Evidence that the Principal Co^{II}-Binding Site in Human Serum Albumin Is Not at the N-Terminus: Implication on the Albumin Cobalt Binding Test for Detecting Myocardial Ischemia‡

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ABSTRACT: Human serum albumin (HSA) is the most abundant protein in the blood plasma and is involved in the transport of metal ions. Four metal-binding sites with different specificities have been described in HSA: (i) the N-terminal site provided by Asp1, Ala2, and His3, (ii) the site at the reduced Cys34, (iii) site A, including His67 as a ligand, and (iv) the nonlocalized site B. HSA can bind Co^{II}, and HSA was proposed to be involved in Co^{II} transport. Recently, binding of Co^{II} to HSA has attracted much interest due to the so-called albumin cobalt binding (ACB) test approved by the Food and Drug Administration for evaluation of myocardial ischemia. Although the binding of Co^{II} to HSA is important, the binding of Co^{II} to HSA is not well-characterized. Here the binding of Co^{II} to HSA was studied under anaerobic conditions to prevent Co^{II} oxidation. Electronic absorption, EPR, and NMR spectroscopies indicate three specific and well-separated binding sites for Co^{II} in HSA. Co^{II} ions in all three sites are in a high-spin state and coordinated in a distorted octahedral geometry. Competition experiments with Cd^{II} (known to bind to sites A and B) and Cu^{II} (known to bind to the N-terminal site) were used to identify the sites of binding of Co^{II} to HSA. They revealed that the first two equivalents of Co^{II} bind to sites A and B. Only the third may be bound to the N-terminal site. The repercussions of these results on the understanding of the ACB test and hence the myocardial ischemia are discussed.

Human serum albumin (HSA,[1] 66.5 kDa), a single chain of 585 amino acids, is the most abundant protein in blood plasma, typically present at concentrations of ∼0.6 mM (*1*). It consists of three structurally homologous, largely helical (67%) domains [I, II, and III (*2*)]. Each domain consists of two subdomains, A and B. Like other mammalian albumins, human albumin contains 17 disulfide bridges and a free thiol at Cys34 (*1*).

HSA has been proposed to be involved in metal ion transport and has a variety of metal sites with different specificities (*3*, *4*). Four different binding sites can be readily distinguished (Figure 1). The best-characterized metal site on albumin is that for Cu^{II} and Ni^{II}. These metal ions bind strongly to a square-planar site of four nitrogen ligands from Asp1, Ala2, and His3 at the N terminus (*6*−*8*). This motif exists in different native peptides and proteins and was dubbed the amino-terminal Cu^{II}-, Ni^{II}-binding (ATCUN) motif (*9*).

The second binding site is at Cys34, where Au^{+} (from antiarthritic drugs) binds to the thiolate sulfur (*10*). The third and fouth metal-binding site are called sites A and B, based on ^{113}Cd NMR experiments demonstrating the existence of two strong Cd^{II}-binding sites (*4*, *5*, *11*). Recently, the A site has been identified (*12*). It has been clearly demonstrated that His67 is a ligand to Cd^{II} in this site A. Molecular modeling and sequence comparison suggested as further ligands Asn99, His247, Asp240, and H_2O. The latter can be replaced with Cl^{-} at higher concentrations. This site A is also the preferential site of Zn^{II}. Zn^{II} has been shown to supplant Cd^{II} from site A, suggesting that Zn^{II} binds more strongly than Cd (*4*, *5*, *11*). This is also in line with the report that the first equivalents of Cu^{II} and Zn^{II} do not bind to the

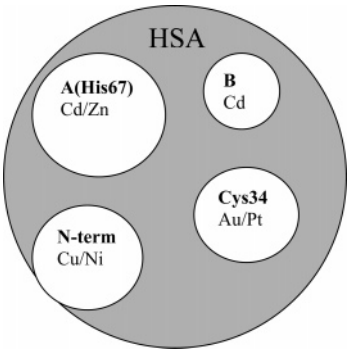

FIGURE 1: Scheme of the metal-binding sites in HSA: N-terminal site, site A with His67 as a ligand; site B, so far not localized; site with the ligand Cys34. Cu^{II} and Ni^{II} bind preferentially to the N-terminal site. Cd^{II} binds to sites A and B; Zn^{II} prefers site A, and Au^{I} and Pt^{II} bind to Cys34.

‡ This article is dedicated to Jean-Jacques Bonnet.

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[1] Abbreviations: ABC test, albumin cobalt binding test; ATCUN, amino-terminal Cu^{II}-binding, Ni^{II}-binding; DAHK, Asp-Ala-His-Lys tetrapeptide; EPR, electron paramagnetic resonance; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; HSA, human serum albumin.

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same site (*13*). NMR and circular dichroism studies suggest that site A is also a secondary (weaker) binding site for Cu^{II} and Ni^{II} (*3*, *11*). The second Cd^{II} site, called site B, exhibited a Cd^{II} chemical shift of ~20 ppm, indicating not more than one nitrogen ligand is involved (*12*). This site has not yet been localized. A possible Zn^{II} site at residues 35−37, consisting of Asp, Glu, and His, has been discussed, on the basis of studies with model tripeptides (*14*). As a summary, the different sites are given in Figure 1. We refer to the different binding sites as the N-terminal site, site A, site B, and the cysteine-binding site. In terms of metal, the first equivalent of Cu^{II} or Ni^{II} will bind to the N-terminal site and the second equivalent to site A. Cd^{II} binds to sites A and B with approximately the same affinity.

