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REVIEW

Physiological and pathological changes in the redox state of human serum albumin critically influence its binding properties

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Binding and transport of a number of endogenous and exogenous compounds is an important function of the main plasma protein, albumin. *In vivo* and *in vitro*, albumin may be oxidatively modified in different ways with different agents at different sites. These modifications have various consequences on the physiological functions of albumin. Diabetes mellitus, liver diseases and nephropathy are just a few examples of disorders in which oxidative stress is involved and altered albumin functions have been described. This review is focussed on the consequences of oxidative modification on the binding properties of albumin. These range from no effect to decreased or increased binding affinities depending on the ligand under investigation and the type of modification. Indicators for modification include glycosylation, disulphide formation or the content of carbonyl groups. The redox state of albumin can affect the binding properties in several ways, including altered conformation and consequently altered affinities at binding sites and altered binding when the binding reaction itself is redox sensitive. The physiological or pathophysiological concentrations of different oxidatively modified albumin molecules vary over a wide range and are crucial in assessing the clinical relevance of altered ligand binding properties of a particularly modified albumin species in various disease conditions.
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Abbreviations: Cys34, cysteine34; HMA, human mercaptalbumin; HNA, human nonmercaptalbumin; SNO-alb, *S*-nitroso-albumin

Introduction

The regulation of oncotic pressure, the binding and transport of endogenous and exogenous compounds and antioxidant functions comprise a set of quite diverse functions. A single molecule is involved in all of these functions: albumin. Oxidative stress is believed to play an important role in the pathogenesis of critical clinical conditions, such as sepsis, end-stage renal disease or liver failure. Oxidative damage of albumin may impair its function. The aim of this review is to describe oxidative damage of albumin in various disease conditions, its relation to disturbed function and the possible role of damaged albumin in the pathophysiology of these conditions with special regard to liver failure.

The compounds to be bound and transported by albumin are quite diverse and include bilirubin, fatty acids, metal ions and therapeutic agents (Peters, 1996). Binding of a drug to albumin has different important effects on its pharmacokinetics. Thus, the free drug may be removed from circulation more rapidly but the free drug is the pharmacologically active form while the bound form is inactive (Fasano *et al.*, 2005).

The binding properties of albumin depend on the three dimensional structure of the binding sites, which are distributed over the molecule. The protein consists of a single polypeptide chain, which is organized in three domains, I, II and III, each consisting of two subdomains, A and B (Peters, 1996). Detailed figures of albumin and its drug binding sites have been published by Ghuman *et al.* (2005) and Figure 1 shows the structure of albumin with different binding sites based on the protein data bank files provided by this group. In principle, each modification of the protein can be expected to modify its conformation and hence its binding properties. Plasma concentrations of albumin are high. Therefore, reactive oxygen and nitrogen species and products of their reaction with other biomolecules, such as lipid peroxidation products, occur physiologi-

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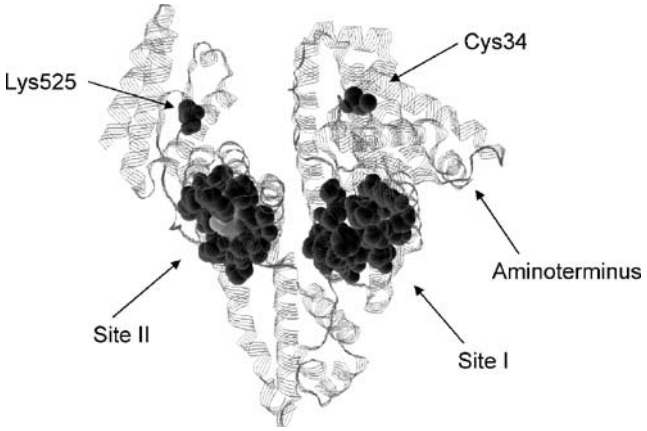

Figure 1 Albumin binding sites: the structure of albumin is shown as strands using a PDB-file of Ghuman *et al.* (2005). Amino acids of the major binding sites I and II (according to Peters, 1996), Cys34 and Lys525 are depicted in space-filling representation. The ligand diazepam within site II is shown in light grey (PDB ID: 2BXF).

cally and pathophysiologically in the vicinity of albumin. These reactive species as well as other physiological compounds like glucose lead to modifications of albumin (Halliwell and Gutteridge, 1999; Szapacs *et al.*, 2006). As these modifications are likely to cause conformational changes of the protein structure, the consequence of oxidation reactions may be changes in the binding properties of albumin even if they occur at a distance from a binding site. Oxidative stress is believed to play a pathophysiological role in different diseases (Halliwell and Gutteridge, 1999) and it is readily conceivable that the binding properties of albumin may be altered during the development of these pathologies.

