

Crystal Structure and Biological Implications of a Bacterial Albumin Binding Module in Complex with Human Serum Albumin*

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Many bactericide species express surface proteins that interact with human serum albumin (HSA). Protein PAB from the anaerobic bacterium *Finegoldia magna* (formerly *Peptostreptococcus magnus*) represents one of these proteins. Protein PAB contains a domain of 53 amino acid residues known as the GA module. GA homologs are also found in protein G of group C and G streptococci. Here we report the crystal structure of HSA in complex with the GA module of protein PAB. The model of the complex was refined to a resolution of 2.7 Å and reveals a novel binding epitope located in domain II of the albumin molecule. The GA module is composed of a left-handed three-helix bundle, and residues from the second helix and the loops surrounding it were found to be involved in HSA binding. Furthermore, the presence of HSA-bound fatty acids seems to influence HSA-GA complex formation. *F. magna* has a much more restricted host specificity compared with C and G streptococci, which is also reflected in the binding of different animal albumins by proteins PAB and G. The structure of the HSA-GA complex offers a molecular explanation to this unusually clear example of bacterial adaptation.

Numerous Gram-positive bacterial species, including human pathogens, express surface proteins that interact with host proteins like human serum albumin (HSA)¹ and IgG with high specificity and affinity (1). Among IgG-binding bacterial proteins, protein A of *Staphylococcus aureus* (2) and protein G of group C and G streptococci (3, 4) are the most well known; both interact with the constant region (*F_c*) of IgG. Protein G also interacts with HSA, and the regions responsible for IgG and HSA binding have been found to be separately located on the molecule (5).

The anaerobic bacterium *Finegoldia magna* (formerly *Peptostreptococcus magnus*) is present in the indigenous flora of the skin, the oral cavity, and the gastrointestinal and urogenital tracts. However, these bacteria are also important human

pathogens connected with conditions such as soft tissue abscesses and deep wound infections (6). Some isolates of *F. magna* bind HSA to their surface, and the molecule responsible for this is called protein PAB (7). Protein PAB contains a domain showing high sequence homology (60%) to the albumin binding domains (ABDs) of protein G. This ABD, the GA module, was found to have been transferred from protein G into the gene-encoding protein PAB through a recombination event including a conjugational plasmid. Although the biological function(s) of the GA module is not known in detail, the acquisition of the GA module seems to add selective advantages to the bacterium in terms of growth and also increases its virulence (8, 9).

HSA is the most abundant protein in plasma, where it acts as a transporter of an exceptionally broad spectrum of compounds that are predominantly fatty acids but also amino acids, bile acids, and steroids (10). It is also capable of binding and transporting a wide range of therapeutic substances. Its binding abilities have been probed in a number of studies, and crystal structures are available for HSA in complex with fatty acids (11, 12), hemin (13, 14), and local anesthetics (15). In this paper we present the first crystal structure for a protein-protein complex of HSA, the HSA-GA complex. This complex is formed when the GA module from protein PAB binds to HSA. The data provide insights into factors influencing the affinity and specificity of the interactions between albumins from different animal species and bacterial albumin-binding proteins, with interesting evolutionary implications. The structure might also prove useful in the study of HSA in the context of structure-aided drug design.

MATERIALS AND METHODS

Purification and Complex Formation—HSA was purchased from Octapharma AG (Sweden) and purified according to the protocol of Curry *et al.* (11) to remove dimers and multimers. The protein was concentrated to 100 mg/ml with a Millipore spin filter (10,000 Da cutoff) in 50 mM potassium phosphate, pH 7.5, and 150 mM NaCl and subsequently frozen in aliquots at –85 °C. Purified and lyophilized GA module protein (residues 213–265 of protein PAB) was purified as described (16). For crystallization, a mixture of HSA and GA module (molar ratio 1:1) was incubated for at least 30 min at room temperature.

