Water Proton Relaxation Rate Enhancements and Association Constants for Mn(II) to Serum Proteins Determined by NMR T_1 Measurements

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The water proton relaxation rate enhancement of Mn(II) bound to bovine serum albumin (BSA) and the association constant for manganese to BSA have already been determined, but such determinations have not been done for human serum albumin (HSA) and other human serum proteins and also for human serum. In this work, NMR T_1 values in aqueous solutions of serum proteins and serum were measured versus increasing concentration of Mn(II). Proton relaxation rate enhancements (ε^*) caused by different manganese concentrations were determined for each solution and $1/\varepsilon^*$ was fitted against concentrations of Mn(II). Proton relaxation rate enhancements (ε_b) of Mn(II) bound to albumin, γ-globulin, (α+β)-globulins and serum were found to be 13.69, 3.09, 8.62, and 10.87, respectively. Free and bound manganese fractions, resulted from each addition of Mn(II) to the sample, were determined by using corresponding (ε^*) and the ε_b values. Association constants for Mn(II) to HSA and γ-globulin were calculated as $1.84 \times 10^4\ \text{M}^{-1}$ and $2.35 \times 10^4\ \text{M}^{-1}$, respectively. Present data suggest that the proton relaxation rate enhancement of Mn(II) in serum is caused by Mn(II) bound to various serum constituents. Data also suggest that association constants for Mn(II) to γ-globulin are nearly the same as that to HSA.

Key words: Water Proton, Enhancement, NMR Relaxation, Association Constant

Introduction

Total material content of body fluids, which consists of several individual materials, influences the spin-lattice relaxation time (T_1) of water protons (Raeymaekers *et al.*, 1988; Herring *et al.*, 1990; Olszewski and Baranowska, 1993; Timmer *et al.*, 1998; Erol *et al.*, 2004). Also an increase or decrease in the concentrations of individual materials, caused by diseases, results in an increase or decrease in the relaxation time (Singer, 1978; Koivula *et al.*, 1982; Schuhmacher *et al.*, 1987, 1990; Yılmaz *et al.*, 1998). In addition, if paramagnetic ions or complexes were added to a body fluid, they can be bound to individual materials which cause an effective decrease in the relaxation time (Kang *et al.*, 1984; Barnhart *et al.*, 1985).

Mn(II) is a suitable probe for NMR investigations of biological macromolecules (Reuben and Cohn, 1970: Jones *et al.*, 1974). Therefore, the influence of Mn(II) on relaxation times of biological fluids has been studied from time to time (Kang *et al.*, 1984; Barnhart *et al.*, 1985; Barnhart and Berk, 1986; Angtuaco *et al.*, 1986; Plowchalk *et al.*, 1987; Ling, 1989: Yılmaz *et al.*, 1999; Pouliquen and Gallois, 2001). These studies were also informative for the development of contrast agents. In these studies, the influence of Mn(II) on blood, plasma, protein solutions containing albumin, γ-globulin and human serum albumin (HSA), dog bile, human placenta, amniotic fluid and hemoglobin solutions were analyzed in terms of Mn(II) concentrations. Such an analysis reflects the effect of Mn(II) only on the observed $1/T_1$ in solution. However, blood, plasma and mixed protein solutions contain various macromolecules, and the relaxation rate of such solutions can be analyzed in terms of concentrations of individual molecules and their efficiency to reduce T_1 (Yılmaz *et al.*, 2004). When Mn(II) is added to a solution containing different molecules, Mn(II) may be bound to several molecules and the changes in the observed $1/T_1$ may be caused by contributions of individual molecules binding Mn(II). Therefore, a study on binding of Mn(II) to main serum proteins and on the relaxation rate enhancement caused by protein-Mn(II) complex should give more details for the influence of Mn(II) on the relaxation time in biological fluids. On the other hand, Mn(II)-based contrast agents introduced to body reach a target tissue by different ways (Robinson *et al.*,

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1995; Aime *et al.*, 2000; Thomsen *et al.*, 2004; Schmidt *et al.*, 2002). When it is intravenously introduced, a Mn(II) complex in blood circulation interacts with individual blood constituents including serum proteins. Therefore, a study on interaction of Mn(II) with serum proteins may be useful for contrast agent studies in MRI. Binding of Mn(II) to bovine serum albumin (BSA) and the association constant for manganese to BSA have already been studied (Mildvan and Cohn, 1963; Sherry *et al.*, 1973), but such studies have not been done for HSA and other serum proteins yet.

In this work, T_1 values in protein solutions, serum and distilled water were measured versus increasing concentrations of Mn(II). The relaxation rate ($1/T_1$) enhancement (ε^*) of Mn(II) was determined for each concentration. By using the enhancements, the relaxation enhancement of Mn(II) bound to proteins (ε_b) and the fractions of bound and free Mn(II) were determined first. Then the association constants for Mn(II) to albumin and γ-globulins were determined.

