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A Magnetic Resonance Study of Concanavalin A. Identification of a Lanthanide Binding Site†

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ABSTRACT: The solvent proton relaxation enhancement technique has been utilized to demonstrate that the plant lectin concanavalin A (Con A) exhibits a single tight binding site ($K_D \cong 15\ \mu M$) per 27,000 molecular weight subunit for the paramagnetic lanthanide Gd^{3+}. It was also found that Gd^{3+} could not be competitively displaced from its binding site on demetallized Con A by the addition of Zn^{2+} to occupy the transition metal ion site S1, nor by the subsequent addition of excess Ca^{2+} to occupy site S2. When both Mn^{2+} and Gd^{3+} were added to demetallized Con A, a combined solvent relaxation enhancement effect was observed. The individual contributions of each ion to the combined enhancement were extracted from the data and were found to be identical with their contributions in the absence of the other ion. The Ca^{2+} effect on the relaxation enhancement properties of the Con A bound Mn^{2+} was shown to be completely intact in the presence of Gd^{3+}. Thus, Gd^{3+} binds to a separate site on Con A, distinct from the transition metal ion site (S1) and the Ca^{2+} binding site (S2). We propose to designate this Gd^{3+} binding site as S3. Gd^{3+} binding to site S3 was found to be effectively competed for by the lanthanides Sm^{3+}, Eu^{3+}, and Tb^{3+} as well as Pb^{2+}. On the basis of the crystallographic locations previously determined for the Pb^{2+} binding sites and also the Sm^{3+} binding sites on Con A, a tentative proposal is advanced for the location of the single tight Gd^{3+} binding site observed in solution. The location of the Gd^{3+} binding site on the Con A monomer provides a second paramagnetic metal ion reference point on the protein structure which can be utilized in high resolution nuclear magnetic resonance studies of this lectin in solution.

In view of the numerous recent applications of the plant lectin Con A[1] as a probe of cell surface structure and dynamics (Nicolson, 1971, 1973; Inbar and Sachs, 1973; Edelman et al., 1973), there is now considerable interest in the detailed structural features of the protein and exactly how these relate to its interaction with a cell surface receptor (Gunther et al., 1973; Edelman et al., 1973; Cuatrecasas, 1973).

It has been established that Con A is a metalloprotein (Agrawal and Goldstein, 1968; Kalb and Levitzki, 1968; Shoham et al., 1973) requiring the prior occupation of two metal ion binding sites in order to exhibit specific monosaccharide binding activity (Kalb and Levitzki, 1968). The "site induction" model for the metal ion requirements of Con A was first advanced by Kalb and Levitzki (1968). They proposed that each Con A monomer has a site (labeled S1) able to bind a variety of transition metal ions (Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+}) which must be occupied before the second metal ion site (S2), specific for Ca^{2+}, is available. It was also found that both sites S1 and S2 must be occupied in order for Con A to bind methyl α-D-glucoside. A structural rationale for the induction of site S2 by the occupation of S1 is offered by the recent X-ray crystal structure of Con A (Becker et al., 1975; Hardman and Ainsworth, 1972), in which the Mn^{2+} (site S1) and Ca^{2+} are found to be approximately 5 Å apart and sharing two aspartate residue ligands (Asp-10 and -19). Recent results obtained in this laboratory (Barber and Carver, 1973, 1975) indicate that nuclear magnetic resonance (NMR) techniques can be usefully applied to characterize such ligand-induced (i.e., Ca^{2+} and methyl α-mannoside) structural perturbations for Con A in solution.

In this paper we wish to report a third metal ion binding site per Con A monomer, most highly specific for Gd^{3+}, but which also binds the lanthanides Tb^{3+}, Eu^{3+}, and Sm^{3+} less tightly. We propose to refer to this site as site S3 in keeping with the metal ion site nomenclature of Kalb and Levitzki

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[1] Abbreviations used are: Con A, concanavalin A; PRE, solvent proton relaxation enhancement.

(1968). Specific competition experiments employing the PRE properties of the Con A-Gd^{3+} complex, combined with information regarding heavy metal derivatives of Con A (Quiocho et al., 1971; Edelman et al., 1972; Becker et al., 1975), allow us to limit the possible locations of the Gd^{3+} binding to two sites. Our data clearly indicate that site S3 is structurally distinct from sites S1 and S2, in contrast to the previously reported conclusions of Sherry and Cottam (1973) on lanthanide binding to Con A but in agreement with the more recent work of Sherry (Sherry et al., 1975). This additional defined paramagnetic ion site per Con A monomer has proved to be a valuable reference point in the high resolution NMR studies of Con A in solution (B. J. Fuhr, B. H. Barber and J. P. Carver, manuscript submitted for publication). As well, the fact that Mn^{2+} and Gd^{3+} occupy independent sites on the protein enables the simultaneous observations of a solvent relaxation enhancement from each of the paramagnetic ions. The resolution of this mixed enhancement experiment into the individual enhancement parameters is dealt with in this study and applied to an investigation of possible S1–S3 site interaction.