A very interesting interaction between Cd^{II} binding in site A and fatty acid binding has been reported (*12*). Stewart et al. suggested that fatty acid binding disrupts Cd^{II}/Zn^{II}-binding site A, i.e., that Cd^{II}/Zn^{II} and fatty acid have a negative allosteric interaction. The interaction of Zn^{II} with HSA is physiologically relevant since the concentration of Zn^{II} in blood plasma is ~19 μM. Most of this Zn is bound to HSA (affinity constant log $K_d = -7.53$) (*13*, *15*).

Not much is known about the binding of Co^{II} to HSA despite the fact that HSA has been suggested to be a main transporter of Co^{II} (*16*) and binding of Co^{II} to bovine serum albumin was studied 50 years ago (*17*−*19*). ^{1}H NMR of HSA showed that Co^{II} perturbed the resonances of the N-terminal Asp-Ala-His-Lys motif (ATCUN motif). This suggested that Co is able to bind to the N-terminal site (*8*). Moreover, it has been shown that Co^{II} binds to small peptides of the N-terminal region containing the ATCUN motif (*20*, *21*).

Recently, much interest was paid to binding of Co^{II} to HSA due to the so-called albumin cobalt binding (ACB) test (*22*−*25*; for a review, see ref *26*). This test is approved by the Food and Drug Administration (FDA) for evaluation of myocardial ischemia (*27*). The test is based on the idea that changes in the structure of albumin occur in myocardial ischemia which affects Co^{II} binding. The test was first described by Bar-Or et al. (*22*). It consists of incubating exogenous Co^{II} with blood serum (at a ratio of ~1.5 equiv of Co^{II} per HSA). Then an excess of dithiothreitol (in H_2O) is added, which is supposed to chelate the unbound Co^{II}, forming a brown precipitate. This unbound Co^{II} can be quantified by measuring the absorption of Co-bound DTT at 470 nm. It is thought that the structure of HSA is changed in myocardial ischemia, which leads to a weaker Co^{II} binding capability and as a consequence to higher concentrations of unbound Co^{II}. The latter is detected with dithiothreitol. The weaker Co^{II} binding has been ascribed to structural change in the N-terminal binding site (*21*, *22*).

In light of the importance of the ACB test and the novel and interesting insights into the binding of Cd^{II} or Zn^{II} to HSA, let us reinvestigate the binding of Co^{II} to HSA. In particular, the question of whether Co^{II} really binds to the N-terminal site as suggested was addressed. Preferential binding of Co^{II} to this site is not expected from Co coordination chemistry. Co^{II} prefers octahedral, penta, or tetrahedral geometries, like Zn^{II} and Cd^{II}, and not square planar, like the Cu in the N-terminal site of HSA.

MATERIALS AND METHODS

Sample Preparation

Essentially fatty acid free human serum albumin (HSA) was used if not otherwise stated (Sigma). HSA was used without further purification. Only in the case of NMR experiments was HSA purified over a Superdex 75 10/300 GL column with an AKTA instrument (Amersham Pharmacia Biotech) under isocratic conditions [30 mM phosphate buffer (pH 7.0)].

HSA was dissolved in 40 mM HEPES and adjusted to pH 8.0. HSA concentrations was determined by using an ϵ at 280 nm of 33 000 cm^{-1} M^{-1} (*28*). The usual concentrations of HSA were between 0.35 and 0.7 mM. The tetrapeptide Asp-Ala-His-Lys (DAHK) was purchased from Bachem, dissolved in 40 mM HEPES, and adjusted to pH 8.0. The concentration of DAHK was determinded by weight (molecular mass of 469.5 Da). The concentrations of HSA and DAHK were confirmed by a titration with Cu^{II} based on the knowledge that both can bind 1 equiv of Cu^{II} with the typical absorption maximum at 520 nm [in the case of HSA, the fact that ~5% of the N-terminus is truncated and thus not available for Cu binding has been taken into account (*29*)]. The metal ions were added from a concentrated metal solution of $CoCl_2$, $CdCl_2$, or $CuCl_2$ in H_2O. If not otherwise stated, all the experiments including Co were conducted under anaerobic conditions (argon atmosphere). The solutions of $CoCl_2$ and HSA were degassed by three cylcles of freezing and thawing under argon. Co^{II} concentrations were determined after the experiments by acidification with 12 M HCl below pH 2. This leads to a $CoCl_4$ complex with typical d−d transitions at 624 nm ($\epsilon = 407$ cm^{-1} M^{-1}), 662 nm ($\epsilon = 594$ cm^{-1} M^{-1}), and 691 nm ($\epsilon = 635$ cm^{-1} M^{-1}). Dialysis experiments were performed with 100 μL of HSA at a concentration of ~0.7 mM by adding 10 equiv of $CoCl_2$ per HSA. The buffer was 40 mM HEPES (pH 8.0) with or without 10 mM citrate. The samples were dialyzed overnight at 4 °C against 1 L of 40 mM HEPES buffer (pH 8.0).

