^{1}H and ^{113}Cd NMR Investigations of Cd^{2+} and Zn^{2+} Binding Sites on Serum Albumin: Competition with Ca^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}

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^{1}H and ^{113}Cd NMR studies are used to investigate the Cd^{2+} binding sites on serum albumin (67 kDa) in competition with other metal ions. A wide range of mammalian serum albumins possess two similar strong Cd^{2+} binding sites (site A 113−124 ppm; site B 24−28 ppm). The two strong sites are shown not to involve the free thiol at Cys34. Ca^{2+} influences the binding of Cd^{2+} to isolated human albumin, and similar effects due to endogenous Ca^{2+} are observed for intact human blood serum. ^{1}H NMR studies show that the same two His residues of human serum albumin are perturbed by Zn^{2+} and Cd^{2+} binding alike. Zn^{2+} displaces Cd^{2+} from site A which leads to Cd^{2+} occupation of a third site (C, 45 ppm). The N-terminus of HSA is not the locus of the two strong Cd^{2+} binding sites, in contrast to Cu^{2+} and Ni^{2+}. After saturation of the N-terminal binding site, Cu^{2+} or Ni^{2+} also displaces Cd^{2+} from site A to site C. The effect of pH on Cd^{2+} binding is described. A common Cd^{2+}/Zn^{2+} binding site (site A) involving interdomain His residues is discussed.

Introduction

Human albumin (66.5 kDa) is the most abundant protein in blood serum with a concentration of ca. 0.63 mM. It is a single polypeptide chain of 585 amino acids with a largely-helical triple-domain structure involved in the binding, transport and delivery of a range of endogenous small molecules, as well as drugs and xenobiotics.[1−3] Other mammalian albumins are highly homologous with human albumin, all of which contain 17 disulfide bridges.

Albumin has also been implicated in the transport[4,5] and storage[6] of many metal ions. In particular, the strong binding of Cu^{2+} and Ni^{2+} at the N-terminus of human serum albumin (HSA)[7] has been well characterized,[8−10] and the only free thiol at Cys34 is known to be the major site for Au^{+} antiarthritic drugs.[11−13] In contrast, the binding sites for Zn^{2+}, Cd^{2+}, and Ca^{2+} on albumin have not been specifically located.

Three-fourths of the Zn^{2+} in plasma (ca. 14 μM) is bound to HSA.[14,15] This accounts for as much as 98% of the exchangeable fraction of Zn^{2+} in serum[16] and is known to be associated with the transport of zinc.[5,14,16,17] For example, albumin has been shown to modulate Zn^{2+} transport into endothelial cells.[18] About 90% of Cd^{2+} in blood is bound to serum albumin and α_2-macroglobulin,[19] and 50% of this is bound tightly to α_2-macroglobulin and is nonexchangeable,[20] whereas the remainder is bound to albumin and is readily exchangeable.[21] Of the total Ca^{2+} in plasma (ca. 2.2 mM), 50−60% is thought to be present as low M_r forms, and one-third to one-half of Ca^{2+} is bound to albumin.[22]

^{113}Cd ($I = 1/2$, 12.8% natural abundance) has been used to study the binding sites for Cd^{2+} and as a probe for other metals in particular Ca^{2+} and Zn^{2+} in proteins. The sensitivity of ^{113}Cd, 1.35×10^{-3} relative to ^{1}H, can be improved by enrichment. The chemical shift range is large (ca. 1000 ppm) and diagnostic of the types of bound ligand.[23−25] The first ^{113}Cd NMR study of Cd^{2+} binding to albumin (BSA) was reported by Martins and Drakenberg[26] who characterized two strong Cd^{2+} sites with ^{113}Cd NMR shifts of 28 and 140 ppm and suggested the involvement of oxygen ligands in the former and His ligands (His3 and His9) in the latter site. Competition studies between Cd^{2+} and 1 mol equiv of Zn^{2+} or Cu^{2+}

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(7) Abbreviations used: BSA, bovine serum albumin, globulin-free and fatty-acid-free; bfBSA, bovine serum albumin blocked at Cys34 and Cys, endogenous bound fatty acid also present; ESA, equine serum albumin, globulin-free and fatty-acid-free; fCSA, canine serum albumin, with endogenous bound fatty acids; fSSH, sheep serum albumin, with endogenous bound fatty acids; HSA, human serum albumin, globulin-free and fatty-acid-free; pH*, pH meter reading in D_2O solutions; PSA, porcine serum albumin, globulin-free and fatty-acid-free; TOCSY, total shift correlation spectroscopy; TSP, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4.

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suggested that there is a common binding site for Cu^{2+}, Zn^{2+}, and Cd^{2+}, with a higher affinity for Cu^{2+} and Zn^{2+} than Cd^{2+}. In contrast, Göumakos et al.[27] reported that Cd^{2+} is not displaced from its strong binding sites by 1 mol equiv of Cu^{2+}. Other workers have also suggested the involvement of His residues in the binding of Cd^{2+} and Zn^{2+} to albumin.[28,29] However, Sukki et al.[30] and recent reviews on albumin[1,3] have suggested that Cd^{2+} binds to the free thiol at Cys34. The same suggestion has also been made for Zn^{2+}.[31] Yongqia et al.[32] have even suggested that Zn^{2+}, Cd^{2+}, and Hg^{2+} bind to the disulfide bridges of albumin. Studies of model tripeptides have indicated the possibility of Zn^{2+} binding to the N-terminus to HSA.[1,33]

There have been several studies of the affinities of Zn^{2+}, Cd^{2+}, and other metals for albumin, most recently by Masuoka et al.[34] Göumakos et al.[27] have determined by equilibrium dialysis that there is one strong binding site for Zn^{2+}, log K 6.4, and two weaker sites, log K 5 on HSA. For Cd^{2+} binding to HSA they found two strong sites, log K 5.3, and one much weaker site. Zn^{2+} competition for only one of the strong Cd^{2+} binding sites was also reported. Ca^{2+} binds much more weakly to HSA than Cd^{2+} or Zn^{2+}, and a binding affinity of log K 2.9 for one Ca^{2+} ion has been suggested, with additional weaker sites.[35]

In view of the important role of albumin in zinc and cadmium transport in blood, and the disagreements in the literature as to the nature of their binding sites, we have used ^{1}H and ^{113}Cd NMR to investigate Cd^{2+} complexation to a variety of mammalian albumins, the competitive effects of Ca^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, and to detect Cd^{2+} binding to albumin directly in intact blood serum.

