Modified high-affinity binding of Ni^{2+} , Ca^{2+} and Zn^{2+} to natural mutants of human serum albumin and proalbumin

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High-affinity binding of radioactive Ni^{2+} , Ca^{2+} and Zn^{2+} to six genetic albumin variants and to normal albumin isolated from the same heterozygote carriers was studied by equilibrium dialysis at pH 7.4. The three cations bind differently to albumin. Ni^{2+} binds to a site in the N-terminal region of the protein which is partially blocked by the presence of a propeptide as in proalbumin (proAlb) Varese ($\text{Arg}^{-2} \rightarrow \text{His}$), proAlb Christchurch ($\text{Arg}^{-1} \rightarrow \text{Gln}$) and proAlb Blenheim ($\text{Asp}^1 \rightarrow \text{Val}$) and by the presence of only an extra Arg residue (Arg^{-1}) as in Arg-Alb and albumin (Alb) Redhill. The association constants are decreased by more than one order of magnitude in these cases, suggesting biological consequences for the ligand. The additional structural changes in Alb Redhill have no effect on Ni^{2+} binding. Finally, the modification of Alb Blenheim ($\text{Asp}^1 \rightarrow \text{Val}$) reduces the

binding constant to 50 %. Ca^{2+} binding is decreased to about 60–80 % by the presence of a propeptide and the mutation $\text{Asp}^1 \rightarrow \text{Val}$. Arg^{-1} alone does not affect binding, whereas Alb Redhill binds Ca^{2+} more strongly than the normal protein (125 %). In contrast with binding of Ni^{2+} and Ca^{2+} , albumin shows heterogeneity with regard to binding of Zn^{2+} , i.e. the number of high-affinity sites was calculated to be, on average, 0.43. The binding constant for Zn^{2+} is increased to 125 % in the case of proAlb Varese, decreased to 50–60 % for proAlb Christchurch and Alb Redhill but is normal for proAlb Blenheim, Alb Blenheim and Arg-Alb. The effects of the mutations on binding of Ca^{2+} and Zn^{2+} indicate that primary binding, when operative, is to as yet unidentified sites in domain I of the albumin molecule.

INTRODUCTION

Bisalbuminaemia is characterized by the presence of more than one type of albumin in blood plasma, and it is commonly believed that the condition is not associated with disease. However, in order to obtain a more profound insight into the potential biological importance of albumin isoforms, it is necessary to examine the functional properties of purified forms of these proteins.

About 50 structurally different genetic variants of human serum albumin or proalbumin have been identified and sequenced at the DNA and/or protein level. Many of the mutants have amino acid changes in three short segments of the molecule: in the propeptide and the N-terminus, and in subdomains IIB and IIIB. As the variants were originally detected by electrophoretic techniques, this apparent clustering of the mutations may reflect a folding of the polypeptide chain that makes these regions more exposed to the solvent and therefore potentially important for the unique ligand-binding properties of albumin [1].

The present binding studies were performed with six different proteins from the first group of variants [2–6]. Thus, because the type and position of the molecular changes are known, the experimental findings should be of not only biological but also protein-chemical relevance. For this study, Ni^{2+} , Ca^{2+} and Zn^{2+} were chosen as ligands, because albumin is the major, or sole, carrier of these cations [7]. The data obtained by using radioactive metal ions and equilibrium dialysis reveal that all the protein preparations were heterogeneous with respect to high-affinity binding of Zn^{2+} but not Ni^{2+} and Ca^{2+} . Furthermore, all of the variants showed a substantial decrease in affinity for Ni^{2+} ,

whereas the effects of the mutations on binding of Ca^{2+} and Zn^{2+} were more diverse.

