Chapter 1
Human Serum Albumin: A Multifunctional Protein

Ulrich Kragh-Hansen

Abstract Human serum albumin is synthesized in the liver and continuously secreted into the bloodstream. Several receptors are strongly involved in the following distribution and metabolism of the protein. The receptor-albumin interactions can be modified by specific mutations, a finding which could be of pharmaceutical and medical interest.

The largest pool of albumin is found in the extravascular spaces although at a lower concentration than in the bloodstream. The higher concentration in the circulation is the main contributor to plasma's colloid osmotic pressure and to the Gibbs-Donnan effect in the capillaries.

Albumin seems to be the quantitatively most important circulating antioxidant, and it has enzymatic properties which are so pronounced that they most probably are of biological importance. The protein's ability to bind ligands and thereby to serve as an important depot and transport protein for numerous endogenous and exogenous compounds is well studied. Recent work has given much new information about the location and structure of binding sites and about potential ligand interactions. Structural information is also useful when designing new drugs whether the aim is to avoid binding or to make use of the protein's depot function. Nonbinding therapeutics can get improved stability and benefit from the long biological half-life of albumin by forming complexes with it. The complex formation can take place by enriching the therapeutic with an organic molecule which can bind reversibly or covalently to the protein. If the therapeutic is a polypeptide or protein, fusion proteins can be produced.

Albumin also shows promises for targeted drug delivery. This process can be passive and based on the enhanced permeability and retention effect. The effect can be increased by using dimers, polymers, or albumin-based nanoparticles. The targeting process can also be active and based on an interaction between albumin carrying a targeting ligand and cellular receptors.

Keywords Albumin-receptor interactions • Ligand binding • Stability • Half-life • Drug targeting

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1.1 Introduction

Human serum albumin (HSA) is a multifunctional protein exclusively synthesized by liver hepatocytes and continuously secreted into the circulation. Here, it is the most abundant protein and comprises 60–65 % of total plasma protein. Many observations propose the existence of an important link between the concentration of HSA and health (Peters 1996). Due to a large number of acidic (98 Glu + Asp) and basic residues (83 Lys + Arg), the protein is highly soluble in aqueous media. Thus, its concentration in plasma is ca. 0.6 mM (4 % w/v), but solutions of 20 % can be made for clinical use. Actually, it is possible to make preparations of up to 50 % (Peters 1996). The presence of the many titratable amino acid residues also implies that HSA has an important buffering capacity. The uneven content of acidic and basic residues results in a net charge of ca. −15 at physiological pH, a fact that renders HSA important for the Donnan effect in the capillaries. Finally, under certain circumstances, the protein can serve as a source of amino acids or energy.

1.2 Synthesis and Structure

HSA is a member of the albumin superfamily, which also includes the transport proteins α-fetoprotein, vitamin D-binding protein (Gc-globulin), and afamin (α-albumin) (Kragh-Hansen et al. 2013). In addition, the superfamily includes the α-fetoprotein-related gene, but due to multiple mutations, this gene is an inactive pseudogene in humans. All the genes are single-copy genes, and the four active ones in the human are expressed in a codominant manner, ie, both alleles are translated. The genes lie on chromosome 4, near the centromere for the long arm, at position 4q11−13. The albumin gene is 16,961 nucleotides long from the putative cap site to the first poly(A) addition site. It is split into 15 exons that are symmetrically placed within the three domains thought to have arisen by triplication of a single primordial domain.

The mRNA for HSA encodes for a precursor protein (pre-pro-albumin) of 609 amino acids. The N-terminal pre-peptide of 18 amino acids guides the nascent albumin peptide chain from the ribosome, where it was synthesized, through a receptor on the membrane of the endoplasmic reticulum into the lumen of the reticulum. Afterward, it is rapidly cleaved off. The N-terminal, basic pro-peptide of six amino acids, is cleaved in one of the last steps before secretion of the mature protein into the space of Disse and the hepatic sinusoid. Thus, HSA consists of 585 amino acids; the molecular mass is ca. 66.5 kDa.

Normally, wild-type pro-albumin is not secreted from the liver cells in a detectable amount. However, it can be found in the circulation in certain pathological conditions (Kragh-Hansen et al. 2013).

HSA is produced as a simple, monomeric protein, ie, without prosthetic groups and covalently bound lipid or carbohydrate. The three-dimensional structure of the single polypeptide chain, and of its recombinant version (rHSA), has been determined crystallographically, and the structure is now known to a resolution of 2.3 Å (He and Carter 1992; Sugio et al. 1999; Hein et al. 2010). The polypeptide chain forms a heart-shaped protein with approximate dimensions of 80 × 80 × 80 Å and a thickness of 30 Å. It has about 67 % α-helix but no β-sheet and can be divided into three homologous domains (I–III). Each of these is comprised of two subdomains (A and B). The A and B subdomains have six and four α-helices, respectively, connected by flexible loops. All, but one, Cys34, of the 35 cysteine residues are involved in the formation of 17 stabilizing disulfide bonds. Small-angle X-ray scattering studies of HSA in solution show general agreement with the crystal structure (Olivieri and Craievich 1995). Also, a combined phosphorescence depolarization-hydrodynamic modeling study has proposed that the overall conformation of HSA in neutral solution is very similar to that observed in crystal structures (Ferrer et al. 2001).

In addition to HSA, the crystal structure of albumin from cattle, horse, rabbit, and hare has been determined (Bujacz 2012; Majorek et al. 2012). Although a number of differences were found in the binding pockets, as well as variations in surface structure and charge distribution, structural alignments of the crystal structures with HSA showed strong structural similarities between the albumins. This finding is probably mainly due to a conserved set of disulfide bridges.

At present, 70 mutations of the HSA gene are known which result in a circulating variant of pro-albumin or albumin (alloalbumins) (Kragh-Hansen et al. 2013; The Albumin Website). Because both alleles of the gene are translated, most genetic variants have been detected in heterozygotes, ie, in persons having both a variant and wild-type (normal) HSA. In addition to single-amino acid substitutions, glycosylated variants, N-terminally and C-terminally modified alloalbumins, have been found. Mutations can also compromise the protein synthesis to such an extent that HSA is completely absent or strongly decreased in affected individuals leading to the condition known as analbuminemia. To date, 22 such molecular defects have been reported (Minchiotti et al. 2013; The Albumin Website).

Because alloalbumins do not seem to be associated with disease, they can be used as markers of migration and provide a model for study of neutral molecular evolution. They can also give valuable molecular information about binding sites, antioxidant and enzymatic properties, as well as in vivo and in vitro stability. Mutants with increased affinity for endogenous or exogenous ligands could be therapeutically relevant as antidotes, both for in vivo and extracorporeal treatment. Variants with modified biodistribution could be used for drug targeting. In most cases, the desired function can be further elaborated by producing site-directed, recombinant mutants.

