Cadmium(II), Zinc(II), and Copper(II) Ions Binding to Bovine Serum Albumin. A ^{113}Cd NMR Study

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The interaction between bovine serum albumin and the metals Cd(II), Zn(II) and Cu(II) was studied by ^{113}Cd NMR. A ^{113}Cd titration of the protein revealed two strong Cd(II) binding sites. Competition between Zn(II) (or Cu(II)) and Cd(II) for the strong sites indicates that there is a common strong binding site for Cu(II), Zn(II) and Cd(II), with higher affinity for Cu(II) and Zn(II) than for Cd(II). Competition between serum albumin and NTA for Cd(II) did not allow conclusive evaluation of the intrinsic binding constants, probably due to the formation of ternary complexes NTA–Cd(II)–protein. Chemical shifts are in good agreement with histidyl residue involvement in one site, and probably oxygen ligands in a second site. The site responsible for strong binding of Zn(II) contains two histidyl residues, but not thiol groups.

Introduction

The importance of serum albumin as the main carrier of metal ions in the blood is well established [1]. Transition metal interaction with albumin attracted wide interest in the past. In most of these studies, however, it has been assumed that several identical binding sites exist. Binding constants for these sites have been obtained using various methods. Binding of zinc, for instance, has been subject to numerous studies [2–15], but as far as we have found in the literature only two attempts have been made to determine the binding constant for specific high-affinity sites. Österberg [11] has found that there is one high affinity site whose intrinsic binding constant can be determined only when the pK of the displaced proton is known. Constants with values ranging from pK_a = 9.6 and downward can be calculated with the highest value where the thiol group is involved. From a competition between serum albumin and various amino acids for zinc ions, Giroux and Henkin [12] obtained a binding constant pK_a = 6.98.

Metal NMR has for some time been used to study metal binding to small ligands, as well as to proteins. ^{113}Cd NMR has proven to be very suitable for such studies, especially because cadmium can replace both zinc, in several enzymes [16], and calcium in structural calcium binding proteins [17]. We therefore decided to study metal binding to serum albumin by means of ^{113}Cd NMR, and in this work we will present a study on cadmium binding to serum albumin, as well as the competition between cadmium and zinc (or copper).

Experimental

Crystalline bovine serum albumin was obtained from Sigma (A-6003, fraction V, essentially fatty acid free). Solutions of the protein in 0.16 *M* NaCl (Merck, *Supra pur.*) were dialyzed against EDTA and subsequently dialyzed several times against distilled water. Cadmium, zinc and copper solutions were prepared from the respective perchlorate salts.

NMR measurements

The ^{113}Cd NMR measurements have been performed on a home-made spectrometer [18] using a 6T Oxford superconducting magnet, giving a ^{113}Cd resonance frequency of 56.55 MHz. Normally 2 m*M* albumin samples have been used, with varying concentrations of cadmium and other metal ions. 100,000 transients with a flip angle of *ca.* 45° (8 μs) required *ca.* 8 h of accumulation. The samples were in 17 mm O.D. ampoules inserted horizontally. The ^{113}Cd NMR chemical shifts are given relative to that of an aqueous 0.1 *M* $Cd(ClO_4)$ solution, with high frequency (low field) shifts taken as positive.

Results and Discussion

Cd binding

The addition of ^{113}Cd(II) ions to a 2 m*M* solution of serum albumin gives rise to two ^{113}Cd NMR signals, with chemical shifts at +150 ppm and +28 ppm. Both resonances show excessive broadening, at 700 Hz and 450 Hz, respectively. The broadening is not dependent on the temperature, which indicates that it is not due to metal exchange. Furthermore the lack of temperature dependence shows that the life-time of the bound cadmium ions is $>10^{-2}$ s. Figure 1 shows that the two strong Cd(II) sites (δ = +150 and +28 ppm) have similar binding constants since the

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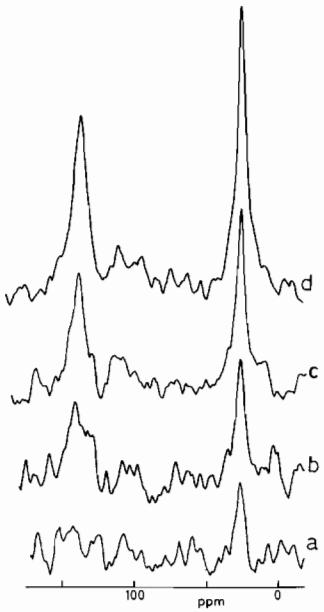

Fig 1. ^{113}Cd NMR spectra showing the effect of increasing amounts of ^{113}Cd(II) to a 2 m*M* serum albumin solution. pH 6.7, 0.16 *M* NaCl. a) 1 m*M* Cd(II), b) 2 m*M* Cd(II), c) 3 m*M* Cd(II), d) 4 m*M* Cd(II).