MATERIALS AND METHODS

Chemicals

The genetic variants and their normal counterpart, endogenous albumin (Alb A), were isolated from serum samples taken from heterozygote carriers and structurally characterized as previously described [2–6]. After isolation, the albumins were also checked by electrophoresis; no denaturation or cross-contamination was detected. A commercial preparation (pooled fraction) of human serum albumin proposed to be Alb A (97 % pure according to the manufacturer) was obtained from AB Kabi (Stockholm, Sweden). All the proteins were delipidated by treatment with a hydroxyalkoxypropyl dextran (Sigma Chemical Co., St. Louis, MO, U.S.A.) in dilute H_2SO_4 , pH 3.0, at 2–3 °C [8]. After defatting, the albumins were dialysed extensively against deionized water, lyophilized and stored at –20 °C until use.

Newly prepared batches of $^{63}\text{NiCl}_2$ (20 mCi/mg of nickel) were supplied by Amersham International (Amersham, Bucks., U.K.), $^{45}\text{CaCl}_2$ (22 mCi/mg of calcium) by New England Nuclear (Boston, MA, U.S.A.) and $^{65}\text{ZnCl}_2$ (1.3 mCi/mg of zinc) by Research Center Risø (Roskilde, Denmark). According to the manufacturers, no significant radionuclidic impurities could be detected in any of the batches used in this study. The salt $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ was a product of highest purity from Merck (Darmstadt, Germany). All other chemicals were of the purest grade available.

Equilibrium dialysis

Binding of radioactive plus non-radioactive Ni^{2+} and radioactive Zn^{2+} was studied at 20 °C in 50 mM Tris/HCl buffer, pH 7.4. Solutions of different concentrations of Ni^{2+} (typically 0.89–6.62 μM or 4.19–167 μM) or Zn^{2+} (0.80–9.89 μM) and a constant concentration (0.1 %, w/v) of albumin were prepared. Metal ions were added, with magnetic stirring, to the protein-containing solutions as small volumes of concentrated stock solutions.

Degree of binding was determined by using Dianorm equilibrium dialysers (Dianorm Geräte, München, Germany) with half-cell volumes of 250 μl . The dialysis membranes had a molecular-mass cut-off of 5000 Da (Diachema dialysis membranes, type 10.14). Samples (175 μl) containing protein and ligand were pipetted into the left side of the cells, and the same volume of buffer alone was pipetted into the right side. After being closed, the equilibrium dialysers were placed in a temperature-controlled water bath, and the cells were rotated around their horizontal axis at a speed of 12 rev./min for 17–18 h (Ni^{2+}) or 22–23 h (Zn^{2+}). The half-cells were then emptied, and portions were used for liquid-scintillation counting.

A slightly different experimental protocol was used to study Ca^{2+} binding, because it has previously been found that this ligand interacts with the Tris anion and because the ligand is weakly bound to albumin [9]. Thus binding of radioactive Ca^{2+} was examined in 50 mM Hepes buffer, pH 7.4. Solutions of different concentrations of Ca^{2+} (0.108–1.44 μM) and a constant concentration (0.3 %, w/v) of albumin were prepared in essentially the same way as for the two other metal ions. Degree of binding was determined using Plexiglass dialysis cells with half-cell volumes of 1.2 ml. They were equipped with cellulose membranes cut from dialysis tubing (Union Carbide Corp., Chicago, IL, U.S.A.). Samples (750 μl) containing protein plus ligand and buffer alone were injected into the left and right sides of the cells respectively. After being closed, the cells were rotated for 4 h in a cabinet at a constant temperature of 20 °C.

As the radioactive cations were delivered in 0.1 M HCl, the pH of the final solutions was measured and adjusted if necessary before pipetting into the dialysis cells. Albumin concentrations were checked by the method of Lowry et al. [10] using the commercial protein as a standard. Control experiments ensured that equilibrium was established within the periods used, and that protein leakage could be neglected. Furthermore, no quenching of radioactivity by albumin was observed.

On the basis of previous studies with Ca^{2+} [9], the existence of a significant Donnan effect could be excluded at the low protein concentrations used.