Materials and Methods

Samples

Serum proteins [albumin, γ-globulins and ($\alpha + \beta$)-globulins] were purchased from Sigma (St. Louis, MO, USA). Aqueous solutions of 5 g/100 ml of albumin and 2.5 g/100ml of each of γ-globulin and (α+β)-globulins were prepared. Serum was collected from healthy volunteers. 2 ml of each solution was transferred into 10 mm O.D.NMR tubes. In addition, a stock solution of Mn(II) was also prepared by dissolving $MnCl_2$ in deionized water. The relaxation measurements were made against stepwise addition of 20 μl stock solution to 2 ml of samples. The concentration of Mn(II) in samples ranged from 0.036 to 0.182 mM.

T_1 measurements

T_1 measurements were carried out on a JEOL FX-60Q FT NMR Spectrometer (JEOL LTD, Tokyo, Japan) operating at 60 MHz for proton. The inversion recovery pulse sequence (180°-τ-90°) was used with delay time τ varying from 0.05 to 5 s. The pulse repetition time was set at 20 s. The magnetization decay curve was found to be a single exponential. Probe temperature was kept at 20 °C ± 0.5 °C, by using a VT-3C automatic temperature controller unit. The experimental error for T_1 measurements was about ± 0.03 s.

Calculation of the enhancements and the association constants

The enhancement (ε^*), caused by each concentration of manganese, was calculated from the following equation given in earlier studies (Mildvan and Cohn, 1963; Sherry *et al.*, 1973):

$$\varepsilon^* \frac{1/T_1^* - 1/T_{10}^*}{1/T_1 - 1/T_{10}}, \tag{1}$$

where $1/T_1$ is the observed relaxation rate in the presence of manganese without proteins and $1/T_{10}$ the observed relaxation rate in the absence of manganese. The terms with asterisk represent the same parameters in the presence of proteins. The ε_b of each protein was determined from extrapolation of the least square fit of $1/\varepsilon^*$ versus total manganese on $1/\varepsilon^*$-axis (Mildvan and Cohn, 1963).

The observed enhancement (ε^*) is a weighted average of that due to free ([Mn_f]) and bound manganese ([Mn_b]) and it can be expressed as follows (Mildvan and Cohn, 1963):

$$\varepsilon^* = \frac{[Mn_f]}{[Mn_t]}\varepsilon_f + \frac{[Mn_b]}{[Mn_t]}\varepsilon_b, \tag{2}$$

where [Mn_t] denotes the concentration of total manganese in mM. ε_f, the enhancement of free manganese, is equal to 1 by definition, and ε_b is the enhancement of manganese bound to protein. The molar fractions of Mn_f and Mn_b were determined from (3) and (4) which were solved from (2) in terms of total manganese and the enhancement parameters (Mildvan and Cohn, 1963):

$$[Mn_f] = \left(\frac{\varepsilon_b - \varepsilon^*}{\varepsilon_b - 1}\right)[Mn_t], \tag{3}$$

$$[Mn_b] = \left(\frac{\varepsilon^* - 1}{\varepsilon_b - 1}\right)[Mn_t]. \tag{4}$$

The association constants for manganese to HSA and γ-globulin (K_a) were determined from the following equation, assuming one to one binding site for each protein (Mildvan and Cohn, 1963):

$$K_a = \frac{[Mn_b]}{[Mn_f][P - Mn_b]}, \tag{5}$$

where [P] denotes the molar concentration of protein, which was 7.3×10^{-4} M for albumin and 1.7×0^{-4} M for γ-globulin.

Results and Discussion

Table I shows the $1/T_1$ in distilled water, the serum proteins and serum versus increasing concen-

Table I. The relaxation rates in the solutions studied versus increasing concentrations of Mn(II). T_1 is given in seconds, whereas the concentration of Mn(II) is given in mM.

[Mn(II)]	$1/T_{1(\text{water})}$	$1/T_{1(\text{albumin})}$	$1/T_{1(\gamma\text{-globulin})}$	$1/T_{1[(\alpha+\beta)\text{-globulins}]}$	$1/T_{1(\text{serum})}$
0	0.36	0.38	0.38	0.39	0.61
0.036	0.68	4.54	1.26	2.87	3.87
0.073	0.90	7.44	1.80	4.10	5.90
0.109	1.25	11.51	2.39	6.07	8.77
0.146	1.50	13.95	3.06	7.69	10.43
0.182	1.79	17.20	3.18	9.54	12.62

trations of Mn(II). As it is seen, the $1/T_1$ for each solution increases with increasing concentrations of Mn(II) added. The data in Table I are consistent with all the previous studies revealing a linear increase of $1/T_1$ in biological fluids versus increasing concentrations of manganese (Kang *et al.*, 1984; Barnhart *et al.*, 1985; Barnhart and Berk, 1986; Angtuaco *et al.*, 1986; Plowchalk *et al.*, 1987; Ling, 1989; Yılmaz *et al.*, 1999).

