ESR studies on the effect of ionic radii on displacement of Mn²⁺ bound to a soluble β-galactoside binding hepatic lectin

Rajeev Kayestha, Sumati, Krishnan Hajela*

School of Life Sciences, Devi Ahilya Vishwavidyalaya, Vigyan Bhawan, Khandwa Road Campus, Indore, 452 001 India

Abstract Binding of divalent metal ions to hepatic soluble β-galactoside binding lectin was studied using electron spin resonance (ESR) spectroscopy. The Mn^{2+} bound to hepatic lectin could be displaced by Mg^{2+}, Cu^{2+}, Ni^{2+} and Ca^{2+} but not by Sr^{2+}. As the ionic radii of Mg^{2+} (0.65 Å), Cu^{2+} (0.73 Å) and Ni^{2+} (0.72 Å) are appreciably smaller than Ca^{2+} (0.99 Å), it appears that the Mn^{2+} binding site is more accessible to Mg^{2+}, Cu^{2+}, and Ni^{2+} as compared to Ca^{2+}, the ionic radius of Mn^{2+} being 0.80 Å. Sr^{2+} with an ionic radius of 1.13 is thus unable to displace bound Mn^{2+}. Surprisingly, the presence of specific sugars like α-lactose, or α-D-galactose facilitated the displacement of bound Mn^{2+} by metal ions whereas non-specific sugars, i.e. α-D-glucose, β-D-fructose and α-D-ribose had no effect. It appears that minor perturbations in the saccharide binding site significantly affect the ability of the metal binding site to ligate bivalent metals.

Key words: β-Galactoside binding lectin; Metal binding; EPR

1. Introduction

Lectins are generally considered to be non-enzymatic and non-immune proteins which selectively bind to a specific carbohydrate structure [1]. The animal lectins are broadly classified into two major categories, the C type (Ca^{2+}-dependent) animal lectins which are structurally related to the asialoglycoprotein receptor while the S type (thiol-dependent) animal lectins form a distinct group which are generally β-galactoside binding [2]. Embryonic and differentiating tissues of various vertebrates from teleosts [3,4], to amphibians [5], birds [6,7] and mammals [8–10], contain a β-D-galactoside binding lectin that agglutinates trypsinized rabbit erythrocytes and is specifically inhibited by β-D-galactopyranosyl β-D-thiogalactopyranoside and lactose. The major β-galactoside binding lectin occurring in vertebrate tissues is a dimer with a subunit mass of about 14 kDa and requires thiol but not divalent metal ions for its haemagglutination activity, and exhibits considerable sequence homology among various species [11].

Besides the 14 kDa lectin, other β-galactoside binding lectins have been found in mammalian tissues which include sheep, goat and buffalo liver having molecular weights of 18, 22 and 24 kDa, respectively [12]. Of these three β-galactoside binding hepatic lectins, only sheep lectin required 2-mercaptoethanol for its haemagglutination activity; in addition it requires Ca^{2+}. In contrast to major soluble β-galactoside specific lectins, goat and buffalo soluble hepatic lectins show haemagglutination activity against trypsinized rabbit red blood cells only in the presence of Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} or Sr^{2+} [12] thus differing

significantly in cofactor requirement from the known major mammalian β-galactoside specific lectins [13,14]. We thus find it important to confirm the presence of a metal binding site of these lectins. This paper describes to our knowledge for the first time the ESR characterization of binding of the metal ions to a soluble β-galactoside specific lectin from goat liver. The importance of this study lies in the fact that the majority of the known soluble β-galactoside specific lectins are independent of metal ion requirement.

The ESR spectrum of Mn^{2+} is highly responsive to changes in coordination symmetry [15]. We have thus used ESR spectroscopy to investigate changes in the transition metal site of soluble β-galactoside specific goat hepatic lectin on binding to various metal ions in the presence or absence of specific and non-specific sugars. The ESR spectrum was first used for the study of low molecular weight Mn^{2+} complexes of biochemical interest. The method was then used for the investigation of an interaction of Mn^{2+} with proteins [15–19]. In most of these cases, the phenomenon of the absence of any observable ESR signal of the bound form of Mn^{2+} to protein was used for the determination of the dissociation constant of the binding site and their number. The observed ESR signal served as a measure of the equilibrium concentration of free Mn^{2+} ions [15–19].