Experimental Section

NMR Samples. In general, 2 mM solutions of albumin in 0.1 M phosphate buffer were used for both ^{113}Cd (3 mL) and ^{1}H NMR (0.7 mL) samples. Deuteriated phosphate buffers were prepared by freeze-drying D_2O solutions containing $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 2H_2O$ (BDH, AnalaR).

Mammalian albumins were obtained from Sigma as follows: human (HSA; catalog no. A-3782), fatty-human (fHSA; A-8763), bovine (BSA; A-0161), fatty-Cys34-blocked with Cys, (bfBSA; A-0161), fatty-canine (fCSA; A-9263), porcine (PSA; A-1173), equine (ESA; A-5280), fatty-sheep (fSSH; A-4289), where "fatty" implies the presence of endogenous bound fatty acids, and the remainder are essentially fatty-acid-free. Typically the thiol content of the commercial albumins indicates that up to 40% of Cys34 is blocked, largely as a disulfide with cysteine.[13]

Serum samples were obtained from a healthy human volunteer after fasting. The blood samples were allowed to clot, and serum was obtained via centrifugation. The serum samples were frozen (−20 °C) for storage. Small aliquots of 0.1 M NaOH were added to the serum to adjust the samples to pH 7 when necessary. D_2O (total 20% v/v) was added as a lock signal for NMR experiments. It was assumed that the albumin concentration in normal serum is ca. 0.63 mM.[3]

Additions of metal ions to albumin samples were made as small aliquots of 20−100 mM stock solutions of $CuCl_2 \cdot 2H_2O$, $CdCl_2$, $NiCl_2 \cdot 6H_2O$, and $ZnCl_2$. (AnalaR Grade) in D_2O. Stock solutions of $^{113}CdCl_2$ (40 mM) were made up by dissolving ^{113}CdO (>95% ^{113}Cd) in the minimum amount of DCl and were diluted with D_2O.

The pH* values of the protein solutions and serum were carefully monitored in the NMR tube before and after metal additions and NMR measurements using an ultrathin micro combination electrode (Aldrich) and a Corning 145 pH meter. Where appropriate, additions of 0.1 M NaOD or 0.1 M DCl were used to readjust the pH* during the titrations.

Multi-elemental analysis of the mammalian albumins used here was investigated by ICP-MS, using a VG Plasma Quad spectrometer, with direct injection nebulization of 1 μM solutions of albumin in water spiked with 50 ppb indium as internal standard. No major differences in the levels of detected elements were found between mammalian albumins studied. Typical levels of first row transitional elements were 1 μmol of metal ion per mmol of albumin. Most heavier elements such as Cd, Au, Hg, and Pb were present at concentrations of less than 0.1 μmol per mmol albumin, while I and Ba levels were typically 10 μmol per mol of albumin. Typical levels of Ca, Mg, and K were 100 μmol per mmol of albumin, and higher concentrations of Na and Cl were observed.

^{113}Cd NMR. ^{113}Cd NMR spectra were obtained at 22 °C on JEOL GSX-270 and 500 spectrometers at 59.92 and 110.93 MHz, respectively, using 10 mm tubes. Typically a spectral width of 400 ppm was used, centered at 125 ppm (relative to $Cd(ClO_4)_2$), with 16k data points, a pulse width of 10 μs (50°), and total repetition time (acquisition plus delay) of between 0.7 and 1.0 s. Gated broad-band ^{1}H decoupling (during acquisition) was used to avoid negative NOEs. Reasonable signal-to-noise ratios were usually obtained from 50 000 transients. Typically spectra were processed using 75 Hz exponential line broadening functions. Shifts are referenced to $Cd(ClO_4)_2$ (0.1 M in D_2O, external).

^{1}H NMR. 600 MHz ^{1}H NMR spectra were recorded at 37 °C on a Varian VXR600 spectrometer, typically using 50° pulses, a spectral width of 13 ppm, acquisition time of 0.7 s, relaxation delay (not including acquisition time) of 2.3 s, and accumulation of 256 transients. The FID was zero-filled to 32k points. The 2D TOCSY spectra were acquired using a spin-lock time (MLEV-17) of ca. 60 ms with 2k data points in the t_2 dimension and 256 increments in the t_1 dimension. This was zero-filled to 4k × 1k points. Typically 48−128 transients were acquired for TOCSY experiments. The residual HOD resonance was suppressed when necessary by presaturation during the relaxation delay. An optimal combination of exponential and unshifted sine−bell functions was used for resolution enhancement of 1D spectra.[36,37] Shifted Gaussian functions were used for processing 2D data in both dimensions. Chemical shifts are referenced to sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 (TSP) via internal dioxane (3.764 ppm).

Electronic Absorption Spectroscopy. UV/visible spectra were obtained on a double-beam Perkin-Elmer Lambda 5 spectrophotometer using 1 cm path length cells.

Results

^{113}Cd NMR. ^{113}Cd NMR spectra were obtained from a number of mammalian albumins, containing 1 mol equiv of $^{113}Cd^{2+}$, Figure 1A, each giving peak A in the range 113−124 ppm and B from 24 to 28 ppm, except for sheep albumin which has peak A at 85 ppm. In all cases the relative peak areas are close to 1:1 except for porcine albumin, for which peak B is much more intense, A:B ratio 0.2:0.8. Addition of a second molar equivalent of ^{113}Cd to HSA caused the ^{113}Cd NMR signals to double in intensity. Addition of a third molar equivalent of ^{113}Cd (in the presence of 100 mM phosphate buffer) had no effect on the spectrum, and a cadmium phosphate precipitate was observed.

Addition of 1 mol equiv of $^{113}Cd^{2+}$ to BSA, which had its only free thiol at Cys34 blocked with cysteine, gave rise to two similar ^{113}Cd NMR peaks as those observed for Cd_1-BSA itself, Figure 1A, except that peak A was shifted to higher field by 15 ppm for Cys34-blocked BSA.

