# Allosteric Inhibition of Cobalt Binding to Albumin by Fatty Acids: Implications for the Detection of Myocardial Ischemia

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ⓈSupporting Information

ABSTRACT: The biomarker "ischemia-modified albumin" (IMA), measured by the albumin−cobalt-binding assay (ACB assay), is the only FDA-approved biomarker for early diagnosis of myocardial ischemia. On the basis of the hypothesis that high levels of free fatty acids are directly responsible for reduction in cobalt binding by albumin, chemically defined model systems consisting of bovine serum albumin, Co^{2+}, and myristate were studied by isothermal titration calorimetry, ^{111}Cd NMR spectroscopy, and ACB assays. Significantly reduced Co^{2+} binding to albumin, as demonstrated by an increase in the absorption of the Co-dithiothreitol adduct, elicited by adding ca. 3 mol equiv of myristate, was comparable to that observed in clinical ACB assays. Levels of free fatty acids are elevated during myocardial ischemia but also in other conditions that have been correlated with high IMA values. Hence, IMA may correspond to albumin with increased levels of bound fatty acids, and all clinical observations can be rationalized by this molecular mechanism.
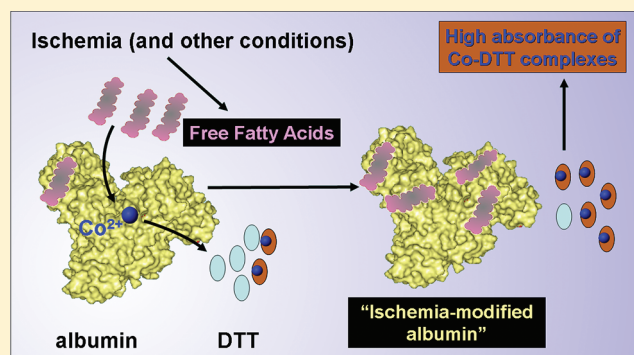
■ INTRODUCTION

An intriguing biomarker termed ischemia-modified albumin (IMA) has been attracting much attention in recent years.[1−4] IMA, as measured by the albumin−cobalt-binding assay (ACB assay[5]), is the only Food and Drug Administration (FDA)-approved early biomarker for myocardial ischemia,[3] a contributing factor to acute coronary syndrome that may lead on to a heart attack. Plasma from ischemic patients displays reduced Co^{2+}-binding ability as compared to healthy controls;[6] this can be detected colorimetrically via complex formation between Co^{2+} and dithiothreitol (DTT). However, while the ACB assay has been found to have a high negative prognostic value[6,7] and helps to exclude ischemia as a cause of chest pain, its specificity is low.[3,8] Accordingly, increased IMA values have been detected in a plethora of other conditions including fatty liver, cancer, infections, renal disease, preeclampsia, diabetes, stroke, and even sustained exercise.[2−4,8−10] Hence, several recent studies have questioned the usefulness of the ACB assay.[11,12]

Despite over 70 studies involving over 15000 patients, no conclusive explanation for the performance characteristics of the ACB assay has been offered, hampering efforts to improve its prognostic and/or diagnostic value. Most strikingly, perusal of IMA-related literature reveals that the actual identity of this biomarker is unclear. "IMA" has so far only been defined in terms of its decreased Co-binding capacity, but the actual nature of the putative ischemia-induced modifications has remained elusive. Identifying these modifications is important as it would not only provide an insight into the mechanism involved in the formation of IMA and help to understand how and why IMA correlates with certain diseases but also aid in the understanding of the chemical basis for the ACB assay, which might lead to improvements in its performance.

Most efforts to understand the mechanism of action of the ACB assay have centered on the N terminus of albumin,[5,13,14] which has been assumed to be the main Co^{2+}-binding site. Following the failure to demonstrate a correlation between N-terminal modifications and IMA, more recent suggestions have included lower plasma pH (acidosis) and altered redox balance,[5,14] and although changes in these conditions are plausible as factors influencing the speciation of Co^{2+} during the ACB assay, their impact has not been verified experimentally.