1.3 Distribution and Circulatory Half-Life

HSA is solely synthesized in the liver. By contrast, its sites of degradation are widespread. Most of the protein is hydrolyzed in the muscle and skin, but some leaks into the gut, some is taken up by Kupffer cells of the liver, and a small amount is degraded elsewhere or lost with shed dermis, saliva, sweat, tears, or milk (Fig. 1.1) (Peters 1996).

In healthy adults, ca. 13.8 g is made per day and secreted into the bloodstream. This amount corresponds to ca. 25 % of the protein synthesis activity of the liver. However, under physiological circumstances, only 20–30 % of the hepatocytes produce albumin, and synthesis can therefore be increased on demand by a factor of 200–300 % (Evans 2002). From the liver, HSA is distributed in the bloodstream but also to several extravascular spaces, some of which are poorly accessible; these are mainly found in the skin (Fig. 1.1). The total amount of HSA in the body is ca. 360 g, of which about two-thirds is outside the bloodstream and about one-third is in the bloodstream. However, the concentration of HSA is higher in the bloodstream, and that is why the protein can contribute with ca. 80 % of the colloid osmotic pressure of plasma (ca. 15 mm of Hg).

HSA leaves the intravascular space in different ways. For example, fenestrated capillaries and, especially, sinusoidal capillaries allow the protein to pass. The latter are mainly found in the liver and spleen but also in bone marrow, lymph nodes, and adrenal glands. In other situations, the escape is transcellular and mediated by a receptor.

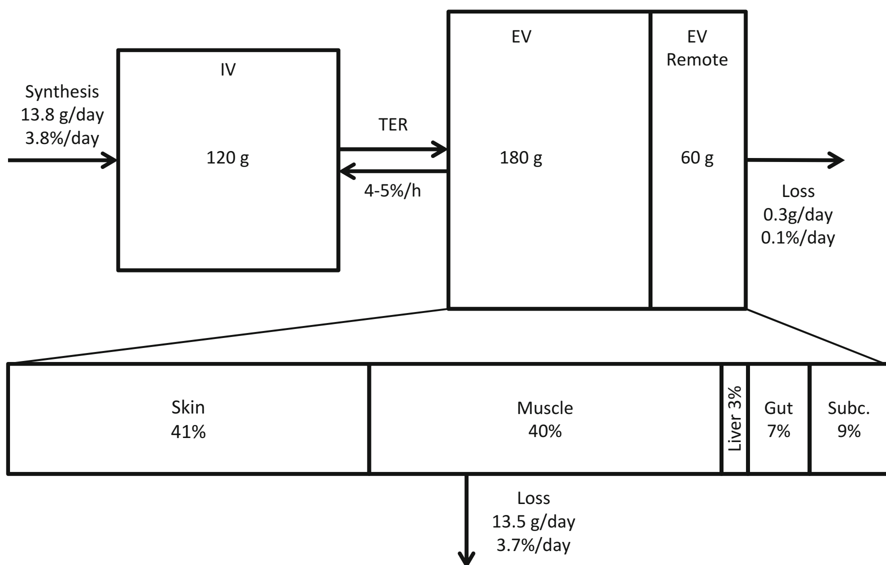

Fig. 1.1 Distribution and dynamics of HSA in a healthy person of 70 kg. *IV* intravascular, *EV* extravascular, *TER* transcapillary escape rate, *subc*, subcutaneous (The illustration is based on information found in Peters (1996))

Thus, the protein can interact with the receptor gp60 (60-kDa glycoprotein), also called albondin, situated in the plasma membrane of continuous endothelium (except for the brain) and alveolar epithelium (Sleep 2014; Merlot et al. 2014). Binding results in internalization of the complex by a caveolin-dependent endocytotic process and ultimately to transcytosis of albumin. It has been proposed that ca. 50 % of albumin leaves the capillary lumen in this way. Despite extensive studies, the gene for and the structure of gp60 are still unknown, and the molecular mechanisms of this transcytosis are still poorly understood.

HSA can most probably also leave the bloodstream by a process involving the intracellular receptor FcRn (neonatal Fc receptor) (Fig. 1.2a). This type of transcytosis is initiated by pinocytosis of HSA at the luminal membrane of the endothelial or epithelial cell (Bern et al. 2015). FcRn is placed in the membrane, but at physiological pH, there is no significant interaction between receptor and protein. After being taken up, HSA enters early endosomes. From there, the protein is transferred to acidified endosomes which have FcRn in their membrane, and at that pH (5–6), HSA binds strongly to FcRn. The endosomes with the protein-receptor complexes can now fuse with the basolateral side. This event results in exocytosis of HSA, because the pH of the cellular surroundings is neutral. Interestingly, IgG can be transcytosed by the same mechanism (Bern et al. 2015).

When albumin is saturated with fatty acids, transcytosis is two to three times higher than that of defatted albumin (Galis et al. 1988). The preferential transcytosis of cargo-carrying albumin is an interesting aspect, because it could help albumin to transport cargo into extravascular compartments.

HSA has an approximate plasma half-life of 19 days (Peters 1996). This half-life is extraordinary long for a circulating protein and is partly due to a return from the extravascular space to the circulation via the lymphatic system. The return amounts to 4–5 % of intravascular albumin per hour (Fig. 1.1), and the protein makes ca. 28 "trips" in and out of the lymphatic system during its lifetime (Peters 1996). Another contributing factor to the long circulatory half-life is that normally HSA is not lost in the urine. One reason for this is that, due to its size and charge, the filtration of HSA in the glomeruli is low. In addition, any protein filtered is reabsorbed in the proximal tubuli and transferred to the bloodstream via endocytosis by a receptor complex formed by cubilin and megalin (Merlot et al. 2014; Bern et al. 2015). Interestingly, results of animal studies have suggested an important role of FcRn for the renal retrieval of albumin (Sand et al. 2015; Bern et al. 2015).

FcRn is expressed in multiple cell types and tissues and is most important for the half-life of HSA, because it protects the protein (and IgG) from degradation in the lysosomes. Actually, FcRn rescues as much albumin as the liver produces (Bern et al. 2015). The initial steps in the protection are similar to those leading to transcytosis (Fig. 1.2a): the protein is taken up by the cell in question by pinocytosis and ends up as FcRn-bound in acidified endosomes. Now, in the present situation, the endosomes migrate to the membrane, where HSA originally was taken up, and release it by exocytosis. Thus, HSA is recycled back to the circulation. Albumin that does not bind to FcRn in the acidified endosomes, because it is conformationally

