Structure, Properties, and Engineering of the Major Zinc Binding Site on Human Albumin*

Claudia A. Blindauer†,†, Ian Harvey♭,‡, Kerr E. Bunyan¶, Alan J. Stewart‖, Darrell Sleep¶¶, David J. Harrison♭♭, Stephen Berezenko¶¶, and Peter J. Sadler‡‡

From the †Department of Chemistry, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, United Kingdom, the ‡Council for the Central Laboratory of the Research Councils Daresbury Laboratory, Warrington WA4 4AD, United Kingdom, the ¶Division of Pathology, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh EH4 2XR, Scotland, United Kingdom, ¶¶Bute Medical School, University of St. Andrews, St. Andrews, Fife KY16 9TS, Scotland, United Kingdom, and **Novozymes Biopharma Ltd., Castle Boulevard, Nottingham NG7 1FD, United Kingdom

Most blood plasma zinc is bound to albumin, but the structure of the binding site has not been determined. Zn K-edge extended x-ray absorption fine structure spectroscopy and modeling studies show that the major Zn$^{2+}$ site on albumin is a 5-coordinate site with average Zn-O/N distances of 1.98 Å and a weak sixth O/N bond of 2.48 Å, consistent with coordination to His$^{67}$ and Asn$^{99}$ from domain I, His$^{247}$ and Asp$^{249}$ from domain II (residues conserved in all sequenced mammalian albumins), plus a water ligand. The dynamics of the domain I/II interface, thought to be important to biological function, are affected by Zn$^{2+}$ binding, which induces cooperative allosteric effects related to those of the pH-dependent neutral-to-base transition. N99D and N99H mutations enhance Zn$^{2+}$ binding but alter protein stability, whereas mutation of His$^{67}$ to alanine removes an interdomain H-bond and weakens Zn$^{2+}$ binding. Both wild-type and mutant albumins promote the safe management of high micromolar zinc concentrations for cells in cultures.

Zinc is not only required for hundreds of essential extra- and intracellular proteins and enzymes but is also recruited by toxins such as anthrax lethal factor (1) and staphylococcal enterotoxin (2). There is a need to understand how zinc transport and distribution is controlled (3).

Although considerable progress has been made in the identification and study of membrane-bound zinc transporters, the molecular mechanism of extracellular zinc transport is still obscure. The total concentration of zinc in blood is high, ~15–20 μM (4), and plasma zinc concentrations are maintained at a relatively constant level, except during periods of dietary zinc depletion and acute responses to stress or inflammation, when they are depressed (5). In humans, ~98% of so-called "exchangeable" zinc in blood plasma (9–14 μM) is bound to serum albumin (6). Studies on perfused rat intestine have implicated albumin in the transport of newly absorbed zinc in portal blood, from the intestine to the liver (5). Albumin has also been shown to promote zinc uptake by endothelial cells, with receptor-mediated endocytosis as the most likely mechanism (7).

Albumin, the most abundant protein in blood plasma (~40 mg ml$^{-1}$ and 0.6 mM), is synthesized in the liver and secreted into the blood stream as a 585-residue, single-chain protein after loss of a 24-residue propeptide (8). The protein is largely α-helical and folds into three structurally homologous domains (I, II, and III), each of which contains two subdomains (A and B) (see Fig. 1A) (9). There are 35 Cys residues that form six disulfide bridges in each domain, except for domain I, which contains only five bridges and a free thiol at Cys$^{34}$.

Zinc binding to albumin has also been demonstrated in vitro, and the so-called high-affinity site for zinc on albumin displays a binding constant of $K_a \approx 10^7$ M$^{-1}$ (10–13). Although over 50 x-ray structures of albumin have been reported to date (9, 14–16), no experimental structural data are available for the zinc site on albumin. On the basis of $^{111}$Cd NMR studies, site-directed mutagenesis, and molecular modeling, we have proposed that the major zinc site on albumin, termed site A, is located at the interface of domains I and II and is formed by the side chains of His$^{67}$ and Asn$^{99}$ from domain I and by His$^{247}$ and Asp$^{249}$ from domain II (Fig. 1B) (17, 18).

Here, we report the first direct structural characterization of a zinc site on albumin, using Zn K-edge x-ray absorption fine structure (EXAFS)$^2$ spectroscopy. We also explored the possibility of engineering albums with increased or decreased zinc-binding affinity by mutating the postulated zinc-binding ligands. NMR methods were used to identify mutated and zinc-binding histidines and to probe the effects of zinc binding on the conformational dynamics of the protein. Finally, we show that albumin at physiological concentrations promotes the culture of hepatocytes at otherwise toxic zinc concentrations.

**EXPERIMENTAL PROCEDURES**

All albumin samples were prepared from purified, recombinant proteins produced as described previously (17, 27).

---

* This work was supported by the Biotechnology and Biological Sciences Research Council (Collaborative Award in Science and Engineering) (to A. J. S.), Novozymes Biopharma Ltd., a European Community Marie Curie Fellowship (to C. A. B.), the Council for the Central Laboratory of the Research Councils Synchrontron Radiation Source (to A. J. S.), the Wellcome Trust (Edinburgh Protein Interaction Centre), and the Wolfson Foundation, and an Olga Kennedy Fellowship from the Royal Society (to C. A. B.).