Another intriguing link between the ischemic conditions and the Co-binding capacity of plasma has emerged recently. In 2008, a relationship between fatty acids and IMA levels was suggested.[9] It is known that the plasma concentration of "free" fatty acids (FFAs) is increased during myocardial ischemia.[4,15] Indeed, serum fatty acids may themselves serve as a marker of myocardial injury.[2,16] Crucially, so-called FFA are not free but predominantly bound to serum albumin (Figure 1).[17,18]
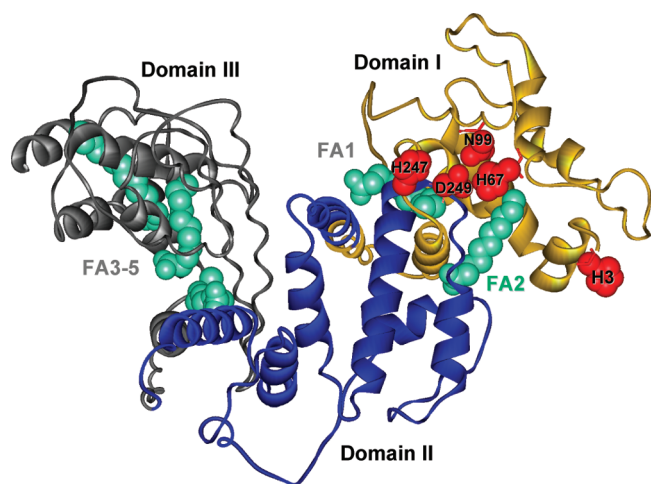
Figure 1. Location of fatty acid- and metal-binding sites on mammalian albumins. Myristate molecules in the five major fatty acid-binding sites (FA1−5) are shown in green. The figure is based on PDB 1bj5.[18] His3 from the N-terminal ATCUN (amino-terminal Cu-Ni binding) motif,[25] and the four residues forming the major Zn-binding site A (H67, N99, H247, and D249) are shown in red. Both metal and fatty acid-binding sites are distributed asymmetrically over the molecule. Of particular importance is the domain I/II interface, harboring the interdomain site FA2, and metal site A. Fatty acid-binding to site FA2 communicates allosterically with metal-binding to sites A and B;[19] the location of site B remains unknown.

Recently, Bhagavan et al.[16] compared IMA levels with FFA levels in the sera of patients with acute myocardial infarction and controls. A "plausible but not causative relationship" between these two parameters was revealed—an increase in FFA levels resulted in a similar increase in IMA and hence lower Co^{2+}-binding capacity.

On the basis of the hypothesis that metal binding to the major zinc-binding site A and fatty acid binding to site FA2 are mutually exclusive, we have recently demonstrated that myristate severely impairs the Zn- and Cd-binding capacity of albumin.[19] Following their spectroscopic studies, which established that the N terminus is not the principal binding site for Co^{2+} on albumin, Mothes and Faller had suggested earlier that Co^{2+} is likely to occupy the same binding sites as Zn^{2+} and Cd^{2+} and hence, that similarly, Co^{2+} binding could be directly influenced by fatty acids,[20] but so far, this theory has not been tested.

Therefore, to test the hypothesis that elevated fatty acid levels alone are sufficient to decrease the cobalt-binding capacity of albumin, without the need to invoke oxidative stress or more complex mechanisms, we have studied the interactive effects of cobalt and fatty acid binding to albumin in vitro, using chemically well-defined preparations of bovine serum albumin (BSA), cobalt, and myristate. Mature BSA and human serum albumin (HSA) show 76% sequence identity (and 89% sequence similarity), and crucially, both their metal and fatty acid-binding properties are very similar.[17,21,22] We show that physiologically relevant concentrations of a long-chain fatty acid are sufficient to reduce the cobalt-binding affinity and capacity of albumin to an extent consistent with the effects observed in clinical ACB assays. Together with our recent studies on allosteric regulation of metal sites A and B by fatty acid binding to albumin,[19] this direct molecular link provides a new basis for understanding the molecular identity

of IMA and the basis of the ACB assay, with wide-ranging implications for their clinical utility.