2. Materials and methods

2.1. Reagents

Neuraminidase, sugar substrates, phenylmethyl-sulfonyl fluoride (PMSF) and fetuin-agarose were purchased from Sigma Chemical Co., MO, USA. The glass capillaries used were from TOP Syringe Manufacturing Co., Bombay, India. All the other reagents were of analytical grade.

2.2 Isolation of soluble hepatic lectin

The soluble goat hepatic lectin was isolated essentially as described by Ali and Salahuddin [12] with some modifications. Goat liver (25 g) was homogenised in 10 mM sodium phosphate buffer pH 7.5, containing 2 mM PMSF. The debris was removed by centrifugation and the clear supernatant thus obtained was incubated with DEAE-cellulose gel equilibrated in the same buffer. After washing unbound protein, the bound protein was eluted with 0.5 M NaCl. This was then applied on an asialofetuin-agarose column (1.5 × 10 cm) equilibrated in 10 mM Tris HCl buffer (pH 7.5) containing 150 mM NaCl, and 5 mM $CaCl_2$. The bound protein was specifically eluted with 0.5 M lactose. The lectin was then rechromatographed on a fresh asialofetuin-agarose column. The eluted lectin was first dialysed against 10 mM EDTA in 0.01 M Tris HCl buffer pH 7.5 to remove bound metal ions and then extensively dialysed against 0.01 M Tris HCl buffer pH 6.8 to remove EDTA. The lectin thus obtained was used throughout the study. The lectin exhibited a single band on SDS-PAGE thus indicating its purity. To prepare asialofetuin-agarose column, fetuin-agarose was treated with neuraminidase as described by Ali and Salahuddin [12].

2.3. ESR spectroscopy

In an experiment, 100 μl of soluble goat hepatic lectin (0.5 mg/ml as determined spectrophotometrically taking absorbance at 280 nm) and

---

*Corresponding author.

$MnCl_2$ (final concentration 0.25 mM) in 0.01 M Tris HCl buffer pH 6.8 (50 μl) and 50 μl of the same buffer were mixed and ESR spectra recorded on a Varian ESR spectrometer model E 104A at 100 kHz field modulation. Instrument parameters were as follows: field 3237 gauss, scan range 1000 gauss, modulation amplitude 2 gauss, receiver gain 2.5×10^3, time constant 0.5 s, scan time 2 min, microwave power 10 mW and frequency 9.01 GHz. The peak-to-peak amplitude of the fourth peak from the low field side of the spectrum was measured for Mn^{2+} concentration [15]. Nanomoles of Mn^{2+} can be detected easily without the distortion of peaks. The peak amplitude shows a linear increase with increase in concentration of Mn^{2+} (calibration curve not shown). All measurements were made at room temperature. For displacement studies, either $CuSO_4$, $MgCl_2$, $NiCl_2$ (final concentration 0.3 mM to 0.8 mM) or $CaCl_2$ (final concentration 25 mM to 60 mM) and $SrCl_2$ (final concentration 100 mM) were also added. To study the effect of inhibitory and non-inhibitory sugars, α-lactose, α-D-galactose, α-D-glucose, β-D-fructose and α-D-ribose (45 mM final concentration) were also added, adjusting the volume accordingly. The amount of sugars added was 45 fold higher than that required to produce 50% reduction in haemagglutinating activity.