Calculations

The radioactivity of ligand-containing solutions that had not been dialysed was taken to represent the known concentration of total ligand. Concentrations of bound and free ligand were calculated by using the radioactivity of samples taken from the albumin-containing half-cells, representing bound plus free ligand, and from the corresponding albumin-free half-cells which represents free ligand.

Association constants were computed by an iteration procedure based on the following general equation:

$$\bar{v}_L = \sum_{i=1}^j \frac{n_i K_i [L]_f}{1 + K_i [L]_f}$$

where $\bar{v}_L$ is the average number of mol of ligand L bound/mol of albumin (molecular mass 67 kDa), n_i and K_i are the number of

sites and the corresponding binding constant in the i th binding class respectively and $[L]_f$ is the concentration of the unbound form of ligand.

Except for binding of high concentrations of Ni^{2+} to commercial Alb A, all results were plotted as Scatchard plots before being inserted into the above equation. For Ca^{2+} and Zn^{2+} binding it was found that the experimental results could be described very well by linear plots. In the case of Ni^{2+} , the preliminary analysis revealed some secondary binding, which was corrected for when calculating the high-affinity binding constants. Thus for all the ligands it was possible, by using the present low ligand/protein molar ratios, to focus on primary binding to the genetic variants and endogenous Alb A. This implied that it was fairly simple to calculate high-affinity binding constants. Therefore it was decided to use the available amount of the genetic variants by performing binding experiments with a relatively few ligand–protein combinations but to repeat the experiments as many times as possible.

For experiments performed five or more times, the values of the binding constants are given as means $\pm$ S.D. In the remaining examples binding constants were reproducible to within 20 %.

RESULTS

Natural mutants

Table 1 summarizes the molecular characteristics of the six genetic variants of albumin and proalbumin used in this work. The three proalbumins are all the result of point mutations in the structural gene; they have amino acid substitutions at positions –2 (proAlb Varese), –1 (proAlb Christchurch) and 1 (proAlb Blenheim). The amino acid changes in these variants prevent proteolytic cleavage of the propeptide, which is why they, in contrast with wild-type proalbumin, are secreted unprocessed by liver cells into the bloodstream. ProAlb Blenheim has a normal propeptide sequence and is produced by a single amino acid substitution in the mature albumin molecule. Partial cleavage in the circulation leads in this case to two circulatory products, proAlb Blenheim and Alb Blenheim. Arg-Alb arises from an amino acid substitution in the prosegment ($\text{Arg}^{-2} \rightarrow \text{Cys}$) which results in abnormal hydrolysis of the prepropeptide. Alb Redhill is unique because it is the only example so far of an albumin variant with two mutations. One is the same as that in Arg-Alb ($\text{Arg}^{-2} \rightarrow \text{Cys}$), and the other is a neutral amino acid change in

Table 1 Molecular characterization of albumin and proalbumin mutants

Alb A and its propeptide consist of 585 and six amino acids respectively. The numbering of the latter is from –6 to –1 (the juxtaposition to albumin).

Name of variant	Structural change(s)	Reference
ProAlb Varese	$\text{Arg}^{-2} \rightarrow \text{His}$	[2]
ProAlb Christchurch	$\text{Arg}^{-1} \rightarrow \text{Gln}$	[3]
ProAlb Blenheim	Alb Blenheim to which the normal propeptide is still bound	[4]
Alb Blenheim	$\text{Asp}^1 \rightarrow \text{Val}$	[4]
Arg-Alb	Alb A possessing an extra Arg at the N-terminus (Arg^{-1} from the propeptide)	[5]
Alb Redhill	An extra Arg at the N-terminus plus a point mutation ($\text{Ala}^{320} \rightarrow \text{Thr}$) plus an oligosaccharide (2.5 kDa) bound to Asn^{318}	[6]

Table 2 High-affinity binding of metal ions to natural mutants of human serum albumin and proalbumin

The binding constants for the commercial protein are means for five to eleven experiments, whereas those for the remaining proteins are means for two to four experiments. The number of high-affinity binding sites for Ni^{2+} and Ca^{2+} was assumed to be equal to one, whereas that for Zn^{2+} binding was calculated to be 0.43. N.D., not determined.