Figure 1B shows the pH* dependence of the ^{113}Cd NMR spectrum of Cd_1-HSA. It can be seen that at pH* values below 7, both peaks A and B broaden markedly such that by pH* 5

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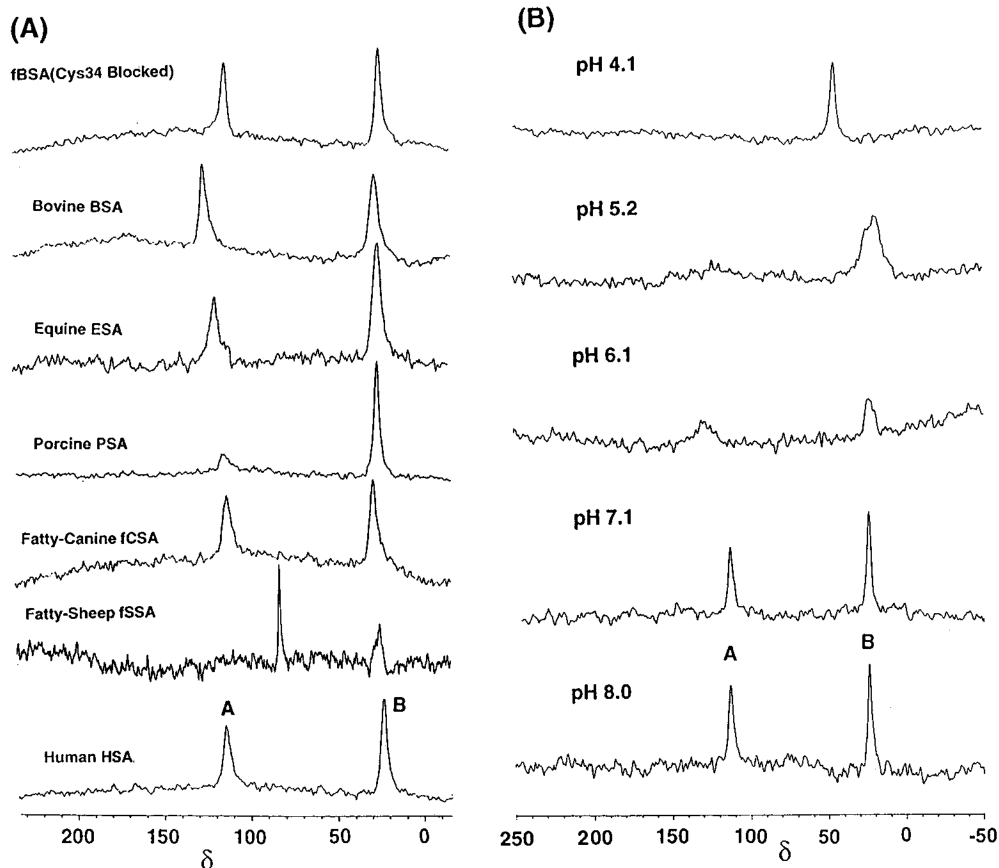

Figure 1. (A) 59.92 MHz ^{113}Cd NMR spectra of mammalian albumins (2 mM), in the presence of 1 mol equiv of $^{113}CdCl_2$, 0.1 M phosphate buffer, at pH 7 (except sheep recorded at 110.9 MHz). The chemical shifts for peaks A and B, respectively, are as follows: human 115, 24 ppm; bovine 124, 25 ppm; bovine blocked at the Cys34 109, 25 ppm; equine 118, 25 ppm; porcine 116, 27 ppm; canine 113, 28 ppm; sheep 84, 25 ppm. (B) 59.92 MHz ^{113}Cd NMR spectra of HSA (2 mM), in the presence of 1 mol equiv $^{113}CdCl_2$ at pH* 4, 5, 6, 7, and 8 in 0.1 M phosphate. The chemical shifts (in ppm) and line widths (in Hz in brackets) for peaks A and B, respectively, are as follows: pH 8.0, 115 (220) 24 (190); pH 7, 115 (230), 24 (180); pH 6.1, 125 (760), 24 (420); pH 5.2, broad, 24 (780); pH 4.1, not observed, 45 (230).

peak A has almost broadened beyond detection. However, at pH* 4, one relatively sharp peak with a shift of 45 ppm is observed.

Addition of 1 mol equiv of Ca^{2+} had no detectable effect on the ^{113}Cd NMR spectrum of peaks A and B of Cd_1-HSA. However there was progressive broadening of both of these Cd^{2+} resonances at higher levels of Ca^{2+}, 10−60 mol equiv, Figure 2. Peak A (115 ppm) shifted to 125 ppm after addition of 10 mol equiv of Ca^{2+}, and with 60 mol equiv of Ca^{2+} present, peak A shifted further downfield to 155 ppm.

The ^{113}Cd spectrum of human blood serum, at pH 7.0, with either 2 (Figure 2) or 4 mol equiv of ^{113}Cd added also showed two signals of similar intensity at 25 and 130 ppm. A ^{113}Cd NMR signal typical of free Cd^{2+} was not observed.

Addition of Zn^{2+} to Cd_2-HSA caused ^{113}Cd peak A to decrease in intensity, as previously reported,[27] such that with 1.5 mol equiv of Zn^{2+} present, the signal was not observed, Figure 3A. The intensity and line width of peak B was little affected, but a new broad resonance appeared at ca. 45 ppm, $\Delta\nu_{1/2}$ 900 Hz, and progressively increased in intensity with Zn^{2+} addition (Peak C). Addition of 1 mol equiv of Zn^{2+} to HSA loaded with only 1 mol equiv of Cd^{2+} caused a loss of intensity of peak A but no new peak C was observed.

Addition of 1 mol equiv of Cu^{2+} or Ni^{2+} had no significant effect on the ^{113}Cd NMR spectrum of Cd_1-HSA (Supplementary Figure S1). The electronic absorption spectra of Cd_1Cu_1HSA and Cd_1Ni_1HSA solutions gave bands at 420 and 525 nm, respectively. The addition of 1 mol equiv Cu^{2+} also had no effect on the ^{113}Cd NMR spectrum of Cd_1-BSA, in contrast to a previous report.[26] On further addition of Cu^{2+}, ^{113}Cd peak A

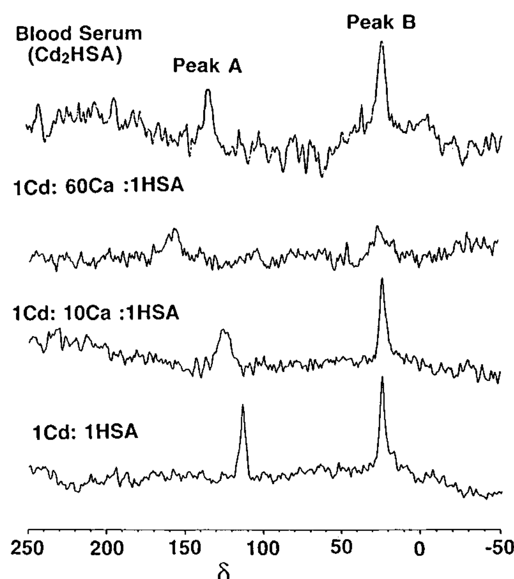

Figure 2. 59.92 MHz ^{113}Cd NMR spectra of HSA (2 mM) in the presence of 1 mol equiv of $^{113}CdCl_2$ added, at pH 7, after the addition of 0, 10, and 60 mol equiv of Ca^{2+}, in the absence of phosphate buffer, and a ^{113}Cd NMR spectrum of blood serum at pH 7 (20% D_2O, 2 mol equiv of $^{113}CdCl_2$ with respect to albumin).