3. Results and discussion

The spectra of Mn^{2+} ion bound to the goat hepatic soluble β-galactoside specific lectin in aqueous solution are presented in Fig. 1, a–p. Spectra were recorded as described above. Taking into consideration the broadening of the lines, the signal intensity of the same molar concentration of Mn^{2+} in hepatic lectin was grossly lower (almost flat spectra) than that of Mn^{2+} in aqueous solution. It has been suggested that the disappearance of the hyperfine structure of the complex indicates the formation of a covalent bond [15]. It could also be a function of the tumbling rate and the size of anisotropy in the resonance and this would provide a case for 'immobilization' of Mn^{2+} by the protein moiety [16]. This immobilization would be analogous to those observed in spectra of organic free radicals bound to proteins in solutions [15]. These spectra approach the spectra observed in polycrystalline and powder samples because of the incomplete averaging of anisotropic interactions resulting from a slow molecular tumbling of protein bound ions. In some cases this 'immobilization' is the dominant effect of the macromolecules in the changes of the Mn^{2+} EPR spectra [15].

Addition of different metal ions (each added independently in increasing concentrations) resulted in an increase in the intensity of the resolved spectra as well as narrowing of the components due to release of Mn^{2+} from the metal binding site (Fig. 1, a–p). The amount of Mn^{2+} displaced from the metal binding site was found to be different for different metal ions (Fig. 2). Addition of Mg^{2+} (0.03 mM to 0.875 mM final concentration) released 69% of the bound Mn^{2+}, i.e. 0.172 mM out of 0.25 mM added. Addition of Ni^{2+} (0.03 mM to 1.2 mM final concentration) released 84% of the bound Mn^{2+}. Addition of Cu^{2+} (.03 mM to 1.25 mM final concentration) released 96% of the bound Mn^{2+}. In all these cases total amount of bound Mn^{2+} was not displaced even if higher concentrations of metal ions were used. On the contrary Ca^{2+} release 100% of the bound Mn^{2+} on addition of 25 to 50 mM of $CaCl_2$. However, the final concentration of Ca^{2+} added (i.e. 50 mM) to displace 100% of the bound Mn^{2+} is 40 fold higher than that of Cu^{2+} (1.25 mM) and about 57–61 fold higher than the final concentration of Mg^{2+} (0.875 mM) and Ni^{2+} (0.813 mM) used respectively, for displacement of bound Mn^{2+}.

This differential release of Mn^{2+} by various metal ions used could be directly correlated with the ionic radii of these ions.
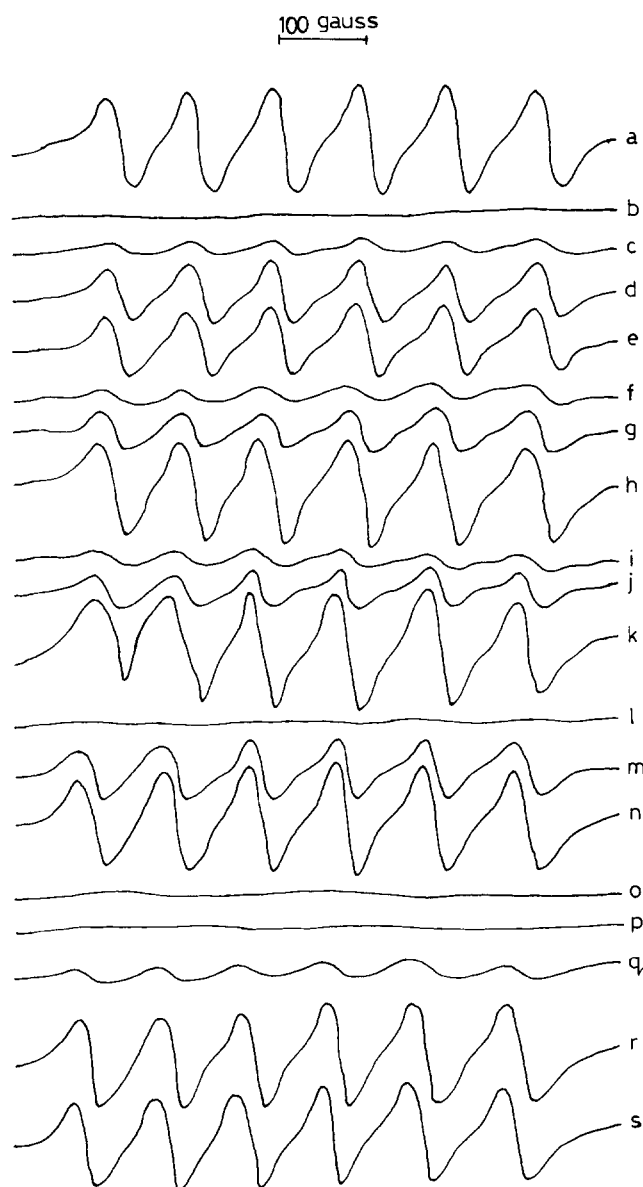