RESULTS AND DISCUSSION

Determining the preferred binding sites for Co^{2+} is a prerequisite for understanding the molecular basis of the ACB test and the identity of IMA, and has been the subject of recent controversy. Most mammalian albumins contain at least three binding sites for the metal ions Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, and Cd^{2+} (Figure 1), and different metal ions bind preferentially to different sites.[23,24] For Cu^{2+} and Ni^{2+}, the N-terminal amino-terminal copper- and nickel-binding (ATCUN) motif, which requires a histidine residue in position 3, is the primary binding site.[25] The second site, termed site A, is the primary binding site for Zn^{2+}[26,27] and a secondary site for Cu^{2+} and Ni^{2+},[23] whereas Cd^{2+} binds with similar affinity to both sites A and B—the third site.[28−30] While the location of site A has been established using mutagenesis and extended X-ray absorption spectroscopy,[27] site B is only defined by its chemical shift in $^{111/113}$Cd nuclear magnetic resonance (NMR) spectra, and its location remains unknown. Co^{2+} has recently been shown by CD spectroscopy and competition with other metal ions to bind preferentially to site B in HSA, followed by site A, and only then the N terminus.[31] This assignment is consistent with conclusions from a previous detailed study that employed absorption spectroscopy[20] but contradicts earlier suggestions[32−34] that were used as the basis of attempts to explain the mode of action of the ACB assay.[13,14] Because of this disagreement, we first studied the competition between Co^{2+} and other metal ions with unambiguous binding site locations, employing isothermal titration calorimetry (ITC) and ^{111}Cd NMR spectroscopy.

Before ITC competition experiments were initiated, the binary system Co^{2+}/BSA was studied under experimental conditions that were identical to those previously employed for Zn^{2+}/BSA[19] to allow direct comparisons between these two metal ions (Figure S1 in the Supporting Information). Two caveats have to be taken into account when studying Co^{2+} binding to albumin: (i) if coordinated to nitrogen-containing ligands, Co^{2+} is easily air-oxidized to Co^{3+}. Considerable oxidation to Co^{3+} in the presence of albumin has been noted by Mothes and Faller.[20] Solutions for ITC were carefully degassed, and no further exposure to air occurred during the titrations. It was noted that the first few additions produced large exothermic peaks even in the absence of albumin; it is conceivable that this may have been due to either residual oxygen or Co^{3+} in the reaction vessel, and these titration points were discarded. (ii) An hysteretic effect for Co^{2+} binding to albumin has been noted previously.[34] Care was taken that there was no residual heat evolution before each subsequent addition of Co^{2+}. With these precautions in place, consistent data were obtained. Under the conditions employed, the binding reaction of Co^{2+} with BSA was exothermic (Figure S1 in the Supporting Information). Apparent binding constants for Co^{2+} binding to BSA (log $K_{app(Co-BSA)}$) were ca. 1 order of magnitude lower than those determined previously for the highest affinity Zn^{2+} site,[19] irrespective of the binding model chosen. The ITC data can be fitted well with a simple model of one set of binding sites; these fits converged robustly and gave an average log $K_{app(Co-BSA)}$ = 4.6 ± 0.3, together with a binding site stoichiometry of 2.4 ± 0.4. This log K value lies between the reported values (4.9, 4.0, and 3.9) for individual binding sites in HSA.[31] At this point, it should be noted that a difference of less than 1 log unit means

that the binding equilibria for the various sites will substantially overlap, rendering resolution of individual constants difficult. Furthermore, the overall low binding constants also mean that significant amounts of Co^{2+} will remain unbound to albumin during the titrations, as well as in the ACB test, since only 25 μM BSA was employed in our work, and ca. 90−100 μM HSA is present in the final ACB assay solutions. For example, it can be calculated from the published equilibrium constants[31] that under typical ACB test conditions[6] but in the absence of DTT, 46% of Co will be bound to the primary site and about 16% to each of the other two sites, with 22% remaining unbound or bound to other plasma components. Unfortunately, the theoretical distribution in the presence of DTT cannot be calculated because no log K values for the Co−DTT system are available due to precipitation of the complexes formed.[35]

