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# Probing of the Surface of High-Density Lipoproteins with Mn(II) Ions and EPR Spectroscopy

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Negatively charged sites on the surface of two human high-density lipoproteins, $HDL_2$ and $HDL_3$, have been studied using EPR spectroscopy. Like for LDL, there are two types of binding sites for the Mn(II) ions on each HDL class, characterized by essentially the same binding constants as those for LDL. The small number of »strong« sites ($n$ = 2), attributed to the negatively charged amino-acid residues, indicates that only a small fraction of these residues is available for cation binding. The binding constants for »weak« sites and the number of these sites in LDL and in the two HDL subfractions studied are consistent with the idea that these sites relate to the phospholipid domains on the lipoprotein surface.

## INTRODUCTION

Considerable work has been done on the use of the Mn(II) ions in conjunction with the electron paramagnetic resonance (EPR) for monitoring the surface of low-density lipoprotein (LDL) of human[1–5] and porcine plasma.[6] The Mn(II) ions were used not only as suitable, paramagnetic analogues of other, biologically more relevant, divalent cations, Ca(II) and Mg(II), which are absolute requirement for the receptor LDL binding,[7] but also for probing the distribution of the negatively charged ions and possibly zwitter-ionic regions on the LDL surface.

One of the key steps in the LDL metabolism is its internalization by various types of cells via specific cell-surface receptors, B/E receptors. Chemically modified LDL is recognized by another, less specific receptor, scavenger receptor, and metabolized in a different way.[8] It is clear that for the recognition by receptors, a special arrangement of the constitutive protein, apoprotein B, or distribution of the charged residues on the protein moiety of the LDL surface, is expected. It has been demonstrated that not

only the total charge, but also the number of negatively charged residues on the LDL is significantly increased upon oxidation,[4] or upon other, more specific, ways of chemical modifications.[5] The conclusion is reached that apoprotein B in native LDL makes a compact conformation, with only eight negatively charged groups, presumably aspartic and glutamic acid residues, on the particle surface. Oxidation or other chemical modifications induce loosening of the LDL structure, with exposure of additional, already existing negatively charged residues on the particle surface.[4,5]

This study is aimed at probing the surfaces of the two subfractions of human high-density lipoprotein, $HDL_2$ and $HDL_3$. Specifically, one of the goals is to see whether there are one or more types of binding sites on the HDL surfaces. Since the HDL class lipoproteins contain different apoproteins than LDL, it will be examined whether only the local environment, like individual amino acids, or a protein as a whole determine the binding properties. In addition, the number of binding sites on protein is expected to give some general information on the apoprotein arrangement.

## EXPERIMENTAL

### *Sample Preparations*

$HDL_2$ and $HDL_3$ were prepared from pooled fresh human plasma by ultracentrifugation. The cut-off densities (1.063 and 1.125 g/mL for $HDL_2$, and 1.125 and 1.210 g/mL for $HDL_3$) were adjusted by adding NaBr. Each centrifugation was performed at 50 000 rev./min for at least 24 hours, at 5 °C, in a Beckman 70 Ti rotor. All solutions were protected against peroxidation by EDTA (1 g/L) present in the medium through all preparation steps. After isolation, the HDL solutions were dialysed against degassed 0.1 M Tris-HCl buffer with EDTA (1 g/L), pH 7.4, and stored at 4 °C until used.

Prior to the EPR measurements, EDTA was removed by extensive dialysis against degassed Tris-HCl buffers (pH 7.4) of various selected buffer concentrations (selected ionic strengths). The manganese stock solutions were prepared in the same buffers.

### *EPR Measurements*

EPR measurements were performed with a Varian E-109 spectrometer, operating at 9.3 GHz, equipped with a double cavity resonator. The intensity of the EPR signal of the sample, located in one compartment, was compared with the signal of a standard, located in the other compartment of the resonator. The measurements were made either at 37 °C with the use of the Brucker temperature control unit, or at ambient temperature. As already pointed out,[6] the amplitude of the well known sextet pattern of free Mn(II) is very sensitive to temperature and it is found that after a certain period of warm-up, the ambient temperature measurements gave more reproducible results.