Protein	Association constant (M^{-1})		
	Ni^{2+} ($10^{-5} \times K_1$)*	Ca^{2+} ($10^{-3} \times K_1$)	Zn^{2+} ($10^{-6} \times K_1$)
Commercial Alb A	8.6	1.1	1.2
Alb A Varese	4.7	1.1	1.2
ProAlb Varese	0.29	0.87	1.5
Alb A Christchurch	4.9	1.3	1.4
ProAlb Christchurch	0.33	0.74	0.71
Alb A Blenheim	6.7	1.2	1.1
ProAlb Blenheim	0.35	N.D.	1.1
Alb Blenheim	3.5	0.88	1.2
Alb A Redhill	10.6	1.1	1.3
Arg-Alb	0.63	1.1	1.2
Alb Redhill	0.49	1.4	0.73

* Before calculation of K_1 , the experimental results were corrected for secondary binding by using $n_2 = 1$ and $K_2 = 0.25 \times 10^5 \text{ M}^{-1}$.

domain II of the molecule ($\text{Ala}^{320} \rightarrow \text{Thr}$) which leads to glycosylation of the protein at Asn^{318} .

Binding studies

The experiments examined the binding of one ligand to commercial Alb A, used as a standard, to endogenous Alb A and to one or two variants. The proteins, except for the commercial one, were always isolated from the same person. After the binding experiments with a particular ligand were finished, the average high-affinity association constant for commercial Alb A was calculated, and the other high-affinity binding constants were related to that value. Thus all the constants given for each of the three ligands in Table 2 can be compared with each other.

Binding of Ni^{2+}

Figure 1 shows representative results for normal albumin and four different variants. Ni^{2+} binding to the commercial protein and to the two preparations of endogenous Alb A are evident and comparable. Binding to proAlb Blenheim (Figure 1a), Arg-Alb and Alb Redhill (Figure 1b) are also similar to each other but the level of binding is much lower than normal. The extent of ligand binding to proAlb Varese and proAlb Christchurch (not illustrated) was about the same as that found for the variants just mentioned. Finally, Alb Blenheim possesses an intermediate affinity for Ni^{2+} (Figure 1a).

Albumin- Ni^{2+} interactions were quantified in terms of binding sites and association constants as described in the Materials and methods section. As it is well known from several types of study that human serum albumin possesses one high-affinity binding site for Ni^{2+} [1,7,11,12], n_1 was set equal to one. Furthermore, when the high-affinity binding constants were calculated, the experimental results were corrected for secondary binding (see below). The average constant thus computed for the commercial protein was $8.6 (\pm 3.1) \times 10^5 \text{ M}^{-1}$ ($n = 11$). This value is comparable with those determined for the different preparations of

Figure 1 Binding of Ni^{2+} to commercial Alb A (X), endogenous Alb A (O) and different genetic variants

The variants in (a) are Alb Blenheim (●) and proAlb Blenheim (▲) and those in (b) Alb Redhill (●) and Arg-Alb (■). When the binding curves were constructed both primary and secondary ligand binding were taken into account; for further information see the text. The symbols $\bar{v}$ and $[\text{Ni}^{2+}]_f$ represent the average number of mol of ligand bound/mol of albumin and the concentration of free Ni^{2+} respectively.

endogenous Alb A (Table 2). The present constants for Ni^{2+} binding to normal albumin are of the same order of magnitude as that ($3 \times 10^5 \text{ M}^{-1}$) published by Callan and Sunderman [11], who also used equilibrium dialysis and radioactive Ni^{2+} . The primary binding constants determined for all the genetic variants were decreased (Table 2).