The ε^* values, obtained from (1) by using the data in Table I, are given in Table II for each protein studied and serum. The fits of the $1/\varepsilon^*$ versus Mn(II) concentration are given in Fig. 1 for the studied solutions except for $(\alpha+\beta)$-globulins. For $(\alpha+\beta)$-globulins, the $1/\varepsilon^*$ versus Mn(II) is shown in Fig. 2. Fig. 1 indicates a linear relationship for each solution. Also the $1/\varepsilon^*$ in Fig. 2 increases linearly with increasing concentration of Mn(II) at lower concentrations, but at 0.11 mM of Mn(II) it appears to saturate all binding sites of $(\alpha+\beta)$-globulins. By extrapolating the lines in Figs. 1 and 2 on the $1/\varepsilon^*$-axis (Mildvan and Cohn, 1963), the ε_b values of albumin, γ-globulin, $(\alpha+\beta)$-globulins and serum can be determined as 13.69, 3.09, 8.62, and 10.87, respectively. The present enhancement determined for Mn(II) bound to proteins in serum is

[Mn(II)]	$\varepsilon^*_{\text{albumin}}$	$\varepsilon^*_{\gamma\text{-globulin}}$	$\varepsilon^*_{(\alpha+\beta)\text{-glubulins}}$	$\varepsilon^*_{\text{serum}}$
0.036	13.00	2.75	7.75	10.19
0.073	13.07	2.63	6.87	9.80
0.109	12.50	2.26	6.38	9.17
0.146	11.90	2.35	6.40	8.61
0.182	11.76	1.96	6.40	8.40

Table II. Proton relaxation rate enhancements (ε^*) calculated from Equation (1) by using the data in Table I.

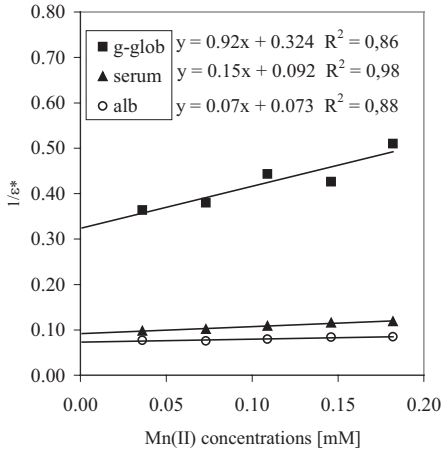

Fig. 1. The inverse of the observed enhancements ($1/\varepsilon^*$) versus total concentrations of Mn(II). The order of formulas is the same as that of symbols.

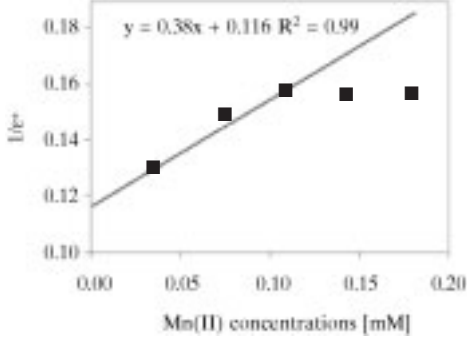

Fig. 2. The inverse of the observed enhancements ($1/\varepsilon^*$) versus total concentrations of Mn(II) for $(\alpha+\beta)$-globulins. The formula corresponds to the fit at lower concentrations.

consistent with an earlier result, being about 11.5 (Kang *et al.*, 1984).

Tables III and IV show the fractions of Mn_f and Mn_b [determined from (3) and (4)], and P_f (P_f = $P-Mn_b$ for one to one binding) and K_a values [calculated from (5)] for HSA and γ-globulin, respectively. K_a values of HSA and γ-globulin were found to be 1.84×10^4 м$^{-1}$ and 2.35×10^4 м$^{-1}$, respectively. The mean K_a value for HSA is consistent with that for BSA (Mildvan and Cohn, 1963; Sherry *et al.*, 1973).

Human serum consists of water (90–92%), proteins (7–8%) and ions (1–2%). The basic serum proteins are albumin, γ-globulin and (α+β)-globulins. The amount of other proteins in serum can be neglected. Using the electrophoretic analysis, the ratios of albumin, γ-globulin and (α+β)-globulins to total protein(TP) in the examined serum were found to be 0.6, 0.2, and 0.19, respectively. The ε_b rate of serum sample can therefore be written as

$$\varepsilon_b = 0.6\varepsilon_{b(\text{albumin})} + 0.2\varepsilon_{b(\gamma\text{-globulin})} + 0.19\varepsilon_{b[(\alpha+\beta)\text{-globulin}]}. \quad (6)$$

If the ε_b value of each protein, calculated from Fig. 1, is introduced into (6), a value of 10.47 can be calculated for the mean ε_b value of serum. It is seen that the mean serum ε_b is in agreement with 10.87 obtained from extrapolation of the serum line on the $1/\varepsilon^*$-axis in Fig. 1.