**The on-line version of this article (available at http://www.jbc.org) contains supplemental experimental data, Tables S1 and S2, and Figs. S1–S13.

† To whom correspondence should be addressed: Dept. of Chemistry, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, United Kingdom. E-mail: c.blindauer@warwick.ac.uk.

‡ The abbreviations used are: EXAFS, x-ray absorption fine structure; N-to-B, neutral-to-base transition; rHA, recombinant human albumin.
Mutant albumin sequences were prepared from a native human albumin cDNA by site-directed mutagenesis.

$^1$H NMR Spectroscopy—Amide proton resonances were eliminated by repeated incubation of samples with $D_2O$ (99.9%; Aldrich). One-dimensional and two-dimensional $^1$H NMR experiments were routinely carried out at 310 K on a Bruker Avance 600 spectrometer operating at 599.82 MHz using a Z-gradient triple-resonance ($^1$H, $^{13}$C, and $^{15}$N) probe head using 1 mM albumin solutions in $D_2O$ at pH = 7.3–7.4 (pH$^+$ corresponds to the pH meter reading for a solution in $D_2O$ using H$_2$O-based buffers for calibration, without further correction. These can be converted to pD by adding 0.4), containing 50 mM NaCl, 50 mM Tris-Cl and sodium formate (1 mM, as internal calibration standard; 8.48 ppm relative to sodium 3-(trimethylsilyl)propionate). Typically, 512 transients were acquired on 1 mM solutions of mutant albumins in 50 mM Tris-Cl, pH 7.3–7.4 (pH$^+$), and sodium formate (1 mM, as internal calibration standard; 8.48 ppm relative to sodium 3-(trimethylsilyl)propionate). Typically, 512 transients were acquired for the one-dimensional spectra (90° excitation pulse, 9 kHz sweep width, and 8,192 time domain data points) using a presaturation pulse sequence for residual water suppression. Two-dimensional data are shown and described in supplemental Figs. S4–S8.

$^{111}$Cd NMR Spectroscopy—$^{111}$Cd NMR experiments were acquired on 1 mM solutions of mutant albumins in 50 mM Tris-Cl, pH 7.1, 50 mM NaCl, and 10% D$_2$O, 90% H$_2$O containing 2 mol eq of $^{111}$CdCl$_2$, prepared by dissolving $^{111}$CdO (95.11% isotopic purity; Oak Ridge National Laboratory) in the minimum amount of 1 mM HCl. One-dimensional $^{111}$Cd($^1$H) NMR spectra (106.04 MHz, Bruker DMX500) were acquired at 295 K using a 10-mm Broad Band Observe (BBO) probe head, with 0.1 mM Cd(ClO$_4$)$_2$ (0 ppm) as an external standard.

EXAFS Data Acquisition—Albumin samples were 1 mM in 50 mM HEPES, which had been demetallated using Chelex resin (Bio-Rad) prior to albumin addition. Aliquots of metal chlorides (Zn$^{2+}$, Cd$^{2+}$, and Cu$^{2+}$) were added from 0.2 M stock solutions. Zn K-edge EXAFS data were recorded on EXAFS stations 9.3 and 16.5 at Daresbury Laboratory Synchrotron Radiation Source at 15 K and in fluorescence mode. Total data collection times were between 11 and 19 h per sample.

Model Building and Refinement—The EXAFS data were processed and analyzed as described previously (19) using full (three-dimensional) multiple scattering. We used our model of the zinc site (17), based on a published crystal structure at 2.5 Å of ligand-free (apo)albumin (Protein Data Bank code 1AO6 (9)), as a starting point for EXAFS data refinement. The model included the side chains of His$^{67}$, Asn$^{99}$, and Asp$^{249}$, as well as the side chain and backbone of His$^{247}$ and all atoms of Gly$^{248}$, including the peptide bond to Asp$^{249}$ (30 atoms). Analysis required application of the full curved wave theory. In addition, it was necessary to iterate between EXAFS data fitting and energy minimization within the molecular modeling package MOE (version 2004.03), using an in-house customized version of the AMBER94 force field. The final model of zinc-albumin satisfies protein-specific constraints as well, validated by PROCHECK (20) and WHAT_CHECK (21).

The starting models of the mutants were generated by analogy to the wild-type starting model (17), using Sybyl (version 6.8), employing a customized TRIPOS force field incorporating zinc-specific parameters. Molecular graphics representations were generated using MOLMOL (version 2000 K) (22) and WebLab Viewer (Accelrys).

Equilibrium Dialysis and Stability Comparisons—The affinity of albumin for zinc was determined using an adapted published procedure (23). A 1-ml aliquot of 100 μM recombinant human albumin (rHA) was dialyzed for 72 h at 310 K against 250 ml of zinc-containing buffer (25 mM HEPES and 100 mM NaCl). The free zinc concentration was adjusted by using varying concentrations of dipicolinic acid (Sigma). The exact free zinc concentrations were calculated using the “Species” module of the IUPAC Stability Constants Database (29), and the published stability constants for the Zn$^{2+}$-dipicolinic acid system (24). After dialysis, the rHA samples were subjected to rapid gel filtration (<3 min; 25 mM HEPES, pH 7.4; Sephadex G25M, pD 10, GE Healthcare) to remove any unbound or loosely associated zinc; therefore, the measured zinc contents represent zinc that remained bound to albumin under these conditions. Subsequently, the albumin:zinc ratio was determined spectrophotometrically. The albumin concentration was determined by measuring the absorbance at 279 nm, using a molar extinction coefficient of 0.558 mg$^{-1}$ cm$^{-1}$ (25). The zinc concentration was determined using the zinc indicator 4-(2-pyridylazo)resorcinol (Sigma). Calibration standards (1–25 μM Zn$^{2+}$ in 25 mM HEPES) were always run in parallel to albumin samples. The data presented are neither background-corrected nor normalized; the error bars are based on estimated errors in albumin absorbance measurements.