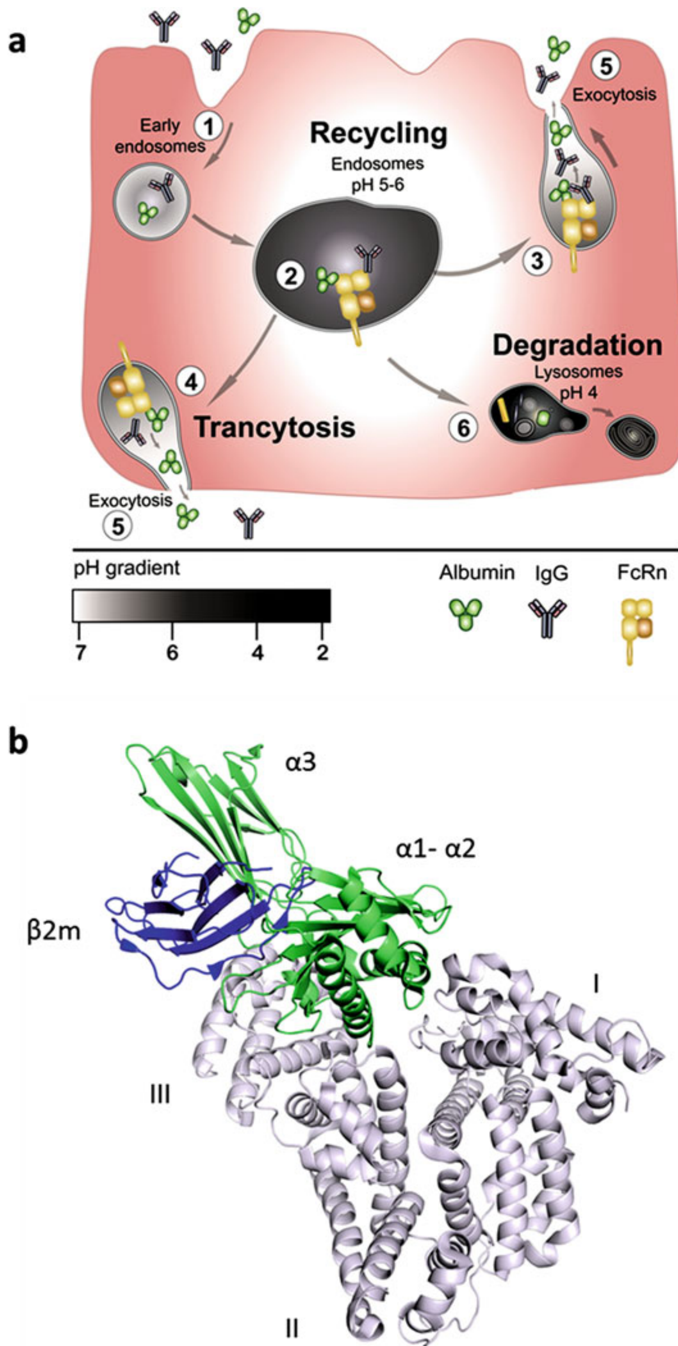

Fig. 1.2 (**a**) pH-dependent, FcRn-mediated cellular transport of HSA (and IgG). Initially, HSA is taken up by pinocytosis from the blood or luminal space of the cell and enters early endosomes (step *1*). From there it is transferred to acidified endosomes having FcRn in the membrane. At this pH of 5–6, the protein binds strongly to the receptor at a 1:1 stoichiometry (step *2*). At this point, three possible fates seem to exist for HSA. First, it can be exocytosed back to the blood or

modified or in surplus, will neither be transcytosed nor recycled but will be destined for lysosomal degradation.

In contrast to the other receptors interacting with albumin, the crystal structure of FcRn, and of its complex with HSA, is known (Fig. 1.2b). This detailed information is of great pharmaceutical and pharmacological interest, because on the basis of this knowledge, it is possible to construct albumin mutants with modified affinities for the receptor (Sand et al. 2015; Bern et al. 2015). Thus, it should be possible, for example, to design albumins with improved recycling and thereby increased circulatory half-life. Such a possibility is of great clinical impact, because in addition to increase the half-life of albumin itself, it should also increase very much the half-life of any bound cargo. An increased affinity for FcRn could perhaps also facilitate transcytosis of HSA with or without bound drugs or therapeutics. These possibilities are increasingly explored, but the influence of other factors has to be addressed. For example, the affinity of albumin for FcRn is strongly species dependent, a fact that has to be taken into account when using laboratory animals in preclinical investigations. In addition, engineered HSA mutants may become immunogenic, and the albumin-FcRn interaction could be influenced by protein-bound drugs or therapeutics. Thus, although very promising, there is still a long way to go before manipulating the interaction with FcRn can be used in the clinic.

1.4 Binding to Other Receptors

Several types of cancer secrete a glycoprotein known as secreted protein acidic and rich in cysteine (SPARC). Among its functions is albumin binding, a function which results in enhanced accumulation of albumin in the tumors, but apparently not in increased uptake of the protein into the tumor cells (Merlot et al. 2014). The albumin-receptor binding could also result in accumulation of albumin-bound drugs and therapeutics in the tumors and thereby result in better therapeutic effects. For example, the response of human head and neck cancers to nab-paclitaxel (nanoparticle albumin-bound paclitaxel) was reported to correlate with SPARC expression (Desai et al. 2009).

Fig. 1.2 (continued) luminal space from where it was taken up. Such an event results in recycling of the protein (steps *3* and *5*). Second, the exocytosis can take place at the basolateral membrane resulting in transcytosis (steps *4* and *5*). Finally, HSA which does not bind to FcRn in the acidified endosomes goes to degradation in the lysosomes (step *6*). Whether the HSA-FcRn complexes go to the luminal or to the basolateral membrane depends on, among other factors, binding of intracellular proteins such as adaptor protein-2 and calmodulin to sorting motifs of the cytoplasmic tail of the α-3 subunit (Sand et al. 2015). Principally the same mechanisms exist for IgG. IgG and HSA bind independently and noncooperatively to FcRn (The illustration is a modification of Fig. 2 in Bern et al. (2015).). (**b**) Crystal structure of the HSA-FcRn complex (Oganesyan et al. 2014). The domains (I–III) of HSA are indicated. FcRn is composed of a long α-chain of 44 kDa (domains α1–α3) and a short β2-microglobulin unit of 12 kDa (β2m). Of these structures, only α3 has a transmembrane fragment and a cytosolic part. Domain III of HSA plays the essential role for binding, but domain I is also necessary. The figure was made with PyMOL on the basis of the atomic coordinates (PDB ID: 4N0F) available at the RCSB Protein Data Bank

The membrane-bound receptors gp18 (18-kDa glycoprotein) and gp30 (30-kDa glycoprotein) are widely distributed scavenger receptors which bind and internalize chemically modified albumin. Thus, the receptors recognize damaged or changed albumin and target it for lysosomal degradation (Merlot et al. 2014).

1.5 Clinical and Pharmaceutical Uses

Huge amounts of HSA, ca. 500 ton per year worldwide, are used for improving clinical conditions such as shock, burns, trauma, surgical blood loss, hypoalbuminemia, decompensated cirrhosis, cardiopulmonary bypass, and acute respiratory distress (Mendez et al. 2005). HSA can also be used as a part of a hemodialysis regimen especially in patients with hepatic failure. Several such systems exist, but the molecular adsorbent recirculating system (MARS) is currently the most effective liver support device and can effectively remove protein-bound and water-soluble substances (Mitzner 2011). The efficiency of the system can most probably be increased by using tailor-made mutants of HSA itself or of one of its domains (Minomo et al. 2013).