## DETERMINATION OF BINDING PARAMETERS

Binding of manganese ions to the HDL particles is determined from the estimates of the free Mn(II) concentration in solution, with and without HDL, in the same way as described earlier for LDL. [2–6] It is possible to have more than one type of binding sites on the HDL surface. For such a system, in a buffer of a specified electrolyte concentration (defined ionic strength), the binding data are expected to obey the following relation:

$$\frac{[\mathrm{Mn}]_B}{[\mathrm{Mn}]_T} = 1 - \left(1 + \sum_k \frac{K_k\, n_k\, [\mathrm{HDL}]}{1 + K_k\, [\mathrm{Mn}]_F}\right)^{-1}, \qquad (1)$$

where $K_k$ refers to the binding constant and $n_k$ to the number of binding sites of the kind $k$. $[Mn]_B$, $[Mn]_F$ and $[Mn]_T$ denote bound, free and total manganese ion concentrations, respectively.

$$[Mn]_T = [Mn]_B + [Mn]_F. \tag{2}$$

The numbers of each kind of binding sites, $n_k$, are independent of the ionic strength of the electrolyte. In contrast, the binding constants $K_k$ vary with the electrolyte concentration. The directly measured, »apparent« binding constants for the binding sites of the kind $k$ for different electrolyte concentrations are expected to be mutually related *via* the »intrinsic« binding constant, $K_k^i$, and the electric potentials near the surface, $\varphi_k^O$, for specific electrolytes[2]:

$$K_k = K_k^i \exp(-2e\varphi_k^O / kT). \tag{3}$$

For calculation of potential $\varphi^O$, it is assumed that the binding sites are independent hydrated point charges on the HDL surface, and that the closest approach of the Mn(II) ion to any kind of the binding sites is 0.6 nm.[9] Therefore, the problem of calculation of the binding constants $K_k$ is reduced to the evaluation of the electric potential $\varphi^O$ at a sphere of radius 0.6 nm around a point charge. For such a system, $\varphi^O$ is calculated from the linearized Poisson-Boltzmann equation:[2,10]

$$\frac{d^2(r\varphi)}{dr^2} = \frac{2e\,C(\infty)}{\varepsilon_O\,\varepsilon_r\,k\,T} \cdot r\,\varphi(r) \tag{4}$$

$\varepsilon_O$ is the permittivity of vacuum, $\varepsilon_O$ is the relative permittivity of water and $C(\infty)$ is the concentration of the 1:1 electrolyte far from the HDL surface. The solution is:

$$\varphi(r) = \frac{q}{4\pi\,\varepsilon_O\,\varepsilon_r} \cdot \frac{1}{r} \cdot \exp(-r/\lambda_O). \tag{5}$$

Here, $q$ is the charge of a binding site and $\lambda_O$ the Debye length,

$$\lambda_O = \left(\frac{\varepsilon_O\,\varepsilon_r\,k\,T}{2e^2\,C(\infty)}\right)^{1/2}. \tag{6}$$

For each electrolyte concentration, $C(\infty)$, a specific value $\varphi^O = \varphi$ (0.6 nm) is easily calculated.

## RESULTS

A general procedure of data collecting is the measurement of $[Mn]_B/[Mn]_T$ *vs.* $[Mn]_T$ for each HDL fraction, for several different electrolyte concentrations. The collected data for each titration curve (for one series of measurements with constant HDL and electrolyte concentrations and varying $[Mn]_T$) could be fitted to a curve defined by eq. (1), with $K_k$ and $n_k$ as adjustable parameters. The experiments were carried out for three different preparations of each HDL subfraction. For each preparation, the $[Mn]_T$ titrations were carried out for at least five different values of the electrolyte

(buffer) concentrations, ranging from 0,02 to 0.25 mol/L. The selected experimental data for $HDL_2$ are shown in Figures 1 and 2. It is obvious that, by assuming an arbitrary number of binding sites, it is possible to fit the data to curve (1) in more than one way. Therefore, some additional restrictions have been introduced for the evaluation of binding parameters.