In addition to the high-affinity site, human serum albumin possesses several binding sites of a lower affinity for Ni^{2+} [11]. Therefore it was decided to investigate whether the binding constants (0.29×10^5 – $0.63 \times 10^5 \text{ M}^{-1}$) calculated for the genetic variants (except for Alb Blenheim) are reduced to such an extent that they become comparable with that characterizing normal secondary binding. Therefore binding of high concentrations of Ni^{2+} to commercial albumin was studied. The results of a representative experiment are shown in Figure 2; it is apparent that binding increases abruptly at low ligand concentrations but flattens out at $\bar{v}$ values of about 1. Such a tendency indicates the existence of one or more sites of lower affinity. The average binding data for six experiments were, assuming the simpler binding model: $n_1 = 1$, $K_1 = 7.7 (\pm 2.1) \times 10^5 \text{ M}^{-1}$, $n_2 = 1$, $K_2 = 0.25 (\pm 0.07) \times 10^5 \text{ M}^{-1}$, i.e. the secondary constant is somewhat lower than the primary constants found for the variants.

The data given for normal secondary binding were also used to correct the results from all the experiments for this type of binding, and the high-affinity constants thus calculated are given in the text and Table 2. In performing this correction it was assumed that secondary binding is unaffected by the different

Figure 2 Binding of Ni^{2+} to commercial Alb A

The binding curve is a theoretical one, and the number of sites and corresponding association constants are given in the text. For further information, see the legend to Figure 1.

Figure 3 High-affinity binding of Ca^{2+} to commercial Alb A (x), endogenous Alb A (O) and different genetic variants

The variant in (a) is proAlb Christchurch (●) and those in (b) are Alb Redhill (●) and Arg-Alb (■). The binding curves are theoretical ones; the upper curve in (b) is for Alb Redhill, whereas the lower curve is common to commercial Alb A, endogenous Alb A and Arg-Alb. The symbols $\bar{v}$ and $[\text{Ca}^{2+}]_f$ represent the average number of mol of ligand bound/mol of albumin and the concentration of free Ca^{2+} respectively.

mutations and by the presence of the propeptides. The argument for this assumption is that all the changes that affected binding are very close to the N-terminus of albumin, the position at which Ni^{2+} is known to bind with high affinity [1,7,12].

It can be seen in Table 2 that variants with more amino acid residues than Alb A bind Ni^{2+} with a primary constant that is at least one order of magnitude lower than that of their normal counterpart and only slightly higher than that found for normal secondary binding. Alb Blenheim binds the ligand with a higher affinity than the other genetic variants, i.e. the high-affinity binding constant is about 50 % of normal.

Binding of Ca^{2+}

In contrast with the great majority of ligands [1], albumin does not possess a typical high-affinity binding site for Ca^{2+} . Instead, Ca^{2+} interacts with several 'sites' which differ only slightly in their affinity towards the ligand [9]. Therefore, in an attempt to focus on the primarily bound Ca^{2+} , investigations were performed at very low $\bar{v}$ values (Figure 3). This was possible by carrying out experiments in the absence of non-radioactive Ca^{2+} and because radioactive Ca^{2+} of high specific radioactivity and high radiochemical purity was available.

It is apparent from the representative results in Figure 3 that the effects of the mutations on Ca^{2+} binding are more diverse than those found for Ni^{2+} , i.e. all possible types of effect were observed: increased binding to Alb Redhill (Figure 3b), normal binding to Arg-Alb (Figure 3b) and diminished binding to proAlb Christchurch (Figure 3a).

As in all cases the data could be described very well by linear Scatchard plots, binding to only one site was assumed, and the primary association constants computed are compiled in Table 2. The constant calculated for Ca^{2+} binding to Arg-Alb is similar to those determined for commercial Alb A [$1.1 (\pm 0.1) \times 10^3 \text{ M}^{-1}$, $n = 5$] and for the different preparations of endogenous Alb A. In contrast, the constants for Alb Blenheim and the proalbumins are reduced to 60–80 % of the normal value, whereas that for Alb Redhill is increased to about 125 %.