HSA is also widely used as a stabilizing agent in pharmaceutical and biological products like vaccines, recombinant therapies, drug formulations, and coatings for medical devices (Chuang et al. 2002). Furthermore, the protein is used as a component in serum-free cell culture media, as a component for imaging agents, and, possibly, for therapeutic apheresis where plasma exchange might be desirable (Chuang et al. 2002).

Traditionally, HSA is obtained by fractionating human plasma. The risk by using this approach is that such preparations can be contaminated with blood-derived pathogens, for which reason the preparations have to be heated at 60 °C for 10 h in the presence of sodium octanoate and N-acetyl-L-tryptophanate. Of these ligands, octanoate has the greatest stabilizing effect against heat, whereas the presence of N-acetyl-L-tryptophanate diminishes oxidation of the protein (Anraku et al. 2004). However, recent studies have revealed that N-acetyl-L-tryptophanate should be replaced by N-acetyl-L-methionine, because the latter is a superior antioxidant and protects HSA against light and thereby photo-irradiation (Kouno et al. 2014). Although this pasteurization procedure is very effective, the potential risk of the presence of pathogens still exists, and the associated screening costs are substantial. In addition, in several countries, supplies of human plasma are limited. Therefore, many and large efforts are being made to produce rHSA as a substitute. rHSA can be highly expressed in various hosts including bacteria, yeast, and transgenic animals and plants (Chen et al. 2013). Of these, yeast (*Pichia pastoris*) and plants (Asian rice) seem to be the most promising for large-scale production. However, challenges like purity and production costs have to be met. For example, HSA expressed in rice has extensive glycation which showed supplier-to-supplier and lot-to-lot variability (Frahm et al. 2014). Therefore, it has not yet been possible to do large-scale production for clinical uses. Nevertheless, rHSA is currently used for

different pharmaceutical purposes. This is the case with, for example, Recombumin® from Novozymes Biopharma, Albagen™ from New Century Pharmaceuticals, and recombinant human albumin from Akron Biotech and Sigma-Aldrich.

1.6 Ligand Binding

HSA serves as a depot protein and transport protein for numerous endogenous and exogenous compounds. In this way, the protein has a major impact on the pharmacokinetics and pharmacological effects of drugs (Yamasaki et al. 2013). Albumin binding can also affect the ligands in a more individual manner. Thus, binding can result in an increased solubility in plasma of the compound, in a reduced toxicity, or in protection of the ligand against oxidizing agents.

Although the ligand-binding properties of HSA are very versatile, it mainly binds organic anions and inorganic cations. This unique ability of the protein is due to a favorable combination of hydrophobic pockets and side-chain charges and to a pronounced flexibility, which, to a large degree, is caused by fairly long interdomain and intradomain polypeptide linkers and flexible loops.

Detailed molecular information about the binding sites is very helpful in the assessment of displacement effects. However, it should be born in mind that displacement effects may as well be caused by ligand-induced conformational changes of the protein. Structural information is also useful when designing new drugs whether the aim is to avoid binding or to make use of the protein's depot function.

For comprehensive tabulations of ligands, see the reviews of, for example, Kragh-Hansen (1981), Peters (1996), Kragh-Hansen et al. (2002), and Fanali et al. (2012). In the following, the known binding sites and regions for high-affinity binding will be presented.

1.6.1 N-terminal End

Cu^{2+} and Ni^{2+} are strongly bound in a square-planar ligand arrangement formed by the three N-terminal amino acids Asp1, Ala2 and His3. The metal ions are held tightly in a chelate ring involving the α-amino nitrogen of Asp1, the first two peptide nitrogens, and the N^1 imidazole nitrogen of His3. Co^{2+} binds with a lower affinity to principally the same site in an octahedral environment but with the β-COO^- group of Asp1 and the ε-group of Lys4 axially contributing to the metal ion coordination sphere (Fanali et al. 2012; Bal et al. 2013).

Co^{2+} binding to HSA from patients with cardiac ischemia is diminished. Perhaps it is not the site at the N-terminal end but other sites which are affected (Bal et al. 2013). Anyway, the observation has led to the development of assays for the condition. However, albumin's affinity for Co^{2+} can also be affected by several other conditions (Gaze 2009).

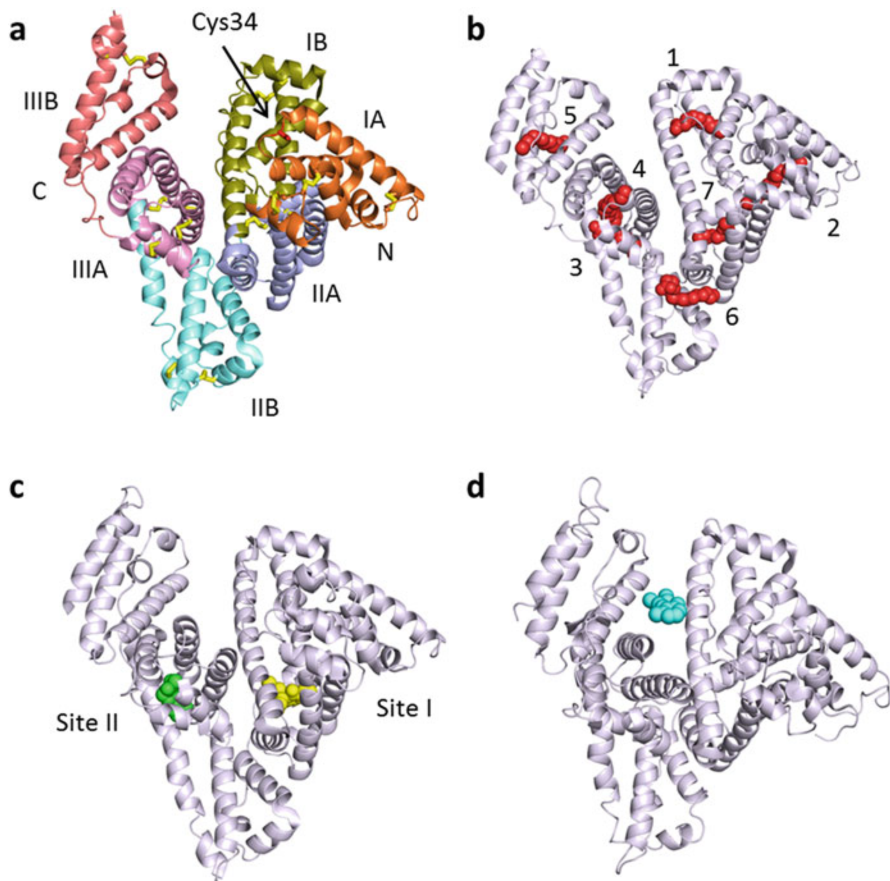

Fig. 1.3 (**a**) Crystal structure of HSA with the position of Cys34 indicated in *red* and the 17 disulfide bridges marked in *yellow*. The subdivision of the protein into domains (I–III) and subdomains (**a**, **b**) is shown. *N* and *C* represent the N-terminal and C-terminal ends, respectively. PDB ID: 1BM0. (**b**) Locations of the seven binding sites common to fatty acid anions using palmitate as an example (*red*). PDB ID: 1e7h. **(c)** Binding site for warfarin (PDB ID: 2bxd) in site I (*yellow*) and for diazepam (PDB ID: 2bxf) in site II (*green*). (**d**) Lidocaine binding (*magenta*) in the central, interdomain crevice. PDB ID: 3JQZ