Cell Culture, Death, and Number Assessment—WRL-68 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in 80 cm$^2$ tissue culture grade flasks (Cellstar) in Dulbecco’s modified Eagle’s medium (containing l-glutamine; Invitrogen) supplemented with 10% newborn calf serum (Invitrogen), penicillin and streptomycin, and non-essential amino acids (Invitrogen), which include the zinc-binding histidine and cystine at a concentration of 0.2 mM each.

Cells were grown at 310 K, 5% CO$_2$ in an incubator and supplemented with fresh medium every 2–3 days or as required. Once cells became confluent, they were harvested using trypsin and EDTA.

Cells were seeded onto 12-well cell culture plates (tissue-culture-treated polystyrene, Nunclon) at 0.099–0.1 × 10$^6$
cells/ml using 0.5 ml (for assessment of albumin/zinc effects) (see Fig. 6) or 1 ml (for assessment of zinc toxicity, see supplemental Fig. S10) per well and left overnight to equilibrate. The medium was then aspirated off, and the cell layer was washed once with phosphate-buffered saline. Cells were treated with 0.5 ml of medium supplemented with 0, 60, 300, or 600 μM ZnCl₂, in the absence or presence of wild-type rHA or H67A mutant albumin with medium alone as a control. Following a 48-h incubation, the plates were analyzed using flow cytometry. Propidium iodide (1 μg/ml) was added to samples immediately prior to counting to detect cell death. Samples were vortexed and run using a Beckman Coulter EPICS cell counter. The total number of events after 60 s was recorded to determine cell numbers for comparison between groups. All treatments were carried out in triplicate on plates. Each plate was set up in triplicate as well.

RESULTS

To elucidate the residues involved in high-affinity zinc binding on albumin, we studied the metal-binding properties of wild-type rHA and the three mutants H67A, N99D, and N99H by Zn K-edge EXAFS spectroscopy, equilibrium dialysis, and ¹¹¹Cd and ³H NMR spectroscopy. The H67A mutant corresponds to a deletion of one metal ligand and was expected to bind zinc less strongly and in a different mode than wild-type rHA, and the two Asn⁹⁹ mutants were designed to replace the weak ligand asparagine (amide oxygen) with the stronger ligands aspartate (carboxylate oxygen) or histidine (imidazole nitrogen).

EXAFS Spectroscopy—For the wild-type rHA, we analyzed Zn K-edge data for three different preparations, namely Zn-rHA, Zn,Cd-rHA, and Zn,Cd,Cu-rHA (see “Experimental Procedures” and Fig. 2A). Because it is known that albumin contains several metal-binding sites (10, 13), it was important to establish that the various methods monitor zinc binding to its high-affinity site, rather than mixtures of different species. Based on previously published data (10, 11, 13, 26, 27), the latter mixed-metal preparation is expected to yield albumin with Cu²⁺ bound to the N-terminal ATCUN motif (28), Cd²⁺ bound to the so-called site B (27), and Zn²⁺ to site A (17, 27). The EXAFS spectra for these differently metallated albumins are essentially identical, indicating that under the conditions applied, the first equivalent of Zn²⁺ binds to the high-affinity site (A), rather than mixtures of different species. Based on previously published data (10, 11, 13, 26, 27), the latter mixed-metal preparation is expected to yield albumin with Cu²⁺ bound to the N-terminal ATCUN motif (28), Cd²⁺ bound to the so-called site B (27), and Zn²⁺ to site A (17, 27). The EXAFS spectra for these differently metallated albumins are essentially identical, indicating that under the conditions applied, the first equivalent of Zn²⁺ binds to the high-affinity site (A), rather than mixtures of different species. Based on previously published data (10, 11, 13, 26, 27), the latter mixed-metal preparation is expected to yield albumin with Cu²⁺ bound to the N-terminal ATCUN motif (28), Cd²⁺ bound to the so-called site B (27), and Zn²⁺ to site A (17, 27). The EXAFS spectra for these differently metallated albumins are essentially identical, indicating that under the conditions applied, the first equivalent of Zn²⁺ binds to the high-affinity site (A), rather than mixtures of different species.
 Förings of the protein, a refined model of the site was obtained. Therefore, the positions of the backbone atoms of residues 247–249 shown in Fig. 3 were representative of a family of conformers which provides a reasonable fit to the data (R = 23%).

The EXAFS spectrum of the H67A mutant (Fig. 2B, inset) differs considerably from the spectrum of wild-type rHA and is consistent with a 4(+1)-coordinate site. The loss of one ligand is also reflected in the lower intensity of the first shell peak in the Fourier-transformed spectrum. In contrast, spectra for both the Asn99 mutants exhibit comparatively small, yet significant differences form the spectrum (Fig. 2A) of wild-type rHA. All data for the mutants were well fitted using optimized models based on the in silico mutation of the respective residues (supplemental Fig. S1).