Binding properties of albumin

The modes, specificities and capacities of ligand binding to albumin are as diverse as the compounds, which can be bound (Peters, 1996). The modes of binding include complex formation with metals like copper, hydrophobic and electrostatic interactions in high affinity binding and covalent binding to different amino acids. Several binding sites are distributed over the molecule according to different marker molecules (Kragh-Hansen, 1990). For fatty acids alone, seven binding sites have been described (Simard *et al.*, 2006). The most prominent sites for drugs are site I and site II located on the subdomains IIA and IIIA, respectively (Sudlow *et al.*, 1976; Peters 1996, Figure 1). Complex formation and high affinity binding are principally reversible, whereas covalent binding to albumin may occur in a reversible or irreversible manner. Different compounds show a large variation in their association constants to albumin. Most drugs have association constants in the range of 10^3–$10^6\,M^{-1}$ but constants over a wide range of 10^2 up to $10^{11}\,M^{-1}$ have been recorded (Peters, 1996; Table 1). The ligand binding properties of albumin have been reviewed recently (Bertucci and Domenici, 2002; Kragh-Hansen *et al.*, 2002; Fasano *et al.*, 2005; Ghuman *et al.*, 2005; Otagiri, 2005; Ascenzi *et al.*, 2006; Simard *et al.*, 2006). The outcome of these reviews has been that the choice of the methods used for preparation of albumin and the different types of binding assay (both factors are discussed below in greater detail) were crucial determinants of the results and therefore makes it difficult to compare results from different groups.

Table 1 Binding constants of different ligands

Binding site	*Ligand*	*K [M^{-1}]*	*Reference*
Site I	Bromcresol green	7×10^5	Peters (1996)
	Phenylbutazone	7.0×10^5	Bertucci *et al.* (1998)
	Warfarin	3.3×10^6	Peters (1996)
Site II	Ibuprofen	2.7×10^6	Peters (1996)
	Ketoprofen	2.5×10^6	Kragh-Hansen *et al.* (2002)
	Tryptophan	1.4×10^4	Peters (1996)
Aminoterminus	Co^{2+}	6.5×10^3	Kragh-Hansen (1981)
	Cu^{2+}	1.5×10^{11}	Masuoka *et al.* (1993)
	Ni^{2+}	3×10^5	Kragh-Hansen (1981)

The redox state of albumin

Albumin contains a total of 35 cysteine residues. Thirty-four of these are involved in intramolecular disulphide bonds while cysteine34 (Cys34) remains free (Peters, 1996, Figure 1). In plasma, there exist several sulphydryl/disulphide couples including the Cys34 of albumin. In healthy adults, about 70–80% of the Cys34 in albumin contains a free sulphydryl group (human mercaptalbumin, HMA); 25% of the Cys34 forms a disulphide with small sulphydryl compounds like another cysteine, homocysteine or glutathione (human nonmercaptalbumin1, HNA1); and a small fraction of the Cys34 is more highly oxidized to the sulphinic or sulphonic acid form (human nonmercaptalbumin2, HNA2) (Hayashi *et al.*, 2000). The sulphenic acid form was described as a reactive intermediate (Claiborne *et al.*, 1999; Carballal *et al*, 2003). Another possible oxidative modification of Cys34 is the nitrosylation by nitric oxide (NO). Nanomolar amounts of nitroso-albumin have been reported *in vivo* (Jourd'Heuil *et al.*, 2000; Marley *et al.*, 2001; Stamler, 2004; Zhang and Hogg, 2005). An overview of the different derivatives of Cys34 is given in Figure 2.

Albumin comprises the largest thiol pool in plasma. However, other thiol containing compounds with a low molecular weight exist (Figure 2a). This pool of compounds in plasma gives rise to thiol exchange reactions leading to a number of disulphides (Figure 2b) and mixed disulphides including albumin (Figure 2c). The extracellular redox potential of the cysteine/cystine redox couple was reported to be kept constant and important for regulation of intracellular functions (Jones, 2006). The plasma concentration of albumin (up to 700 μM) is far higher than the plasma concentrations of cysteine (30–50 μM) or glutathione sulphydryl (low micromolar range) (Jones *et al.*, 2002; Moriarty-Craige and Jones, 2004). Whether the redox potential of Cys34 and its disulphides is regulated or is merely a consequence of protein aging, or whether it serves as a kind

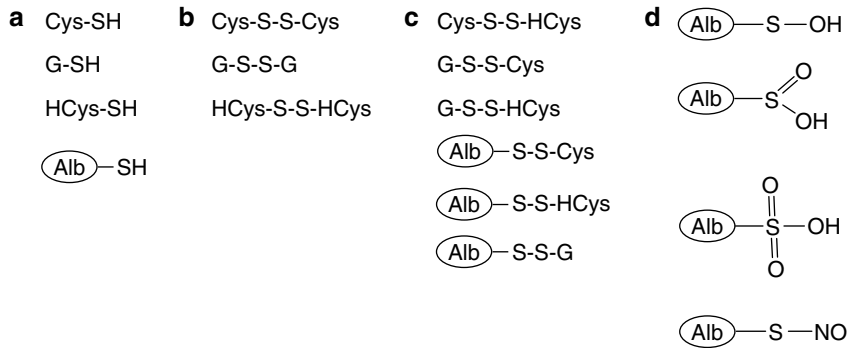

Figure 2 Major sulphur compounds in plasma: (**a**) reduced forms; (**b**) disulphides; (**c**), mixed disulphides; (**d**) higher oxidized forms of albumin (sulphenic acid, sulphinic acid, sulphonic acid, SNO-alb. Alb, albumin; Cys, cysteine; G, glutathione; HCys, homocysteine.

of redox pool regulating the redox potential of the less concentrated small sulphydryl compounds remains unclear.