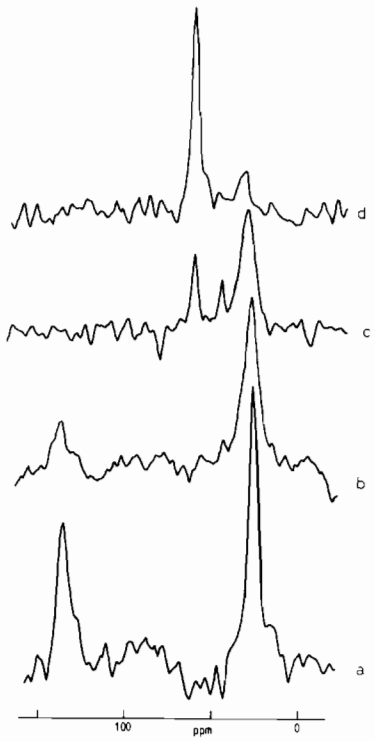

Fig. 2. ^{113}Cd NMR spectra (4 m*M* Cd(II) and 2 m*M* serum albumin, pH 5.7) showing the effect of addition of NTA. a) No NTA, b) 2 m*M* NTA, c) 6 m*M* NTA, d) 12 m*M* NTA

intensities of the two resonances increase almost in parallel with increasing cadmium concentrations. When an excess of cadmium (more than two equivalents per albumin molecule) is used, a very broad ^{113}Cd NMR signal at about +80 ppm appears. This signal is temperature dependent and broadens beyond detection at +8 °C. This indicates that this resonance is due to ^{113}Cd ions with a lifetime on the protein site of *ca.* 10^{-5} s.

Titration shows that the two strong Cd(II) sites (δ = +150 ppm and +28 ppm) are pH dependent, in the range 5–7. Above pH 7 the two sites appear insensitive to pH changes, and below pH 5 only one ^{113}Cd signal with a chemical shift (of +80 ppm) is observed. The present data cannot be used to calculate the cadmium–serum albumin binding constant. We have, however, tried to estimate it in two different ways.

At first, the mere fact that the exchange of cadmium from two sites on serum albumin is sufficiently slow to give rise to resolvable, non-exchange broadened resonances, indicates that the binding constants for these sites are large. Assuming that the Cd(II) on-rate is diffusion limited, $k_{on} \sim 10^9$ s^{-1} (this is an assumption which we have no proof for; however, it is often assumed that the metal on-rate is diffusion limited [19], but there are cases where the on-rate is significantly slower than the diffusion limit [20]) then, we find $K_a > 10^7$ M^{-1} for these two strong sites, using the above estimated lower limit for the lifetime, 10^{-2} s. Similarly, the binding constant for the weaker sites giving rise to the resonance observed (+80 ppm) can be estimated to $K_a \cong 10^4$ M^{-1}.

Secondly, we tried to perform a competition experiment using NTA (nitriloacetate). As shown in Fig. 2, the ^{113}Cd spectrum is drastically affected by the addition of NTA. However, the disappearance of the ^{113}Cd signal at +150 ppm is not followed by the appearance of a new signal of the Cd(NTA) or Cd(NTA)$_2$ complexes, which is around +10 ppm (unpublished results). Sarkar and Wigfield [21] have shown that ternary complexes can be formed between serum albumin, Cu(II) and amino acids (histidine and threonine). An obvious explanation to our results is therefore that mixed complexes can also be found between serum albumin, Cd(II) and NTA, even though the complexing properties of Cu(II) and Cd(II) are different. The ^{113}Cd NMR signal observed at +60 ppm could thus be tentatively assigned to the ternary complex. Moreover the absence of a signal corresponding to Cd(NTA)$_2$, even when a large excess of NTA is present, shows that the ternary complex must be stronger than Cd(NTA)$_2$. These observations corroborate the above estimated K_a value for the two strong cadmium sites. The ^{113}Cd NMR chemical shifts of the two strongly bound cadmium ions indicate that there is no sulphur involved in the cadmium binding, since it has been shown that sulphur binding results in chemical shifts which are of several hundred ppm

higher frequency than those observed here [22]. The +150 ppm shift agrees quite well with that found for the enzymes carboxypeptidase, alkaline phosphatase and superoxide dismutase, where the cadmium ion is supposed to be bound to two or three histidyl residues [16]. There are very few ^{113}Cd chemical shifts for protein bound cadmium in the range 0–100 ppm, which makes it difficult to assign (even tentatively) the binding site corresponding to +28 ppm. However, the fact that there is a competition, although weak, with Ca(II) for this site indicates that only oxygen is involved in the binding of the metal ions.