(115 ppm) decreased in intensity and a new peak appeared at 34 ppm (peak C′, $\Delta\nu_{1/2}$ ca. 340 Hz), Figure 3B, such that when 3 mol equiv of Cu^{2+} were present, peak A had disappeared. A similar effect was observed with Ni^{2+} but the new peak C″ at ca. 35 ppm was much broader ($\Delta\nu_{1/2}$ ca. 1 KHz), Figure 3b.

The line widths of peak A and B for HSA increase from ca.

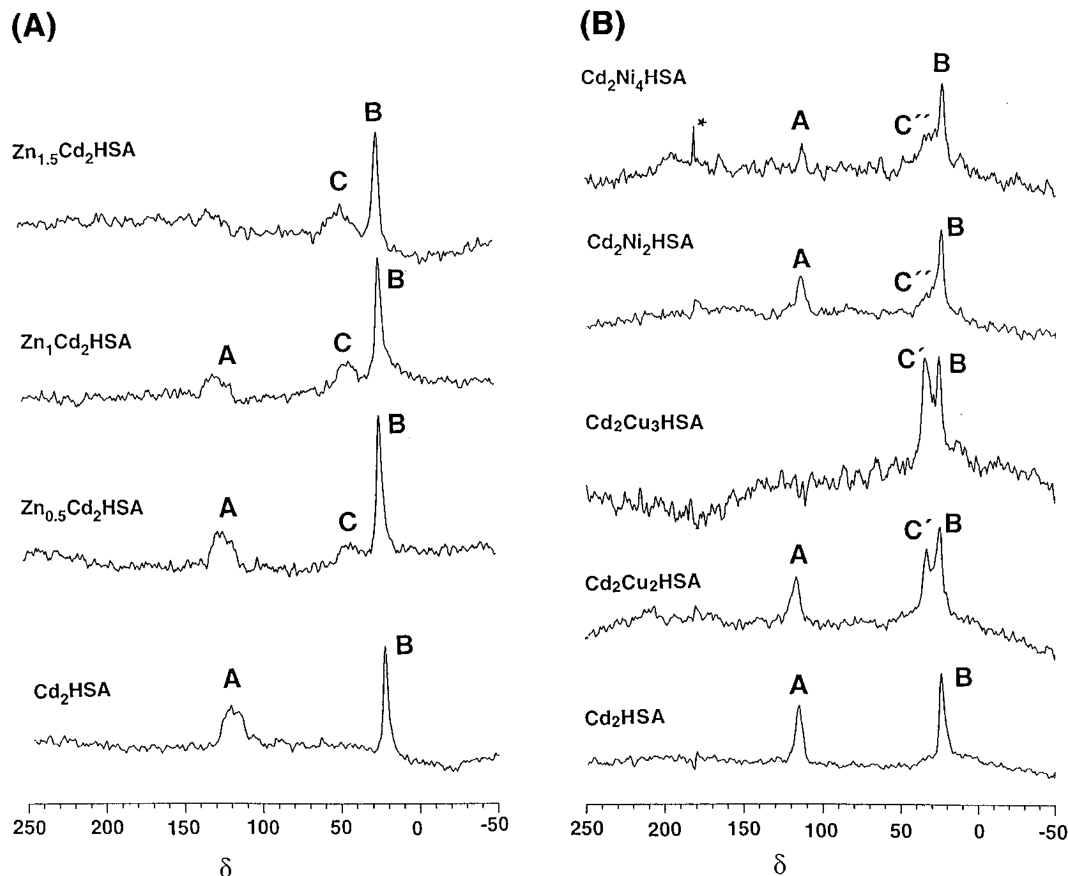

Figure 3. (A) 59.92 MHz ^{113}Cd NMR spectra of HSA (2 mM), in the presence of 2 mol equiv of $^{113}CdCl_2$, at pH 7, before and after the addition of 0.5, 1.0, and 1.5 mol equiv of Zn^{2+}. The areas of peaks A (115 ppm), B (25 ppm), and C (45 ppm), respectively, relative to the unchanged peak B, are as follows: no Zn^{2+} added, 1.3, 1.0, 0.0; $^1/_2$ equiv of Zn^{2+}, 0.6, 1.0, 0.2; 1 equiv of Zn^{2+}, 0.4, 1.0, 0.6; $1^1/_2$ equiv of Zn^{2+}, 0.0, 1.0, 1.0. (B) 59.92 MHz ^{113}Cd NMR spectra of HSA (2 mM), in the presence of 2 mol equiv of $^{113}CdCl_2$, at pH 7, before and after the addition of 2 and 3 mol equiv of Cu^{2+} and 2 and 4 mol equiv of Ni^{2+} (∗, spike at 183 ppm is an artifact).

256 and 205 Hz, respectively, at 59.92 MHz, to 410 and 319 Hz, at 110.93 MHz. Different batches of defatted HSA tended to give rise to different line widths for peak A (ranging between 250 and 800 Hz). A similar solution of Cd_1-fHSA (i.e. with endogenous bound fatty acid) gave rise to a greatly broadened and reduced relative intensity for peak A.

^{1}H NMR. Increasing amounts of Cd^{2+} (0.125, 0.25, 0.5, 1.0, and 2.0 mol equiv) were titrated into 2 mM solutions of HSA in 0.1 M phosphate buffer at pH* 7.0, and also at pH* 6.7, because of overlap between the previously-assigned His3 ϵCH resonance (labeled His-V) and that of His-VI at pH* 7.