Equilibrium Dialysis—The affinity of the mutated albumins for zinc was determined by equilibrium dialysis. The moderate, i.e. high nanomolar, zinc affinity of albumins reported in the literature (log K = 6.1–7.5) (10–11,12,13) might be attributable to both the exposed nature of the postulated site and the presence of the amide side chain of asparagine as one of the four ligands, a binding group that is relatively weak and uncommon in proteins. Mutation of Asn99 to His or Asp, both of which have high affinities for zinc, was therefore predicted to increase zinc affinity, whereas the removal of the His67 ligand was expected to decrease affinity.

Fig. 4A shows a comparison of zinc affinities obtained from the equilibrium dialysis experiments. A value of log K = 7–8 can be estimated for the conditional binding constant of the first molar equivalent of Zn2+ to wild-type rHA at pH 7.4, a value in the same range as those reported previously for human serum albumin (10–11, 12, 13). Importantly, all three mutations have a pronounced effect on the affinity of albumin for zinc. The relative affinities follow the order: N99D, N99H > wild type > H67A. The binding constants of the Asn99 mutants are ~5 times greater than that of wild-type rHA, whereas that of the H67A mutant is ~10 times less. These data are in agreement with the changes expected on introducing side chains with stronger affinity than Asn (Asp and His) on the one hand and lower affinity (Ala), on the other, into the postulated zinc binding site (29).

Analysis of the EXAFS data provided a structural model of the zinc site in wild-type albumin. Single scattering analysis of the first shell (ligand or nearest neighbor) atoms for wild-type rHA containing 1 mol eq of bound Zn2+ (Zn-rHA) categorizes the zinc environment as a 5-coordinate O/N site with an average Zn-O/N distance of 1.98 (±0.02) Å, plus another more distant O/N at 2.48 Å. A theoretical molecular model based on Protein Data Bank code 1AO6 was built, in which zinc is bound by the four postulated ligands and a water molecule. The theoretical EXAFS data for this model display an R-factor of 28% with respect to the experimental data, even prior to any refinement. Using this model as the starting point and applying full multiple scattering EXAFS data fitting (Fig. 3A), combined with force-field-based energy minimization of the surrounding portions of the protein, a refined model of the site was obtained (Fig. 3B).

The imidazole Nε of His67 (1.99 Å), the side chain amide oxygen of Asn99 (2.03 Å), imidazole N-δ of His247 (1.98 Å), and carboxylate oxygen of Asp249 (1.99 Å), together with a water (1.91 Å) provide the first ligand shell, whereas the backbone carbonyl oxygen of His247 provides a sixth, more weakly bound ligand (2.48 Å). Attempts to fit the data by alternative models, e.g. by defining the δ-oxygen or nitrogen of Asp249 or Asn99 as the sixth ligand, did not yield satisfactory fits.

Intriguingly, although ligand backbone atoms and non-bonding residues are usually omitted in this method, it was necessary to include all atoms of residues His247–Gly248–Asn249 within 5 Å of the Zn2+ ion in this case, to account for all contributions to the data. However, even though highly constrained, unique positions could not be determined from the EXAFS data alone for a number of the atoms, and a small number of closely related models with similar structures were obtained. Therefore, the positions of the backbone atoms of residues 247–249 shown in Fig. 3B are representative of a family of conformers which provides a reasonable fit to the data (R = 23%).
Zinc Binding to Albumin

**FIGURE 4.** A, comparison of the affinity for zinc of wild-type (○) and mutant albumins (■, H67A; ●, N99D; □, N99H) by equilibrium dialysis (310 K; 72 h). The lines are drawn to guide the eye and do not represent quantitative fits. The results demonstrate that mutations of His97 and Asn99 affect the affinity of albumin for zinc in the manner predicted for these ligands. B, one-dimensional $^{111}$Cd spectra of wild-type and mutant human albumins (106.04 MHz; 295 K (except for the N99D spectrum, which was taken at 310 K); 1 mM Cd$_2$rHA, 10% D$_2$O, 50 mM Tris-Cl, 50 mM NaCl, pH 7.0). The effects of His67 and Asn99 mutations on cadmium binding to site A are striking and different for each mutant. Whereas deletion of His67 leads to the complete disappearance of the peak at 106 ppm (106.04 MHz; 295 K) (Fig. 4A), in contrast to wild-type rHA, for which complete disappearance is not observed. The peak at 106 ppm is in the chemical shift range expected for a site with one nitrogen and three to five oxygen donors (31). The temperature dependence of the line widths suggests that exchange processes occur in the N99D mutant, which are at an intermediate rate on the NMR time scale at 295 K.

We used $^1$H NMR spectroscopy to identify His residues of interest and to probe the effect of Zn$_{2+}$ on His residues of wild-type and mutant rHAs. High resolution $^1$H NMR studies of albumin are complicated by peak overlap and broadening. Sequential assignments are difficult to make, but with the aid of resolution enhancement techniques, two-dimensional methods, and site-directed mutagenesis, some assignments of His H-ε1 peaks in the low field region are possible.