As the major plasma protein, albumin is expected to be a target of modification during oxidative stress. Only recently, it was reported that the *in vitro* exposure of albumin to peroxyl radicals leads to an increase in the disulphide content of albumin (Ogasawara *et al*., 2007). The fraction of HNA is increased during the course of several pathologies like renal dysfunction (Soejima *et al*., 2004; Terawaki *et al*., 2004), various liver diseases (Sogami *et al*., 1985; Oettl *et al*., 2006), coronary artery disease (Kadota *et al*., 1991), diabetes mellitus (Suzuki *et al*., 1992), senile cataract (Hayashi *et al*., 2000), invasive surgery (Hayakawa *et al*., 1997), and during intensive exercise (Imai *et al*., 2002) and aging (Era *et al*., 1995). The fraction of HNA was negatively correlated with creatinine clearance in patients with renal dysfunction (Terawaki *et al*., 2004) and, during intrauterine growth restriction, the fraction of HNA2 in maternal plasma was about twice as high as in control plasma (Bar-Or *et al*., 2005).

Besides Cys34, other amino acids are prone to attack of reactive species. An important parameter for the oxidative modification of a protein and a widely used marker for oxidative stress is the content of carbonyl groups (Levine, 2002; Dalle-Donne *et al*., 2003b), which may arise from direct reaction of a protein with reactive species or with intermediates of lipid peroxidation. Different diseases such as liver failure, diabetes mellitus, sepsis, Alzheimer's disease, chronic renal failure or rheumatoid arthritis lead to an increase of the carbonyl content of plasma proteins (Dalle-Donne *et al*., 2003a; Oettl *et al*., 2006). In some cases, the treatment of a disorder becomes a source of oxidative stress, as in the intravenous administration of iron in haemodialysis patients, which was shown to increase both the carbonyl content of plasma proteins, especially fibrinogen and albumin, and the fractions of HNA1 and HNA2 (Anraku *et al*., 2004).

Glycosylation of a protein occurs via the reaction of the sugar's carbonyl group with the protein's amino group in a reversible manner (Halliwell and Gutteridge, 1999). The second reaction step is a slow but less reversible rearrangement, which leads to a so-called Amadori product. By this reaction, a carbonyl group is introduced into the protein and by the determination of carbonyl groups, a glycosylated protein is detected as being oxidatively modified. The fraction of glycosylated albumin in healthy persons varies from about 1% (Peters, 1996) up to 10% (Shaklai *et al*., 1984) and, during diabetes mellitus, this proportion increases two- to threefold (Bourdon *et al*., 1999). The quantification of glycosylated albumin has been suggested as a marker for glucose control in diabetes, as an alternative to HbA1c (Chujo *et al*., 2006).

Under normal conditions, albumin has a mean lifetime of about 27 days (Peters, 1996), the turnover rates of albumin are altered by several disease conditions: for example, albumin synthesis is decreased in liver disease, whereas increased loss of albumin occurs in nephrotic syndrome or protein-losing enteropathy. During aging of the albumin molecule, different oxidation reactions may occur at different locations. Based on the presence of carbonyl groups, albumin and fibrinogen have been reported to be the main protein targets of oxidative stress in plasma (Shacter *et al*, 1994; Himmelfarb and McMonagle, 2001; Michelis *et al*., 2003). The oxidative modifications of albumin have different consequences for the functions of the molecule. The pharmacokinetics of albumin itself are changed, as oxidized forms are removed faster from circulation (Bito *et al*., 2005; Iwao *et al*., 2006a, b). Antioxidant functions of albumin are disturbed as the free sulphydryl group of Cys34 plays an important role for this function (Bourdon *et al*., 1999; Bourdon *et al*., 2005) and the drug binding properties of albumin are changed in different ways as discussed below.

Binding properties and oxidation of albumin

High affinity binding

Most of the available studies on the binding characteristics of oxidized albumin were performed using *in vitro* oxidized albumin. Different methods for oxidations were used as well as different methods to monitor oxidation. Incubations with ascorbic acid in the presence of oxygen and metal ions (trace amounts or μM concentrations added), hydrogen peroxide, chloramine T, glucose, cystine or homocystine and NO have all been used as methods of generating oxidized albumin. Indicators for the redox state of albumin were the content of

SH-groups, the fractions of HMA and HNA, the content of carbonyl groups, formation of tyrosine dimers, glycosylation and the content of *S*-nitroso groups. The methods used for determination of binding range from quantification of unbound ligand by high-performance liquid chromatography to titrations and determination of binding capacity and binding constants by fluorescence quenching techniques, circular dichroism, nuclear magnetic resonance or rate of dialysis techniques. In Table 2, the changes in high affinity binding of ligands to oxidized albumin and the different methods of oxidative modification are summarized.