Experimental Section

Materials. The Con A used in this study was obtained from Pharmacia (Batch No 3059 and 4000) or was the generous gift of Dr. Dean Sherry. The protein was demetallized by a modification of the acid dialysis procedure of Kalb and Levitzki (Kalb and Levitzki, 1968). Details of the procedure are discussed elsewhere (Barber and Carver, 1975). The efficiency of the demetallization procedure was monitored by atomic absorption analysis for Mn^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+} and in all cases the levels of contamination were found to be less than 0.1 mol/mol of Con A monomer. Prior to the demetallization procedure the Con A was enriched in "fragment-free" molecules by the 1% NH_4HCO_3 incubation described by Cunningham et al. (1972). The buffers used in this study, 0.05 *M* sodium acetate and 0.2 *M* NaCl, were demetallized by passage through a column of Chelex 100 (Bio-Rad Laboratories) prior to the final pH adjustment. Samples were prepared by lyophilization of the stock solution and addition of the appropriate buffer. Con A concentrations were determined spectrophotometrically using the $E_{1cm}(1\%)$ of 12.4 (Yariv et al., 1968). Monomer molar concentrations were determined on the basis of a subunit molecular weight of 27,000 (Wang et al., 1971). Zn^{2+} was supplied as $ZnCl_2$ (Fisher Certified), Ca^{2+} as $CaCl_2$ (BDH Analar), and Pb^{2+} as $PbCl_2$ (Fisher Certified), from stock solutions prepared by weight in glass distilled H_2O. The lanthanides $GdCl_3$, $SmCl_3$, $TbCl_3$, and $EuCl_3$ were obtained from Alpha Inorganics and used as stock solutions prepared by weight in glass-distilled H_2O or prepared from their oxides by addition of HCl. Concentrations of the Gd^{3+} stock solutions were monitored by measuring the water proton T_1. All experiments were performed in buffer solutions between pH 5.0 and pH 5.6 under conditions for which Con A is known to exist as a dimeric species of approximately 55,000 molecular weight (McKenzie and Sawyer, 1973) and well below the precipitation point for lanthanide hydroxides (Reuben, 1971a).

NMR Measurements. The spin-lattice relaxation time, T_1, values were determined using a semilogarithmic plot of signal amplitude following a 180°-τ-90° pulse sequence as a function of the pulse spacing. All relaxation times were measured on a Bruker variable frequency (4-62, 90 MHz) pulsed spectrometer (B-KR322s) operating at the ambient probe temperature of 24 ± 1°. Samples were typically of 50–150 μl in volume and were examined in acid-washed glass tubes (Wilmad 513B-IPP).

Electron Spin Resonance (ESR) Measurements. The ESR spectra reported were obtained on a Varian E-6 spectrometer operating at 9.12 GHz and a room temperature of 24°. Samples were contained in 50-μl Corning disposable microsampling pipets and positioned in the microwave cavity in a reproducible fashion.

Theoretical Treatment

Interpretation of a Mixed PRE Experiment. Not all paramagnetic ions can be usefully applied as solvent relaxation enhancement probes. In fact, the requirement for a significant change in the dominant correlation time upon binding of the ion to a macromolecule restricts the list to the few with sufficiently long τ_s values so as to make τ_R the dominant contribution in the aquo ion. Dwek (1972) has discussed the relative merits of various metal ions in this application and Mn^{2+} and Gd^{3+} clearly rank first and second in their suitability as PRE probes. The relaxation times measured in a solvent relaxation enhancement experiment can be used to define the enhancement parameter, ϵ^*_1, as represented by the expression

$$\epsilon^*_1 = \frac{\left|\frac{1}{T_1}\right|^*_{\text{metal ion}} - \left|\frac{1}{T_1}\right|^*_0}{\left|\frac{1}{T_1}\right|_{\text{metal ion}} - \left|\frac{1}{T_1}\right|_0} \quad (1)$$

where the * indicates the presence of the macromolecule. The utility of expressing the T_1 values in terms of the enhancement parameter ϵ^*_1 lies in the possibility of relating ϵ^*_1 to ϵ_{b1}, a value which is characteristic of the structural and dynamic features of the binary paramagnetic ion macromolecular complex (Dwek, 1972). ϵ^*_1 can be expressed as

$$\epsilon^*_1 = X_b\epsilon_{b1} + (1 - X_b) \quad (2)$$

in which X_b represents the fraction of the total paramagnetic ion in solution which is bound to the macromolecule.