100 gauss

Fig. 1. Electron spin resonance (ESR) spectra of Mn^{2+} bound to soluble β-galactoside binding goat hepatic lectin. Instrument parameters are as described in section 2. $MnCl_2$ (0.25 mM final conc.) was added to soluble β-galactoside binding goat hepatic lectin (0.5 mg/ml final conc. as determined spectrophotometrically). Increasing concentration of various metal ions were then added to displace bound Mn^{2+}. (a) Free $MnCl_2$, 0.25 mM; (b) $MnCl_2$ bound to hepatic lectin. Displacement by addition of (c) 0.063 mM $MgCl_2$ (d) 0.25 mM $MgCl_2$ (e) 0.875 mM $MgCl_2$ (f) 0.063 mM $NiCl_2$ (g) 0.25 mM $NiCl_2$ (h) 0.813 mM $NiCl_2$ (i) 0.063 mM $CuSo_4$ (j) 0.25 mM $CuSO_4$ (k) 0.813 mM $CuSo_4$ (l) 25 mM $CaCl_2$ (m) 40 mM $CaCl_2$ (n) 50 mM $CaCl_2$ (o) 25 mM $SrCl_2$ (p) 100 mM $SrCl_2$ (q) 0.028 mM $MgCl_2$ in presence of 45 mM α-lactose (r) 0.227 mM $MgCl_2$ in presence of 45 mM α-lactose (s) 0.875 mM $MgCl_2$ in presence of 45 mM α-lactose. Spectra in presence of other varying concentrations of metal ions or sugars are not shown due to lack of space.

The ionic radius of Mn^{2+} is 0.80 Å [20], Mg^{2+}, Ni^{2+} and Cu^{2+} with ionic radii of 0.65 Å, 0.72 Å and 0.73 Å [21] displace 67%, 84% and 96% of bound Mn^{2+}, respectively. Ca^{2+} with an ionic radius of 0.99 Å which is larger than that of Mn^{2+} displaces 100% of bound Mn^{2+}. However, as 40 to 60 fold higher concentrations of Ca^{2+} are required as compared to Mg^{2+}, Ni^{2+} and

Cu^{2+}, it appears that the binding site is more accessible to these ions as compared to Ca^{2+}. This possibility is confirmed by the fact that Sr^{2+} with a ionic radius of 1.13 Å is totally unable to displace the bound Mn^{2+}, even when added to 100 mM final concentration. Although Mg^{2+}, Ni^{2+}, Cu^{2+} and Ca^{2+} were able to displace Mn^{2+}, it is clear that the binding site is more specific for Mn^{2+} in comparison to these ions as 3.5, 3.25, 5 and 200 fold higher concentrations than Mn^{2+} were required, respectively, to displace bound Mn^{2+} which was added in a final concentration of 0.25 mM. This is also confirmed by the fact that addition of displacing metals (Mg^{2+}, Ni^{2+}, Cu^{2+} or Ca^{2+}) prior to the addition of $MnCl_2$ to hepatic lectin or after the addition of $MnCl_2$ to hepatic lectin does not make any difference in their ability to displace Mn^{2+} (data not shown).