In a study by Meucci *et al*. (1991), *in vitro* oxidized albumin had a higher affinity for bromcresol green compared with the unmodified protein. This finding would have implications for the routinely performed quantification of albumin, if *in vivo* oxidized albumin were to have similar properties. In addition, HNA was found to have an increased binding capacity for bromcresol purple compared with HMA and the lower the SH-content of albumin, the higher was the albumin concentration determined by the bromcresol purple method (Muramoto *et al*., 1999). Pitfalls of the quantification of albumin by the bromcresol green method in samples from cirrhotic patients have been reported (Watanabe *et al*., 2004). Moreover, the capacity for bilirubin binding and the affinity constant for progesterone were decreased in oxidized albumin (Meucci *et al*., 1991).

A number of studies concerning structure and function of oxidized albumin came from Otagiri and Kragh-Hansen. The effect of oxidation on ligand binding turned out to be dependent on the oxidant used. The binding of warfarin, which is a ligand for site I was not affected by albumin oxidation with ascorbic acid/$FeCl_2$ (Anraku *et al*., 2001; Iwao *et al*., 2006b). In contrast, albumin oxidized with chloramine T or albumin of haemodialysis patients showed a decreased binding of warfarin compared with native control albumin or albumin from control subjects, respectively (Anraku *et al*., 2003; Mera *et al*., 2005). Surprisingly, glycosylation of recombinant human albumin resulted in a slight increase of warfarin binding (Nakajou *et al*., 2003). On the other hand, the binding of ketoprofen, a site II ligand, was decreased by all modes of albumin oxidation and also in albumin from haemodialysis patients. Generally, the binding properties of site II were more affected by oxidation compared with those of site I.

Although albumin is not a classical glycoprotein, in healthy persons about 1–10% of albumin is non-enzymically

Table 2 Oxidation of albumin and its effect on high affinity binding

Mode of oxidation	*Indicator of oxidation*	*Ligand used for binding*	*Change*	*Reference*
Ascorbic acid	Carbonyl groups	Bromcresol green	↑	Meucci *et al.* (1991)
	Tyrosine dimers	Bilirubin	↓	
		Progesterone	↓	
Ascorbic acid/$FeCl_2$	Carbonyl groups	Warfarin	±	Anraku *et al.* (2001)
Chloramine T		Ketoprofen	↓	
H_2O_2				
Ascorbic acid/$FeCl_2$	Carbonyl groups	Warfarin	±	Iwao *et al.* (2006a, b)
		Ketoprofen	↓	
Chloramine T	Carbonyl groups, fraction of HNA2	Warfarin	±, ↓[a]	Anraku *et al.* (2003)
		Ketoprofen	±, ↓[a]	
Haemodialysis	Carbonyl groups	Warfarin	↓	Mera *et al.* (2005)
	HMA/HNA ratio	Ketoprofen	↓	
Glucose	% glycosylated albumin	Dansylsarcosine	↓	Wörner *et al.* (1990)
Glucose	% glycosylated albumin	L-tryptophan	±	Bohney and Feldhoff (1992)
Glucose	Mol glucose/ mol albumin	Dansylamide	±	Okabe and Hashizume (1994)
		Warfarin	±	
		Phenylbutazone	↓	
		Dansylproline	↓	
		Ibuprofen	↓	
		Flufenamic acid	↓	
Glucose	% glycosylated albumin	Warfarin	↑	Nakajou *et al.* (2003)
		Dansylsarcosine	↓	
Liver failure		Dansylsarcosine	↓	Klammt *et al.* (2001)
Cystine	HNA1	L-tryptophan	↓	Kawakami *et al.* (2006)
		Cefazoline	↓	
		Verapamil	↑	
Homocysteine	Protein bound homocysteine	Salicylic acid	±	Perna *et al.* (2006)
		Warfarin	±	
		Diazepam	↓	
NO	SH-content	Palmitate	↑, ↓[b]	Burczynski *et al.* (1995)
NO	SH-content	Phenolsulphophthalein	↓	Kashiba-Iwatsuki *et al.* (1997)
Ethacrynic acid	Molecular mass	Diazepam	±	Bertucci *et al.* (1998)
		Phenylbutazone	±	
		Bilirubin	↑	

Abbreviations: HMA, human mercaptalbumin; HNA, human nonmercaptalbumin; NO, nitric oxide.
[a]Depending on concentration of oxidant.
[b]Depending on concentration of ligand.