The overall appearance of the wild-type and mutant spectra is very similar (Fig. 5A), but there are substantial changes to two low field-shifted peaks, labeled 1 and 4, and their corresponding cross peaks in the two-dimensional spectra (supplemental Figs. S4–S7 and Table S1), for all three mutant proteins, suggesting that these peaks correspond to the two histidines in the mutated zinc site, His67 and His247. As seen in Fig. 5A (bottom spectrum; see also supplemental Fig. S8 for a two-dimensional total correlation spectrum), the peaks for at least four His residues are severely broadened (or shifted) by Zn$_{2+}$ binding to rHA, most notable being the disappearance of peaks 1, 4 (His67 and His247), 6 (not assigned), and 11 (His3). In contrast, the peak for H-ε1 of His99 (labeled 7) appears to sharpen on Zn$_{2+}$ binding to rHA. Such effects could arise from chemical exchange, changes in mobility (including the direct effects of Zn$_{2+}$ binding), or changes in solvent accessibility. It is noteworthy that these spectral perturbations are also observed on addition of substoichiometric amounts of zinc (supplemental Fig. S9).

The effects of Zn$_{2+}$ on the His H-ε1 peaks of the H67A mutant (supplemental Fig. S9) were similar to those for wild-type rHA, consistent with zinc binding to the same site, as suggested by the EXAFS data. However, studies of both Asn99 mutants were hampered by reversible, temperature-dependent Zn$_{2+}$-induced protein precipitation at 310 K. This result suggests that there is a complicated relationship between Zn$_{2+}$-induced structural changes at the interface between domains I and II and the thermostability of the protein fold.

**Zinc Binding to Albumin in Mammalian Cell Culture**—To investigate the Zn$_{2+}$-binding ability of albumin in the presence of mammalian cells, we studied the growth of human WRL-68 cells on standard cell culture plates. This human epithelial hepatoploid liver cell line exhibits a morphology similar to primary hepatocyte cultures. Zn$_{2+}$ concentrations above 72 μM were toxic to cells cultured in Dulbecco’s modified Eagle’s medium...
supplemented with 10% newborn calf serum (supplemental Fig. S10, A and B), as seen by cell rounding, loss of adherence, and necrotic cell death, effects similar to those of Zn\(^{2+}\) on other cell types (32, 33). Physiological concentrations of rHA (600 \(\mu\)M) were sufficient to completely abolish the adverse effects of Zn\(^{2+}\) on hepatocytes, even at Zn\(^{2+}\) concentrations as high as 600 \(\mu\)M (Fig. 6, A and B), suggesting that the zinc-albumin complex is stable under \textit{in vitro} conditions and capable of controlling Zn\(^{2+}\) distribution in cell cultures. It should be noted that although the cell culture medium used in these experiments contains the amino acids cystine and histidine (both 0.2 mM), which can chelate Zn\(^{2+}\) with micromolar affinity (log \(K_{\text{a}}\) of 6.5 (histidine) or 6.7 (cystine) (29)), the differences observed in Fig. 6 (A and B) are due to the presence of albumin only. All albumin mutants studied were also well tolerated by cultured cells, both in the presence and absence of added Zn\(^{2+}\).

**DISCUSSION**

The combination of site-directed mutagenesis, molecular modeling, and EXAFS has provided new insight into the structure of the important high-affinity zinc site on human albumin. The residues forming this site are conserved in all known mammalian albumin sequences (supplemental Table S2 and Fig. S12) except for that of the guinea pig, which conspicuously lacks both domain I residues. No human albumin variants with mutations of any of these four residues are known. These findings further emphasize the importance of this site with its peculiar combination of moderate affinity but high specificity for zinc, properties likely to relate to its biological transport function.

The coordination sphere of albumin-bound zinc can be described as distorted octahedral, if the backbone carbonyl oxygen of His\(^{247}\) is included as the sixth ligand. Hence, the zinc coordination sphere can be considered as saturated. The zinc-amino acid distances are within the ranges observed for small molecule zinc complexes and protein structures (34, 35). Interestingly, the zinc-oxygen (water) distance is relatively short (1.91 Å) compared with those for small molecule complexes (2.09 ± 0.05) but has numerous precedents in zinc sites in proteins, as an analysis of Zn-water distances in the MESPEUS Database shows (35). This might suggest that this bound aqua ligand might be highly polarized and have a low \(pK_a\), a feature of some catalytic zinc sites (e.g. carbonic anhydrase). Albumin is known to exhibit esterase-like activity, but this has previously
been assigned to Lys^{199}, Trp^{214}, and Tyr^{411} (36). The catalytic activity of zinc albumin has not yet been studied; perhaps this possibility should be further investigated.

A limited number of examples of metal sites with the same set of ligands as the zinc site in albumin are found in enzymes, in particular some phosphatases; in the latter examples, the pentacoordinate zinc site forms part of a binuclear site with bridging carboxylate and hydroxyl groups and displays a geometry different to that represented in Fig. 3B. A mononuclear site with the same set of four protein ligands is found in the zinc-dependent endopeptidase LytM in its inactive form (37). In contrast to albumin, in LytM, there is no space for coordination of a fifth ligand for zinc. The active form of the enzyme is formed only after dissociation of the Asn residue, which essentially provides a switch for enzyme activation.