Spectroscopy

UV−*Vis.* UV−visible absorption spectra were recorded at room temperature on a Spectrometer Cary 2300 instrument in a sealed quartz cuvette (path length of 1 cm). Due to the small extinction coefficient of the d−d bands of Co^{II}, the absorption changes were relatively small and thus difficult to measure. All the Co^{II} titrations were repeated several times. The obtained extinction coefficient (ϵ) and maximal absorption (λ_{max}) were reproducible in the limits given in Table 1 and after a baseline correction was performed. This correction included the subtraction of a linear baseline, which intersected the spectrum before and after the d−d transitions (i.e., at the trough around 420 and 700−800 nm).

EPR. X-Band electron paramagnetic resonance (EPR) data were recorded using an Elexsys ESP 500 device, operating at a microwave frequency of ~9.5 GHz. All spectra were recorded using a microwave power of 2 mW across a sweep width of 7000 G (centered at 3500 G) with a modulation amplitude of 10 G. Samples were frozen in a quartz tube by adding 10% glycerol as a cryoprotectant in 50 mM HEPES. Experiments were carried out at 4 K using a liquid helium cryostat.

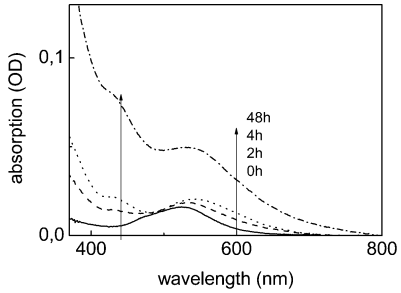

FIGURE 2: Time course of the electronic absorption difference spectrum of Co_1−HSA vs HSA upon exposure to air. Conditions: 0.35 mM HSA in 40 mM HEPES (pH 8.0). Co was added to HSA under argon and exposed to air at time zero.

NMR. After a purification step (gel filtration; see above), HSA in phosphate buffer was freeze-dried and subsequently dissolved in D_2O. Freeze-drying and dissolving in D_2O were repeated to completely exchange H_2O with D_2O. The pH* (pH meter readings in D_2O) was adjusted to 7.0 with NaOD. This corresponds to a pD of 7.4. The final concentration of HSA was ~2 mM in ~0.1 M phosphate buffer.

NMR spectra were collected using a Bruker Avance 500 spectrometer equipped with a 5 mm triple-resonance inverse Z-gradient probe. All chemical shifts for ^1H are relative to tetramethylsilane. ^1H NMR spectra were recorded at 310 K in D_2O.

RESULTS

Co^{II} Oxidation. Initial Co^{II} titrations to HSA were conducted under an aerobic atmosphere. A change in the spectrum with time was observed. This was assigned to partial oxidation of Co^{II} to Co^{III} (30−40% after a few minutes) as suggested by Co^{II} quantification in the sample. Figure 2 shows the absorption changes of Co^{II}-bound HSA prepared under argon upon exposure to air. The difference spectrum of Co^{II}-bound HSA versus HSA (Figure 2, solid line) showed the d−d transition of Co^{II} at 527 nm with a shoulder at ~475 nm. After exposure to air, the difference spectrum of Co^{II}-bound HSA versus HSA changed with time (Figure 2). Two new and more intense bands around 430 and 550 nm appeared. Similar bands have been observed for octahedral Co^{III} complexes with N and O ligands (see, e.g., ref *30*). When titration experiments were performed under argon, no Co^{II} oxidation occurred. Thus, for the rest of the study, titrations were performed under an anaerobic atmosphere.

Co^{II} Titration to HSA. One to four equivalents Co^{II} was titrated to HSA. Figure 3 shows the difference spectra induced by each equivalent of Co^{II} added (obtained by subtraction of the spectrum before addition of Co^{II} from the spectrum after addition of Co^{II}). Each equivalent of Co^{II} that was added induced a band typical for the d−d transitions. The absorption maximum and extinction coefficient of the difference spectra induced by each equivalent of Co^{II} are given in Table 1.

In general, each equivalent Co^{II} that was added (from 1 to 4) induced a d−d transition around 520 nm with an extinction coefficient (ϵ) of $<40\ M^{-1}\ cm^{-1}$. Such an ϵ below $50\ M^{-1}\ cm^{-1}$ is typical for an octahedral coordination geometry of high-spin Co^{II}. Pentacoordinated and tetrahedral Co^{II} complexes exhibit higher extinction coefficients, i.e., $50\ M^{-1}\ cm^{-1} < \epsilon < 300\ M^{-1}\ cm^{-1}$ and $\epsilon > 300\ M^{-1}\ cm^{-1}$, respectively (*31*). Although 4 equiv of Co^{II} is in an octahedral environment, their d−d absorption signatures were significantly different (Table 1). The first 3 equiv of Co^{II} has a higher ϵ, and their maximal absorption is red-shifted compared to that of Co in the buffer (in the absence of HSA). This indicates that the first 3 equiv of Co^{II} was bound to HSA. In contrast, the spectroscopic features (ϵ and λ_{max}) of the fourth equivalent of Co^{II} bound to HSA did not differ significantly from those of Co^{II} in the buffer. These indicate that HSA has three specific Co-binding sites.

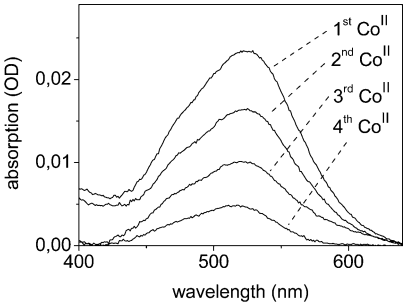

FIGURE 3: Electronic absorption difference spectrum of Co^{II} titration to HSA: first Co^{II}, Co_1−HSA vs HSA; second Co^{II}, Co_2−HSA vs Co_1−HSA; third Co^{II}, Co_3−HSA vs Co_2−HSA; and fourth Co^{II}, Co_4−HSA vs Co_3−HSA. Conditions: 0.63 mM HSA in 40 mM HEPES (pH 8.0).