glycosylated (Shaklai *et al*., 1984; Peters, 1996) and glycosylated albumin accounts for about 80% of circulating glycosylated protein (Cohen, 2003). Shaklai *et al*. (1984) reported that glycosylation alters the conformation of albumin and its binding functions. While haemin affinity was unchanged the affinity of glycosylated albumin to bilirubin was reduced to about 50% compared with untreated albumin. In another paper of that time, the binding of monoacetyldiaminophenyl sulphone – a ligand binding at the bilirubin binding site – was found to be diminished in glycated albumin while diazepam binding (site II) was not affected (Karp *et al*., 1985). Meanwhile, different effects of glycosylation on ligand binding of albumin have been found. Binding of dansylsarcosine to site II was diminished in affinity and kinetics in albumin with a degree of glycosylation comparable with that of diabetic patients (Wörner *et al*., 1990). The binding of tryptophan – also a site II ligand – was not disturbed by glycosylation of albumin (Bohney and Feldhoff, 1992). Okabe and Hashizume (1994) used a number of ligands for the investigation of binding properties of glycosylated albumin with about 2 mol glucose bound to 1 mol albumin. Binding of the site I ligands, dansylamide and warfarin, was the same in modified and unmodified albumin while phenylbutazone was less bound compared with unmodified albumin. Binding of the site II ligands, dansyproline and ibuprofen, was found to be markedly decreased and that of flufenamic acid only slightly decreased. In contrast, Nakajou *et al*. (2003) reported glycosylation, with up to about 9 mol glucose/mole albumin, enhanced warfarin binding to recombinant albumin.

These last reports demonstrate the importance of a careful and detailed assessment of how the albumin was prepared and the methods used to measure ligand binding. Thus, Wörner *et al*. (1990) prepared glycosylated albumin from fatty acid-free human albumin and quantitated bound glucose by affinity chromatography, which gives the percentage of glycosylated albumin (5–16%). They measured ligand binding by a kinetic fluorescence stop flow method. Bohney and Feldhoff (1992) prepared glycosylated albumin after defatting and refatting it, characterized the amount of glycation by a thiobarbituric acid method giving mol ketoamine bound per mol albumin (up to 0.82 in glycosylated vs 0.15 in untreated albumin) and measured binding by the rate of dialysis method. Okabe and Hashizume (1994) used commercially available glycosylated albumin without defatting (2 mol glucose/mol albumin) and measured binding by a fluorescence method. Nakajou *et al*. (2003) prepared glycosylated albumin after defatting, measured the fraction of glycosylated albumin with affinity chromatography (71%) and performed ligand binding by a fluorescence technique.

Glycosylation of albumin gives heterogenous products, depending on the starting material used and the conditions applied. Even the same modified albumin species showed different behaviour when different probes for the same binding site were used. This demonstrates the impact of sample preparation and methods and the difficulties in comparing results of different groups. Moreover, Koizumi *et al*. (1998) reported that an early stage of glycosylation does not influence drug binding of albumin, whereas decreased binding occurs due to further glycosylation for furosemide, procaine, phenylbutazone, salicylic acid, sulphamethoxazole, tolbutamide and warfarin, but not for naproxen.

Koyama *et al*. (1999) found a correlation of carbamazepine binding in serum with the fraction of non-glycated albumin, which decreases with age and concluded that carbamazepine preferentially binds to the non-glycated form of albumin. Despite that correlation, it must be remembered that other modifications of albumin also take place with increasing age, for instance, the fraction of HNA or the carbonyl content of plasma proteins are increased (Era *et al*., 1995; Levine, 2002).

In a study using bovine serum albumin, a threefold increase in the binding capacity of copper and iron ions was found owing to *in vitro* glycation (Eaton and Qian, 2002). It was further shown that glycation of bovine albumin resulted in an increase of NO release from S-NO-cysteine.

Completely different forms of glycosylated albumin are genetic variants, which carry an oligosaccharide moiety. Several variants have been described and found to have altered fatty acid-binding properties compared with the recombinant wild-type human serum albumin (Kragh-Hansen *et al*., 1996, 2001; Nielsen *et al*., 1997). Altered drug binding properties of these variants are to be expected but no data are found in the literature.

Very recently, the binding properties of albumin samples with different fractions of HMA have been investigated (Kawakami *et al*., 2006). A decrease of the fraction of fully reduced albumin from about 70–80% to 55% by an *in vitro* reaction was accompanied by a slightly decreased binding of L-tryptophan and a pronounced decrease of cefazolin binding (site I). In contrast, binding of verapamil (site I and II) was slightly enhanced. In this study, oxidation of albumin was carried out by simple incubation at 37°C under aerobic conditions. Contrary to that report, albumin that was homocysteinylated *in vitro* had the same binding properties for the site I ligands, salicylic acid and warfarin, but showed significantly decreased binding of the site II ligand, diazepam (Perna *et al*., 2006). Possible explanations of this discrepancy are the preferred binding of homocysteine to Lys525 rather than Cys34 and the location of Lys525 within the diazepam binding site, together with the possibility of different effects resulting from cysteinylation vs homocysteinylation.

In patients with excretory liver failure, binding of dansylsarcosine was found to be less than half of that in control subjects (Klammt *et al*., 2001). Oxidative stress is involved in different forms of liver disease, particularly in acute-on-chronic liver failure, and the HMA/HNA ratio of albumin from patients with liver disease has been reported to be shifted to the more oxidized form (Oettl *et al*., 2006).

Formation of *S*-nitrosoalbumin (SNO-alb) is accepted as an important pathway for NO metabolism *in vivo*. Almost no data are available concerning the influence of S-nitrosylation of albumin on its ligand-binding properties. As the concentration of SNO-alb is quite low, compared with the large amount of albumin in plasma, this modification should not be relevant to the *in vivo* functions of albumin. It should be noted that palmitate binding to bovine serum albumin was reduced by S-nitrosylation at the first high affinity binding site but enhanced at the second low affinity site (Burczynski *et al*., 1995) and the high affinity

binding of phenolsulphophthalein was clearly reduced by nitrosylation (Kashiba-Iwatsuki *et al*., 1997). The importance of albumin for the transport and availability of NO is further discussed under 'Covalent binding'.