Binding of Zn^{2+}

For all experiments, extrapolation of the linear Scatchard plot intersected the abscissa at a $\bar{v}$ value well below 1. Such a phenomenon indicates heterogeneity in high-affinity binding of Zn^{2+} [13], and some representative examples are given in Figures 4(a) and 4(b). By heterogeneity it is meant that part of the albumin does not possess the high-affinity site for Zn^{2+} . In Figures 4(a) and 4(b), it can be seen that for all the albumin types shown the points describe a straight line, the extrapolation of which intersects the abscissa at about 0.4. Analysis of all the experimental data obtained for this ligand revealed $n_1 = 0.43 \pm 0.10$ ($n = 36$).

After this preliminary analysis, theoretical binding curves were produced by using the average value of n_1 (0.43). As seen in Figures 4(c) and 4(d), this approach gave a good fit to the data. The binding constant computed for commercial Alb A is $1.2 (\pm 0.2) \times 10^6 \text{ M}^{-1}$ ($n = 10$), and the different constants are all in the order of 10^6 M^{-1} (Table 2). These values are slightly higher than those calculated for binding of Ni^{2+} and about a thousandfold higher than those determined for Ca^{2+} .

As indicated by the association constants presented in Table 2, binding of Zn^{2+} is least affected by the mutations. The affinity of the ligand for three of the variants (proAlb Blenheim, Alb Blenheim and Arg-Alb) was comparable with that for normal albumin. However, the binding constant for proAlb Varese was increased to about 125 %, whereas the constants for proAlb Christchurch and Alb Redhill were decreased to 50–60 % of the normal value.

Figure 4 High-affinity binding of Zn^{2+} to commercial Alb A ($\times$), endogenous Alb A ($\circ$) and different genetic variants

The data are shown as Scatchard plots in (a) and (b) and as ordinary binding plots in (c) and (d) respectively. The variant in (a) and (c) is proAlb Christchurch ($\bullet$) and those in (b) and (d) are Alb Redhill ($\bullet$) and Arg-Alb ($\blacksquare$). The regression lines in (a) and (b) were calculated by the method of least squares. The binding curves in (c) and (d) were constructed by using $n_1 = 0.43$, and the association constants calculated are included in Table 2. The symbols $\bar{v}$ and $[\text{Zn}^{2+}]_f$ represent the average number of mol of ligand bound/mol of albumin and the concentration of free Zn^{2+} respectively.

DISCUSSION

The present work examined high-affinity binding of Ni^{2+} , Ca^{2+} and Zn^{2+} to a 'family' of genetic variants of human proalbumins and serum albumins modified at the N-terminus. The results clearly show that binding of the three ligands was affected differently by the mutations. This suggests that these cations bind to different sites in the albumin molecule.

Binding of Ni^{2+}

High-affinity binding of Ni^{2+} takes place at a specific site in the N-terminal region of albumin. This binding site, which has been investigated in great detail, involves the α -amino nitrogen, the first two peptide nitrogens (mainly deprotonated at physiological pH), the N^1 imidazole nitrogen of the histidine residue in the third position, and the side-chain carboxyl oxygen of the aspartic acid residue in position 1 [12]. Our data show that the presence of a propeptide is able to greatly decrease Ni^{2+} binding, i.e. the association constants for the proalbumins are reduced by more than one order of magnitude compared with those for binding to Alb A. This was found to be the case whether the propeptide was modified (proAlb Varese and proAlb Christchurch) or not (proAlb Blenheim). The site does not seem to be completely blocked, because the binding constants, not corrected for secondary binding, were more than double (0.55×10^5 – $0.62 \times 10^5 \text{ M}^{-1}$) that calculated for secondary binding to commercial Alb A ($0.25 \times 10^5 \text{ M}^{-1}$). This is in contrast with binding of Cu^{2+} , a ligand that has the same high-affinity site as Ni^{2+} [1,7,12]. Brennan and Carrell [14] studied its binding to proAlb

Christchurch and found spectrophotometric evidence for total blockade of the high-affinity binding site in that genetic variant.