Having established the behavior of the binary system, competition with other metals was studied. The N-terminal site was blocked with Cu^{2+}: the binding constant of BSA or HSA for Cu^{2+} is 5−6 orders of magnitude larger than those measured for Co^{2+}, so it is thermodynamically impossible for Co^{2+} to displace Cu^{2+} bound to the ATCUN motif unless added in 100000-fold excess. The ITC data obtained were indistinguishable from those recorded in the absence of Cu^{2+} (Figure 2a). Similar conclusions were previously drawn based
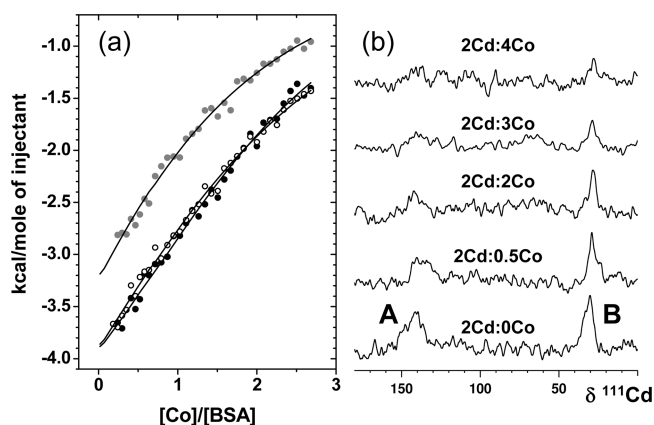

Figure 2. Competition between Co^{2+} and other metal ions for binding sites on albumin. (a) ITC data in the presence and absence (O) of 1 mol equiv of Cu^{2+} (●) or Zn^{2+} (gray circle) (25 μM BSA, 333 μM Co^{2+}, 50 mM Tris-Cl, and 50 mM NaCl, pH 7.4). Zn^{2+} clearly decreases Co^{2+}-binding capacity and affinity, and Cu^{2+} has no significant effect. (b) ^{111}Cd NMR spectra of 1.5 mM Cd$_2$BSA (310 K, 50 mM Tris-Cl and 50 mM NaCl, pH 7.4) with increasing amounts of Co^{2+} added. The two peaks corresponding to binding sites A and B are labeled, and both are affected by the addition of Co^{2+}. Even though the disappearance of ^{111}Cd NMR peaks alone does not necessarily allow the conclusion that Co^{2+} displaces Cd^{2+}, the combined observations by ITC and NMR suggest strongly that Co^{2+} competes for sites A and B.

on UV−vis spectroscopic studies on Co^{2+}−HSA complexes in the presence and absence of 1 mol equiv of Cu^{2+}, which also were not significantly different.[20] In contrast, occupation of site A with Zn^{2+} had a clear effect on Co^{2+} binding and reduced the overall observed stoichiometry from 2.0 to 1.3. Although these data sets capture the binding of up to 2 mol equiv of Co^{2+} only, these observations suggest that site A is more significant than the N terminus in governing the Co-binding capacity of albumin.