Cys34 may be modified by other reagents like ethacrynic acid or penicillin (Bertucci *et al*., 1998, 2001). In the case of ethacrynic acid, the binding constant of recombinant albumin for bilirubin was slightly increased while binding of diazepam and phenylbutazone were not affected. This is an example for the modification of albumin by drug binding and how drug binding interferes with the high affinity binding of other compounds. However, drug–drug interactions are beyond the scope of this article.

Albumin has binding sites for a number of metal cations including copper, nickel, calcium, magnesium, zinc, cadmium, mercury, aluminium, manganese and cobalt (Peters, 1996). Copper, nickel and cobalt deserve special attention as binding is specific and, especially for copper, the affinity constants are quite high (Table 1). The amino terminus of albumin is a high affinity complex formation site for Cu^{2+}, Ni^{2+} and Co^{2+} (Peters, 1996; Bar-Or *et al*., 2001a). During ischaemia/reperfusion, the structure of the amino terminus of albumin is changed in a way that causes the loss of its Co^{2+} binding capacity leading to the formation of an 'ischaemia-modified albumin' (Bar-Or *et al*., 2001b). Although the mechanism of this modification is not fully understood, the loss of Co^{2+} binding during ischaemia/reperfusion may be of clinical relevance, as it is the basis of an assay for the assessment of myocardial ischaemia (Bhagavan *et al*., 2003; Apple *et al*., 2005). It is likely that free radicals and other reactive species are involved in the formation of ischaemia-modified albumin, as its amount correlated with the amount of carbonyl groups in plasma proteins from systemic sclerosis patients (Borderie *et al*., 2004).

Iron binding is normally not a function of albumin. However, during different pathologies, the plasma level of non-transferrin-bound iron may rise (Pootrakul *et al*., 2004; Le Lan *et al*, 2005; Cabantchik *et al*., 2005; Lee *et al*., 2006). The chemical nature of non-transferrin-bound iron is not clear. Iron is said to be bound to a number of ligands including albumin (Hider, 2002). On the other hand, iron ions together with a redox cycler like ascorbate are used for *in vitro* oxidation of proteins (Meucci *et al*., 1991; Ogino and Okada, 1995; Anraku *et al*., 2001; Gryzunov *et al*., 2003; Baron *et al*., 2006; Temple *et al*., 2006). Albumin itself with its free sulphydryl group may obviously serve as such a redox cycler of iron (Chung *et al*., 2005). Iron overload therefore may switch albumin from an antioxidant to a pro-oxidant molecule. In addition, intravenous iron administration during haemodialysis treatment was shown to lead to an *in vivo* oxidation of plasma proteins, predominantly fibrinogen and albumin (Michelis *et al*., 2003; Anraku *et al*., 2004).

Covalent binding

Ethacrynic acid as mentioned above is an example of a drug, which is bound covalently to albumin, to Cys34, although there are also other binding sites for this compound (Bertucci *et al*., 1999). In addition, cysteine, lysine, serine and arginine have been described as covalent binding sites for drugs (Qiu *et al*., 1998). In most cases, the redox situation of albumin is not expected to be relevant for binding. In contrast, if a compound binds to Cys34, the redox situation including the reactions of Cys34 with small SH-compounds becomes important for binding. Obviously, thiol-containing drugs are of special interest in this context.

In plasma, thiol exchange reactions take place between the Cys34 of albumin and small molecular weight thiols like cysteine, homocysteine and glutathione and their disulphides and mixed disulphides (Figure 2). Thiol-containing drugs enter these reactions and binding of the compound to albumin might involve the formation of a mixed disulphide of the drug before the reaction with Cys34. The redox situation of Cys34 depends on the concentration of small molecular weight thiol compounds and the reaction of a thiol drug with Cys34 of albumin is influenced by the concentration of these compounds.

Again, Otagiri has contributed a lot of work to this topic. It was shown that a derivative of bucillamine – a cysteine derivative – had a higher reactivity with albumin in sera from rheumatic, hepatic and haemodialysis patients compared with controls (Narazaki and Otagiri, 1997). After dialysis treatment, significantly less bucillamine was albumin bound compared with the situation before the treatment. The fraction of HMA negatively correlated with the binding of the drug. However, it should be borne in mind that albumin from patients differs from albumin of control subjects in other respects than the fraction of HMA. Similarly, albumin from patients with renal failure showed different reaction kinetics with *N*-acetylcysteine and an improved binding of the drug was determined (Harada *et al*., 2004). The situation becomes even more complex if a drug binds to albumin covalently in addition to high affinity binding. For captopril, a thiol-containing angiotensin converting enzyme inhibitor, binding to albumin as a mixed disulphide was reported and, in addition, high-affininty binding was suggested (Narazaki *et al*., 1997). However, the influence of different redox states of albumin has not been investigated in detail.