1.6.2 Cys34

Cys34 in subdomain IA (Fig. 1.3a) is located in a crevice on the surface of the protein and does not participate in any disulfide bridges. Despite a limited accessibility, the sulfhydryl group can bind Hg^{2+}, Ag^{+}, Au^{+}, and Pt^{2+} (Fanali et al. 2012). The residue can also interact with nitric oxide and 8-nitro-cGMP and thereby form *S*-nitrosothiol and *S*-cGMP-HSA, respectively (Ishima et al. 2012a, b). In the circulation, about half of HSA has Cys34 coupled to low molecular weight thiols such as cysteine, glutathione, and homocysteine. The thiol group can also interact with

several drugs such as bucillamine derivatives, D-penicillamine, captopril, meso-2,3-dimercaptosuccinate, N-acetyl-L-cysteine, aurothiomalate, auranofin, and ethacrynate (Kragh-Hansen et al. 2002).

1.6.3 Subdomains IA and IIA

Palmitate has been reported to bind with a high affinity to an enclosed site located between subdomains IA and IIA (Simard et al. 2006). The methylene tail of the fatty acid anion binds in a nearly linear conformation within a narrow hydrophobic cavity formed by residues of the two subdomains, while the carboxyl forms specific salt bridge interactions with Tyr150, Arg257, and Ser287 (site 2 in Fig. 1.3b). Apparently, no other ligands, with the exception of other medium- and long-chain fatty acid anions, bind in this site.

HSA is the major Zn^{2+} transporter in plasma, and in the fatty acid-free protein, the metal ion binds primarily to an essentially preformed, 5-coordinate site located at the interface of domains I and II involving His67 and Asn99 from the former and His247 and Asp249 from the latter domain (Blindauer et al. 2009). The site is also a primary binding site for Cd^{2+} and a weak site for Cu^{2+} and Ni^{2+}. Therefore, the site is often called the multi-metal-binding site (Bal et al. 2013). Binding of a fatty acid to site 2 results in conformational changes which disrupt the site.

1.6.4 Subdomain IB

This subdomain houses a fairly large, L-shaped cavity with charged residues such as Arg117 and Arg186, capable of making hydrogen bondings, at its entrance (Zunszain et al. 2008). High-affinity binding of bilirubin and fusidic acid within this pocket causes only minor conformational changes in the site. By contrast, high-affinity binding of hemin and low-affinity binding of a fatty acid anion (site 1 in Fig. 1.3b) induce a significant conformational rearrangement of the subdomain. Wang et al. (2013) found that the subdomain is a major binding site for complex heterocyclic molecules such as the oncology agents camptothecin, 9-amino-camptothecin, etoposide, teniposide, bicalutamide, and idarubicin. The authors also observed that the large binding cavity has two access sites.

1.6.5 Subdomain IIA

The pioneering work of Sudlow et al. (1975), which was based on displacement of fluorescent probes, revealed that most drugs bind with a high affinity to one of two sites, called site I and site II. Of these, site I is placed in subdomain IIA (Fig. 1.3c).

The site is adaptable, because it can bind structurally very diverse ligands. However, usually they are dicarboxylic acids and/or bulky heterocyclic molecules with a negative charge localized in the middle of the molecule. The site is also large, because several examples have been found of independent binding of two different compounds (Kragh-Hansen et al. 2002). Several studies have shown that the site is composed of flexible subsites. Thus, Yamasaki et al. (1996) reported independent, high-affinity binding of acenocoumarol, dansyl-L-asparagine, and n-butyl p-aminobenzoate to three subsites which were named Ia, Ib, and Ic, respectively. Of these, Ia and Ib correspond to the warfarin and azapropazone binding regions, respectively.

X-ray crystallography has revealed many details about the site. Ghuman et al. (2005) found that it was a preformed binding pocket within the core of the subdomain including the lone tryptophan residue of the protein (Trp214). In the defatted protein, the interior of the pocket is predominantly apolar but contains two important clusters of polar residues, an inner one toward the bottom of the pocket (Tyr150, His242, and Arg257) and an outer cluster at the pocket entrance (Lys195, Lys199, Arg218, and Arg222). The large binding cavity is comprised of a central zone from which extend three distinct compartments. Warfarin, phenylbutazone, oxyphenbutazone, and the renal toxin 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid bind in the center of the pocket and partly into the compartments. Iophenoxic acid binds with a high affinity in the main chamber (central zone) and makes crucial hydrogen bonds to the side chains of Tyr150 and Arg257 (Ryan et al. 2011a). The ligand binds in a similar position and orientation to other compounds that are composed primarily of iodinated aromatic rings such as triiodobenzoic acid, diiodosalicylic acid, and iodipamide. The dansylated forms of L-asparagine, L-arginine, and L-glutamate are fluorescent marker ligands for the site, and they make essentially the same set of interactions with the binding pocket (Ryan et al. 2011b).

1.6.6 Subdomain IIIA

Sudlow's site II, also called the indole-benzodiazepine site, is situated in subdomain IIIA (Fig. 1.3c). Ligands binding to the site are often aliphatic or aromatic carboxylic acids with a negatively charged acidic group at one end of the molecule away from a hydrophobic center. Site II seems to be smaller, more selective, and less flexible than site I (Kragh-Hansen et al. 2002). Among the ligands known to bind to the site with a high affinity are L-tryptophan, diazepam, and several nonsteroidal antiinflammatory drugs. Octanoate and fatty acids with a longer chain length also bind to site II with a high affinity (Kragh-Hansen et al. 2006; Simard et al. 2006).

Crystallographic analyses have shown that, like site I, site II comprises a largely preformed hydrophobic cavity with distinct polar features in defatted HSA (Ghuman et al. 2005). However, differences between the sites also exist, among them being that site II is smaller than site I and without preformed sub-compartments. Furthermore, site II has only one main cluster of polar residues near the pocket

entrance; it is composed of Arg410, Tyr411, Lys414, and Ser489. Diflunisal, diazepam, ibuprofen, and the renal toxin indoxyl sulfate were found to bind in the center of the binding pocket. Propofol also binds to the site but in a different conformation due to its hydroxyl group in the center of the molecule. The flexibility of the site is less pronounced than that of site I, because binding is often strongly affected by stereoselectivity (Kragh-Hansen et al. 2002). However, site II possesses some flexibility, because it can bind diazepam, which has a large, branched structure, and two molecules of myristate (Ghuman et al. 2005). Ryan et al. (2011b) have studied in detail the binding of the marker ligands dansyl-L-phenylalanine, dansyl-L-norvaline, and dansylsarcosine.