Crystallization of the HSA-GA Complex—Crystallization was achieved by vapor diffusion at 18 °C using the hanging drop method with a crystallization solution consisting of 2.2 M (NH₄)₂SO₄ and 0.1 M citrate, pH 6. The crystals belong to space group C22₁ with unit cell dimensions of *a* = 96.3 Å, *b* = 134.8 Å, *c* = 122.5 Å, and $\alpha = \beta = \gamma = 90^\circ$ and one complex per asymmetric unit. Crystals were frozen in liquid nitrogen using mineral oil as a cryoprotectant.

Crystallographic Data Collection and Structure Determination—Data statistics are summarized in Table I. The data were indexed using Denzo and XdisplayF and scaled with Scalepack (17). A molecular replacement search in MOLREP (18) using chain A of the apo form of HSA (Protein Data Bank entry 1A06) as a search model gave the solution for the albumin part of the complex with an *R*-factor of 48.9 and

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The atomic coordinates and structure factors (code 1TF0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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¹ The abbreviations used are: HSA, human serum albumin; GA, protein G-like albumin binding module; ABD, albumin binding domain.

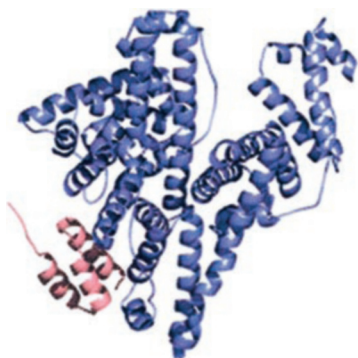


FIG. 1. **Schematic structure of the HSA-GA complex.** The GA module binds in a novel site on albumin. The HSA molecule is shown in *blue* and the GA module in *pink*. The same color code is used for all figures in this paper.

a correlation coefficient of 56.2. After cycles of rigid body refinement in REFMAC5 (19), maps were generated using xdlMAPMAN (20). Manual inspection of normalized positive difference density maps ($|F_o| - |F_c|$) at a σ level of 3 in the program O (21) indicated the location of the GA three-helix bundle. The minimized average NMR structure of the GA module (Protein Data Bank entry 1PRB) was manually built into the positive difference density. The resulting complex was subsequently subjected to several cycles of restrained refinement in REFMAC5 using the CCP4i interface (22) and manual rebuilding in O. Toward the end of model building, TLS refinement in REFMAC (version 5.2) was also carried out. Attempts were made to refine the model without geometric restraints. However, at this limited resolution such attempts not only result in fitting to noise (higher free R -factor), but also to improbable torsion angles for the main chain and side chains. Therefore, the restraints were kept throughout the refinement. The model is available through the Protein Data Bank, accession code 1TF0 (RCSB022596). An additional map calculation was performed using ARP/wARP (23). Model validation was carried out using PROCHECK (24). Molecular graphics illustrations were generated with PyMol (25).

RESULTS

Overall Structure of the HSA-GA Complex—Fig. 1 shows the structure of the 1:1 complex formed between the GA module of protein PAB and HSA. The complex crystallizes in the centered orthorhombic space group C22₁, a crystal form previously unreported for HSA. The structure was solved by molecular replacement, and the resulting model of the complex was refined using TLS and restrained refinement to a resolution of 2.7 Å. TLS refinement of the complex proved to be beneficial and was carried out using four TLS groups, with HSA residues 1–197 in the first, residues 198–385 in the second, residues 386 to 569 in the third, and GA residues 1–53 in the fourth TLS group. The HSA chain is disordered between residues 77 and 88 and beyond residue 573. These regions have been omitted from the model. The protein has satisfactory bond angles and bond lengths, and 87.9% of the residues in the complex fall within core regions in the Ramachandran plot. The two residues in the disallowed regions, Lys-541 in HSA and Asp-3 in GA, are involved in crystal packing interactions, which may account for the unusual torsion angles. The data and refinement statistics are summarized in Table I.