The effect of the propeptides on Ni^{2+} binding is probably not the result of a simple physical blockade of the site but is rather due to the proximity of a positively charged arginine residue, because Arg-Alb (and Alb Redhill) binds Ni^{2+} to the same low extent as the proalbumins. In contrast, the second mutation and the oligosaccharide of Alb Redhill do not affect binding.

As mentioned above, bound Ni^{2+} interacts with four different nitrogen atoms and a single oxygen atom in the albumin molecule. We have been able to shed light on the importance of the oxygen atom, because in Alb Blenheim the aspartic acid residue in position 1 has been replaced by an amino acid residue without a carboxy group, i.e. valine, which reduced the binding constant to about 50 % of normal.

The reduced Ni^{2+} -binding capacity of proalbumins has been used to distinguish this type of genetic variant from Alb A and albumin variants with mutations C-terminal of position 3. Thus Takahashi et al. [15] observed lower metal ion binding by a series of proalbumin variants by electrophoresis, followed by radio-autography, of heterozygote sera to which $^{63}\text{Ni}^{2+}$ had been added. In contrast with the results of Takahashi et al. [15] and the present investigation, Brennan and co-workers [4,5,14], using the same technique as Takahashi et al. [15], were not able to detect $^{63}\text{Ni}^{2+}$ binding at all by Arg-Alb and proalbumin variants.

Binding of Ca^{2+}

Primary binding of Ca^{2+} apparently does not take place at the well-characterized high-affinity site for Ni^{2+} and Cu^{2+} . This

agrees with the fact that Ca^{2+} is much larger (99 pm) than Ni^{2+} and Cu^{2+} , which are almost the same size (about 70 pm). It is proposed that Ca^{2+} binding takes place at one or more amino acid residues of subdomain IA which are not closely associated with the N-terminus. Such a position is suggested, because binding to proAlb Varese, proAlb Christchurch and Alb Blenheim was moderately decreased, whereas binding to Arg-Alb was normal. Therefore, the effects on binding of the three former genetic variants are probably not caused by short-range electrostatic influences but are rather the results of conformational changes in the subdomain.

In contrast with Arg-Alb, Alb Redhill exhibits modified Ca^{2+} -binding properties, and this effect must be brought about by the mutation at position 320 (Ala→Thr) and/or by the carbohydrate linked to Asn-318. In the albumin molecule, these positions are located far from subdomain IA [16], and therefore it is likely that the increased Ca^{2+} binding to this variant is caused by electrostatic attraction to the negatively charged oligosaccharide side chain [6].

Binding of Zn^{2+}

The 11 different albumin preparations used in this work all showed heterogeneity with respect to high-affinity binding of Zn^{2+} , i.e. the number of primary sites were found to be less than 1.0. This means that there must be at least two proteins in the albumin preparation, one (43%) with high affinity for Zn^{2+} binding and the other (57%) exhibiting low affinity or no binding at all. The present study is the first to report heterogeneity with regard to reversible binding of an inorganic ligand to albumin. However, reports have appeared that describe heterogeneity of binding of organic ligands. Moehring et al. [17] found that their human serum albumin preparations consisted of at least two conformers, with 37% binding deuteroporphyrin IX with a very high association constant and 63% binding the ligand with a constant that was more than one order of magnitude lower. Furthermore, Honoré and Brodersen [13] detected heterogeneity of human serum albumin with regard to binding medium-chain fatty acid anions (hexanoate, octanoate and decanoate), about 65% of the protein having high binding affinity.