If Con A had two nonidentical binding sites for Mn^{2+} and Gd^{3+} per monomer, it would offer the possibility of detecting a combined proton relaxation enhancement from the two paramagnetic ions simultaneously. The resolution of the results from this type of experiment, into individual ϵ_{b1} values for the two ions, enables a unique approach to the study of the interaction between metal ion binding sites on this protein. For a PRE experiment with a single paramagnetic species present, the spin–lattice relaxation time enhancement parameter, ϵ^*_1, is defined by the expression in eq 1. If we extend this expression, by analogy, to include the observed relaxation times in the simultaneous presence of two paramagnetic ions (e.g., Mn^{2+} and Gd^{3+}), we can define the calculated enhancement parameter, ϵ^{**}_1, in this situation as

$$\epsilon^{**}_1 = \frac{\left|\frac{1}{T_1}\right|^*_{Mn^{2+},Gd^{3+}} - \left|\frac{1}{T_1}\right|^*_0}{\left|\frac{1}{T_1}\right|_{Mn^{2+},Gd^{3+}} - \left|\frac{1}{T_1}\right|_0} \quad (3)$$

"*" refers to the presence of the metal binding macromolecule. The paramagnetic ions are present as indicated. Making the assumption of rapid chemical exchange between bound site H_2O molecules and the bulk solvent, as well as

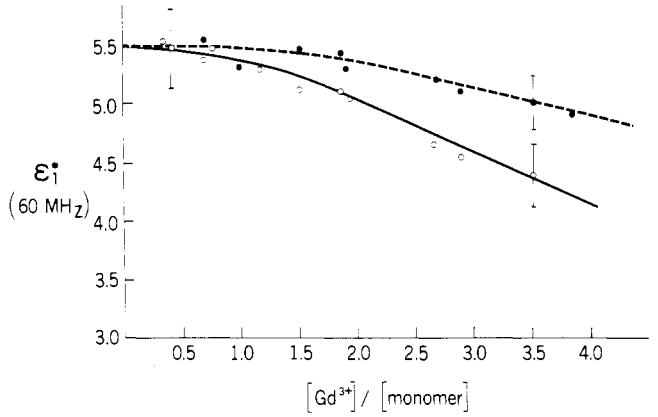

FIGURE 1: A plot of the change in the enhancement parameter ϵ^*_1 at 60 MHz for the titration of demetallized Con A and the Con A-Zn^{2+}-Ca^{2+} complex with Gd^{3+}. The values for demetallized Con A are represented by ●, and those for Con A-Zn^{2+}-Ca^{2+} by ○ ([Zn^{2+}] = 0.98 mM and [Ca^{2+}] = 6.8 mM). Con A monomer concentrations were determined spectrophotometrically as described in the text and ranged from 0.605 to 0.505 mM over the titration.

assuming the mole fraction of total solvent in the hydration sphere of the paramagnetic ions is small, enables one to rewrite ϵ^{**}_1 as

$$\epsilon^{**}_1 = [X_b^{Mn}\epsilon_{b1}^{Mn} + (1 - X_b^{Mn})]\phi_{Mn} + [X_b^{Gd}\epsilon_{b1}^{Gd} + (1 - X_b^{Gd})]\phi_{Gd} \quad (4)$$

where ϕ_{Mn} and ϕ_{Gd} are the multiplication factors required to interpret the relaxation times in terms of the ϵ_{b1} parameters in a mixed PRE system. Implicit in the derivation is the fact that the $\Sigma_i \phi_i = 1$ for the paramagnetic species, i, present in the PRE experiment. By expressing in detail the terms contributing to each relaxation time in eq 3, and rearranging the expression into the form of eq 4, it can be shown that

$$\phi_{Mn} = \frac{1}{1 + \dfrac{T_{1M_fMn}[Gd^{3+}]_T 9}{T_{1M_fGd}[Mn^{2+}]_T 6}} \quad (5)$$

$$\phi_{Gd} = \frac{1}{1 + \dfrac{T_{1M_fGd}[Mn^{2+}]_T 6}{T_{1M_fMn}[Gd^{3+}]_T 9}} \quad (6)$$

In these expressions, T_{1M_f} values refer to the spin-lattice relaxation times for the H_2O protons in the first hydration sphere of the respective aquo ions, $[Mn^{2+}]_T$ and $[Gd^{3+}]_T$ indicate the total concentration of the respective ions in solution, and the free aquo ion hydration numbers of 6 and 9 have been assumed for Mn^{2+} and Gd^{3+}, respectively (Dwek, 1972; Reuben, 1971a). The ϕ values require the knowledge of T_{1M_fMn} and T_{1M_fGd} as well as the relative concentrations of the paramagnetic ions, and an estimate of their hydration numbers in the free aquo ion. If these factors are known, or can be determined, the mixed PRE data can be reduced to an expression involving individual ϵ_{b1} parameters which can be compared to similar values measured in the absence of other PRE metal ions. This then allows a direct measure of possible metal ion site interactions between sites to which a PRE ion can bind and exhibit an enhancement. Such a treatment of the mixed PRE system for Mn^{2+} and Gd^{3+} has been undertaken for Con A and the results will be presented at a later point in this paper.