The GA module binds to HSA at a site in domain II of the albumin molecule close to a cleft bounded by helices 2 and 3 in domain IIA, helices 7 and 8 in domain IIB, and the loop region before helix 7 in IIB (Fig. 2A). In the GA module, residues from the second helix and the two loops surrounding it are involved in binding. The GA helices pack outside the cleft at an angle of $\sim 60^\circ$ against HSA helix 3 in domain IIA. Although this arrangement is almost identical to the orientation reported for the all α -helical affibody- Z_{SPA} complex (26), the respective binding sites on the Z domain and the GA module do not overlap. The three-helix bundle Z domain of protein A and the

TABLE I
Crystallographic statistics

Data collection	
Wavelength (Å)	0.99989
Beamline	ID14-EH4
Resolution (Å)	2.7
Number of unique reflections	22263
Completeness for range (%)	100
Redundancy	10.2
R_{merge}	0.07 (0.27) ^a
$I/\sigma(I)$	14.0 (9.4) ^a
Refinement	
Resolution range (Å)	65–2.7
No. of reflections	
Total	21137
Test set	1103
R_{work} (%)	24.9 (32.4) ^a
R_{free} (%)	29.5 (38.8) ^a
No. of non-hydrogen protein atoms	4902
Residues in model	
Albumin	5–76, 89–572
GA	1–53
Wilson B -factor (Å ²)	65.5
Average B -factor (Å ²)	
Main chain	31.3
Side chain	33.1
Ligands	68.3
RMSD ^b stereochemistry	
Bonds (Å)	0.016
Angles (°)	1.7

^a Values for highest resolution shell (2.77–2.70 Å) are in parentheses.

^b RMSD, root mean square deviation.

GA module share a striking similarity in their overall structure. Even so, it is helix 1 of the Z domain that packs against the affibody helices, not the second and third as in the HSA-GA case. This illustrates the versatility inherent in an all-helical binding surface.

The Structure of the Albumin Molecule—Binding the GA module leaves the albumin chain largely unchanged compared with a fatty acid complex of HSA (PDB code 1BJ5), apart from local side chain rotations. The model of the complex also contains two molecules of fatty acid, which had been co-purified with the albumin prior to complex formation and crystallization. Judging from the markedly lower quality of the electron density in domain I of the HSA molecule, this region seems to be more flexible and disordered than the rest of the complex.

The Structure of the GA Module—No significant conformational changes in GA occur upon HSA binding, as judged by comparison of the present crystal structure and the previously reported solution structure (27). The three helices of the GA module form a tight three-helix bundle with a distinct hydrophobic core. Both the N and C terminus of the GA module are well ordered in the crystal. The N-terminal residue Thr-1 is hydrogen bonded to the side chain of a symmetry-related Lys-536, whereas the penultimate residue at the C-terminal end, His-52, is stabilized by a hydrogen bond to the main chain oxygen of Ala-21 in the GA module.

The main chain of GA helix 1 was corrected, and side chain orientations throughout the entire chain also had to be amended. The rebuilding resulted in that almost 90% of the GA residues in the crystal structure came within core regions in the Ramachandran plot, compared with 64% in the NMR solution structure.

The HSA-GA Interface—The interface is a predominantly flat surface of ~ 700 Å² involving one-fourth of the total GA surface area (Fig. 2B). The binding surface consists of a hydrophobic core flanked by two hydrogen bond networks.

The hydrophobic core of the interface is lined with residues Phe-228, Ala-229, Ala-322, Val-325, Phe-326, and Met-329 from HSA and residues Phe-27, Ala-31, Leu-44, and Ile-48 from

FIG. 2. **The GA binding site.** A, the GA helices pack at almost right angles to helices 3, 4, and 7 (*h3*, *h4*, and *h7*) in HSA domain II. B, surface representation of the rather flat GA binding site on albumin, where the main interaction area on albumin is shown in white. Residues (represented as single-letter amino acid abbreviations with position numbers) from the GA module participating in binding surface interactions are shown as sticks. C, in the hydrophobic core of the interface, protruding from the second GA helix, Phe-27 (*F27*) is buried in the hydrophobic pocket formed by Met-329 (*M329*), Phe-309 (*F309*), and Phe-326 (*F326*) in HSA. The $2|F_o| - |F_c|$ map is shown at a σ level of 1.