Table 1: Titration of Co^{II} to HSA

	extinction coefficient ($M^{-1}\ cm^{-1}$)	absorbance maximum (nm)
first Co^{II}−HSA	34 ± 5	527 ± 2
second Co^{II}−HSA	28 ± 5	523 ± 2
third Co^{II}−HSA	15 ± 3	519 ± 2
fourth Co^{II}−HSA	8 ± 3	515 ± 3
Co^{II} in HEPES buffer	5 ± 2	512 ± 2
Co^{II}−DAHK	15 ± 3	515 ± 3

Dialysis experiments revealed that between 1 and 2 equiv of Co^{II} was bound to HSA after dialysis. The addition of 10 mM citrate to the buffer did not decrease the number of Co atoms bound to HSA, indicating that these binding sites were stronger than the binding to citrate. The high uncertainty surrounding the 1−2 equiv of Co^{II} bound to HSA was due to the partial oxidation of Co^{II} to Co^{III}. This occurred because dialysis experiments had to be done aerobically (see above). Thus, the 1−2 equiv of Co^{II} bound to HSA is only a lower limit. Together with the absorption experiments, this indicates that HSA has two to three specific Co^{II}-binding sites, from now on designated as first Co^{II}−HSA, second Co^{II}−HSA, and third Co^{II}−HSA.

EPR Spectroscopy. To further characterize the binding sites, EPR spectroscopy was applied. The Co^{II} in all three binding sites (i.e., first Co^{II}−HSA, second Co^{II}−HSA, and third Co^{II}−HSA) exhibited a broad axial signal with a $g_\perp$ of ~4.4 and a $g_{||}$ of ~2.6 (latter not resolved) (Figure 4). No hyperfine structure was observed (^{59}Co which has 100% abundance and an I of $^7/_2$). Similar features have been observed for other high-spin Co^{II} complexes (see, e.g., refs *32*−*34*; for a review, see ref *35*). They are also in line with the conclusions from the observed d−d transitions in electronic absorption, i.e., that all three Co^{II} ions are high-spin and in a distorted octahedral environment (see above). Due to the poor distinction of the three Co^{II} sites by EPR,

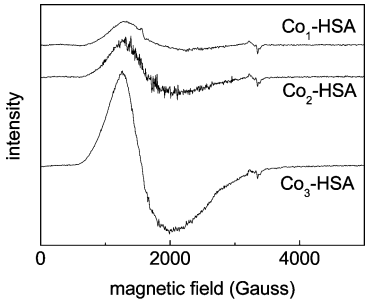

FIGURE 4: EPR spectra of HSA with 1, 2, and 3 equiv of Co^{II} added. Conditions: 4 K with a microwave power of 2 mM.

Table 2: Titration of Co^{II} to Cd^{II}_2−HSA and Cu^{II}_1−HSA

	extinction coefficient (M^{-1} cm^{-1})	absorbance maximum (nm)
first Co^{II}−Cd^{II}_2−HSA	18	518
second Co^{II}−Cd^{II}_2−HSA	14	515
third Co^{II}−Cd^{II}_2−HSA	11	516
first Co^{II}−Cu^{II}_1−HSA	34	526
second Co^{II}−Cu^{II}_1−HSA	33	523
third Co^{II}−Cu^{II}_1−HSA	16	519
fourth Co^{II}−Cu^{II}_1−HSA	9	517

the spectra were not further analyzed. The intensity of the EPR signal increased upon each addition of each equivalent of Co^{II} to HSA,[2] indicating that there was no strong ferromagnetic coupling among the three Co centers (see also below).

Identification of the Three Co^{II}-Binding Sites of HSA. On the basis of the literature, four metal-binding sites were well-known (see Figure 1 and the introductory section). The binding of Co^{II} to Cys34 could be readily excluded, because binding of Co to cysteine induces an intense ligand to metal charge transfer band around 320 nm ($\epsilon \sim 1000$ M^{-1} cm^{-1}) (see, e.g., ref *36*). Such a band has not been observed for first Co^{II}−HSA, second Co^{II}−HSA, and third Co^{II}−HSA (see above).[3] To investigate which other binding site (A, B, or N-terminal) Co^{II} binds, competition experiments with Cd^{II} and Cu^{II} were performed.

Competition with Cd^{II}. HSA is known to bind two Cd^{II} ions to the binding sites called A and B (*12*). The first binding site is located at His67, and the second one has not been clearly identified yet. We tested if Cd^{II} and Co^{II} were in competition for the same binding sites. The titration of Co^{II} to a sample of Cd^{II}_2−HSA (HSA which had previously incubated with 2 equiv of Cd^{II}) was performed. The ϵ and λ_{max} of the d−d bands induced by binding of each equivalent of Co^{II} are given in Table 2. The addition of the first equivalent of Co induced a d−d band with an ϵ of 18 M^{-1} cm^{-1} and a λ_{max} at 518 nm. These parameters do not agree with the parameter of first Co^{II}−HSA or second Co^{II}−HSA, but rather with third Co^{II}−HSA. This indicates that Cd occupied and hence blocks the first two binding sites for

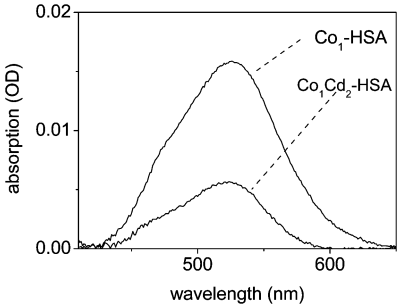

FIGURE 5: Effect of addition of Cd to Co_1−HSA. The spectrum Co_1−HSA shows the d−d band of Co^{II} bound to HSA before addition of Cd^{II} and the Co_1−Cd_2−HSA spectrum that after 2 equiv of Cd had been added.