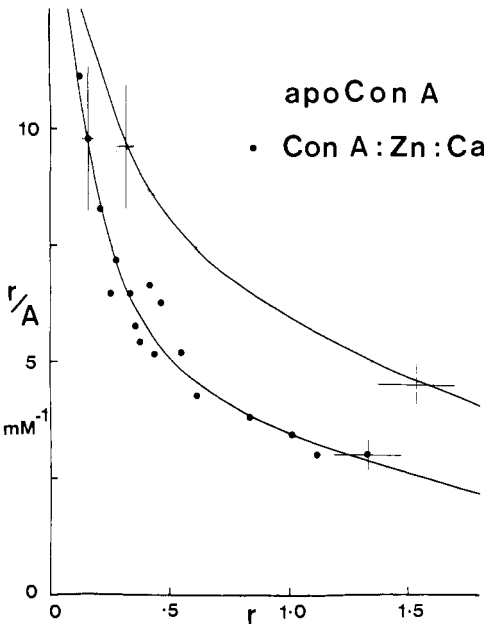

FIGURE 2: A Scatchard plot of the binding of Gd^{3+} to demetallized Con A and to Con A in the presence of excess Zn^{2+} and Ca^{2+} at pH 5.6. The points of demetallized Con A (○) and the Con A-Zn^{2+}-Ca^{2+} complex (●) are based on a measured value of 7.1 for the ϵ_{b1} of the Con A-Gd^{3+} complex at 35 MHz. r represents the concentration of Gd^{3+} bound to Con A divided by the concentration of Con A monomers (27,000 mol wt) and A represents the free Gd^{3+} concentration. The estimated error as a result of the uncertainty in ϵ^*_1 has been included for representative points.

Results

Gd^{3+} Binding to Con A. Gd^{3+}, in the presence of Con A, exhibits a proton relaxation enhancement effect which can be usefully exploited to explore the stoichiometry and details of the Gd^{3+} interaction with Con A. Figure 1 presents a comparison of the change in the observed enhancement parameter ϵ^*_1 measured at 60 MHz upon titration of Con A with Gd^{3+} in the presence and absence of saturating Zn^{2+} and Ca^{2+}. It can be seen that the enhancement due to Gd^{3+} is somewhat reduced when sites S1 and S2 are occupied by Zn^{2+} and Ca^{2+}. Employing the known relationship of ϵ^*_1 to ϵ_{b1} and X_b, the fraction of the added Gd^{3+} bound to Con A (eq 2), it is possible to construct a Scatchard (1949) plot for the interaction of Gd^{3+} with Con A. In order to construct the plot from titration at 35 MHz, an ϵ_{b1} value of 7.1 was used (as determined by the measurement of ϵ^*_1 at 35 MHz under conditions of limiting Gd^{3+} concentrations for which $X_b = 1$) for all Gd^{3+} sites on the monomer; 35 MHz was chosen since ϵ_{b1} has a maximum at this frequency (Barber, 1974). This value is clearly valid for the primary tight site for Gd^{3+} on Con A but could be in error for the additional binding sites. The precise number and affinity of the peripheral Gd^{3+} binding sites will depend upon the accuracy of this assumption. Such Scatchard plots are presented in Figure 2 for apo-Con A and Con A in the presence of saturating Zn^{2+} and Ca^{2+}. The data have been fitted on the assumption that there are two noninteracting classes of binding sites (Klotz and Hunston, 1971) using a nonlinear least-squares curve fitting program (Carver, 1966). The solid lines in Figure 2 correspond to calculated data for the parameter values shown in Table I.

Two solutions are given, the first is the solution obtained when no interactions between sites of the same class is permitted. The second solution is obtained when an exponen-

Table I[a]

Sample	RESMS	n_1	n_2	$k_{D_1}(\mu M)$	$k_{D_2}(mM)$	ω_2
Apo-Con A	8.4×10^{-6}	0.11 ± 0.05	26 ± 40	11 ± 10	3.8 ± 6	0.2
	5.7×10^{-6}	0.20 ± 0.06	3.6 ± 0.5	20 ± 8	0.56 ± 0.31	0
Con A–Zn–Ca	6.5×10^{-6}	0.15 ± 0.04	18 ± 30	11 ± 5	4.8 ± 9	0.2
	5.3×10^{-6}	0.19 ± 0.04	3.3 ± 0.8	15 ± 5	0.83 ± 0.28	0

[a] RESMS is the residual mean square deviation of the weighted nonlinear least-squares fit. n_1 and n_2 are the numbers of sites per monomer in the first and second classes, respectively. k_{D_1} and k_{D_2} are the dissociation constants for the first and second classes of sites. ω_2 is the exponent in the exponential interaction of k_{D_2} values. The actual expression fitted to the data was: $r/A = [n_1/(K_{D_1} + A)] + [n_2/(K_{D_2}' + A)]$, where $k_{D_2}' = k_{D_2} \exp(2r\omega_2 - \omega_2)$ (Scatchard, 1949).