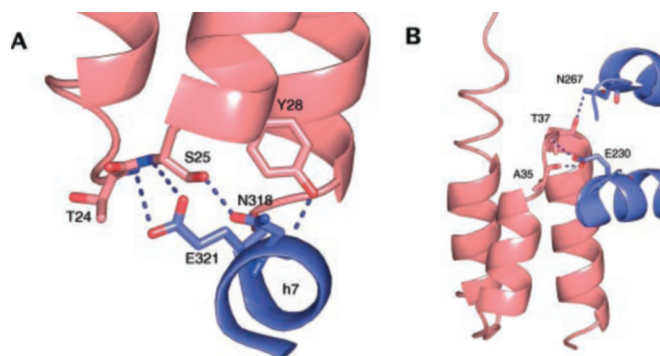
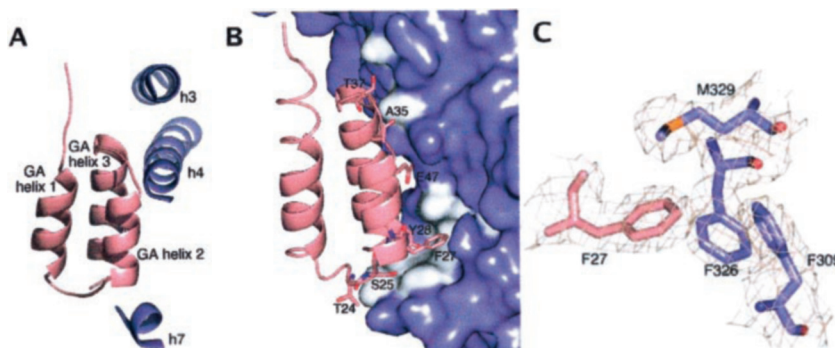


FIG. 3. **The hydrogen bond networks of the interface.** A, the complex is stabilized by a hydrogen bond network between residues (represented as single-letter amino acid abbreviations with position numbers) at the loop between the first and the second GA helix and HSA residues in helix 7 (*h7*) of domain IIB. B, the second hydrogen bond network involves residues at the loop between the second and third GA helix and HSA residues in helices 2 and 3 of domain IIA.

GA. In this context it is worth mentioning the phenyl side chain of Phe-27, which is buried in a cleft formed by the HSA residues Phe-309, Phe-326, and Met-329 (Fig. 2C). In addition, this interaction extends the aromatic cluster of HSA domain II, which consists of Phe-309, Phe-326, Phe-330, Phe-374, Phe-377, Tyr-334, and Tyr-353, to also include Phe-27 and Tyr-28 of GA.

Adjacent to the hydrophobic core of the interface is a hydrogen bond network between helix 7 in the HSA domain IIB and the loop preceding helix 2 in GA (Fig. 3A). HSA residue Glu-321 forms two hydrogen bonds, one each with the main chain nitrogens of Thr-24 and Ser-25 in GA. Furthermore, the side chain hydroxyl group of Ser-25 in GA forms another hydrogen bond with the side chain oxygen of Asn-318 from HSA. The main chain oxygen of Asn-318 is, in turn, involved in a hydrogen bond with the side chain hydroxyl group of Tyr-28 in the GA module.

At the opposite end of the binding interface, a second hydrogen bond network is formed between residues in the loop connecting helices 2 and 3 in GA and residues in helices 2 and 3 in HSA domain IIA (Fig. 3B). Here, HSA residues Glu-230 and Asn-267 form hydrogen bonds to the main chain nitrogen and the side chain hydroxyl group of Thr-37, respectively. Glu-230 also interacts with the main chain oxygen of Ala-35.