Co^{II} (Cd is a d_{10} metal; no d−d transitions are observed). In agreement with this, the second Co^{II} added to Cd_2−HSA had an ϵ of 14 M^{-1} cm^{-1} and a λ_{max} at 515 nm resembling fourth Co^{II}−HSA, i.e., the fourth Co^{II}-binding site (in the absence of Cd^{II}). They are not in line with the first two binding sites of HSA, which have an ϵ of >23 M^{-1} cm^{-1} and a λ_{max} of >520 nm (see Table 1). These results suggest that the 2 equiv of Cd^{II} binds to Co-binding sites 1 and 2 and blocks the binding of Co^{II}, i.e., due to a higher binding affinity of Cd^{II}.

To confirm that, the opposite experiment was performed; i.e., Cd^{II} was added to Co^{II}_1−HSA. One equivalent of Co^{II} was added to HSA which formed Co^{II}_1−HSA (Figure 5). Co^{II}_1−HSA exhibited typical values: $\epsilon \sim 35$ M^{-1} cm^{-1} and $\lambda_{max} \sim 526$ nm. Upon addition of 2 equiv of Cd^{II}, the d−d bands of the Co^{II} changed drastically to an ϵ of ~12 M^{-1} cm^{-1} and a λ_{max} of ~520 nm. This resembles the third Co^{II} site. This suggests strongly that Cd^{II} binds to their sites A and B and pushes out the Co^{II} due to the higher affinity. The Co^{II} thus liberated binds to the next unoccupied Co^{II} site, which is third Co^{II}−HSA.

Similar experiments were conducted with the addition of 2 equiv of Cd to Co^{II}_2−HSA (not shown). In this case, the spectra indicated that Cd replaced both Co ions in HSA, and the Co thus released binds to the third and fourth Co sites, i.e., third Co^{II}−HSA and fourth Co^{II}−HSA.[4] Taken together, these experiments suggest that the first two binding sites of Co^{II} are the same as those for Cd^{II}, i.e., the sites called A and B (see Figure 1). Moreover, it indicates as well that Cd^{II} binds more strongly than Co^{II} to either of the two sites.

Competition with Cu^{II}. Cu^{II} has been well-known to bind first to the N-terminus of HSA (Asp-Ala-His). To evaluate if Co^{II} competes with this binding site, we performed a titration of Co^{II} to the Cu^{II}−HSA complex. Figure 6 shows after addition of 1 equiv of Cu^{II} the typical d−d band with an ϵ of ~80 M^{-1} cm^{-1} and an Abs_{max} of ~526 nm. The absorption is at the same place as the Co^{II} d−d transitions; however, the ϵ is higher, and no shoulder is observed. The spectrum of each equivalent of Co^{II} added to the Cu^{II}−HSA

[2] The intensities varied quite a bit between the different titration experiments (±50%). However, a survey of the different titrations suggests that the intensity is steadily increasing. Thus, a weak interaction between two Co centers cannot be excluded. However, a strong interaction such as a dinuclear Co center with a bridging ligand is very unlikely.

[3] So far, only gold and platinum have been shown to bind to this cysteine. Even the relatively soft Cd does not bind to this cysteine at the first place. Hence, the harder Co is not expected to bind this Cys.

[4] Addition of 1 equiv of Cd^{II} to Co^{II}_1−HSA produced no significant change in the Co^{II} d−d bands (not shown). This is in agreement with the binding of Co(II) to site A or B, due to the following. Sites A and B have approximately the same affinity for Cd^{II} [because in Cd^{II}_1−HSA half of the Cd is located in site A and the other half in site B (*11*)]. As a consequence, when 1 equiv of Cd^{II} is added to Co^{II}_1−HSA, Cd^{II} will occupy the free site (i.e., A or B) and not displace Co^{II} from its site. As Co^{II} stays in its binding site, its absorption spectrum should not change.

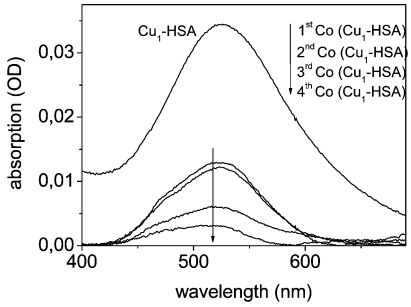

FIGURE 6: Electronic absorption difference spectrum of Co^{II} titration to Cu_1−HSA: first Co^{II} (Cu_1−HSA), Co_1−Cu_1−HSA vs Cu_1−HSA; second Co^{II} (Cu_1−HSA), Co_2−Cu_1−HSA vs Co_1−Cu_1−HSA; third Co^{II} (Cu_1−HSA), Co_3−Cu_1−HSA vs Co_2−Cu_1−HSA; fourth Co^{II} (Cu_1−HSA), Co_1−Cu_1−HSA vs Co_3−Cu_1−HSA. Conditions: 0.38 mM HSA in 40 mM HEPES (pH 8.0).

is depicted in Figure 6 (for a better comparison, the difference spectra are shown). The parameters ϵ and λ_{max} of the difference spectra are given in Table 2.