To further explore the potential of Co^{2+} to interact with sites B and A, ^{111}Cd NMR spectroscopy was used as a complementary technique, as this technique can give information on both sites individually.[26,28−30] The addition of Co^{2+} to BSA in the presence of 2 mol equiv ^{111}Cd^{2+} resulted in a marked decrease in the intensities of both peak A and peak B (Figure 2b). This observation is consistent with the notion that Cd^{2+} and Co^{2+} may compete for these two binding sites, as suggested by others.[20,31] Previous UV−vis experiments on HSA suggested that Co^{2+} binds preferentially to sites B and A; the N terminus only became occupied to a significant extent when sites A and B were blocked by Cd^{2+},[20] and CD spectroscopy has revealed that Cd^{2+} slowly displaces Co^{2+} from its main binding site B.[31] In summary, these ITC and NMR data are fully consistent with conclusions from other techniques;[20,31] sites B and A correspond to the first two binding sites for Co^{2+}, and as emphasized above, their binding equilibria significantly overlap.

Crucially, the preferred interaction of Co^{2+} with sites A and B could explain the suspected correlation with fatty acid levels: We have recently shown that Zn^{2+} and Cd^{2+} binding to both sites A and B is dramatically impaired by the binding of long-chain fatty acids such as myristate.[19] The effect on these two metal ions was so severe that almost no binding was detectable in the presence of 5 mol equiv of myristate by either ITC (for Zn) or ^{111}Cd NMR (for Cd). Because the location of site A is known,[26,27] its complete disruption by fatty acids can be understood by comparing the X-ray crystal structures of fatty acid-bound[18] and -free[36] albumin: Binding of a fatty acid molecule to the interdomain site FA2 (Figure 1) changes the mutual orientation of domains I and II and separates the four residues (His67, Asn99 from domain I, and His247 and Asp249 from domain II) forming the Zn-binding site.[19,24,26,27] The effect on site B (primary site for Cd and secondary site for Zn) had been unexpected but suggested that its structure is also dependent on fatty acid loading.