1.6.7 Subdomain IIIB

Palmitate and other medium- and long-chain fatty acids bind with a high affinity in a hydrophobic channel that spans the width of the subdomain (site 5 in Fig. 1.3b) (Bhattacharya et al. 2000; Simard et al. 2006; Kragh-Hansen et al. 2006). The carboxyl group of the fatty acids interacts with the side chain of Lys525, aided in most cases by Tyr401. So far, no drugs have been reported to bind to this subdomain with a high affinity. However, binding experiments with mutants of HSA and molecular dockings strongly suggest that L-thyroxine binds with a high affinity to a site in the subdomain and that Lys525 is also important for strong binding of this ligand (Kragh-Hansen et al. 2016).

1.6.8 Central, Interdomain Crevice

Only a very few ligands have been reported to bind in the large crevice between domains I and III in defatted HSA. However, two low-affinity sites exist for the binding of decanoate but not for fatty acids of a longer aliphatic chain: one close to the base of the crevice and one further up (Bhattacharya et al. 2000). The positively charged lidocaine binds to a superficially placed site in subdomain IB in the upper part of the interdomain cleft. Arg114, Lys190, and Asp187 are important residues for binding of the drug (Fig. 1.3d) (Hein et al. 2010).

1.6.9 Covalent Binding

Certain metal ions, nitric oxide, and several drugs and other organic compounds can bind covalently to Cys34 (see Sect. 1.6.2).

The metabolism of drugs with a carboxylic acid function involves usually the formation of acyl glucuronides. This type of metabolite is a reactive electrophilic

species and can therefore react covalently with HSA. Several such examples exist, and serine, arginine, or lysine residues, especially Lys159 or Lys199, are involved (Kragh-Hansen et al. 2002).

D-glucose and other reducing monosaccharides can interact with HSA. The interaction is with positively charged amino acids, and especially Lys525 but also Lys199, Lys281, and Lys439 seem to be important in this respect (Nakajou et al. 2003). This nonenzymatic glycation starts by condensation of the carbonyl group in acyclic glucose with a free amino group forming a Schiff base or aldimine intermediate, which undergoes an Amadori rearrangement to form a stable ketoamine that can cyclize to a ring structure. The Amadori product may subsequently undergo oxidative and nonoxidative degradation or rearrangement, giving rise to a heterogeneous group of substances loosely described as advanced glycation end products. Because the ambient glucose concentration is the major determinant for the reaction, glycation is increased in diabetes, and glycoalbumin has been proposed as a useful test for short- and intermediate-term control (2–3 weeks). By contrast, glycohemoglobin (HbA1c) is the favorite test for monitoring long-term (2–3 months) control of blood sugar levels in diabetics. Glycoalbumin may also serve as a biomarker for complications of diabetes such as nephropathy, retinopathy, and cardiovascular disease (Cohen 2013; Furusyo and Hayashi 2013).

1.7 Coupling of Nonbinding Therapeutics to HSA

Several polypeptide and polynucleotide therapeutics are used in the clinic while others are promising in clinical trials. However, the usefulness of such therapeutics can be limited due to very short in vivo half-lives, in the order of minutes, due to, for example, rapid degradation and renal clearance. The short half-lives can be increased very much by coupling the therapeutics to albumin, and the use of albumin for this purpose is a rapidly growing area of research.

1.7.1 Reversible Binding of Modified Therapeutics

A well-known example to illustrate this possibility is Levemir® (Insulin detemir), which is an albumin-binding human insulin analog in which threonine in position B29 has been replaced by lysine to which myristic acid is covalently attached. The modified insulin binds reversibly to HSA, and its half-life is increased from 4–6 min to 5–7 h (Sleep 2014). More recently, Tresiba® (Insulin degludec) has been developed. In this insulin form, palmitic acid is conjugated to the mutated lysine in position B29 through a gamma-L-glutamyl spacer, and in this case the half-life has been increased to more than 24 h in the blood of patients (Sleep 2014). In principally the

same way has the in vivo half-life of the glucagon-like peptide 1 (GLP-1) been extended. In liraglutide (Victoza® and Saxenda®) Lys37 has been substituted by arginine, and palmitic acid has been coupled to Lys26. Liraglutide has a half-life of 11–15 h in contrast to the endogenous hormone that has a half-life of ca. 2 min. Furthermore, semaglutide is a modified version of GLP-1 to which stearic diacid is bound, and this product has a half-life of 160 h allowing once-weekly dosing (Sleep 2014). Attachment of a fatty acid is also able to result in albumin binding of other therapeutics like cisplatin. In addition to fatty acids, also a small organic molecule based upon 4-(p-Iodophenyl) butanoic acid derivatives, called Albutag, and several peptides can imply albumin binding. For example, the binding of Albutag to a single-chain antibody has increased its half-life 40-fold in a tumor-bearing mouse model (Sleep 2014). For more examples, see the review of Bern et al. (2015).

Exogenous double-stranded siRNA can result in potent and sequence-specific, posttranslational gene silencing, but their in vivo use is impeded by a poor stability and short half-life. Bienk et al. (2015) observed that although cholesterol is not a known ligand for HSA, siRNA functionalized with two cholesteryl moieties exhibited high and specific affinity to the protein ($K_{ass} = 10^7\ M^{-1}$) and a sixfold increase in serum half-life in NMRI mice.

1.7.2 Covalent Binding of Modified Therapeutics

Polypeptide therapeutics can be modified by a maleimide- or thiol-containing compound which ensures conjugation to Cys34. This approach has been used in the case of, for example, aldoxorubicin (doxorubicin), insulin, methotrexate, exendin-4, protamine, growth hormone, the antiviral peptide PC-1505, the opioid agonist dynorphin A, YY peptide, and the granulocyte colony-stimulating factor (Elsadek and Kratz 2012; Sleep 2014; Bern et al. 2015). Usually, the half-lives of the therapeutics are increased very much to several hours or even days. Using this approach for increasing half-lives, it is necessary to release the prodrug or the drug itself from HSA. One way of obtaining this release is to use an acid-sensitive linker. In this way the compound can be set free extracellularly in the slightly acidic environment often present in, for example, tumor tissue or intracellularly in acidic endosomes or lysosomal compartments after cellular uptake. Alternatively, the linker can be hydrolyzed by making use of redox enzymes or albumin's enzymatic properties (see Sect. 1.11).

Using this way of extending half-lives and that mentioned in Sect. 1.7.1, one should be aware of the risk that binding of the modified therapeutic could displace albumin-bound drugs, or other ligands, simultaneously given to the patient. In addition, the bound therapeutic could interfere with albumin binding to receptors such as FcRn and thereby interfere with the half-life, distribution, or metabolism of itself and of albumin (see Sect. 1.3).