In general, the amide side chain of asparagine (or glutamine) is only rarely observed as a protein ligand for zinc, or indeed for other metal ions, but zinc-carboxamidine coordination is common during catalysis, for example in the enzyme-substrate complexes of Zn-containing peptidases and proteases, such as carboxypeptidase A (38). In the interdomain zinc site in albumin, Asn^{99} has a dual role; in the absence of zinc, together with the backbone carbonyl oxygen of His^{247}, Asn^{99} provides one of the few, and therefore likely crucial, interdomain hydrogen bonds in albumin. The finding that the yield from albumin expression of the N99D mutant was typically three times lower than for the wild type and other mutants, together with the observation of ^1H NMR peaks for a second conformer, might suggest that this interdomain H-bond is indeed important for stability of the protein fold. The N99D mutation (replacement of a neutral-NH2 group by a negatively charged oxygen atom) abolishes the interdomain H-bond, and instead introduces a charge repulsion between Asp^{99} O-δ and the carbonyl oxygen of His^{247}. (supplemental Fig. 13). In contrast, the N99H mutation allows an H-bond to be preserved in this location (supplemental Fig. S13).

It is clear that albumin provides a large capacity for zinc binding in blood plasma, but it is interesting to note that zinc binding in turn has a substantial effect on the dynamic behavior of albumin, as exemplified by the ^1H NMR data (Fig. 5 and supplemental Fig. S8). The effects of zinc on the ^1H NMR spectra of human serum albumin bear a remarkable resemblance to the effects induced by raising the pH* (see “Experimental Procedures”) above 7.5 (39); both H+ release and Zn^{2+} addition lead to the disappearance of the same His H-ε1 resonances. It has been suggested that these His residues are implicated in the pH-induced N-to-B conformational transition (40). Conformational changes and allosteric effects are important for the ability of albumin to bind and transport hormones and therapeutic drugs (41, 42) and therefore can have direct influence on the pharmacokinetics of these compounds (43).

For example, the N-to-B transition has been shown to influence directly the affinity of albumin toward steroids (44) and warfarin (45, 46), which both bind to Sudlow site I. This site contains a hydrophobic binding pocket located in subdomain IIa, which is also the main binding site for other drugs and hormones including aspirin, phenytoin, pregnenolone, testosterone, and thyroxin (41, 43). Similarly, the environment of Cys^{34}, an important antioxidant and drug binding site, is also affected by this conformational change (47).

Although numerous x-ray structures of albumin in the nitrogen form have been determined in the past decade, no three-dimensional structural data are available for the B conformation. A reexamination of currently available experimental evidence may provide a step toward a better understanding of the molecular mechanisms of the N-to-B transition as well as the effects of zinc binding. It should be noted that chemophysical studies have confirmed that the solution structure of albumin is well represented by the x-ray crystal structures (48).

It is thought that the N-to-B transition is associated with a “loosening up” of the structure localized in domain I with a contribution from domain II (40, 48). A small loss of α-helical content in the B form has been localized to the interdomain linker helices, and it has been suggested that in this form mutual domain movements can take place (49), which may be more restricted in the nitrogen form.

The underlying molecular mechanism of the N-to-B transition is thought to involve the breaking of hydrogen bonds from protonated histidine residues (40). Some of these residues have unusually high pK_a values (25) (supplemental Table S2); these are expected to be protonated, and therefore strong H-bond...
donors, at neutral pH. The loss of H-bonds can explain the findings of reduced proton affinity (50) as well as the increased domain-domain flexibility (49) of the B form.

An inspection of published x-ray structures of albumin reveals that five histidine residues are situated at domain interfaces and have the capacity to form H-bonds involving their side chains: His\textsuperscript{67}, His\textsuperscript{146}, His\textsuperscript{242}, His\textsuperscript{247}, and His\textsuperscript{288} (Fig. 5B and supplemental Table S2). Intriguingly, the number of His residues involved in the N-to-B transition has previously been reported as five (39). All five interdomain histidines are located between domains I and II (supplemental Table S2), an observation consistent with \textsuperscript{1}H NMR spectroscopic studies of peptic and tryptic fragments of albumin (40).

Direct experimental evidence exists not only for the involvement of one of these five residues, His\textsuperscript{242}, in the N-to-B transition but also for its impact on drug binding to Sudlow site I (46). Mutation of His\textsuperscript{242} to a valine shifts not only the midpoint of the N-to-B transition from the wild-type value of 7.8 to a value of 7.1–7.2 but also affects the affinity toward warfarin. His\textsuperscript{242} is located in domain II and forms a hydrogen bond with Gln\textsuperscript{196} (Fig. 5B), which is located in the domain linker helix.

Albumin contains very few interdomain H-bonds that do not involve histidines, and hydrophobic interactions between domains I and II are also scarce. Thus, it is highly likely that the domain I/II interface is indeed subtly controlled by the protonation state of the interdomain His residues, and any manipulation, including zinc binding, of interdomain histidines may have effects on the entire interface.