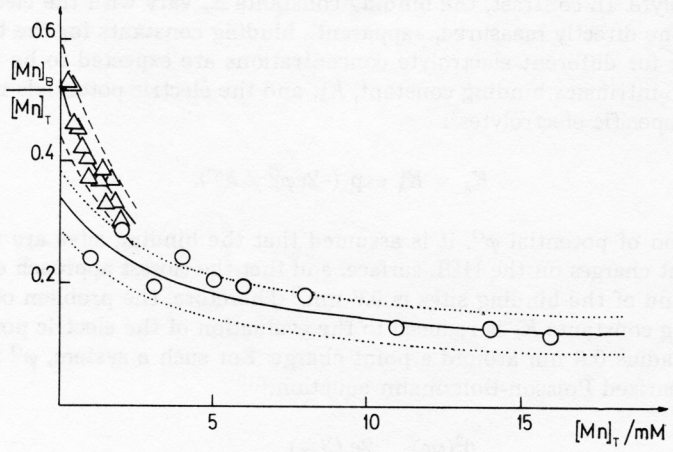


Fig. 1. Binding of Mn(II) ions to $HDL_2$ (0.1 mmol/L) in 0.02 mol/L Tris-HCl buffer (triangles) and in 0.25 mol/L buffer (circles), at 21 °C, pH 7.4. The solid lines represent the theoretical curves obtained by using the parameters listed in Table I. The dashed lines represent the expected binding for $n_1 = 2 \pm 1$, and the dotted lines the expected binding for $n_2 = 40 \pm 10$.

(a) The number of types of binding sites selected is the minimum number required for a reasonable fit; possible corrections brought about by assuming an additional type of binding sites are of the order of the scattering of the experimental data.

(b) The data for all individual titration curves (each for a specified electrolyte concentration) are described by the same values of $n_1$ and $n_2$, and by the binding constants that are mutually related with the same values of $K_1^i$ and $K_2^i$ by eqs. (1–6).

It is easily shown that for the data for $HDL_2$, presented in Figures 1 and 2, condition (a) reduces the binding sites to only two types, »strong« and »weak«, like for LDL.[2] Criterion (b) allows the use of all the data for $HDL_2$ at the same time in search for unique, consistent values of the four binding parameters.

In doing that, first, simultaneous variation of all the four binding parameters for each set of data was allowed in order to find the best fit. Under the condition $q = -e$, the deduced values for $n_1$ were in the range 1–3 and the values for $K_1^i$ varied between 500 and 1200 $mol^{-1}L$, close to the corresponding value of 760 $mol^{-1}L$ for LDL.[2] The border values were generally obtained for the measurements with either only a few or scattered points.

Next, instead of varying all the four parameters, we adopted the LDL value for $K_1^i$ and tried to deduce the remaining three parameters under the condition $n_1$ = in-

teger. It is easily shown that only $n_1 = 2$ satisfies all the titration curves. This is nicely illustrated by the top curve in Figure 1, since the binding data for the samples in electrolytes of low ionic strength are determined predominantly by »strong« binding, represented by $K_1^i$ and $n_1$.

Finally, with fixed $K_1^i$ and $n_1$, $K_2^i$ and $n_2$ were found to fit all the measured $[Mn]_B/[Mn]_T$ *vs.* $[Mn]_T$ curves. The deduced values for all the binding parameters are summarized in Table I. The value for $K_2^i$ (18.9 $mol^{-1}L$) is close to the corresponding value in LDL (17.5 $mol^{-1}L$).[2] Full curves in Figures 1 and 2 are reproduced using eqs. (1–6) and the parameters from Table I. The fit with the corresponding experimental results is good. The dashed lines in Figure 1 represent the expected binding curves for $n_2 = 40 \pm 10$ in the concentrated electrolyte (0.25 mol/L), sensitive to the weak and numerous binding sites, illustrating the reliability of the deduced values.