The stoichiometry of the BSA-Mn(II) complex was studied by NMR T_1 measurements in the early 1960s (Mildvan and Cohn, 1963; Sherry *et al.*, 1973). The studies were based on: (a) titration of manganese with bovine serum albumin, followed by NMR T_1 measurements; (b) titration of bovine serum albumin with manganese, followed by NMR T_1 measurements; (c) the NMR and ESR measurements for a single concentration of metal ion and of protein. Using all three methods described above, K_a values of BSA-Mn(II) complexes are determined and found to be compatible with each other. The K_a value of the γ-globulin-Mn(II) complex, calculated from Table IV, has a close value of that of the HAS-Mn(II) complex. That is, despite different molecular size and shape, the binding preference of Mn(II) to γ-globulin is almost not different than the binding preference of Mn(II) to HSA.

Since (α+β)-globulins contain several globulins with different molecular weights, the K_a value of the (α+β)-globulins-Mn(II) complex can not be determined. Nevertheless, the relaxation increase in Table I and the enhancements in Table II confirm binding of Mn(II) to (α+β)-globulins. The line corresponding to higher concentrations in Fig. 2 can not be attributed to a second class of binding because it has a zero slope.

The present study implies that the relaxation enhancement in serum is the average of the individual enhancements caused by main serum proteins. In this sense, the present data may be more informative for the influence of Mn(II) on the relaxation time in biological fluids containing proteins than in other material. In addition, Mn(II)-based complexes are used as contrast agents for MRI examinations (Robinson *et al.*, 1995; Aime *et al.*, 2000; Thomsen *et al.*, 2004; Schmidt *et al.*, 2002), and therefore studies on the interaction of Mn(II) with proteins are still of scientific interest (Fallis *et al.*, 1998; Aime *et al.*, 2002; Troughton *et al.*,

Table III. Fractions of bound (b) and free (f) manganese calculated from Equations (3) and (4), K_a values calculated from Equation (5) and concentrations of manganese-free protein [$P_f = P-Mn(II)_b$]. The concentration of albumin was 0.73 мм.

	Albumin			
[Mn(II)] [μм]	$[Mn(II)]_f$ [μм]	$[Mn(II)]_b$ [μм]	$P_f \cdot 10^{-4}$ [м]	K_a (м$^{-1}$) $\cdot 10^{-4}$
36	1.9	34.1	6.96	2.58
73	3.5	69.5	6.61	3.00
109	10	99.0	6.31	1.57
146	20	126	6.04	1.04
182	27	155	5.75	1.00
	Average $K_a = (1.84 \pm 0.91) \cdot 10^4$ м$^{-1}$			

Table IV. Fractions of bound (b) and free (f) manganese calculated from Equations (3) and (4), K_a values calculated from Equation (5) and concentrations of manganese-free protein [$P_f = P-Mn(II)_b$]. The concentration of γ-globulin (P) was 0.17 мм.

	γ-Globulin			
[Mn(II)] [μм]	$[Mn(II)]_f$ [μм]	$[Mn(II)]_b$ [μм]	$P_f \cdot 10^{-4}$ [м]	K_a (м$^{-1}$) $\cdot 10^{-4}$
36	5.9	30.1	1.40	3.64
73	16	57	1.13	3.15
109	43	66	1.04	1.48
146	51	95	0.75	2.48
182	98	84	0.86	1.00
	Average $K_a = (2.35 \pm 1.11) \cdot 10^4$ м$^{-1}$			

2004). Since intravenously injected contrast agents interact with individual materials in blood, the enhancements in Table II and the association constants for Mn(II) to serum proteins may also contribute to the studies on the development of intravenous contrast agents. Furthermore, NMR titration studies of proteins showed that the spin-lattice relaxation rate of a protein could be expressed in terms of a fast exchange between three water components, so called bound, structured and bulk water (Fullerton, 1988). Physicochemical properties of structured water in human albumin and γ-globulin solutions were studied in the presence of increasing concentrations of manganese (Pouliquen and Gallois, 2001). The present data imply that molecular dynamics of structured water in (α+β)-globulins and serum may also be studied in the presence of Mn(II).

In conclusion, the present data suggest that the proton relaxation rate enhancement of Mn(II) in serum is caused by Mn(II) bound to serum constituents. The data also suggest that the association constant for Mn(II) to γ-globulin is nearly the same as that to HSA.

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