It is clear that the ϵ and λ_{max} values of the first 2 equiv of Co^{II} (first Co^{II}−$Cu^{II}{}_1$−HSA and second Co^{II}−$Cu^{II}{}_1$−HSA) were not significantly different from those for the titration of Co^{II} to HSA in the absence of Cu^{II}. This is in line with the interpretation that the first two Co^{II} ions bind to Cd^{II} sites A and B and not to the N-terminal biding site (ATCUN). The third equivalent of Co added to Cu^{II}−HSA yielded ϵ and λ_{max} values similar to those in the absence of Cu. This would indicate that the third Co site is not identical with the N-terminal binding site. However, this possibility cannot be excluded because the differences between the third and fourth Co sites are relatively small and Cu^{II} bound to HSA absorbs in the same region as Co^{II}.

To gain insight about the d−d band of Co bound to the N-teminal binding site, the binding of Co to the tetrapeptide Asp-Ala-His-Lys was studied. This peptide is the N-terminal sequence of HSA and has been shown to exhibit metal binding properties very similar to those of HSA (*9*). The Co^{II} bound to the Asp-Ala-His-Lys peptide (DAHK) exhibited an ϵ of 15 M^{-1} cm^{-1} and a $\lambda_{max}\epsilon$ of 515 nm, not in agreement with the first two Co sites in HSA but rather with the third site. Taken together, the data are in line with the binding of the first 2 equiv of Co^{II} to Cd^{II}-binding sites A and B. The question of whether the third Co site is at the N-terminus cannot be answered conclusively, but this solution seems to be the most likely.

EPR Spectroscopy. The absence of significant EPR differences in the Co^{II}-binding sites and Co^{II} in buffer did not allow the measurement of the competition between Co^{II} and Cu^{II} (or Co^{II} and Cd^{II}). On the other hand, EPR measurements were used to investigate if the binding of Co^{II} affects the EPR spectrum of $Cu^{II}{}_1$−HSA and $Cu^{II}{}_2$−HSA. Cu^{II} is generally well characterized by EPR, and in particular, $Cu^{II}{}_1$−HSA has been described well (*9*, *37*−*40*). Thus, it should be easier to detect an interaction between metal centers on the Cu^{II} signal than on the less-characterized Co^{II}−HSA signal. Figure 7 shows the EPR spectra of $Co^{II}{}_1$−HSA, $Co^{II}{}_1$−$Cu^{II}{}_1$−HSA, $Co^{II}{}_1$−$Cu^{II}{}_2$−HSA, and $Co^{II}{}_2$−$Cu^{II}{}_1$−HSA. The EPR signals originating from Co^{II} and from Cu^{II} are relatively well separated (Co^{II} below 2800 G and Cu^{II} above). $Co^{II}{}_1$−$Cu^{II}{}_1$−HSA and $Co^{II}{}_2$−$Cu^{II}{}_1$−HSA exhibit the typical parameters of Cu^{II}−HSA, where Cu^{II} is bound to four nitrogens in a square planar geometry; i.e., $g_\perp = 2.05$, $g_{||} =$

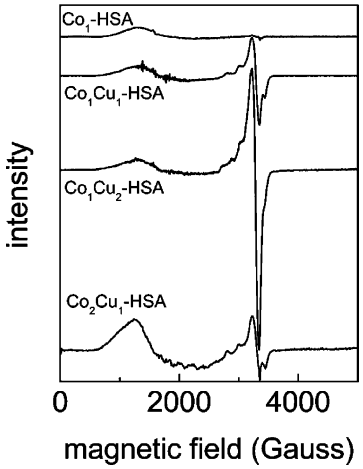

FIGURE 7: EPR spectra of HSA with Co^{II} and Cu^{II} added as indicated. Conditions: 4 K with a microwave power of 2 mM.

2.18, and $a_{||} = 201$ G. There is no indication of a strong magnetic interaction, since the spectrum of Co^{II} is the same in the absence and presence of 1−2 equiv of Cu^{II}. Reversibly, the addition of Co^{II} does not influence the spectrum of $Cu^{II}{}_1$−HSA and $Cu^{II}{}_2$−HSA (see, e.g., ref *3*). This indicates that there is no strong magnetic interaction between the three binding sites. This is also in line with the titration of Co (see above), in which no interaction between the different Co equivalents was observed.

^{1}H NMR Spectroscopy. ^{1}H NMR has been used in the past to investigate the interaction of HSA with different metals (*8*, *11*). Although HSA is a relatively large protein (69 kDa), some of the resonances were sufficiently resolved to be identified. In particular, 11 peaks between 7.5 and 8.5 ppm could be assigned to the ϵ_{C-H} of histidines (named His I−His XI), although some of them are quite broad (*11* and references cited therein). It has been shown that binding of Cu to HSA affects predominantly the His peak V (assigned to His3); in contrast, Cd affects mostly His peaks VII, VIII, and IX (likely His of sites A and B). For Co^{II}, it has been reported that it broadens the resonances of N-terminal residues, including HisV, and it was concluded that Co^{II} binds to the N-terminal site (see also the introductory section) (*8*). This seems to contradict the results presented above which show that Co^{II} binds to sites A and B (but see Discussion). Since the experiment in ref *8* has been carried out under aerobic conditions (leading likely to partial oxidation of Co^{II}), we reinvestigated the paramagnetic effect of Co^{II} on the His resonances of HSA under anaerobic conditions.