A similar procedure was applied to $HDL_3$. The selected binding data are shown in Figures 3 and 4. Again, it was found that $n_1 = 2$ and $K_1^i = 760$ $mol^{-1}L$ satisfy all sets of data, finally yielding $n_2 = 20$ and $K_1^i = 19.7$ $mol^{-1}L$ (see Table I).The reproduced curves with the binding parameters from Table I are represented by the full lines in Figures 3 and 4. For $HDL_3$, too, the fit of all of the experimental data to the theoretical

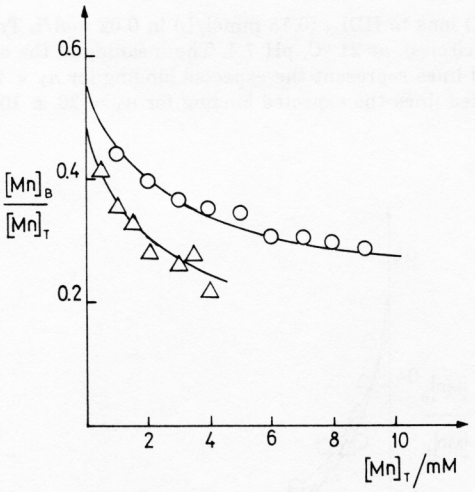


Fig. 2. Mn(II) binding to $HDL_2$ (0.1 mmol/L) in 0.04 mol/L Tris-HCl buffer (triangles) and to $HDL_2$ (0.17 mmol/L) in 0.1 mol/L buffer (circles). The solid lines represent the theoretical curves produced with the parameters from Table I.

TABLE I

*Summary of the binding parameters*

| | $n_1$ | $K_1^i$ / ($mol^{-1}L$) | $n_2$ | $K_2^i$ / ($mol^{-1}L$) |
|---|---|---|---|---|
| $HDL_2$ | 2 | 760 | 40 | 18.9 |
| $HDL_3$ | 2 | 760 | 20 | 19.7 |

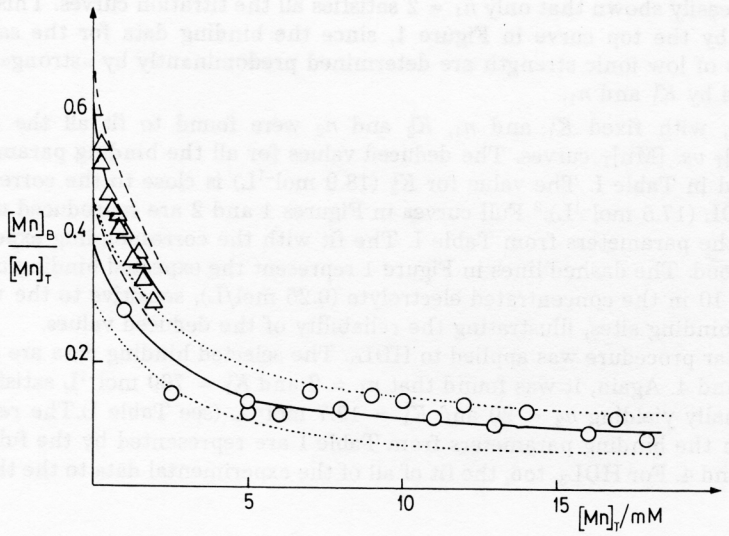


Fig. 3. Binding of Mn(II) ions to $HDL_3$ (0.15 mmol/L) in 0.02 mol/L Tris-HCl buffer (triangles) and 0.25 mol/L buffer (circles), at 21 °C, pH 7.4. The meaning of the solid lines is the same as in Figure 1. The dashed lines represent the expected binding for $n_1 = 2 \pm 1$ in the 0.02 mol/L electrolyte, and the dotted lines the expected binding for $n_2 = 20 \pm 10$ in the 0.25 mol/L electrolyte.

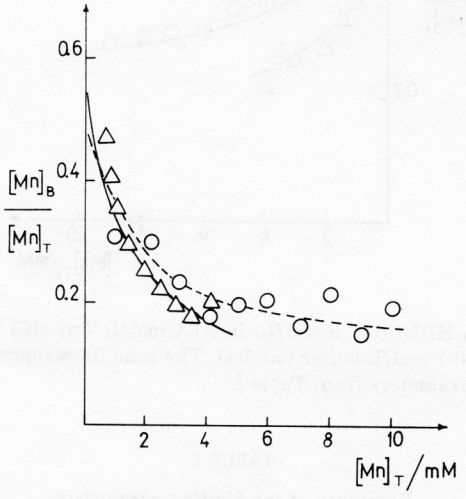


Fig. 4. Relative binding of Mn(II) to $HDL_3$ (0.14 mmol/L) in 0.04 mol/L buffer (triangles) and to $HDL_3$ (0.17 mmol/L) in 0.1 mol/L buffer (circles). The solid lines are reproduced theoretically for the two specified buffer concentrations, using the parameters from Table I.

curve (1) is good. To illustrate the reliability of the deduced parameters, Figure 3 also shows the expected binding curves for $n_1 = 2 \pm 1$ (dashed lines) and $n_2 = 20 \pm 10$ (dotted lines) for the measurements with the two extreme electrolyte concentrations.