There are 16 His residues in HSA, and 11 resonances in the aromatic region have been assigned as His ϵCH peaks via their characteristic pH/chemical shift profiles and 2D TOCSY ϵ/δCH cross-peaks.[38] Seven of these (His-I, II, III, IV, VI, XII, and XIII) are little affected by Cd^{2+} addition, whereas three of them (His-VII, VIII, and IX) decrease in intensity in resolution-enhanced spectra (Figures 4A and 5A). 1D and 2D (TOCSY) spectra showed that a number of other resonances, in addition to His resonances, were perturbed by Cd^{2+} binding. In the aromatic region, peaks assignable to Tyr 3,5H and 2,6H protons shifted progressively to high field by 0.02 ppm and to low field by 0.06 ppm, respectively. In the aliphatic region, a number of high-field-shifted resonances are perturbed. In particular a peak at 0.30 ppm (labeled a in supplementary Figure S2) shifts to 0.32 ppm, while peaks at 0.94 ppm, 0.70 ppm and 0.62 ppm (b, c, and d in Figure S2) decreased in intensity. There was also a progressive sharpening of resonances previously assigned[38] to the N-terminal residues Asp1, Ala2, and His3, and a small shift in the Lys4 ϵCH_2 peak to high field (supplementary Figure S2).

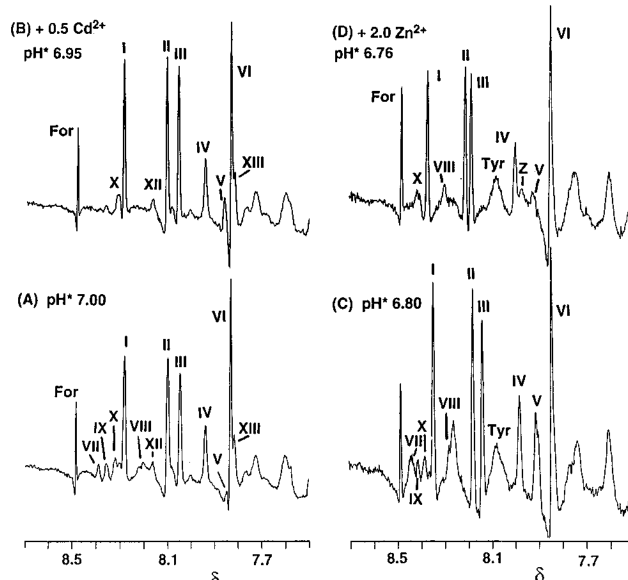

Figure 4. 600 MHz ^{1}H NMR spectra of the His ϵCH region of HSA in 0.1 M phosphate buffer, 37 °C, (A) before and (B) after the addition of Cd^{2+} at pH* 7 and (C) before and (D) after addition of Zn^{2+} at pH* 6.8. See Figure 5 for variations of peak heights with metal concentration. Assignments: I−XIII, His ϵCH; V, His3 ϵCH; For, formate. Peak Z appears on the addition of Zn^{2+}, but is unassigned.

Similar 1D ^{1}H NMR titrations of Zn^{2+} with HSA (2 mM in 0.1 M phosphate buffer) were carried out, at pH* values of 6.8 and 7.0 to clarify overlap of peaks. As in the Cd^{2+} titration, His ϵCH resonances His-VII and -IX were perturbed by the binding of Zn^{2+} whereas the remaining His ϵCH peaks were

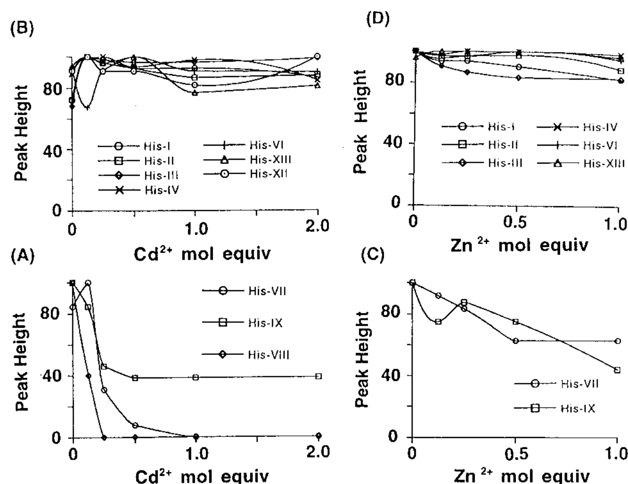

Figure 5. Plot of peak intensities of His ϵCH ^{1}H resonances of HSA vs mole equivalents of Cd^{2+} and Zn^{2+} added at pH* 7.0. Plots B and D represent His ϵCH largely unaffected by metal addition, whereas plots A and C are for His ϵCH resonances broadened by metal addition. Peak heights are normalized to their maximum intensities during the titration (100%). Peak His V (assigned to His3) is not plotted because it is overlapped with His-VI at pH 7.0 (Figure 4). Peak overlap probably accounts for the reduction in intensity of peak IX without total disappearance.

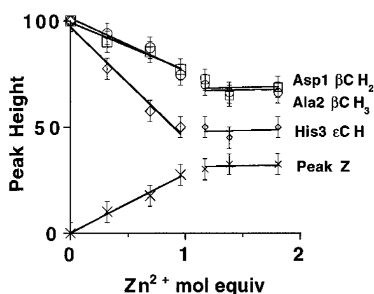

Figure 6. Graph of peak heights of resonances for N-terminal amino acids of HSA vs mole equivalents of Zn^{2+} added. Peak heights are normalized to their maximum intensities (100%). The new resonance Z is normalized to that of His3 ϵCH of apo-HSA.

little affected, Figures 4C,D and 5C,D. Resonances assignable to a Tyr residue which were perturbed by Cd^{2+} binding were also perturbed by Zn^{2+} binding. Also the high-field resonances at 0.30, 0.62, 0.70 and 0.94 ppm (labeled a, b, c and d, respectively, in supplementary Figure S2) were also perturbed by the addition of Zn^{2+}.