First, the well-established effect of Cu^{II} on the resonances of His was confirmed. The results reported in Figure 8 (top panel) shows the ^{1}H NMR of HSA with and without 0.7 equiv of Cu^{II} in the region of ϵ_{C-H} His resonances. It can be clearly seen that the addition of Cu^{II} affects predominantly the resonance of HisV, which broadened below detection. Identical behavior has been reported in the literature (*8*) and is in line with the fact that Cu binds to the N-terminal site. In the case of the addition of 0.7 equiv of Co^{II} to HSA (Figure 9, top panel), the effect was more drastic and less specific. All of the resonances were affected, but to a different degree. His VI and His IV were the least affected. With regard to the effect on the His of the Cu^{II} and Cd^{II} sites, one can observe that HisV (Cu^{II} site) is affected in line with the literature (*8*). But also His VII, VIII, and IX from Cd^{II} sites

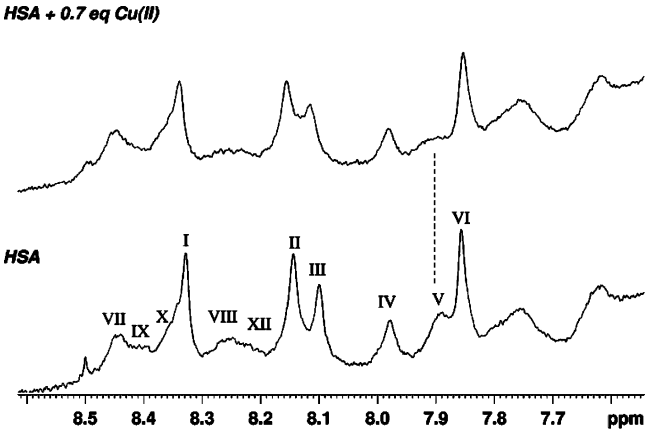

FIGURE 8: ^{1}H NMR spectra of HSA before (bottom spectrum) and after addition of 0.7 equiv of Cu^{II} (top spectrum) in D_2O at pD* 7.0. The resonances were attributed to the ϵ C−H of histidines and identified with Roman numerals (*11*). His V (indicated by a dashed line) corresponds to the histidine of the N-terminal binding site (His at position 3). pD* 7.

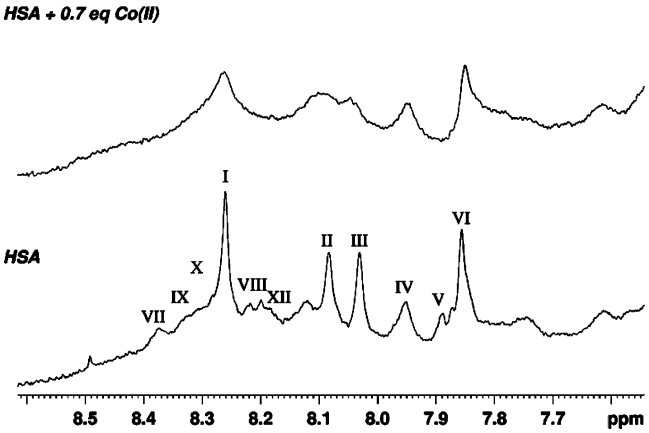

FIGURE 9: ^{1}H NMR spectra of HSA before (bottom spectrum) and after addition of 0.7 equiv of Co^{II} (top spectrum) in D_2O at pD* 7.0. The resonances were attributed to the ϵ C−H of histidines and identified by Roman numerals (*11*). His V corresponds to the histidine of the N-terminal binding site and His (His at position 3). Histidine peaks VII, VIII, and IX have been attributed to sites A and B.

A and B were highly affected by broadening below the detection limit. In general, the peaks of interest were already relatively broad before Co^{II} addition, and the quantification of broadening seems to be very difficult due to the fact that all peaks were broadened. However, the NMR results do not contradict the conclusion from the absorption that Co^{II} binds predominantly to sites A and B, but it indicates that Co^{II} could also partially or transiently bind to the N-terminal site (see also Discussion).

DISCUSSION

The data presented here suggest that at least 3 equiv of Co^{II} can specifically bind HSA at three different binding sites. They bind in an octahedral geometry. Dialysis experiments indicated that 2−3 equiv of Co^{II} was bound relatively firmly, in agreement with the literature (*16*). Co^{II} bound to HSA underwent partial oxidation. The three Co^{II} sites seem not to interact magnetically with each other, indicating that they are relatively distant. UV−vis spectra indicate that the first 2 equiv of Co^{II} binds to the Cd^{II} sites called A and B (see Figure 1). Only the third equivalent of Co^{II} would be available for binding at the N-terminal Cu^{II} site. Indeed, Co^{II} is able to bind to the tetrapeptide with the N-terminal HSA Asp-Ala-His-Lys sequence (*21*) and exhibited similar d−d transitions as the third Co binding site in HSA. It is clear that Co^{II} would bind in a distorted octahedral geometry to the N-terminus, and it is thus not clear if Co^{II} has the same four nitrogen ligands as the square planar Cu^{II} and Ni^{II}.