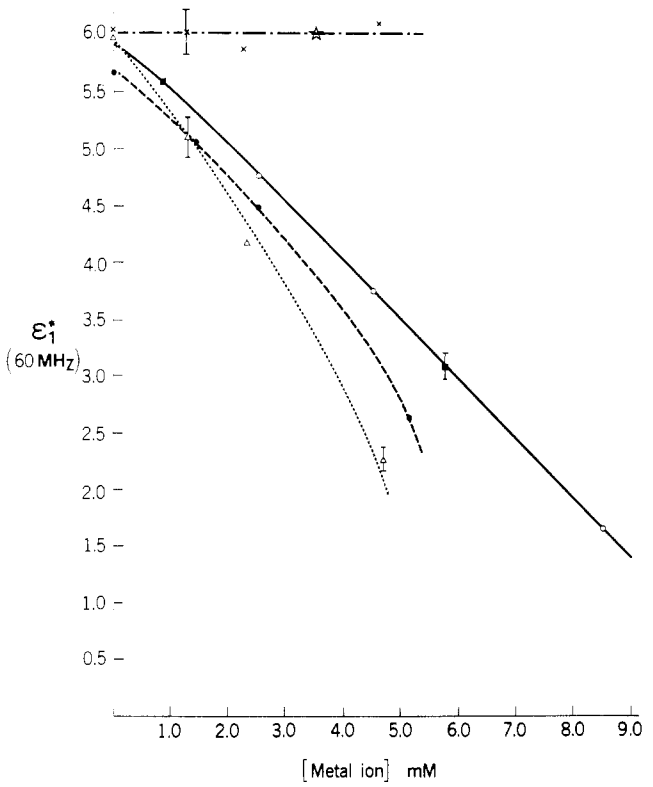

FIGURE 3: A plot representing the ability of the metal ions Sm^{3+}, Eu^{3+}, Tb^{3+}, Pb^{2+}, Zn^{2+}, and Ca^{2+} to reduce the observable enhancement ϵ^*_1 at 60 MHz for Gd^{3+} bound to demetallized Con A. The metal ions are represented as follows: Sm^{3+} (○), Eu^{3+} (●), Tb^{3+} (△), Pb^{2+} (■), and Zn^{2+} (x). A further point (☆) is also included to indicate the subsequent addition of excess Ca^{2+} following the Zn^{2+} titration (final concentrations, $[Zn^{2+}] = 3.5$ mM, $[Ca^{2+}] = 2.5$ mM). In each case the initial Con A monomer concentration was 0.8 mM and the molar ratio of Gd^{3+} to Con A monomer was 0.77:1.

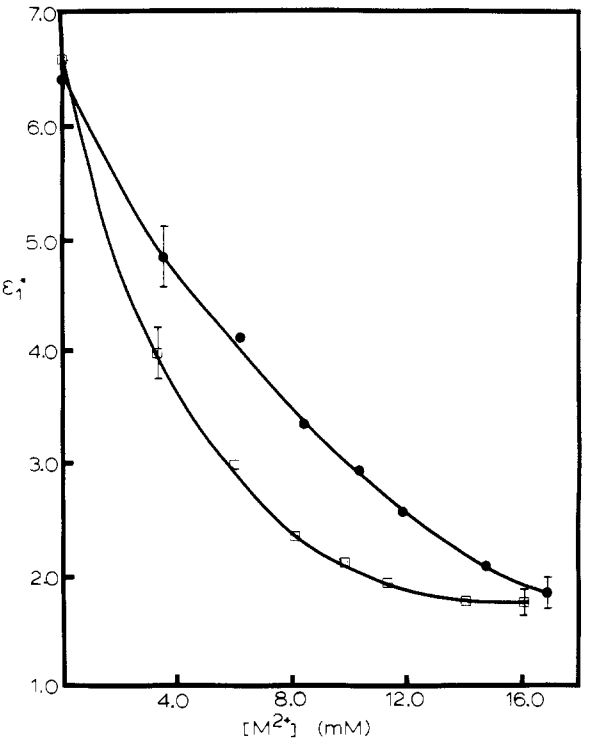

FIGURE 4: A plot representing the ability of the metal ions Sm^{3+} and Pb^{2+} to reduce the ϵ^*_1 at 35 MHz for Gd^{3+} bound to the Con A–Zn^{2+}–Ca^{2+} complex. The points for Sm^{3+} are represented by □, and for Pb^{2+} by ●. The initial Con A monomer concentration was 0.8 mM ($[Zn^{2+}] = 1.2$ mM, $[Ca^{2+}] = 3.2$ mM) and the molar ratio of Gd^{3+} to Con A monomer was 0.2:1.

tial interaction between the binding constants for the weak sites (Scatchard, 1949) is assumed, to compensate for the changing electrostatic interactions with the degree of saturation. In either solution, a fraction of a tight site ($K_D \cong 15$ μM) is found per monomer in addition to many weak sites. The number of weak sites in uncertain, not only because of the errors in the fitting process but also because, as noted above, the ϵ_{b1} value for these sites may differ from that for the tight site. The significance of these findings is further discussed below.

In order to relate the Gd^{3+} binding site to possible metal ion sites previously defined in X-ray crystallographic studies and to determine the degree of specificity for the Gd^{3+} site with respect to other lanthanides, we undertook a series of competition experiments. The results of competition for the Gd^{3+} site on the Con A–Gd^{3+} complex, as determined by the reduction of the observed enhancement, by the lanthanides Tb^{3+}, Eu^{3+}, and Sm^{3+} are presented in Figure 3. Also included are points on the competition curve for the heavy metal ion Pb^{2+}. The competition data in Figure 3 are for Con A in the presence of Gd^{3+} only, at a molar ratio of 0.77:1 with respect to the Con A monomer concentration. Under these conditions approximately 25% of the bound Gd^{3+} is bound at the tight site. As seen in Figure 3 the addition of Zn^{2+} to Con A–Gd^{3+} under similar dilution conditions at this degree of saturation does not perturb the observed enhancement, nor does the subsequent addition of excess Ca^{2+} in the presence of Zn^{2+}. Repeating the competition experiment for Sm^{3+} and Pb^{2+} in the presence of excess Zn^{2+} and Ca^{2+} and at a Gd^{3+}–Con A monomer ratio of 0.2 where more than 50% of the bound Gd^{3+} is bound at the tight site, Figure 4, again indicates that the two ions are similarly effective in decreasing the observed enhancement, presumably by competitively occupying both the tight and weak Gd^{3+} binding sites on Con A.