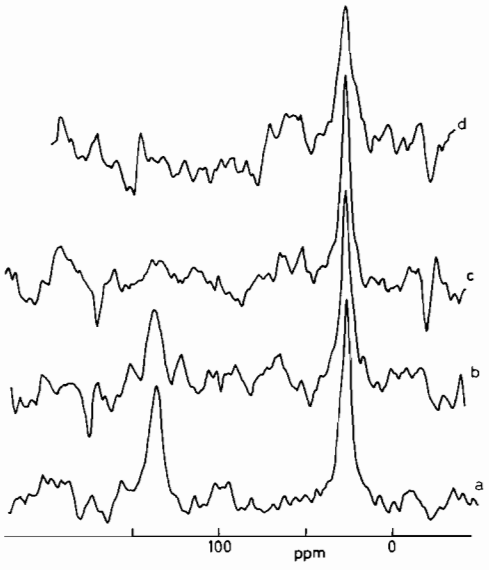

Fig. 3. ^{113}Cd NMR spectra (4 m*M* Cd(II), 2 m*M* serum albumin, pH 7.5) showing the effect of addition of Zn(II). a) No Zn(II), b) 1 m*M* Zn(II), C) 2 m*M* Zn(II), d) 4 m*M* Zn(II).

Metal competition

In order to determine if any of the two strong Cd(II) sites are the same as the Zn(II) or Cu(II) sites, which are normally assumed to be different, we made two competition experiments adding either Zn(II) or Cu(II) ions to the Cd(II)–serum albumin solution. In Fig. 3, the Zn(II) competition is shown. It is obvious that the ^{113}Cd site with a shift of *ca.* +150 ppm is the same as the strong Zn(II) site. The competition with Cu(II) (not shown) gave much the same results as for the Zn(II). The ^{113}Cd signal with a shift of ~+150 ppm disappeared upon the addition of 1 equiv. of Cu(II); with an excess of Cu(II), the second ^{113}Cd signal became slightly broadened. We can state positively that there is a common, strong binding site for Cu(II), Zn(II) and Cd(II). This site has a higher affinity for Cu(II) and Zn(II) than for Cd(II). There is also a second strong Cd(II) site which does not bind either Zn(II) or Cu(II) strongly. It might be worth mentioning that the so called exchangeable copper [23] cannot be that bound to the strong site with a $K_a = 10^{16}$, since this will give (assuming a diffusion limited on-rate) an off-rate of *ca.* 10^{-7} s^{-1}, or a lifetime of *ca.* 3,000 hours. The competition between Ca(II) or Gd(III) with Cd(II) did not give conclusive results. For Ca(II) there is only a minor effect on the ^{113}Cd resonances, even when a 4-fold excess of Ca(II) was added. The intensities of the two signals were slightly reduced, the high field signal (+28 ppm) showing the largest decrease. With Gd(III) both ^{113}Cd signals are affected upon the addition of less than one equivalent of Gd(III) per albumin molecule. However, there is no strong selectivity towards one particular site, at most a slight preference for the site with the high field shifted ^{113}Cd resonance. With two Gd(III) equivalents only a broad ^{113}Cd signal, with a shift of about +80 ppm, could be observed.

Our present results strongly support the conclusion by Giroux and Henkin [12] that an overwhelming proportion of the zinc in normal serum is bound to serum albumin. The site responsible for Zn(II) binding consists most probably of two histidyl residues and not the cysteinyl residue. This site also appears to be the strong Cu(II) site. In all the zinc metalloenzymes so far studied for which X-ray data are available, the active zinc site involves two or three histidyl residues [24]. In metamyoglobin, moreover, it was shown that one equivalent of Zn(II) is bound to a nearby position, probably also involving a histidyl residue. In solution Zn(II) competes with Cu(II) for some of the sites. For serum albumins X-ray data are not yet available, but from amino acid sequence [25, 26] and secondary structure prediction [27] it is possible to recognize only one region, the N-terminus, where two histidyl residues (positions 3 and 10) are sufficiently close to participate in the binding of the same metal ion. For serum albumin, the binding of the first Cu(II) equivalent has been thoroughly studied and one strong site was located at the N-terminus of the peptide chain, involving the histidyl residue in position 3 [18–32], which agrees with our findings that both Zn(II) and Cu(II) replace the same Cd(II) from its binding site. This does not necessarily mean that the binding sites for these three ions are identical but that, *e.g.*, the binding of Cu(II) to its site excludes Zn(II) or Cd(II) from their sites.

A second strong Cd(II) binding site, which also binds Ca(II) but not as strongly as Cd(II), might be a site where the metal ion is bound only to oxygen in less than octahedral geometry.

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