Resonances for Asp1 βCH_2 and Ala2 βCH_3 and His3 ϵCH decreased in intensity with increase in Zn^{2+} concentration in resolution-enhanced spectra, Figures 4C, 4D and S2B. The ϵCH resonance for His3 (labeled V) is split due to the heterogeneity of Cys34.[12,13] Variations in the heights of these peaks with Zn^{2+} additions are plotted in Figure 6. No new resonances associated with Asp1 or Ala2 of Zn-HSA were observed in either 1D ^{1}H NMR or 2D TOCSY ^{1}H NMR spectra of $Zn_{1.3}$-HSA. A new broad resonance (labeled Z) at 7.96 ppm (at pH* 6.8) appeared and increased progressively in intensity with Zn^{2+} addition up to ca. 1 mol equiv commensurate with the reduction in the peak for His3 ϵCH (labeled V, Figures 4D and 6) but no assignment was possible, and no changes in shifts of His3 ϵCH or peak Z resonances were observed. This new peak was not seen with Cd^{2+} addition.

Discussion

Cd Binding. Our ^{113}Cd NMR spectra of BSA and HSA are in general agreement with those published previously.[26,27] There are two Cd^{2+} binding sites with similar peak intensities implying similar affinities. The stoichiometry of the Cd titration implies strong binding with a submillimolar dissociation constant. Site A, 115 ppm (155 ppm in the presence of excess Ca^{2+}) has shifts typical of two or three nitrogen (His) ligands with additional oxygen ligands (Asp or Glu carboxyl, carbonyl or water). For comparison, the cadmium complex of carboxypeptidase A (2 His, bidentate carboxylate, 1 H_2O) has a ^{113}Cd shift of 120 ppm, insulin (3 His, 3 H_2O), 165 ppm, and alkaline phosphatase (3 His, 1−2 H_2O), 117 ppm. A shift of 24 ppm for site B indicates all-oxygen or oxygen ligands plus one nitrogen ligand, and is similar to those reported for concanavalin A (3 carboxylates, 2 H_2O, and 1 His) 46 ppm, alkaline phosphatase (1 His, 2 carboxylates, 1 H_2O) 52 ppm, and alkaline phosphatase (third site, 4 carboxylates), with a shift of 2 ppm.[25]

Effect of Blocking Cys34. It is commonly believed that Cys34 is the preferred binding site for Cd^{2+},[1,3] as it is for Au^{+} and Hg^{2+}. However our data strongly suggest that thiolate sulfur is not a ligand in either of the two strong Cd^{2+} binding sites, since the affinity of Cd^{2+} for BSA is unaffected by the blocking of Cys34; both sites A and B are equally populated after the addition of 1 mol equiv of Cd^{2+}. In addition a change in chemical shift of over 100 ppm would have been expected for the removal of a single thiol ligand on the basis of protein sites with known Cys ligands. For example Cd^{2+} in the catalytic site of horse liver alcohol dehydrogenase complex chelates two thiolate ligands and one imidazole ligand and gives a ^{113}Cd NMR shift of 442 ppm. While Cd^{2+} in the blue copper site of the protein azurin which chelates two imidazoles and only one thiolate gives a chemial shift of 332 ppm.[25] Although Cys34 has a low pK_a, between 5 and 7,[39] it is situated in a crevice[1] and may not be readily accessible. The presence of endogienous fatty acids may account for the 15 ppm shift of peak A. Alternatively the blocking of Cys34 may produce a small conformational change at site A. Commercial albumins are partially blocked at Cys34 as a disulfide with cysteine (ca. 40% blocked for BSA), but separate signals for the blocked and unblocked forms were not observed, suggesting that the blocking has no effect on the ^{113}Cd signals or that there is exchange averaging.

Mammalian Albumins. All the mammalian albumins we investigated had two similar high-affinity Cd^{2+} sites. However, the chemical shift of ^{113}Cd in site A of sheep albumin was ca. 35 ppm to high field of those of the other albumins. The shift to high field would imply a loss of a deshielding His ligand. However this is not readily accounted for by sequence comparisons and consideration of likely His ligands in an interdomain binding site (*vide infra*). In the case of sheep albumin the two domains may not come close enough together to allow binding of all the His ligands. It is also notable that for porcine albumin, site B has a higher affinity than site A (4:1). The presence of trace metals in the albumin samples, at the levels indicated by ICP AES (see Experimental Section), is not likely to have any significant effect on the ^{113}Cd spectra. Addition of one mol equiv of Cu^{2+} or Ni^{2+} had no effect on the spectra (*vide infra*) nor did addition of one mol equiv of Co^{2+}, Al^{3+}, or a La^{3+} (data not shown). The studies on BSA with Cys34 blocked indicate that the oxidation sate of the thiolate does not significantly effect the line width or chemical shift of the ^{113}Cd-albumins studied.

Effect of pH. The two ^{113}Cd NMR resonances for Cd_1-HSA are unchanged between pH 7 and 8, but broaden markedly at lower pH values, such that population of the histidine-rich site A is not observed at pH* 5, and a single peak at 45 ppm is

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observed at pH 4. This can be attributed to protonation of the His residues in the Cd^{2+} binding sites and also to the N to F structural transition of the protein which has a midpoint of pH 4.5.[40-42] The transition involves a localized unfolding of the N-terminal region and possible separation of the domains.[42]

Effect of Ca^{2+}. Ca^{2+} binds almost exclusively to oxygen ligands in proteins, and Cd^{2+} is often a good isomorphous replacement for Ca^{2+} in Ca-binding proteins.[25] Approximately half the serum Ca^{2+} (2.2 mM) is known to be bound to albumin. Our ^{113}Cd NMR studies show that Ca^{2+} is a weak competitor for both strong Cd^{2+} binding sites. Cd^{2+} has been reported to have an affinity for HSA which is 500 times greater than that of Ca^{2+}.[27,35] The progressive low field shift (from 115 to 155 ppm) of the ^{113}Cd resonance A with Ca^{2+} addition may be accounted for by the removal of a shielding carboxylate ligand (Asp or Glu) from the Cd^{2+} coordination sphere, either by direct Ca^{2+} binding to it (possibly within a Cd−Ca cluster) or indirectly via a structural change induced by Ca^{2+} binding at a distant site. Exchange of Cd^{2+} between these two environments at an intermediate rate on the ^{113}Cd NMR time scale could then give rise to the peak broadening which is seen in the presence of Ca^{2+}.

Blood Serum. Our ^{113}Cd NMR spectra of blood serum indicate that the same two Cd^{2+} sites on albumin are populated when Cd^{2+} is added to serum as for isolated albumin. To our knowledge this is the first ^{113}Cd NMR study of intact blood serum. The shift of peak A (125 ppm rather than 115 ppm) suggests that there is a competition between Cd^{2+} and Ca^{2+} binding similar to that observed for isolated HSA. In normal blood serum the Ca^{2+} concentration is ca. 2.2 mM, four times that of albumin.