The ^{1}H NMR data indicate a broadening of almost all ϵ C−H His resonances upon binding of Co^{II}. On the basis of the suggestion that Co^{II} binds to the Cd^{II} sites, one would expect that binding of Co^{II} to HSA affects most strongly His peaks VII, VIII, and IX. Indeed, these three peaks were broadened beyond the detection limit. However, these peaks were already relatively broad before Co^{II} addition, and the quantification of broadening seems difficult due to the fact that all peaks were broadened. Moreover, also peak His V from the N-terminal site was broadened beyond detection. This broadening of His V (together with resonances of Asp1, Ala2, and Lys4) has been also reported by Sadler et al. (*8*). They concluded that Co^{II} binds to the N-terminal site. Their experiment was performed under aerobic conditions, and only 0.125 equiv of Co^{II} was added. Closer inspection of their results revealed also that His VIII and perhaps His VII and XI were affected (*8*). Thus, taken together, the results of addition of either 0.7 equiv of Co^{II} anaerobically or 0.125 of equiv Co^{II} aerobically were quite similar and suggest that Co^{II} perturbs the His from Cd sites A and B as well as the His from the N-terminal site. This does not contradict the results obtained by UV−vis and EPR, which clearly showed that Co^{II} binds preferentially to sites A and B, because the paramagnetic perturbation of the ^{1}H resonances is difficult to quantify and could be due to a partially and/or transient binding. Indeed the addition of 0.125 equiv of Co affected all the His resonances, indicating that Co is rapidly exchanged between the different HSA molecules (otherwise, one would expect that only 12.5% of the resonance are affected and 87.5% are not affected) (*8*).

Taken together, the following can be proposed, which reconciles the present experiments and the results in the literature: Co^{II} is bound predominantly to sites A and B. It exchanges rapidly between the HSA molecules. Co^{II} binds also transiently to the N-terminal site, but the occupation of this site is only minor (not exceeding a few percent). This explains the perturbation of the resonance from His3 in ^{1}H NMR. It is also in line with the competition data monitored by UV−vis, because a minor occupation (<10%) is under the detection limit due to the poor absorption of octahedral Co^{II}.

The model of site A of Cd^{II} constitutes four ligands from amino acid side chains Asn99, His67, His247, and Asp240 and H_2O (which can be replaced with Cl^-) (*12*). They bind Cd^{II} in a distorted trigonal bipyramid (with the two His residues in the axial position). The low ϵ of the d−d transition of Co^{II} indicates a distorted octahedral coordination. To achieve that, an additional ligand has to coordinate to the Co^{II} (e.g., an additional water molecule). The question if Co^{II} binds differently to HSA in site A as Zn^{II}/Cd^{II} or if Zn^{II}/Cd^{II} binds like Co^{II} in an octahedral geometry in site A remains open.

Repercussion on the ACB Test. These data, indicating that Co binds preferentially to sites A and B (and not the N-terminal site), have implications on the ACB test. In the

ACB test, ~1.5 equiv of Co^{II} per HSA was added to blood serum. If Co^{II} added to the serum binds to HSA, it is likely coordinated to site A and/or site B. In that case, the ACB test will measure the difference in the level of binding of Co^{II} at site A or B and not at the N-terminus as proposed (*22*). This is important, because this means that the modification of HSA induced by ischemia is rather around site A or B and not at the N-terminus. Such a putative ischemia-induced modification in HSA (often called ischemia-modified albumin) has not been localized and identified yet. The molecular identification of the modification could help to improve the test for myocardial ischemia. An improvement seems to be necessary since the ACB test helped physicians to correctly rule out myocardial ischemia with an accuracy of only 70% (when the ACB test was used together with an electrocardiogram and a troponin test, with an electrocardiogram and troponin test alone, physicians were 50% accurate in ruling out a myocardial ischemia).

On the basis of the proposal that Co^{II} binds to sites A and B, one could even speculate that the structural changes in HSA and subsequent lower level of Co^{II} binding in myocardial ischemia could be linked to fatty acid binding. The negative allosteric interaction between metal binding in site A and fatty acid binding (*12*) would allow the following hypothesis: A higher fatty acid content in the serum (or a structural change in fatty acid leading to a stronger binding to HSA) could reduce the level of binding of Co to site A and hence an increase in the amount of unbound HSA as detected in myocardial ischemia.

However, several questions regarding the ACB test are still open, and the molecular mechanism is not clear. It has not been proven yet that the test is really based on binding of Co^{II} to HSA. Moreover, Co^{II} could also be oxidized to Co^{III} (as our data indicate) during the test, and the exact reaction with DTT is not well-understood. These results were obtained with commercial HSA from Sigma and pertain to this sample. Future studies are needed to establish that the results apply to other preparations of HSA. It is particularly important for HSA from fresh blood serum, which is used for the ACB test. Differences between such HSA and commercial ones have been reported by using high-performance liquid chromatography coupled to mass spectrometry (*41*). In contrast, commercial and recombinant HSA did not exhibit significant differences in their crystal structure (*42*).

In conclusion, these data strongly suggest that the primary binding sites of Co^{II} are sites A and B and not the N-terminus. It seems important in further studies of the ACB test to take into account sites A and B when binding of Co to HSA is involved.

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