It is striking that two of the five interdomain histidines are part of the zinc-binding site. The zinc ligand His\textsuperscript{247} does not form an interdomain H-bond in fatty acid-free albumin but is H-bonded to Glu\textsuperscript{190} in fatty acid-loaded albumin (9, 14). In either case, His\textsuperscript{247} is usually H-bonded via its N\textsubscript{6} to Asp\textsuperscript{249}, which also forms an interdomain H-bond to Ne of His\textsuperscript{57} in fatty acid-free albumin. The amide-NH\textsubscript{2} group of Asn\textsuperscript{99} is an H-bond donor to the backbone carbonyl oxygen of His\textsuperscript{247} as discussed above. Considering the scarcity of interactions between domains I and II, it is not surprising that the binding of a zinc ion at this interface should have far reaching consequences, as is evident from the \textsuperscript{1}H NMR data. We speculate that zinc can reinforce the interdomain contact and stabilize a specific mutual orientation of the two domains. This would be expected to have an impact on the dynamic behavior of residues at this interface, including the interdomain histidines. Thus, both in the presence and absence of zinc, this particular site is of pivotal importance for stabilizing the contact and relative orientation of domains I and II. Remarkably, a conformational change thought to be similar to the N-to-B transition also occurs when albumin interacts with hepatocyte cell membranes (51, 52), and it has been hypothesized that this may assist in the delivery of ligands, including fatty acids, to liver cells.

Our findings suggest that further work to investigate the possible interactive binding of zinc and drugs, metabolites, and xenobiotics is warranted. Fluctuations in zinc together with fatty acid levels in blood may have important effects on the transport of some classes of these molecules.

Our studies show that physiological concentrations of albumin can protect WRL-68 cells from zinc-induced toxicity (Fig. 6), even at a 1:1 ratio of zinc:albumin. A similar protective action of bovine serum albumin against the toxic effects of zinc has been shown with cerebellar granule neurons (53) and epithelial cells (54). These findings emphasize the requirement for a suitable zinc-binding agent to be present in physiological fluids such as blood plasma and cerebrospinal fluid.

Because the affinity of albumin for zinc is only moderate, albumin-bound zinc has been considered to be part of the so-called exchangeable zinc pool. This moderate affinity is likely to assist the transfer of zinc to target sites. The molecular mechanisms by which this transfer is achieved are not known in detail yet, and it is remarkable that albumin, in addition to many other functions, has evolved as a highly specific zinc "buffer" in plasma. Under physiological conditions, the unique ligand composition of the site is tailored and specific for zinc and appears to bind zinc with the appropriate affinity (and probably dynamics) to allow albumin to safely release and deliver zinc when necessary.

REFERENCES

1. Turk, B. E., Wong, T. Y., Schwarzenbacher, R., Jarrell, E. T., Leppa, S. H., Collier, R. J., Liddington, R. C., and Cantley, L. C. (2004) Nat. Struct. Mol. Biol. 11, 60–66
2. Petersson, K., Håkansson, M., Nilsson, H., Forsberg, G., Svensson, L. A., Liljas, A., and Walse, B. (2001) EMBO J. 20, 3306–3312
3. Ford, D. (2004) Proc. Natl. Acad. Sci. 63, 21–29
4. Caroli, S., Alimonti, A., Coni, E., Petrucci, F., Senofonte, O., and Violante, N. (1994) Crit. Rev. Anal. Chem. 24, 363–398
5. Counis, R. J. (1986) Clin. Phys. Biochem. 4, 20–30
6. Giroix, E. L., and Henkin, R. I. (1973) Bioinorg. Chem. 2, 125–133
7. Rowe, D. J., and Bobilya, D. J. (2000) Proc. Soc. Exp. Biol. Med. 224, 178–186
8. Peters, T. Jr. (1996) All about Albumin: Biochemistry, Genetics and Medical Applications, pp. 9–54, Academic Press, New York
9. Sugio, S., Kashima, A., Mochizuki, S., Noda, M., and Kobayashi, K. (1999) Protein Eng. 12, 439–446
10. Masuoka, J., and Saltman, P. (1994) J. Biol. Chem. 269, 25557–25561
11. Goumakos, W., Laussac, J. P., and Sarkar, B. (1991) Biochem. Cell Biol. 69, 809–820
12. Ohyoshi, E., Hamada, Y., Nakata, K., and Kohata, S. (1999) J. Inorg. Biochem. 75, 213–218
13. Bal, W., Christodoulou, J., Sadler, P. J., and Tucker, A. (1998) J. Inorg. Biochem. 70, 33–39
14. Curry, S., Mandelkow, H., Brick, P., and Franks, N. (1998) Nat. Struct. Biol. 5, 827–835
15. Carter, D. C., He, X. M., Munson, S. H., Twigg, P. D., Gernert, K. M., Broom, M. B., and Miller, T. Y. (1989) Science 244, 1195–1198
16. Ghuman, J., Zunzain, P. A., Pettipas, I., Bhattacharya, A. A., Otagiri, M., and Curry, S. (2005) J. Mol. Biol. 353, 38–52
17. Stewart, A. J., Blindauer, C. A., Berezensko, S., Sleep, D., and Sadler, P. J. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 3701–3706
18. Guo, M., Harvey, L. Yang, W., Coghill, L., Campopiano, D. J., Parkinson, J. A., MacGillivray, R. T., Harris, W. R., and Sadler, P. J. (2003) J. Biol. Chem. 278, 2490–2502
19. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Cryst. 26, 283–291
20. Hoft, R. W., Vriend, G., Sander, C., and Abola, E. E. (1996) Nature 381, 272–277
21. Koradi, R., Billeter, M., and Wuthrich, K. (1996) J. Mol. Graphics 14, 51–55
22. McCall, K. A., and Fierke, C. A. (2004) Biochemistry 43, 3979–3986
23. Tichane, R. M., and Bennett, W. E. (1957) J. Am. Chem. Soc. 79, 1293–1296
24. Christodoulou, J. (1997) NMR Studies of Human Albumin PhD Thesis,
Zinc Binding to Albumin