The binding of Ca^{2+} to S2 in the presence of S1 occupa-

Table II: The Mixed Mn^{2+} and Gd^{3+} PRE Experiment.

Sample (m*M* Concn)	$\epsilon^*_{1\,obsd}$[a]	$\epsilon^{**}_{1\,obsd}$[a]	$\epsilon^{**}_{1\,calcd}$[a]
Con A (0.29)[b]	3.4[c]		
Mn^{2+} (0.24)			
Con A (0.29)[b]	5.0[c]		
Gd^{3+} (0.23)			
Con A (0.28)[b]			
Mn^{2+} (0.23)		4.0	4.2
Gd^{3+} (0.23)			
Con A (0.24)[b]			
Mn^{2+} (0.20)			
Gd^{3+} (0.19)		3.0	2.9
Ca^{2+} (3.94)			

[a] All enhancement parameters have been measured at 60 MHz. [b] Con A concentrations are expressed in terms of a 27,000 mol wt monomer. [c] These values have been used in conjunction with the measured ϵ_{b1} values (see text) to determine each X_b (eq 2).

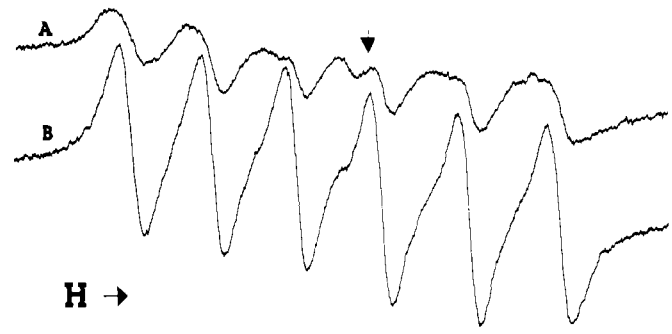

FIGURE 5: The ESR spectrum for Mn^{2+} in the presence of Con A with and without added $GdCl_3$. Part A of this figure depicts the Mn^{2+} ESR spectrum of 330 μM $MnCl_2$ in the presence of 643 μM Con A monomer at pH 5.3 in 0.05 *M* NaOAc–0.2 *M* NaCl buffer. Part B indicates the spectrum recorded after the addition of $GdCl_3$ to a final concentration of 653 μM ([Mn^{2+}] = 330 μM, [Con A] = 643 μM). The spectra was recorded sequentially under the same spectrometer conditions: attenuation, 50 mW; modulation amplitude, 8 G, receiver gain, 3.2×10^3.

tion induces the formation of a monosaccharide inhibitor binding site (Kalb and Levitzki, 1968). It is known that the addition of methyl α-mannoside to occupy this site perturbs the Mn^{2+} PRE in the Con A–Mn^{2+}–Ca^{2+} complex (Barber and Carver, 1973). In a similar experiment with Gd^{3+} occupying S3 on the Con A–Zn^{2+}–Ca^{2+} complex, no perturbation of the Gd^{3+} PRE (pH 5.2) was observed upon the addition of excess methyl α-mannoside. In the presence of Gd^{3+}, methyl α-D-mannoside displaceable Con A binding to Sephadex is not affected. Hence it is unlikely that the lack of perturbation of the Gd^{3+} PRE by the monosaccharide is due to a failure of the latter to bind in the presence of Gd^{3+}.

Mixed PRE Experiment. The results of the analysis applied to the measurement of a PRE effect in the combined presence of Mn^{2+} and Gd^{3+} for Con A are presented in Table II. Also included for comparison are relevant ϵ_{b1} values obtained from the analogous single paramagnetic ion PRE experiments. The $\epsilon^{**}_{1\,calcd}$ parameters were calculated using the previously determined (60 MHz) ϵ_{b1} values of 5.8 for Con A–Gd^{3+} and 5.2 for Con A–Mn^{2+} (Barber and Carver, 1975), coupled with the observation of ϵ^*_1 under identical conditions of Con A and paramagnetic ion concentrations for each PRE ion individually, in order to determine the appropriate X_b (eq 2). In the presence of excess Ca^{2+}, a value of 1.1 was applied for the Mn^{2+} ϵ_{b1} (Barber and Carver, 1975) and the unaltered value of 5.8 for Gd^{3+}. The values of ϕ_{Mn} (0.46) and ϕ_{Gd} (0.54) were determined using measured T_{1M_f} values and the procedure described previously.