1.7.3 Genetic Fusion to HSA

If the gene of the therapeutic polypeptide or protein is available, then half-life extension can also be achieved by albumin fusion technology, where the therapeutic of interest is expressed together with albumin as a single polypeptide chain. If sufficient linker space is given, albumin and the therapeutic are able to fold correctly. A favorable feature of this technique is that it allows a simple one-step synthesis process with no need for in vitro chemical linking steps. The safe and general utility of albumin fusions can be seen by considering the number of fusions approved or in clinical development. Some examples can be mentioned here: coagulation factors (FVIIa, FIX, and von Willebrand factor), anticoagulants (hirudin, infestin, and barbourin), growth factors (erythropoietin and G-CSF), cytokines (IL-2, IL-1ra, interferon-α-2b, and interferon-β), GLP-1, insulin, and thioredoxin-1. For reviews, see Sleep (2014) and Bern et al. (2015).

The therapeutic molecule is fused either to the N- or C-terminus of albumin – or simultaneously to both ends. If fused to the C-terminus, the therapeutic can perhaps interfere with the pH-dependent binding of albumin to FcRn and thereby affect the half-life of the product (see Sect. 1.3). By contrast, nothing indicates that fusion to albumin potentiates the immune response to the therapeutic polypeptide or protein or to albumin itself.

1.8 Targeting

In addition to increasing the stability and half-life of drugs and therapeutics, native and modified HSA can act as a versatile and effective delivery tool for them and thereby increase their therapeutic effect and reduce unwanted side effects. The function can be exerted in three principally different manners, namely, by passive accumulation at disease sites, by adding a tag to the protein which can be recognized by specific receptors, or by mutating the protein.

1.8.1 Passive Targeting

HSA, with or without cargo, accumulates within tumor interstitium due to the enhanced permeation and retention effect (EPR). This effect occurs due to the extensive vascularization in these tissues and to the combined effects of a leaky, immature capillary network and an impaired lymphatic drainage. A supplementary explanation for albumin accumulation in tumors is based on albumin interaction with receptors. Thus, HSA could transcytose across the endothelium of the capillaries via the gp60 or FcRn receptor and bind to SPARC in the interstitium of the tumor

(see Sects. 1.3 and 1.4). In addition to increased interstitial accumulation, tumor cells seem to have increased uptake and catabolism of albumin.

HSA can also accumulate in other types of diseased tissue such as in rheumatoid arthritis and in other inflamed tissues (Neumann et al. 2010). This type of accumulation is most probable also the result of a local EPR effect and the presence of SPARC (Sleep 2014).

The therapeutic potential of passive targeting of HSA in tumors can be illustrated by using *S*-nitrosated HSA as an example. Thus, Ishima et al. (2012a) found that *S*-nitrosated HSA accumulates in the tumor of C26 tumor-bearing mice due to the EPR effect and causes a pronounced cell death. Experiments with an *S*-nitrosated version of recombinant HSA dimer showed that both NO and the dimer itself enhance the EPR effect and that the modified dimer accumulates more than the corresponding monomer in the tumor. Furthermore, *S*-nitrosated HSA dimer delivers large amounts of cytotoxic NO into the tumor tissue. The antitumor effect of the *S*-nitrosated dimer can be further increased by adding more NO moieties to the protein (or by pegylation): binding of 13.5 mol NO/mol protein, using 2-iminothiolane as a linker, resulted in a ten times higher antitumor activity in the mice as compared to a preparation having only 1.5 mol NO/mol protein (Ishima et al. 2014). As mentioned, in addition to having a good antitumor effect, *S*-nitrosated HSA dimer can increase the EPR effect. This results in a higher concentration of the compound in the tumor. However, it can also increase the accumulation and thereby antitumor effect of other antitumor agents simultaneously given to the mice such as micelles like HPMA-ZnPP and liposomes like Doxil (Kinoshita et al. 2015).

Cell influx of NO mainly takes place by cell-surface protein disulfide isomerase, and apoptosis is mainly the result of ROS induction and activation of enzymes such as caspase-3 and heme oxygenase-1. The administration of *S*-nitrosated albumin is safe, because it has no effect on blood pressure, heart rate, or on several biochemical markers (Kinoshita et al. 2015).

1.8.2 Active Targeting

Targeting to cells and organs can be achieved by adding a targeting ligand to HSA. The ligand is targeted to a receptor in the wanted cell type, and by coupling a therapeutic to the albumin-ligand complex a treatment will become much more specific, and the toxic side effects will be reduced. In this respect, the liver has been one of the most desirable target organs. For example, Kupffer cells in the liver possess mannose receptors, and mannosylated HSA can selectively transfer NO to these cells and thereby be used in the treatment of ischemia/reperfusion injuries (Taguchi et al. 2015). The liver also contains hepatic stellate cells having receptors for mannose-6-phosphate, and HSA with this ligand can target antifibrotic drugs to these cells and thereby to the liver (Taguchi et al. 2015). Coupling of 5-fluorouracil to galactosylated HSA results in a potentially useful complex in the treatment of

liver carcinoma, because it can bind to asialoglycoprotein receptors on the hepatocytes (Cai et al. 2006). A similar approach can be made by using lactosaminated HSA, because the outlying galactose moiety can bind to the same type of receptor. In this way can adenine arabinoside-AMP, fluorodeoxyuridine, and doxorubicin be targeted to the liver with good therapeutic results; see Fiume and Di Stefano (2010) who discuss the possible pros and cons of these treatments.

HSA nanocapsules can be surface modified with folic acid, and this complex can interact with folate receptor beta specifically expressed by activated macrophages. In this way, it should be possible to deliver co-bound therapeutics selectively to the pro-inflammatory cells playing a key role in the development of rheumatoid arthritis without causing toxicity and collateral damage to healthy cells (Rollett et al. 2012). Finally, cationized HSA itself and proteins and nanoparticles conjugated to it can cross the blood-brain barrier (Sleep 2014).

1.8.3 Mutated HSA

Not much work has been carried out for testing whether mutants of HSA can be used for targeting. Perhaps the work of Iwao et al. (2007, 2009) can give some hints in that respect. These authors studied the biodistribution of a large number of genetic variants of the protein in mice. They found that the mutations Cys177→Phe, Lys240→Glu, and Glu321→Lys and two examples of a shortening and modification of the C-terminal end resulted in a 2.2- to 20-fold increase in liver uptake and that the mutations Cys177→Phe, Lys313→Asn, and Lys541→Glu increased kidney clearance by a factor 2.2–4.4. Results like these could be useful when designing recombinant, therapeutic albumins or albumin products with a modified cell or organ uptake. Actually, the recombinant mutation of Arg410 to Ala resulted in a more than 27-fold increase of liver clearance, whereas uptake by kidneys, spleen, lungs, and heart were not significantly affected (Iwao et al. 2006).