The effect of the different mutations on binding showed that the high-affinity site for Zn^{2+} , when present, is different from the 'site' for primary binding of the larger Ca^{2+} . Furthermore, although the size of Zn^{2+} is comparable with that of Ni^{2+} and Cu^{2+} , our results also suggest that the high-affinity site for Zn^{2+} binding is not at the N-terminus of albumin. This proposal is in full accordance with that of Goumakos et al. [18], who studied the binding of Zn^{2+} to human serum albumin and dog serum albumin, which has a tyrosine residue instead of a histidine residue at position 3 [19], in the presence and absence of Cu^{2+} . The different locations of these primary binding sites is probably because Zn^{2+} preferably interacts with four co-ordination sites with tetrahedral stereochemistry, whereas both Ni^{2+} and Cu^{2+} have the co-ordination number five when bound to albumin [12].

Zn^{2+} binds with increased affinity to proAlb Varese, decreased affinity to proAlb Christchurch and Alb Redhill, but exhibits normal, or only slightly changed, affinity for the three remaining variants (Table 2). The increased binding to proAlb Varese could be explained by the presence of the new histidine residue at position -2. Even though the histidine residue in position 3 does not contribute to high-affinity binding of Zn^{2+} , the imidazole group in position -2 could be a point of attachment, because it is well known from studies of many different proteins that

histidine residues are well suited for complexing that metal ion. The molecular change in proAlb Christchurch and those of Alb Redhill placed in domain II reduce Zn^{2+} binding to about the same extent, i.e. 50–60% of the normal level. These changes in binding are most probably the result of indirect effects, and the findings are consistent with the primary site for Zn^{2+} binding being placed in domain I of the albumin molecule. Such positioning could imply that the cysteine residue at position 34 is important for primary Zn^{2+} binding. As only 40% of the thiol groups in defatted albumin preparations are reactive [1], i.e. mercaptalbumin, this would match an n_i value of 0.43 very well. However, according to recent n.m.r. spectroscopic studies of Goumakos et al. [18], the cysteine residue does not seem to be involved in high-affinity binding of Zn^{2+} .

Biological aspects

In the following, we have tried to analyse whether the modified binding found in this study could have an impact on the biological fate of the three metal ions. Binding of Ca^{2+} does not involve a typical high-affinity site but occurs on several 'sites' of low affinity [9]. Furthermore, the plasma concentration of total Ca^{2+} is very high (2.1–2.6 mM [9]), even higher than that of albumin (approx. 600 μM [7]). Therefore a large change in one of the binding constants or even total removal of a binding site would have only a small effect on the concentration of unbound Ca^{2+} . In contrast with Ca^{2+} , the normal plasma concentrations of total Ni^{2+} and Zn^{2+} are much lower than that of albumin, about 0.044 μM [11] and 20 μM [18] respectively. These low concentrations imply that binding of the ions takes place almost exclusively to the respective high-affinity sites. This makes binding very sensitive to changes in these sites. Let us use Ni^{2+} binding as an example. If it is assumed that it binds solely to albumin in plasma, and that the association constants determined here at low albumin concentration also apply to that normally found in plasma, then it is possible to calculate, using the equation given in the Materials and methods section, that, if the primary binding constant is reduced from $7.0 \times 10^5 \text{ M}^{-1}$ to $0.7 \times 10^5 \text{ M}^{-1}$, the concentration of unbound Ni^{2+} would increase from $1.05 \times 10^{-4} \mu\text{M}$ to $1.05 \times 10^{-3} \mu\text{M}$ (if the minimal secondary binding is ignored), i.e. an increment in the concentration by one order of magnitude. Therefore, although this effect is probably smaller in heterozygote subjects and can be modified by ion binding to α_2 -macroglobulin [7]), the finding illustrates that bisalbuminaemia could have greater biological and clinical importance than hitherto believed.

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