Con A–Mn^{2+} ESR. Also bearing on the analysis of the mixed PRE experiment and the investigation of possible S3–S1 metal ion site interaction are the ESR results presented in Figure 5. This experiment involves the observation of the Mn^{2+} ESR signal in the presence of Con A without (A) and with (B) a 1:1 molar ratio of Gd^{3+} to Con A monomer in the system. In Figure 5A, a small amount of free Mn^{2+} signal is visible (fourth peak from low field indicated by the arrow) against the background of the Con A bound Mn^{2+} signal. In the case with Gd^{3+} added, Figure 5B, the free Mn^{2+} signal is increased (~60 μM Mn^{2+} released) and masks the bound Mn^{2+} signal. A further, but smaller, increase in the free Mn^{2+} signal is also observed on doubling the Gd^{3+} concentration to a molar ratio of 2:1 (spectrum not shown). In each case the Con A and Mn^{2+} concentrations were the same and each spectrum was recorded under identical spectrometer conditions.

Discussion

Gd^{3+} is a paramagnetic trivalent lanthanide which, because of its relatively long electronic spin relaxation time, τ_s (relative to the other paramagnetic lanthanides), is able to exhibit a solvent proton relaxation enhancement effect when bound to a macromolecule (Dwek, 1972). The enhancement effect results from a difference in the dominant contribution to the correlation time, τ_c, for the dipolar interaction between the unpaired electronic spin and the protons in the first hydration sphere H_2O molecules of the Gd^{3+}, between the solvent and protein bound sites for Gd^{3+}. The use of Gd^{3+} as a relaxation enhancement probe has been limited, but the potential has been clearly outlined in earlier publications (Reuben, 1971a; Dwek et al., 1971; Morallee et al., 1970).

The results indicate that the solvent proton relaxation enhancement technique can be successfully applied to a study of Gd^{3+} binding to the dimeric form of Con A in the region of pH 5.6. Analysis of the Scatchard plot of the binding data obtained from the relaxation enhancement measurements (Table I) reveals a fractional tight ($K_D \cong 15$ μM) binding site for Gd^{3+} in both the absence of any additional metal ions and the presence of saturating Zn^{2+} and Ca^{2+}. The most likely explanation for the fractional value observed for the number of tight sites per monomer is that the tight site is formed from two carboxyl side chains which are partially ionized at this pH. If Gd^{3+} bound only to doubly ionized sites then a fractional value of n_1 would be expected. Secemski and Lienhard (1974) have reported a similar effect for the binding of Gd^{3+} to the Glu–Asp pair in lysozyme and suggested the above explanation for their results. Using the p*K*'s (Parsons and Raftery, 1972) reported for the Glu–Asp pair of lysozyme (6.01 and 4.49, respectively), the fraction of doubly ionized sites at pH 5.6 would be 0.27. On the basis of the competition experiments (see below) it is proposed that S3 is either at Glu-87–Asp-136 or Asp-80–Asp 83; it therefore seems reasonable to conclude that the fractional value for the number of tight sites per monomer observed for Gd^{3+} binding to Con A arises by a similar effect. We are currently investigating the pH dependence of Gd^{3+} binding to further test this hypothesis; however, the

pH dependent aggregation of Con A complicates matters. The weak Con A binding sites for Gd^{3+} are most likely the remaining single carboxyl side chains. There are 25 free carboxyl side chains per Con A monomer (Becker et al., 1975), four of these are involved in S1 and S2, two more would be expected to be involved in S3 (see below). Thus one might expect approximately 20 weak sites with K_D's of $\sim$7 mM, since this is the K_D reported for Gd^{3+} binding to the carboxyl of acetic acid (Sillen and Martell, 1964). As can be seen in Table I, such values are consistent with the data when an exponential interaction between the binding constants is assumed; however, the large experimental errors involved at high degrees of saturation make accurate determination of these parameters extremely difficult.