As the earlier report suggested that presence of divalent metal ions was necessary for the lectin to exhibit haemagglutination activity [12], we studied the effect of the presence of inhibitory and non-inhibitory sugars on the displacement of bound Mn^{2+} from the hepatic lectin. The spectra for the displacement of Mn^{2+} by Mg^{2+} in the presence of α-lactose is given in Fig. 1, q–s. The ESR spectra for displacement of Mn^{2+} by Mg^{2+} in presence of α-D-galactose, α-D-glucose, β-D-fructose and α-D-ribose are not shown. The amount of Mn^{2+} displaced by Mg^{2+} in the presence of various sugars is depicted in Fig. 3. Surprisingly it was found that presence of a inhibitory sugar α-lactose or α-D-galactose (45 mM final concentration) facilitated the release of bound Mn^{2+}. In the absence of sugars, addition of Mg^{2+} (0.028 mM to 0.875 mM final concentration) displaced about 69% of the bound Mn^{2+} (i.e. 0.153 mM out of 0.22 mM bound). Presence of non-inhibitory sugars like α-D-glucose, β-D-fructose or α-D-ribose made no difference and approximately 69% of the bound Mn^{2+} was released in their presence also. However, presence of α-D-galactose resulted in the displacement of 86% of bound Mn^{2+} and presence of α-lactose resulted in displacement of 91% of the bound Mn^{2+} by Mg^{2+}. The experiments on the displacement of Mn^{2+} by Mg^{2+} in the presence of non-inhibitory and inhibitory sugars were repeated three times and the difference was statistically signifi-
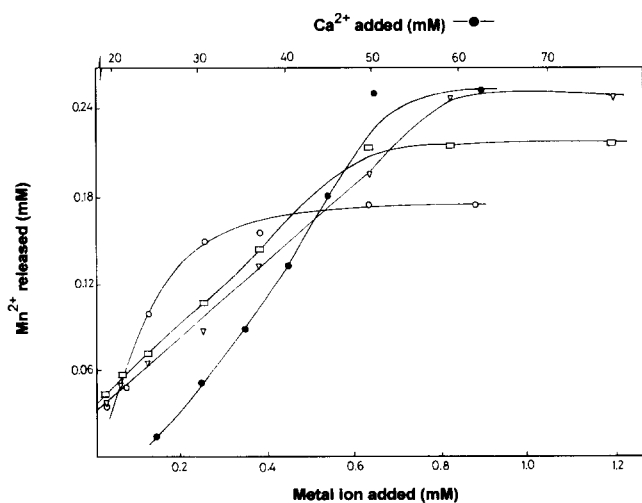
Fig. 3. Release of Mn^{2+} bound to soluble β-galactosidebinding goat hepatic lectin on addition of subsequent concentrations of $MgCl_2$ (●) in the presence of α-D-ribose (○), β-D-fructose (□), α-D-glucose (X), α-D-galactose (▽) and α-lactose (▲).

cant ($P < 0.001$). Thus the soluble goat hepatic β-galactoside lectin was found to be different from goat peripheral blood lymphocyte lectin where presence of inhibitory sugar made no difference in the release of bound Mn^{2+} [22]. The data also suggest that the saccharide binding site is likely to be in close proximity to the metal binding site in the soluble goat hepatic β-galactoside lectin. It is concluded that minor perturbations in the saccharide binding site significantly affect the ability of metal binding site to ligate bivalent metals.

Fig. 2. Release of Mn^{2+} bound to soluble β galactosidebinding goat hepatic lectin on addition of subsequent concentrations of $MgCl_2$ (○), $NiCl_2$ (□), $CuSo_4$ (▽) and $CaCl_2$ (●). The peak to peak amplitude of the fourth peak from the low field side of the spectra shown in Fig. 1 were measured for determining Mn^{2+} concentration.
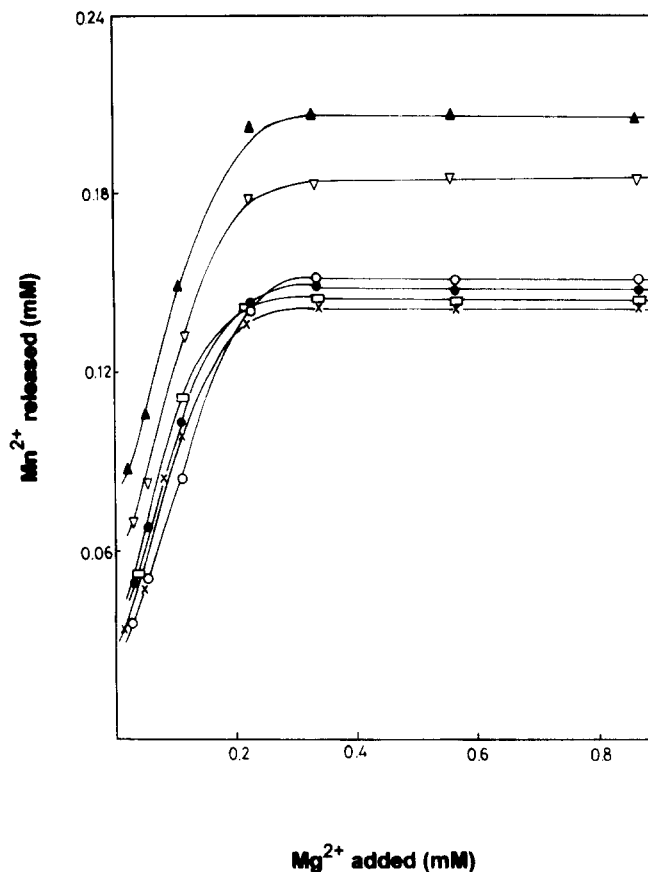
References