In the light of these findings, the influence of fatty acid loading on Co^{2+} binding was studied, employing ITC experiments for Co^{2+} titrated with BSA in the absence and presence of bound myristate (1−5 mol equiv). Myristate was chosen as its albumin binding characteristics are similar to those of the most common physiological fatty acids, stearate and oleate (C16 and C18), and because of its higher solubility in aqueous solution, which was sufficient to fulfill the requirements for ITC experiments. The titration data reveal that overall, Co^{2+} binding decreases in the presence of myristate as indicated by a progressive reduction in overall affinity and binding site availability (Figures 3a and 4a). The effects of myristate on Co^{2+} binding were less dramatic than those observed for Zn^{2+} binding.[19] Significant binding (54% of control) still occurred even with 5 mol equiv myristate, whereas virtually no Zn^{2+} binding was observed under identical conditions.[19] The different effects observed for Co^{2+} and Zn^{2+} can be rationalized by the higher affinity of Co^{2+} (as compared to Zn^{2+}) for both site B (log $K_{app(Zn-BSA)}$ = 4.15)[19] and possibly the N-terminal ATCUN motif. Previous work suggested that abolishing Zn^{2+} binding to a second site, thought to correspond to site B, required higher concentrations of myristate than blocking site A,[19] and considering that Co^{2+} binds several times more strongly[31] to this site than Zn^{2+}, a further diminished effect is in accordance with expectations. A direct comparison of the data shown in Figures 2a and 3a suggests that the effects of 5 mol equiv myristate are more
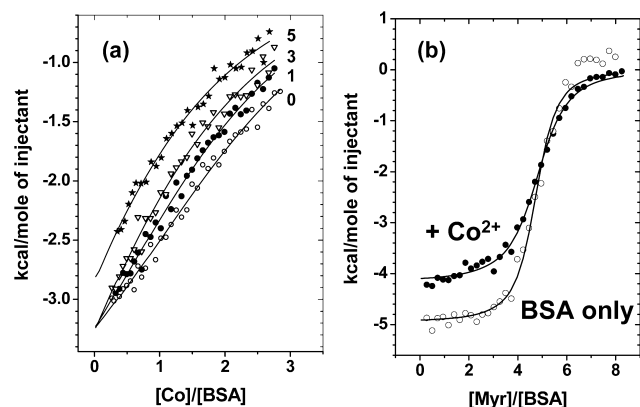
Figure 3. Competition between Co^{2+} and myristate binding to albumin studied by ITC. (a) Titration of Co^{2+} to BSA in the presence and absence of myristate (25 μM BSA, 333 μM Co^{2+}, 50 mM Tris-Cl, and 50 mM NaCl, pH 7.4). The addition of increasing amounts of myristate (○, 0 mol equiv; ●, 1 mol equiv; ▽, 3 mol equiv; and ★, 5 mol equiv) reduces the Co-binding capacity. The curve recorded in the presence of 5 mol equiv myristate indicates a reduction of the binding site concentration by almost 50%. The fits correspond to models involving one set of binding sites. (b) Titration of myristate to BSA in the presence (●) and absence (○) of 1 mol equiv of Co^{2+} (12.5 μM BSA, 500 μM myristate, and H_2O, pH 7.8). The binding reaction with myristate is less exothermic in the presence of Co^{2+}, a behavior similar to that observed with Zn^{2+} (Figure S2 in the Supporting Information).
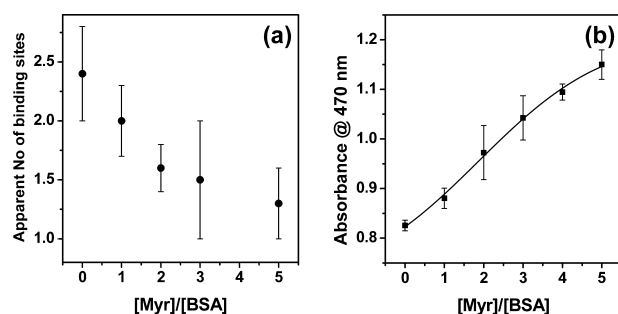