Birkbeck College, University of London

26. Martin, E. O., and Drakenberg, T. (1982) Inorg. Chim. Acta 67, 71–74
27. Sadler, P. J., and Viles, J. H. (1996) Inorg. Chem. 35, 4490–4496
28. Harford, C., and Sarkar, B. (1997) Acc. Chem. Res. 30, 123–130
29. Pettit, G., and Pettit, L. D. (1993) IUPAC Stability Constants Database, IUPAC and Academic Software, Otley, United Kingdom
30. Pearson, R. G. (1963) J. Am. Chem. Soc. 85, 3533–3539
31. Öz, G. L., Pountney, D. L., and Armitage, I. M. (1998) Biochem. Cell Biol-Biochim. Biol. Cell 76, 223–234
32. Rudolf, E., Rudolf, K., Radocha, J., Peychl, J., and Cervinka, M. (2003) Biometals 16, 295–309
33. Cario, E., Jung, S., D’Heureuse, J. H., Sturm, C., Wiedenmann, B., Goebell, H., and Dignass, A. U. (2000) Eur. J. Clin. Investig. 30, 419–428
34. Harding, M. M. (2006) Acta Crystallogr. Sect. D 62, 678–682
35. Hsin, K., Sheng, Y., Harding, M. M., Taylor, P., and Walkinshaw, M. D. (2008) J. App. Crystallogr. 41, 963–968
36. Ma, S. F., Anraku, M., Iwao, Y., Yamasaki, K., Kragh-Hansen, U., Yamaotsu, N., Hirose, S., Ikeda, T., and Otagiri, M. (2005) Drug Metab. Dispos. 33, 1911–1919
37. Odintsov, S. G., Sabala, I., Marcyjaniak, M., and Bochtler, M. (2004) J. Mol. Biol. 345, 775–785
38. Lipscomb, W. N. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3875–3878
39. Labro, J. F., and Janssen, L. H. (1986) Biochim. Biophys. Acta 873, 267–278
40. Bos, O. J., Labro, J. F., Fischer, M. J., Wilting, J., and Janssen, L. H. (1989) J. Biol. Chem. 264, 953–959
41. Fasano, M., Curry, S., Terreno, E., Galliano, M., Fanali, G., Narciso, P., Notari, S., and Ascenzi, P. (2005) IUBMB Life 57, 787–796
42. Yamasaki, K., Maruyama, T., Yoshimoto, K., Tsutsumi, Y., Narazaki, R., Fukuhara, A., Kragh-Hansen, U., and Otagiri, M. (1999) Biochim. Biophys. Acta 1432, 313–323
43. Kratochvil, N. A., Huber, W., Müller, F., Kansa, M., and Gerber, P. R. (2002) Biochem. Pharmacol. 64, 1355–1374
44. Fischer, M. J., Bos, O. J., Van der Linden, R. F., Wilting, J., and Janssen, L. H. (1993) Biochem. Pharmacol. 45, 2411–2416
45. Wilting, J., van der Giesen, W. F., Janssen, L. H., Weideman, M. M., Otagiri, M., and Perrin, J. H. (1980) J. Biol. Chem. 255, 3032–3037
46. Petersen, C. E., Ha, C. E., Curry, S., and Bhagavan, N. V. (2002) Proteins 47, 116–125
47. Wang, X., Guo, L., and Ma, H. (2009) Spectrochim. Acta Part A Mol. Biomol. Spectrosc. Apr 24 (Epub ahead of print)
48. Ferrer, M. L., Duchowicz, R., Carrasco, B., de la Torre, J. G., and Acuña, A. U. (2001) Biophys. J. 80, 2422–2430
49. Dockal, M., Carter, D. C., and Rüker, F. (2000) J. Biol. Chem. 275, 3042–3050
50. Janssen, L. H., Van Wilgenburg, M. T., and Wilting, J. (1981) Biochim. Biophys. Acta 669, 244–250
51. Reed, R. G., and Burrington, C. M. (1989) J. Biol. Chem. 264, 9867–9872
52. Horie, T., Mizuma, T., Kasai, S., and Awazu, S. (1988) Am. J. Physiol. 17, G465–G470
53. Lin, S., Tagliabracci, V. S., Chen, X., and Du, Y. (2005) Neuroreport 16, 1461–1465
54. Okeson, C. D., Riley, M. R., and Riley-Saxton, E. (2004) Toxicol. in Vitro 18, 673–680
55. Christodoulou, J., Sadler, P. J., and Tucker, A. (1994) Eur. J. Biochem. 225, 363–368
56. Stewart, A. J., Blindauer, C. A., Berezenko, S., Sleep, D., Tooth, D., and Sadler, P. J. (2005) FEBS J. 272, 353–362