[1] Sharon, N. and Lis H. (1972) Science 177, 949–959.
[2] Drickamer, K. (1988) J. Biol Chem. 263, 9557–9660.
[3] Teichberg, V.I., Silman, I., Beitsch, D.D. and Resheff, G. (1975) Proc. Natl. Acad. Sci. USA 72, 1382–1387.
[4] Teichberg, V.I. (1987) Methods Enzymol. 50, 291–302.
[5] Bols, N.C., Roberson, M.M., Haywood Reid, P.L., Cerra, R.F. and Barondes, S.H. (1986) J. Cell Biol. 102, 492–499.
[6] Kobiler, D., Beyer, E.C. and Barondes, S.H. (1978) Dev. Biol. 64, 265–272.
[7] Levi, G. and Teichberg, V.I. (1985) Biochem. J. 226, 379–384.
[8] Powell, J.T. (1980) Biochem. J. 187, 123–129.
[9] Childs, R.A. and Feizi, T. (1979) Biochem. J. 183, 755–758.

[10] Hirabayashi, J. and Kasai, K.I. (1984) Biochem. Biophys. Res. Commun. 122, 938–944.

[11] Paroutand, P., Levi, G., Teichberg, V.I. and Strosberg, A.D. (1987) Proc. Natl. Acad. Sci. USA 84, 6345–6348.

[12] Ali, N. and Salahuddin, A. (1989) Biochim. Biophys. Acta 992, 30–34.

[13] Couraud, P., Borocz, D.C., Bringman, T.S., Griffeth, J.,Grogan, M. and Nedwin, G.E. (1989) J. Biol. Chem. 264, 1310–1316.

[14] Wilson, T.J.G., Firth, M.N., Powell, J.T. and Harrison, F.L. (1989) Biochem. J. 261, 847–852.

[15] Tichy, A, Tichy, A and Kocourer, J. (1971) Biochim. Biophys. Acta 229, 63–67.

[16] Reed, G.H. and Cohn, M. (1970) J. Biol. Chem. 245, 662–667.

[17] Mildvan, A.S. and Cohn, M. (1965) J. Biol. Chem. 240, 238–246.

[18] Miller, R.S., Mildvan, A.S., Chang, H.C., Easterday, R.L., Maruyama, H. and Lane, M.D. (1968) J. Biol. Chem. 243, 6030–6040.

[19] Nicolau, C.L., Kalb, A.J. and Yariv, J. (1969) Biochim. Biophys. Acta 194, 71–73.

[20] Wells, A.F. (1975) Bonds in molecular and crystals in structural inorganic chemistry 4th edition, pp. 230–285, Clarendon Press, Oxford.

[21] Parker, S.P. (ed.) (1992) Encyclopedia of chemistry, McGraw Hill, pp. 687 and 1055.

[22] Kayestha, R., Hajela, K. and Bharti, S. (1994) Mol. Immunol. 32, 97–100.