Figure 4. (a) Myristate binding elicits a decrease in total Co^{2+}-binding site availability on BSA. The values for the "apparent number of binding sites" are a result of the fits shown in Figure 3a. (b) Myristate binding to BSA leads to increased Co−DTT complex formation in the ACB assay.

pronounced than those of 1 mol equiv Zn; the corresponding difference likely reflects the impact of myristate on site B. In addition, because the Co^{2+}-binding affinity for the N terminus is only slightly smaller than that for site A,[31] it is possible that "removal" of site A (by addition of either Zn^{2+} or fatty acid) might be compensated by more binding to the N terminus.

The reverse experiment was also carried out; BSA was titrated with myristate in the presence and absence of 1 mol equiv of Co^{2+} (Figure 3b). The presence of Co^{2+} resulted in a decrease in exothermicity, without affecting overall binding stoichiometry. This observation can be rationalized by assuming that the binding of myristate requires the displacement of Co^{2+} from its preferred site(s). Because the Co^{2+}-binding reaction is exothermic, this displacement is an endothermic reaction, leading to a reduction of heat evolved during myristate binding. Very similar observations were made in the case of Zn-BSA, for which a slightly larger effect was registered (Figure S2 in the Supporting Information).[19]

The findings from both competition experiments confirm conclusively that myristate and Co^{2+} binding are interactive and that fatty acids decrease the Co^{2+}-binding capacity of albumin—but does this provide the molecular basis for the ACB assay? To test whether fatty acids alone impact sufficiently on the results of the ACB assay to explain observed differences between normal and ischemic conditions, solutions containing 600 μM BSA and 0−5 mol equiv of myristate were subjected to the ACB assay. The absorbance of the resulting Co−DTT adduct(s) was recorded at 470 nm (Figure 4b). Clearly, there is a positive correlation between the level of myristate and the higher absorbance, and hence less cobalt binding to BSA, consistent with the results from ITC (Figure 4a). Typical absorbance values for individuals without and with myocardial ischemia (MI) reported in the literature are 0.32 ± 0.09 (no MI) and 0.52 ± 0.09 (with MI)[5] or 0.43 ± 0.10 (no MI) and 0.63 ± 0.25 (with MI).[6] Our readings were significantly higher, varying between 0.83 for completely fatty acid-free albumin and 1.15 for BSA in the presence of 5 mol equiv myristate. The lower readings for clinical plasma samples are likely due to Co^{2+} binding to other biomolecules present in plasma; hence, in our "pure" assay, more DTT-bound Co^{2+} was detected. However, more important than absolute values is the difference between what could be considered "normal" and "diseased". The average amount of fatty acids bound to albumin under normal physiological conditions is 0.1−2 mol equiv[17,21] but can rise to up to 4−6 mol equiv[21] under conditions such as extreme exercise, diabetes, heparin treatment, and cardiovascular disease. It can be estimated from Figure 4b that a difference of 0.2 absorbance units, a typical difference observed between healthy and ischemic patients, corresponds to slightly less than three fatty acid molecules per albumin molecule, a value that is in line with observed differences in expected fatty acid loading under normal and ischemic conditions.[37]

■ CONCLUSIONS

In summary, the results presented here, together with the knowledge that ischemia leads to increased plasma fatty acid levels,[4,16,37,38] suggest that the enigmatic modifications that define IMA correspond mainly to an increase in the number of fatty acid molecules bound to otherwise normal albumin, although contributions from acidosis or disturbed redox balance as suggested by others[14] cannot be excluded as further contributing factors and require further study. This would mean that "IMA" is strictly speaking neither a marker for ischemia nor oxidative stress but a proxy for FFA levels. This conclusion can explain virtually all clinical observations. For example, the rapid return to normal IMA values within hours of the ischemic injury is hard to reconcile with any changes to covalent bonds including bond cleavage or oxidative damage, given the large half-life of albumin (19 days), but is fully consistent with expected fluctuations in FFA levels. This allosteric link can also rationalize the low specificity of the ACB test, as any condition that leads to raised FFA levels would also raise the levels of IMA. Such conditions include obesity, diabetes, demanding aerobic physical exercise, prolonged fasting, and indeed other types of ischemia—a compilation that parallels the list of conditions in which elevated levels of IMA have been observed.[3,4,9,10]

Interestingly, the characteristics of a nickel−albumin-binding assay were described recently.[39] The primary binding site of Ni^{2+} is undoubtedly the N terminus;[25] yet, the test performs at least as well as the ACB assay itself. How is this possible if Co^{2+}

and Ni^{2+} have different binding preferences? A possible answer is that the secondary binding site for Ni^{2+} is site A;[23] and under the test conditions, which involve an excess of Ni^{2+} over albumin, the fatty acid-sensitive site A will play a significant role in overall binding.

In terms of improving the prognostic value of IMA, we propose that the ACB assay could be optimized to give an alternative rapid and cost-effective measurement for the FFA/albumin ratio. Besides their potential diagnostic utility in acute coronary syndrome, FFAs have recently been identified as useful prognostic markers that are strongly correlated with mortality in stable coronary heart disease[40] and are also important clinical markers for metabolic syndrome.

■ EXPERIMENTAL SECTION

Sample Preparation. For all experiments, BSA (Sigma, essentially fatty acid free and essentially globulin free) was extensively dialyzed against the desired buffer to remove metal ions and other low-molecular weight impurities.[26] Concentrations were determined using a molar absorptivity of 43824 M^{−1} cm^{−1} at 280 nm.[41] Complexes of BSA with different molar equivalents of myristate (1:1 to 1:5) or competing metal ions (1:1 or 1:2) for ITC, [111]Cd NMR, and ACB experiments were prepared by adding carefully measured amounts of the competing agent to BSA, followed by incubation for 2 h at 310 K. In the case of Cu^{2+},[22] quantitative formation of the complexes can be assumed, whereas the Zn^{2+}, Cd^{2+}, Co^{2+}, and myristate complexes are weaker and will form to a lesser extent. All added metal ions and fatty acids will however participate in the competition reactions.