A Fatty Acid in the Binding Interface—An additional hydrogen bond at the HSA-GA interface occurs between GA residue Glu-47 in helix 3 and HSA residue Lys-212. Adjacent to this region, in the tunnel-shaped cavity previously described as fatty acid binding site 6, we observed density compatible with a medium-length fatty acid. Two molecules of the saturated ten-carbon fatty acid decanoate were built into the model in a

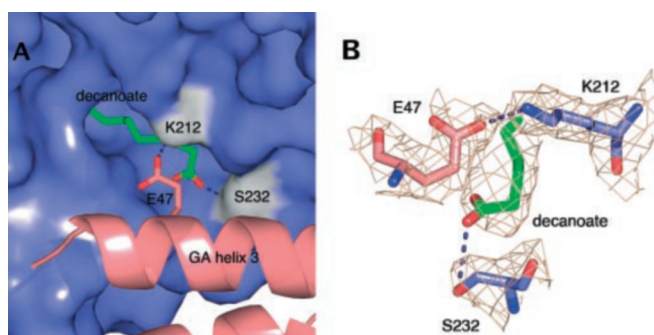


FIG. 4. **A fatty acid at the HSA-GA interface.** A, the fatty acid protrudes from its binding pocket toward the GA binding site. B, the carboxylate head of the fatty acid is ligated by Ser-232 (*S232*). The GA module is anchored by a hydrogen bond between Lys-212 (*K212*) of HSA and Glu-47 (*E47*) of the GA module. The $2|F_o| - |F_c|$ map is shown at a σ level of 1.

linear tail-to-tail orientation. In the final model, the carboxylate head of one of the fatty acids is stabilized by a hydrogen bond with Ser-232 (Fig. 4A). The fatty acid is thereby brought within 4.5 Å from the side chain of Glu-47 in GA. Local side chain rotations occur in HSA upon binding of the fatty acid molecules and the GA module simultaneously. The side chain of Lys-212, which in defatted HSA forms a 3.6-Å salt bridge with the carboxyl group of Glu-208, rotates slightly, pushed by the fatty acid so that its terminal amine group forms a tighter, 2.6-Å ion pair bond to the glutamic acid side chain (Fig. 4B). One molecule of decanoate was also built in fatty acid site 7. To account for density in Sudlow site I, one molecule of citrate was built into this site.

DISCUSSION

Comparison with Previous Binding Experiments—Residues adjacent to the hydrogen bond network at the loop between GA helices 2 and 3 were implicated in albumin binding in the previously reported NMR experiment on an HSA-complexed GA module. The same NMR experiment also suggested that the N terminus of the GA module was involved in binding, which is not visible in the crystal structure. However, because the N terminus of the GA module can be seen participating in crystal packing interactions, a transient association between the GA module and HSA might explain the observed interaction.

A Fatty Acid at the Binding Interface—The presence of fatty acid in the HSA-GA binding interface might influence complex formation, although its precise role in that case is not evident because it is not within hydrogen bonding distance from any of the GA module side chains. However, binding of GA might be influenced indirectly, because the binding of the fatty acid causes the side chain of Lys-212 to be pushed aside slightly into a position that allows it to form a hydrogen bond to GA.



FIG. 5. **Species specificity.** A ClustalW alignment of albumins from several different species, namely human, rhesus monkey, cow, pig, rabbit, mouse, and rat, reveals residue 329 (shaded) to be crucial. A polar (or, as in the case of rabbit serum albumin, basic) residue in this position would not be easily accommodated in the hydrophobic pocket. Swiss-Prot accession numbers are shown.

Whether completely defatted HSA would be able to bind GA is not known. Therefore, it is uncertain whether the presence of a fatty acid is required for or only tolerated in the binding of GA.

Structural Basis for Species Specificity—The GA module in this study is homologous to an ABD of protein G of group G streptococci as a result of it being transferred by exon shuffling from protein G to protein PAB. The binding site for protein G on HSA has been suggested to be located on the second and third domain of HSA and involves a single site consisting of a segment spanning residues 330 to 548 (28). This study demonstrates that HSA residues 212, 309, 318, 321, 326, and 329 are important for GA binding, indicating that there is at least partial overlap between the two sites.

Despite the high sequence homology between the two domains, the wide species affinity displayed by ABD is not retained in the GA module. Whereas affinities for human and rhesus monkey albumin are similar in GA and ABD, the affinity of GA for mouse albumin is more than a hundred times lower than that of ABD (29).

