuable, particularly since they use fragments small enough to be analysed structurally by nuclear magnetic resonance techniques, truncations of the protein may give rise to inadvertent structural perturbations of the drug binding sites. This study, and an earlier structural analysis of HSA complexed with the general anesthetics propofol and halothane (15), demonstrate the feasibility of high-resolution crystallographic studies of the binding of drugs to the intact protein and shows that the structural information obtained provides a richly detailed view of the binding site with which to interpret the biochemical and biophysical data already accumulated on drug interactions with the protein. Further work on other site I
and site II drugs will allow us to build up a much more complete picture of drug interactions with HSA and will provide a structural basis for a more rational approach to drug design either to exploit or avoid the impact of HSA on drug delivery.

Acknowledgements

We thank Delta Biotechnology Ltd for purified recombinant HSA and the staff at DESY Hamburg (Germany) for help with data collection. We are very grateful to Peter Brick for critical reading of the manuscript. AB acknowledges the award of a PhD studentship from the MRC. This work was funded by grant support from the BBSRC.

References


Table 1: Data collection and model refinement statistics

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<th>( \text{S}(-) )</th>
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\(^1\)Values for the outermost resolution shell are given in parentheses.

\(^2\)\( R_{merge} = 100 \times \sum_h \sum_j |I_{hj} - \bar{I}_h| / \sum_h \sum_j I_{hj} \) where \( \bar{I}_h \) is the weighted mean intensity of the symmetry related reflections \( I_{hj} \)

\(^3\)\( R_{model} = 100 \times \sum_{hkl} |F_{obs} - F_{calc}| / \sum_{hkl} F_{obs} \) where \( F_{obs} \) and \( F_{calc} \) are the observed and calculated structure factors respectively.

\(^4\)\( R_{free} \) is the \( R_{model} \) calculated using a randomly selected 5% sample of reflection data omitted from the refinement.
### Table 2a: Potential electrostatic interactions between warfarin and HSA

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<td>O3</td>
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<td></td>
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Table 2b: Van der Waals interactions between warfarin and HSA

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Figure Legends

Fig. 1: Schematic structures of warfarin, phenprocoumon, acenocoumarin. Selected positions in warfarin which are referred to in the text are numbered in parentheses.

Fig. 2: Experimental $F_o$-$F_c$ difference electron density map for (a) R–(+) and (b) S–(–) warfarin in subdomain IIA, where $F_o$ values are the observed HSA-myristate-warfarin amplitudes and $F_c$ are the calculated amplitudes from the protein model from the HSA-myristate structure (11); the phases were derived from the same model. Oxygen atoms in the warfarin molecule are shown in dark grey.

Fig. 3: Structural details of the interaction of the R-(+) enantiomer of warfarin with HSA-myristate. (a) Overview of the HSA-myristate-warfarin complex showing the drug binding to a single locus in subdomain IIA. 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. 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. Warfarin is depicted in the same space-filling representation with yellow carbon atoms. (b) Side-view of warfarin pocket showing a cutaway view of the surface of the binding pocket. The surface is coloured to indicate electrostatic potential with blue representing basic patches. The view is rotated by 180° about a vertical axis relative to the view shown in a.
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The figure was prepared using GRASP (38). (c) Stereo view of the warfarin binding pocket showing the amino acids lining the pocket. The view is rotated by 90° relative to the view in a. Side chain atoms are coloured by atom type (carbon - grey; nitrogen - blue; oxygen - red); water molecules bound adjacent to warfarin are depicted as cyan spheres. (d) Impact of conformational changes induced by fatty acid binding on drug site I and potential implications for warfarin binding. The structures of defatted HSA and HSA-myristate-warfarin were superposed by aligning the positions of residues belonging to domain II (197-383). The figure shows details of warfarin binding to HSA-myristate; superposed on this and shown in semi-transparent mode are helices h2 and h3 of subdomain IB.
Figure 1

Warfarin

Phenprocoumon

Acenocoumarin

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