ITC. Cobalt chloride (Sigma, $CoCl_2 \cdot 6H_2O$), copper chloride (Sigma, $CuCl_2 \cdot 2H_2O$), zinc chloride (Sigma, $ZnCl_2$), myristate, and BSA solutions were prepared with the same buffer stock solution used for dialysis; 50 mM Tris-Cl buffer with 50 mM NaCl (pH 7.4) was employed for the Co^{2+} titration ITC experiments, and Milli-Q water, pH 7.8, for the myristate titration ITC experiments, as the solubility of myristate was too low in Tris and other buffers.

ITC experiments were performed at 298.0 ± 0.1 K on a Microcal VP-ITC calorimeter (GE Healthcare Science). All samples were degassed by being stirred under vacuum before use. In the cell, the BSA solution had a concentration of 25 μM for titrations with Co^{2+} and 12.5 μM for titrations with myristate. In each ITC experiment, 30−35 injections of 6−8 μL of Co^{2+} or myristate ligand solution were made into the cell containing the sample solution. The first injection was 2 μL, and the data point was discarded, as suggested by Microcal's operation manual. The solution in the cell was stirred at 300 rpm by the syringe to ensure rapid mixing. A background titration, consisting of the identical titrant solution but with the buffer in the sample cell, was subtracted from each experimental titration to account for heat of dilution. ITC data were collected automatically and were subsequently analyzed with Microcal Origin 7.0. The fitting curves were calculated using appropriate binding site models (see Figures S1 and S2 in the Supporting Information for details).

[111]Cd NMR Spectroscopy. Metal, myristate, and BSA solution were in 50 mM Tris-Cl buffer with 50 mM NaCl (pH 7.4). Proton-decoupled 1D [111]Cd NMR spectra were acquired on a Bruker DRX500 instrument fitted with a BBO probe-head and operating at 106.037 MHz for [111]Cd. Inverse-gated decoupling during acquisition was achieved using a GARP sequence. Spectra were referenced to an external standard of 0.1 M $Cd(ClO_2)_4$. Data were acquired with 8k complex data points and a spectral width of 300 ppm at 310 K. The data were zero-filled to 32k data points, apodized by exponential multiplication using a line-broadening factor of 90 Hz, and Fourier transformed. Data processing and analysis were carried out using the TOPSPIN 2.1 software package (Bruker BioSpin).

ACB Assay. An aliquot of 50 μL of 0.1% cobalt chloride (Sigma, $CoCl_2 \cdot 6H_2O$) in H_2O was added to 200 μL of extensively dialyzed BSA (600 μM, 10 mM NH_4HCO_3), gently mixed, and incubated for 10 min. Then, 50 μL of DTT (Sigma, 1.5 mg/mL H_2O) was added as a colorizing agent, and the reaction was quenched 2 min later by adding 1.0 mL of 0.9% NaCl. Color development with DTT was compared to a BSA−cobalt blank without DTT at 470 nm using a spectrophotometer (BioMate3, Thermo). This assay was carried out in duplicate.

■ ASSOCIATED CONTENT

ⓈSupporting Information

Figure S1 of raw and processed ITC data for the systems Co^{2+}-BSA and Zn^{2+}-BSA and Figure S2 of ITC data for titrations with myristate. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ACB, albumin−cobalt binding; ATCUN, amino-terminal copper and nickel binding; BSA, bovine serum albumin; DTT, dithiothreitol; FA2, fatty acid-binding site 2; FDA, Food and Drug Administration; FFAs, free fatty acids; HSA, human serum albumin; IMA, ischemia-modified albumin; ITC, isothermal titration calorimetry; K_{app}, apparent binding constant; MI, myocardial ischemia; NMR, nuclear magnetic resonance

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