It is known that Cd^{2+} also binds to α_2-macroglobulin in blood plasma;[20] however, the concentration of this protein in serum is low, ca. 6 μM, well below the detection limits for ^{113}Cd NMR. The ^{113}Cd chemical shifts of Cd_2-transferrin have been reported to be 38 and 44 ppm,[43] but again the concentration of this protein is low (ca. 35 μM). Cd^{2+} could also bind low molecular mass components of blood such as amino acids, citrate, and lactate which have concentrations within the millimolar range[22] and the formation of different types of low molecular mass complexes together with chemical exchange broadening may explain the lack of signal for excess Cd^{2+} added to serum.

Shared Cd^{2+}−Zn^{2+} Binding Site. There is good evidence that one of the strong Cd^{2+} sites (site A, low field resonance) is also the strong Zn^{2+} site. Zn^{2+} displaces Cd^{2+} from site A, as others have also observed.[26,27] In our studies, complete displacement of Cd^{2+} by Zn^{2+} required more than one mol equivalent of Zn^{2+}, which implies that Zn^{2+} has only slightly higher affinity for HSA than Cd^{2+}. Displacement of Cd^{2+} from site A resulted in population of a lower affinity third Cd^{2+} site C (broad peak at 45 ppm).

From the Cd NMR studies alone it is not clear whether Cd^{2+} and Zn^{2+} compete directly for the same site or whether binding of Zn^{2+} elsewhere on the protein causes a structural change and loss of Cd^{2+} binding site A. The ^{1}H NMR studies indicate that Zn^{2+} and Cd^{2+} binding affects the same His peaks; these histidines may well be ligands in the metal ion complex. Binding of either Zn^{2+} or Cd^{2+} to HSA caused reductions in the intensities of the same two His ϵCH ^{1}H NMR resonances (labeled His-VII and His-IX in Figures 4 and 5). We assume that these resonances are exchange-broadened, i.e. exchange between metal-free and metal-bound forms of the protein occurs at an intermediate rate on the ^{1}H NMR time scale. Resonances associated with Tyr spin system were also perturbed by both Cd^{2+} and Zn^{2+} binding, as were certain high-field-shifted resonances. These data suggest strongly that Cd^{2+} and Zn^{2+} cause similar protein conformational changes on binding and are consistent with them sharing a common His-rich binding site.

The primary binding site for Zn^{2+} on HSA is shared with Cd^{2+}, and we will show that the two strong Cd^{2+} sites on HSA are not at the N-terminus; thus, if binding of Zn^{2+} to the N-terminus occurs, it must be as a weaker secondary site. The intensities of resolution-enhanced ^{1}H NMR resonances of the N-terminal residues Asp1−Ala2−His3 decreased on addition of up to 1 mol equiv of Zn^{2+} to HSA, further addition of Zn^{2+} had little effect on the resonances of the N-terminus. In contrast to our ^{1}H NMR studies carried out on the square-planar diamagnetic N-terminal Ni^{2+} complex,[10] a new set of N-terminal resonances for metal-bound HSA was not observed in 1D or 2D spectra, and we interpret this to mean that Zn^{2+} causes an exchange broadening due to a small change in the environment of the N-terminus when Zn^{2+} occupies a distant site (site A). A structural change in albumin as a result of zinc binding is also evident from changes in high-field-shifted resonances, and might be communicated to the N-terminus by movement of the intervening helices. We have previously reported that gold(I) binding to Cys34, which is separated by two helices from the N-terminus, also perturbs the ^{1}H NMR signals of the N-terminal residues.[12,13]

N-Terminal Site. ^{1}H NMR spectra indicate that Cd^{2+} (up to 2 mol equiv) does not bind to the N-terminus of HSA since resonances for Asp1−Ala2−His3− do not shift or broaden. There has been some confusion in the literature as to whether Cu^{2+} inhibits the binding of Cd^{2+} to site A (low field peak) of BSA.[26] Previous ^{113}Cd NMR studies[26] suggested that Cu^{2+} competes for this site in a similar manner to Zn^{2+}. However, we observed no competition between Cu^{2+} (1 mol equiv) and Cd^{2+} bound to HSA or BSA when the pH of the solution was maintained at pH 7. Displacement of protons from the peptide nitrogens and N-terminal amino group would lead to a drop in pH if not sufficiently buffered. The ^{113}Cd NMR spectra of Cd-BSA (by analogy with Cd-HSA) are likely to be highly pH-dependent. Between pH 6 and 5 the low field ^{113}Cd NMR peak (site A) broadens beyond detection even in the absence of Cu^{2+}. Finally our electronic absorption spectra confirmed that Ni^{2+} and Cu^{2+} ions still occupy the N-terminal site[8] in the presence of Cd^{2+}.

Secondary Binding Site for Ni^{2+} and Cu^{2+}. As stated previously, addition of 1 mol equiv of Cu^{2+} or Ni^{2+} had no effect on the occupation of Cd^{2+} sites A or B on albumin. This is consistent with the strong binding of Cu^{2+} and Ni^{2+} to the N-terminal site. However, when a second molar equivalent was added, both Cu^{2+} and Ni^{2+} appeared to displace Cd^{2+} from site A (the His-rich site) and a new ^{113}Cd peak was observed. Zn^{2+}, Cu^{2+}, and Ni^{2+} all displaced Cd^{2+} from site A and then gave rise to the population of a third site (C ca. 34−45 ppm) by Cd^{2+}. This chemical shift is consistent with all oxygen ligands or with oxygen and one imidazole nitrogen ligand. The variation of line width of peak C is presumably due to different Cd^{2+} exchange rates in the presence of the different competing metal ions. Secondary sites for Cu^{2+} on albumin have been reported previously.[44,45] CD studies indicate that Cu^{2+} binds 20 times more strongly than Cd^{2+} to site A.[45] CD and EPR

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data for Cu^{2+} bound to the second site indicate a distorted octahedral geometry and at least 2 His residues as ligands[45] which is consistent with the ^{113}Cd chemical shifts observed for site A.