An interesting aspect of drug binding to albumin as a disulphide is a chemotherapy strategy suggested by Kratz *et al*. (2000), which involves binding of a thiol prodrug to albumin after injection and release of the drug in the acidic environment of the tumour. Albumin binding of different doxorubicin derivatives to albumin has been described (Kratz *et al*., 2002; Di Stefano *et al*., 2006).

Some thiol-containing compounds serve as tumour imaging pharmaceuticals including ^{99m}Tc-cysteine or -homocysteine (Takeda *et al*., 1990) and thiol-containing gadolinium complexes (Raghunand *et al*., 2006). The effect of these compounds depends on the binding to albumin and albumin binding in this case is redox sensitive. ^{99m}Tc-homocysteine was reported to bind to HMA but not to HNA (Takeda *et al*., 1989). In addition, binding to and release from albumin and therefore the pharmacokinetics of thiol drugs are affected by the presence of other thiol compounds (Takeda *et al*., 1991; Raghunand *et al*., 2006). Experiments in mice revealed that the tumour transport of ^{99m}Tc-homocysteine depended on its binding to albumin (Takeda *et al*.,

1989, 1990, 1991) and *in vitro* albumin binding of the drug before its application may be of advantage.

NO is an endogenous compound reacting with low molecular weight thiol compounds and thiol groups in proteins to give *S*-nitroso compounds, besides other products (Mirza *et al*., 1995). In SNO-alb, NO is covalently bound to the SH-group of Cys34. The physiological plasma level of SNO-alb was a matter of debate but meanwhile there exists agreement that the level in healthy humans is in the nanomolar range (Marley *et al*., 2001; Tsikas and Frölich, 2002; Tsikas *et al*., 2002; Ng *et al*., 2004; Stamler, 2004; Zhang and Hogg, 2005), while under pathological conditions, micromolar concentrations of SNO-alb may occur (Ottesen *et al*., 2001; Bayir *et al*., 2003; Ng *et al*., 2004). Because of the low plasma concentrations of SNO-alb, nitrosylation of albumin is not of relevance for its binding of other compounds. However, as NO is bound to a thiol group, the redox state of Cys34 is important for the binding of NO to albumin. The most important small molecular weight substrates for S-nitrosylation *in vivo* are cysteine and glutathione. Although the detailed mechanisms of the metabolism of SNO-alb are poorly understood, a number of S-transnitrosylation reactions in the blood involving albumin/SNO-alb, glutathione/S-NO-glutathione and cysteine/S-NO-cysteine have been described (Scharfstein *et al*., 1994; Jourd'Heuil *et al*., 2000; Tsikas *et al*., 2001). It has been reported that metal ions and reducing agents like ascorbic acid (Singh *et al*., 1996; Scorza *et al*., 1997), compounds like *N*-acetylcysteine (Orie *et al*., 2005) and dithiols like thioredoxin and dihydrolipoic acid (Stoyanovsky *et al*., 2005) stimulate the decomposition of *S*-nitrosothiols. The role of SNO-alb as an NO-donor has been tested in several systems (Hallström *et al*., 2002; Bauer *et al*., 2004; Orie *et al*., 2005; Semroth *et al*., 2005). In clinical conditions with oxidative stress and inflammation, Cys34 will be the target of oxidation as well as nitrosylation following increased NO production by inducible nitric oxide synthase. SNO-alb has important vasodilatory properties (Keany *et al*., 1993) and has been postulated to represent a stable reservoir of NO (Orie *et al*., 2005). It may be hypothesized that oxidation of Cys34 may impair its buffer function for NO resulting in a detrimental release of NO under certain conditions.

There are antiarthritic drugs like auranofin (Et_3PAu-*S*-actylthioglucose), which are based on compounds containing gold(I) complexes and a thiol moiety. Cys34 is the principal binding site of auranofin on albumin and different adducts have been found (Coffer *et al*., 1987; Shaw, 1989; Dhubhghaill *et al*., 1992; Roberts *et al*., 1996). A complex series of reactions is involved in the albumin binding of this group of compounds to HMA and HNA (Shaw, 1989; Roberts *et al*., 1996) including ligand exchange and liberation of acetylthioglucose, which may in turn modify the redox situation on Cys34. Thereby, binding of a drug to an albumin molecule may influence the redox state of another albumin molecule. Another example of the influence of ligand binding on the redox state of albumin with a complete different mechanism is facilitation of oxidation of Cys34 by fatty acid binding (Gryzunov *et al*., 2003).

Other gold complexes like $[Au(S_2O_3)_2]^{3-}$, $[Au(CN)_2]^-$ and $AuPEt_3Cl$ have been reported to react with Cys34 and other amino acids of albumin and to give different adducts (Knisch and Stephan, 1984; Christodoulou *et al*., 1994; Beck *et al*., 2004; Talib *et al*., 2006). The blocking of Cys34 by iodoacetamide and cysteine was shown to diminish the reaction of albumin with gold(I) complexes and the HMA/HNA ratio was said to influence the bioavailability of these drugs.