## DISCUSSION

There is a great analogy between the binding properties of the high-density lipoprotein surfaces and the surface of LDL. In all the lipoproteins studied, there are two distinct types of binding sites. The constants characterizing the binding of the probing ions, Mn(II), to all lipoprotein surfaces are practically the same, indicating the same nature of the binding sites. It has been concluded that for LDL, the »strong« binding sites represent isolated negatively charged amino-acid residues.[5] Since the apoproteins are different in different lipoprotein classes, the equality of the binding constants suggests that only the charge and the local environment of the binding sites, individual amino-acids, determine the strength of interaction. It is interesting to note that from about a hundred negatively charged amino-acids in native $HLD_2$ or $HDL_3$, only two of them are available for divalent cation binding. It is about the same percentage as for LDL.[2–5] This observation means that most of the charged amino-acids are normally involved in the »salt bridge« formation, thus stabilizing the apoprotein and hence the entire lipoprotein structure, and are not free to bind extra ions.

The »weak« binding constants are so small that they could hardly represent the binding of Mn(II) to a site with a net negative charge of $q = -e$. As already pointed out,[5] these binding constants could be attributed to the divalent-ion binding to the zwitter-ionic phospholipid head groups. A non-uniform distribution of the positive and negative charges in the fluid phospholipid pool might locally attract cations for binding. This kind of binding need to be in a defined stoichiometric ratio. Rather, the divalent cations might cluster several phospholipid molecules, reducing the number of binding sites to less than the number of the phospholipid molecules on the surface, or to less than a half of that number. Indeed, from the number of various lipid molecules in various lipoprotein classes[11] and the estimated numbers of the binding sites in HDL (this work) and in LDL,[2,5] the ratio of the number of the phopholipid molecules to the number of the »weak« binding sites, $n_2$, is larger than 2: 2.6 for $HDL_3$, 3.4 for $HDL_2$ and 3.8 for LDL. These different ratios for different lipoproteins might partly come from a possibility that not all phospholipid molecules need be located on the lipoprotein surface. Another, probably more important, contribution to these differences is expected from the fact that the phospholipid domains of various lipoprotein classes differ in their free cholesterol content.[11] A higher free cholesterol concentration in LDL than in HDL makes the LDL phopholipid domain more diluted by cholesterol, which makes the LDL surface lipid domain less susceptible for divalent cations.[12] This fact might be responsible for the lower relative number of »weak« binding sites and slightly, but significantly, lower $K_2^i$ value for LDL (17.5 $mol^{-1}L$) than the corresponding value for $HDL_2$ (18.9 $mol^{-1}L$) or $HDL_3$ (19.7 $mol^{-1}L$).

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## SAŽETAK

### Istraživanje površine lipoproteina visoke gustoće EPR spektroskopijom s Mn(II) ionima

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EPR spektroskopija s paramagnetnim ionima Mn(II) upotrijebljena je za proučavanje negativno nabijenih skupina na površini dvaju lipoproteina velike gustoće, $HDL_2$ i $HDL_3$, iz ljudske krvne plazme. Kao i za LDL, na površini HDL postoje dvije vrste veznih mjesta. Konstante vezanja mangana gotovo su jednake za dvije podskupine HDL kao i za LDL. Mali broj »jakih« veznih mjesta ($n_1 = 2$), koja se pripisuju negativno nabijenim aminokiselinama upućuju na činjenicu da je od ukupnog broja negativno nabijenih aminokiselina u lipoproteinu samo malen broj dostupan za vezanje kationa. Konstante vezanja za »slaba« vezna mjesta i broj takvih mjesta na tri uspoređena lipoproteina slažu se s pretpostavkom da se »slaba« mjesta odnose na fosfolipidne domene na površini lipoproteina.