1.9 Nanoparticles

The use of many drugs, for example, chemotherapy agents and agents for gene therapy, is hampered by low solubility in aqueous media, a pronounced toxicity, and/or fast in vivo degradation. One way of reducing or even solving these problems is to encapsulate the agents in nanoparticles. Albumin seems to be particularly useful in this respect, because it is stable, biodegradable, nontoxic, nonimmunogenic, and can readily bind various drugs. Nanoparticles made of albumin are easy to prepare under soft conditions, and a large variety of protocols for making them exist based on desolvation, emulsification, thermal gelation, nano spray drying, the nab technology, or self-assembly. Because functional groups exist on the nanoparticle surface, it is possible to harden the particles by cross-linking and to attach targeting

ligands or compounds which imply a lower toxicity, because side effects are reduced. Among the two latter types of molecules are galactosamine, folate, polyethylene glycol, surfactants, cationic or thermosensitive polymers, and peptides like Arg-Gly-Asp-containing peptide; even proteins can be covalently bound to the surface of HSA nanoparticles for increasing brain uptake. The first commercial product based on protein nanoparticles in oncology was albumin-bound paclitaxel (Abraxane®) approved by the FDA in 2005. Since then, albumin nanoparticles encapsulating other active compounds have been fabricated, namely, doxorubicin, cisplatin, docetaxel, rapamycin, vinblastine sulfate, mitoxantrone, ferulate, iron oxide, EDTA, and octyl aldehyde. The purposes of making such particles are a potential use in the treatment of different cancer forms and vascular diseases as well as for imaging and chelation therapy. Many of these particles are currently in different phases of clinical trials. For reviews, see Elsadek and Kratz (2012), Elzoghby et al. (2012), and Bern et al. (2015).

1.10 Antioxidant Activities

Free radicals are normal components of cellular oxygen metabolism in mammals, and they often play a central role in signaling processes. However, radicals can be produced in surplus and thereby lead to oxidative stress resulting in reversible and irreversible modifications of sensitive macromolecules. Therefore, it is essential to have well-developed defense mechanisms against this toxicity. In extracellular fluids, a major antioxidant role is performed by albumin, and it can diminish the amounts of different reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Quinlan et al. 2005; Roche et al. 2008; Anraku et al. 2013). In addition to directly interacting with ROS and RNS, albumin can to a certain extent prevent their formation.

HSA can act as a primary antioxidant and prevent ROS and RNS formation by binding transition metal ions. Most importantly, HSA binds copper ions with a high affinity, but the protein can also bind vanadium, cobalt, and nickel ions as well as heme and iron ions, the latter under conditions of iron overload or pronounced hemolysis (Quinlan et al. 2005; Roche et al. 2008). The activities of NO and sulfur-containing organic compounds like homocysteine are reduced through binding to Cys34 of HSA. Interestingly, albumin-bound bilirubin, but not free bilirubin, has antioxidant properties.

HSA can protect ligands against oxidation. For example, polyunsaturated fatty acids bind with a high affinity to the protein, and thereby they become protected from oxidant-mediated damage (Roche et al. 2008).

HSA can also act as a secondary antioxidant and scavenge preformed ROS and RNS. This activity can mainly be attributed to its single thiol group, Cys34, which accounts for ca. 80 % of the reduced thiols in human plasma (Anraku et al. 2013). The thiol group can react with several oxidizing species and become oxidized reversibly to sulfenic (SOH) or sulfinic acid (SO_2H) or irreversibly to sulfonic acid

(SO_3H). Actually, the redox state of Cys34 has been suggested as a useful biomarker for oxidative stress (Anraku et al. 2013). In addition to Cys34, also methionine residues and, probably to a lesser degree, tryptophan, arginine, tyrosine, lysine, threonine, proline, and valine residues contribute to the antioxidant properties of HSA. The involvement of the different amino acid residues depends on their molecular surroundings in the protein and on the type of oxidant (Iwao et al. 2012; Anraku et al. 2015).

1.11 Enzymatic Properties

HSA and some of its ligand complexes possess enzymatic properties which are useful both in vivo and in vitro (Kragh-Hansen 2013). The most pronounced of the many activities of HSA are different types of hydrolysis. Key examples are esterase-like activities involving Tyr411 (Fig. 1.4) or Lys199 and the thioesterase activity of Cys34. In the first case, hydrolysis involves water and both products are released, whereas in the latter cases one of the products is set free, and the other stays covalently bound to the protein. The protein has great impact on the metabolism of, for example, eicosanoids and xenobiotics. HSA is also useful in detoxification reactions, for activating prodrugs and for binding and activating drug conjugates.

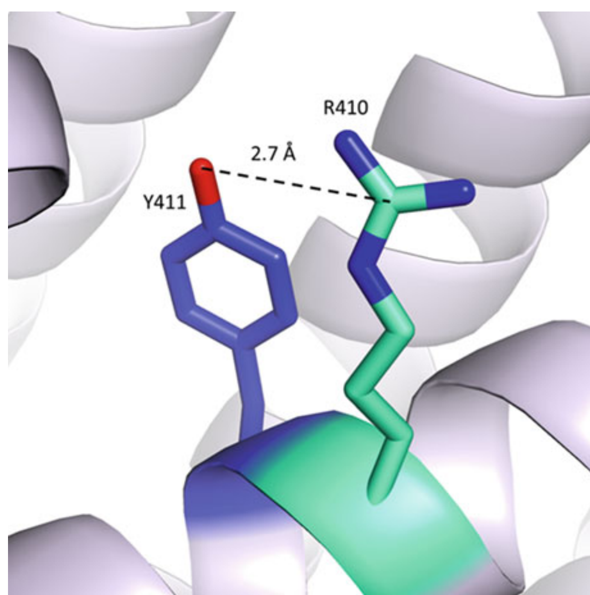

Fig. 1.4 A close-up of the nitrogen atoms of Arg410 and the phenolic oxygen of Tyr411 in subdomain IIIA, which are important for the esterase-like activity of HSA. The interresidue distance is 2.7 Å. PDB ID: 1BM0

The protein can be used to construct smart nanotubes with enzymatic properties useful for biomedical applications. Albumin with a metal ion-containing complex is capable of facilitating reactions involving reactive oxygen and nitrogen species and can be used for nanoparticle formation. Thus, even though the enzymatic properties of HSA are not always appreciated, they can be physiologically relevant and useful for biomedical and pharmaceutical purposes.

1.12 Other Functions

Physiologically, HSA may influence microvascular integrity, aspects of the inflammatory pathway, including neutrophil adhesion, and the activity of cell signaling moieties (Quinlan et al. 2005; Spinella et al. 2016). The protein also has an antithrombotic, anticoagulant effect due to its capacity to bind nitric oxide (Spinella et al. 2016). In addition, it possesses immune-modulating effects. Finally, albumin is a negative acute-phase protein. Perhaps one or more of these activities can be the object for pharmaceutical or pharmacological intervention in the future.

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