Cisplatin is a platin(II) complex, which is extensively used as an anticancer drug. The binding of cisplatin and other platin complexes to albumin has been investigated since the 1980s (Gonias and Pizzo, 1983; Pizzo *et al*., 1988). Up to 10 molecules of cisplatin were found to be bound by one molecule of albumin (Timerbaev *et al*., 2004). Therefore, a number of binding sites have to exist. Cys34 was reported to be one of these (Gonias and Pizzo, 1983; Pizzo *et al*., 1988). However, more recently methionine has been reported to be a more important sulphur binding site for cisplatin compared with Cys34 in recombinant human albumin (Ivanov *et al*., 1998). A platin(IV) complex, *trans*, *cis*-$[Pt(en)(OH)_2I_2]$ reacted only with the sulphydryl form of recombinant human albumin (Kratochwil *et al*., 1999). Thereby, Cys34 was oxidized probably to the sulphenic acid and higher oxidized forms. The reactions of platin complexes with albumin are diverse, depend on the nature of the complex (Kratochwil *et al*., 1999) and are not fully understood.

Nitrogen mustards, highly reactive alkylating agents, are a class of compounds formerly used in cancer chemotherapy (Chabner and Roberts, 2005). These compounds react with albumin resulting in S-alkylation of Cys34 rather than formation of a disulphide (Noort *et al*., 1999; Noort *et al*., 2002). Different reaction rates of HMA and HNA with these compounds and effects of alkylation on binding properties can be expected, although have not been investigated till now.

Clinical implications

The functional alteration of oxidized albumin as outlined above may have important clinical implications in critical conditions associated with high levels of oxidative stress, such as severe sepsis or acute-on-chronic liver failure (Jalan and Williams, 2002). It is very possible that the consecutive alterations of albumin function play an important role in the pathophysiology of multiorgan dysfunction. For example, altered binding of NO to albumin may be involved in the circulatory alterations observed in sepsis and liver failure. On the other hand, a disturbed transport function of oxidized albumin may impair intercellular and interorgan traffic of endogenous compounds such as fatty acids and hormones, but also delivery of drugs that are crucial in the treatment of these conditions (e.g. antibiotics).

The role of albumin in liver failure merits special consideration: first, the rate of albumin synthesis in liver cirrhosis has been found decreased in relation to the degree of liver dysfunction as assessed by the Child–Turcotte classification (Ballmer *et al*., 1993). Second, the function of this reduced pool of serum albumin is probably further hampered by oxidative damage. As mentioned above the highest levels of irreversibly oxidized HNA2 reported to date were found in patients with acute-on-chronic liver failure

(Oettl *et al*., 2006), a condition with very poor prognosis (30-day mortality in excess of 50%). Infusion of 'fresh' albumin (i.e. in its non-oxidized form) should therefore improve the overall functions of serum albumin in patients with liver failure. Interestingly, albumin infusion has been reported to prevent hepatorenal syndrome and increase survival in patients with cirrhosis and spontaneous bacterial peritonitis (Sort *et al*., 1999), which may not only be attributed to the beneficial haemodynamic effects of plasma volume expansion but also be related to an improved transport function of the expanded serum albumin pool. However, attention has to be paid to the properties of pharmaceutical albumin products. As reported by Klammt *et al*. (2001), the site II-binding capacities of commercially available albumin preparations were decreased, compared with a 'de-ligandized' reference albumin and plasma, respectively. This decline was attributed to the presence of the stabilizer, *N*-acetyltryptophan. In addition, it may be that oxidative modification of albumin during preparation also contributes to altered binding properties of commercially available albumin.

Several artificial liver support devices have been developed to improve prognosis in acute and acute-on-chronic liver failure but their clinical efficacy has been limited so far. Recently, cell-free detoxification systems such as the Molecular Adsorbents Recirculating System (MARS) and the Fractionated Plasma Separation and Adsorption (FPSA) system (Prometheus, Fresenius Medical Care, Bad Homburg, Germany) have gained rather wide-spread popularity (Stange *et al*., 2002; Rifai and Manns, 2006). These methods aim at removal of albumin-bound toxins via specific adsorbers. However, the concept of 'cleaning' the patients' own albumin is limited by the fact that part of this albumin is oxidatively damaged and probably not functioning properly even after the treatment. Therefore, considering the likely functional alterations of oxidized albumin in liver failure, other concepts exchanging rather than cleaning the patients' albumin, such as therapeutic plasma exchange (Clemmesen *et al*., 2001) or selective plasma filtration (Rozga *et al*., 2006), may be preferable for support of the failing liver.

Concluding remarks

Age and disease influence the redox state of albumin and this redox state influences the drug binding properties of albumin. This may at least in part explain the altered behaviour of drugs in pathological situations. The diversity of albumin modifications and the diversity of drugs do not allow a general conclusion on how a particular modification will affect the binding of a particular drug. As it was stated recently in a review about the allosteric modulation of drug binding to albumin (Ascenzi *et al*., 2006), cooperative equilibria and competition between ligands make it difficult to interpret ligand binding properties *in vivo* even if the effects on binding *in vitro* are known.

Conflict of interest

The authors state no conflict of interest.

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