Effect of Fatty Acid. It appears that the relative affinity of Cd^{2+} for site A of HSA is reduced when fatty acids are also bound to the protein, since the low field ^{113}Cd peak of fatty acid-bound HSA has a smaller peak area compared to site B. It is known that HSA binds on average between one and two endogenous fatty acid molecules per albumin molecule and can bind up to six with high affinity.[46] Carter and Ho[1] have reported that fatty acid binding causes a slight opening of the interface between the two halves of the molecule and a rotation of domain I. Since site A is also shared by Zn^{2+} it is possible that the degree of fatty acid binding to HSA in blood plasma affects the affinity for Zn^{2+}. However, there appears to be no clear correlation between Zn^{2+} binding affinity and fatty acid content. Resent studies on fatty-acid-free albumin[27] and albumin with endogenous fatty acid present[34] indicate a similar affinity for Zn^{2+}.

Heterogeneity of HSA due to the fatty acid composition probably accounts for the variable line width of peak A for different batches of protein. The field dependence of the line widths may be accounted for by contributions from both heterogeneity and relaxation via chemical shift anisotropy, which is proportional to the square of the magnetic field.[25]

Nature of Cd^{2+} Binding Sites. Finally we discuss the possible identities of the particular amino acid side chains of albumin involved in the Cd^{2+} sites. Since the X-ray coordinates of albumin have not yet been released, we have used the published views of the crystal structure of HSA, together with information on which residues are in helices and loops,[1] to identify a possible region of the structure where two or three (out of the total of 16) His imidazole rings of HSA may be positioned close enough together to form a complex with Cd^{2+} or Zn^{2+}, as predicted for site A (115 ppm, 155 ppm in the presence of Ca^{2+}).

^{113}Cd NMR signals for Cd_1-recombinant domain I (residues 1−194) are too broad to detect[38] which suggests that the two strong Cd^{2+} binding sites of HSA do not lie in this domain alone, although the Cd binding sites could lie between domains I and II. ^{113}Cd NMR spectra of other mammalian albumins appear to possess two similar strong binding sites. Sequence comparisons[1] show that several histidine residues are conserved in all the mammalian albumins studied. There are 14 conserved His residues in human, bovine, and pig albumins. Of these, His3 may be eliminated as a Cd^{2+} ligand, and from views of the crystal structure, it appears that His9, His39, His67, and His146, present in domain I, may also be discounted, as these are distant from His residues in the other domains. There seems to be only one case in which two, or possibly three, His residues lie close in space: His105 and His146 in domain I, and His247 from domain II. Interestingly the regions close to His105 and His247 are relatively rich in Asp and Glu residues (Asp107, Asp108, Asp249, Glu252, Asp255, Asp256, Asp259), which are also potential ligands for Cd^{2+} and Zn^{2+}. A proposal for site A with tetrahedral coordination for Cd^{2+} and Zn^{2+} is shown in Figure 7, although a higher coordination number with additional bound oxygen ligands may also be possible. Added Ca^{2+} may compete for some of the Asp or Glu residues bound to Cd^{2+}.

The high-field ^{113}Cd site B is likely to be rich in oxygen (Asp and/or Glu) ligands. There are 36 Asp and 62 Glu residues in HSA, of which 41 are conserved in the albumins studied. Consequently, a prediction of the high field Cd^{2+} site must be highly speculative and limited to Asp/Glu-rich loops. A classic Ca^{2+} E−F hand motif does not appear to be present within albumin, although there is a loop region between a disulfide bridge formed by Cys559 and Cys567, which contains three conserved carboxylate residues: Asp562, Asp563, and Glu565.

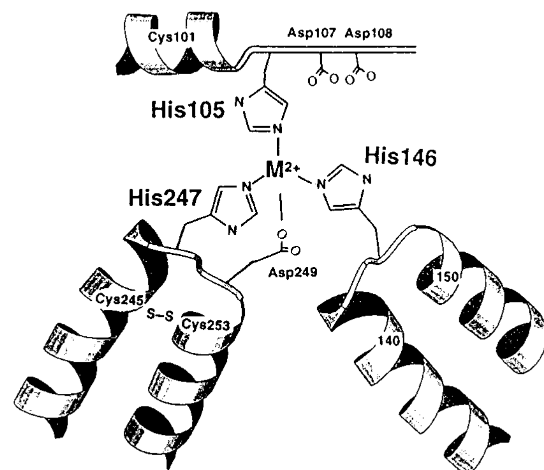

Figure 7. Proposal for the shared Cd^{2+} and Zn^{2+} binding site in HSA (site A), containing His105, His146, His247, and one or more carboxylate ligands (Asp249, Asp107, or Asp108).

Conclusions

^{113}Cd NMR studies of several mammalian albumins reveal two strong Cd^{2+} binding sites with shifts in the ranges 113−124 ppm (site A) and 24−28 ppm (site B). ^{113}Cd NMR studies of Cys34-blocked BSA suggest that the thiol ligand (at Cys34) is not involved in the two strong Cd^{2+} binding sites. Ca^{2+} binding to HSA has a marked influence on Cd^{2+} binding at site A, and this is also observed when Cd^{2+} is added to blood serum. The competitive binding of Zn^{2+} at Cd^{2+} site A[26,27] was confirmed by ^{1}H NMR spectroscopy; similar amino acid residues are perturbed by both Cd^{2+} and Zn^{2+} binding, including His residues which may be in the binding site. One molar equivalent of Cu^{2+} does not compete for the low field Cd^{2+} binding site of BSA or HSA. Our ^{1}H NMR studies suggest that the N-terminus of HSA is not the locus of the two strong Cd binding sites. We have shown that in addition to Zn^{2+}, the metal ions Cu^{2+} and Ni^{2+} have a high affinity for Cd^{2+} site A once the N-terminal site is saturated. As with the primary binding of Zn^{2+}, secondary binding of Cu^{2+} and Ni^{2+} displaces Cd^{2+} from site A into a third site (site C). His residues 247, 146, and 105 together with one or more oxygen (Asp or Glu) residues could form a common interdomain Cd^{2+}/Zn^{2+} binding site, site A.

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Supporting Information Available: Figure S1, 59.92 MHz ^{113}Cd NMR spectra of HSA (2 mM) in the presence of 1 mol equiv of $^{113}CdCl_2$, 0.1 M phosphate buffer, pH* 7, before and after addition of 1 mol equiv of Cu^{2+} or Ni^{2+}, and Figure S2, methyl and methylene regions of 600 MHz ^{1}H NMR spectra of HSA (A) before and after addition of 2 mol equiv of $CdCl_2$ pH* 7.0 and (B) before and after addition of 2 mol equiv of Zn^{2+}, pH* 6.8 (2 pages). Ordering information is given on any current masthead page.

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