Considering that the interaction in the hydrophobic core of the interface between Phe-27 in the GA module and Met-329 in HSA is crucial, we suggest a structural basis for the difference in species selectivity between ABD and the GA module. A superposition of the α carbons in the structures of ABD (Protein Data Bank entry 1GJS) and GA shows that the phenylalanine in GA position 27 corresponds to Tyr-39 in the ABD (not shown). Furthermore, a sequence alignment of albumins of different species shows that the methionine in HSA position 329 corresponds to polar or charged side chains such as serine and lysine in albumins of other species (Fig. 5). As other interactions at the surface seem to be conserved across species, the bootstrapped hydrophobic Met-Phe interaction emerges as a crucial interaction at the HSA-GA interface, and we stipulate that it plays a major part in the species specificity observed for the GA module compared with that of ABD. The polar nature of the hydroxyl group in Tyr-39 of the ABD domain enables it to interact with the range of polar side chains displayed by the albumins from different species, whereas its GA counterpart, Phe-27, is restricted to interact with a hydrophobic side chain, *i.e.* methionine in the case of human and rhesus monkey albumin.

The superpositioning of the ABD domain and the GA module also reveals that the inserted GA residue Thr-24 is situated in a key position in the hydrogen bond network at the loop between GA helices 1 and 2. It has no counterpart in the ABD domain, and although its interactions with HSA can be conserved for albumins of other species (not shown), the possibility that the inserted residue alters affinity by causing a change in the dynamic properties of the interface residues cannot be excluded.

Biological Implications—In 1979, Kronvall *et al.* (30) first described the binding of HSA to bacterial surface structures and found that groups A, C, and G streptococci specifically absorbed HSA from plasma. Subsequently, some strains of *F. magna* were also found to bind HSA (31). In the case of groups C and G streptococci, protein G is responsible for albumin binding, whereas the corresponding protein of *F. magna* is called PAB. Analysis of the gene encoding protein PAB revealed that the HSA binding domain had been transferred from

the protein G gene by the action of a conjugational plasmid from a third bacterial species, *Enterococcus faecalis* (7). This finding represents the first described case of contemporary module shuffling, and the fact that PAB-expressing strains of *F. magna* are tetracycline resistant² suggests that antibiotics provide the selective pressure behind the evolution of this novel HSA-binding protein. As mentioned above, *F. magna* is part of the normal human bacterial flora, but strains that express protein PAB are mostly isolated from patients with localized suppurative infections, suggesting that the binding of HSA to the bacterial surface increases the pathogenic potential of *F. magna*. The study by de Chateau *et al.* (8) did indeed show that HSA enhances the growth rate of streptococcal and *F. magna* strains expressing HSA-binding surface proteins, and the structural data of the present work indicate that the binding of HSA to the GA module could provide growing bacteria with fatty acids and, possibly, other nutrients transported by HSA.

Whereas group C and G streptococci infect most mammalian species, *F. magna* has been isolated only from humans. This finding is reflected also in the albumin binding properties of proteins G and PAB, where protein G has a much broader specificity than PAB, which binds preferentially primate albumins. This observation represents an unusually clear and beautiful example of microbial adaptation to its host(s) at the molecular level. As described in the previous paragraph, the sequence differences between the ABDs of proteins G and PAB and between different albumins, in relation to the HSA-GA interface, help to explain the structural basis for this adaptation.

Implications for the Rational Design of Albumin Ligands—In this study we have obtained reasonably well diffracting crystals of HSA that endure cryoconditions during data collection. This might prove useful, especially in the context of minimizing the HSA affinity of drug molecules by structure-based design. We speculate that the readily available, cryo-enduring HSA-GA complex could be an entry point to enabling high throughput structure determination in the study of HSA-drug interactions. Further studies will be performed to investigate the consistency of HSA binding ligands in the presence of the GA module.

Based on the molecular interactions in the HSA-GA interface, it might be possible to design and screen for compounds that will interfere with the binding of HSA to bacterial surfaces *in vivo*. Several observations have shown that the binding of HSA adds selective advantages to the bacteria and increases their virulence, suggesting that such compounds could be used to treat infections caused by HSA binding bacterial pathogens.

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