During the course of this work, Sherry and Cottam (1973) reported the observation of a PRE effect with Gd^{3+} and Con A. Their analysis indicated a single tight Gd^{3+} binding site (K_D = 40 ± 4 μM) and a weaker Gd^{3+} site (K_D = 200 ± 50 μM) per Con A monomer (27,000 mol wt.). On the basis of a comparison of these binding constants with those of Mn^{2+} for site S1 and Ca^{2+} for S2, they speculated that the tighter and weaker Gd^{3+} binding sites were S1 and S2, respectively. In contrast, our results indicate that the primary Gd^{3+} site on Con A (which we refer to as site S3) is clearly distinct from sites S1 or S2 which is in agreement with more recent studies of Sherry (Sherry et al., 1975). The possibility of a certain degree of Gd^{3+} binding in site S2 at molar ratios of Gd^{3+} to monomer in excess of 1:1, in the absence of Ca^{2+}, cannot be ruled out; however, the data do not indicate a preferred second site after filling of S3. The binding of Gd^{3+} to Con A is not detectably perturbed in the presence of a sixfold excess concentration of Zn^{2+} over the Con A monomer concentration (Figure 3). From published affinity constants for Gd^{3+} and Zn^{2+} binding to acetic acid (6.9 and 93 μM, respectively; Sillen and Martell, 1964) and with the aid of the Coleman and Vallee (1961) treatment for competitive binding, the extent of Zn^{2+} displacement of Gd^{3+} from the weak sites can be shown to be negligible under the conditions of the experiment reported in Figure 3. If Gd^{3+} were binding at S1, then using the measured K_D of 70 μM for Zn^{2+} binding to S1 (Shoham et al., 1973) and the above treatment, it is possible to calculate that Gd^{3+} would have to be binding to S1 with a K_D of less than 0.1 μM in order that the extent of displacement by Zn^{2+} ions, under the conditions of the experiment reported in Figure 3, be less than the experimental error (5%). Similarly, to observe a negligible displacement by Ca^{2+} ions, Gd^{3+} would have to be binding at S2 with a K_D of 1μM or less. The fact that the tightest binding constant found for Gd^{3+} is 15 μM and that this is for a fractional site is strong evidence that the tight Gd^{3+} binding site reported here is neither S1 nor S2. Furthermore, Gd^{3+} is competitively displaced from the tight site by both Sm^{3+} and Pb^{2+} (see below), neither of which demonstrates any affinity for site S1 as determined by the Con A-Ni^{2+} equilibrium dialysis experiments of Shoham et al. (1973). The results of Shoham et al. (1973) also indicate that Sm^{3+} is not competitive for Ca^{2+} bound to Con A, although possible weak binding of Sm^{3+} to S1 and S2 is suggested by the ability of high concentrations of Sm^{3+} to induce sugar binding activity in Con A (Sherry et al., 1975; Shoham et al., 1973).

Gd^{3+} is competitively displaced from Con A by both Pb^{2+}, Sm^{3+}, and other lanthanides. Since these ions have similar affinities for the carboxyl group of acetic acid as Gd^{3+} (Sillen and Martell, 1964), they would be expected to first displace Gd^{3+} from the weak sites to which 75% of the Gd^{3+} is bound under the conditions of the experiment reported in Figure 3. In order to ensure that Pb^{2+} and Sm^{3+} also compete effectively for the tight site, the experiment was repeated at a Gd^{3+}/Con A ratio of 0.2 in the presence of excess Zn^{2+} and Ca^{2+} (Figure 4). Under these conditions 50% of the bound Gd^{3+} is bound at the tight site and yet more than 80% has been displaced at the highest concentration of competing ion. Therefore, the results of the competition experiments indicate that S3, the single tight Gd^{3+} site per monomer, is not coincident with site S1, the transition metal ion site, or S2, the Ca^{2+} binding site. This interpretation is further confirmed by the results of the mixed enhancement experiment discussed below.

The Mn^{2+} ESR signal in a preparation of Con A with Mn^{2+} only present and as well with a 1:1 or 2:1 molar ratio of Gd^{3+} to Con A monomer added has been described (Figure 5). The amount of Mn^{2+} displaced (20%) suggests that, in agreement with the above discussion, Gd^{3+} binding at S3 may be indirectly affecting the S1 affinity for Mn^{2+} through modifications of the protein conformation (although no conformational changes are observed in the 220-MHz proton high resolution spectrum or in the circular dichroism spectrum of the protein). An alternative interpretation, supported by Sherry's most recent results (Sherry et al., 1975), is that Gd^{3+} is causing displacement of Mn^{2+} from S1 by weak binding to S1 in addition to the strong binding to S3 described above. No displacement of Mn^{2+} is observed under the same conditions, but in the presence of Ca^{2+}, where Mn^{2+} is known to bind much more tightly to S1 and Gd^{3+} still binds strongly to S3 (B. H. Barber and J. P. Carver, unpublished results). A similar increase in the free Mn^{2+} ESR signal was reported by Sherry and Cottam (1973) upon the addition of Nd^{3+} to the Con A-Mn^{2+} complex.

The requirement for heavy metal ion crystallographic derivatives in the X-ray crystal structure analysis of Con A has provided a means with which to locate the lanthanide site S3 on Con A. Edelman and coworkers have reported the collection of data on $Pb(NO_3)_2$ and $Sm(NO_3)_3$ heavy-metal derivatives of Con A (Edelman et al., 1972; Becker et al., 1975). The data indicate that for Sm^{3+} there is a single major occupancy site and two minor occupancy ones. For $Pb(NO_3)_2$ two equal occupancy sites are observed per 27,000 molecular weight monomer one of which corresponds to the major Sm^{3+} site, the other corresponds to the weakest Sm^{3+} site. Stimulated by the results of this study, Edelman et al. have examined the $GdCl_3$ derivative of Con A at 2.8-Å resolution in projection and found that the Gd^{3+} ion binds with the same relative occupancies in the same three sites in the crystal as Sm^{3+} (G. M. Edelman, G. N. Reeke, and J. W. Becker, personal communication, Becker et al., 1975). Therefore, the major occupancy Sm^{3+} site, the major Gd^{3+} site, and the major Pb^{2+} site appear to be crystallographically identical in location. This information, coupled with the very similar competition pattern of Sm^{3+} and Pb^{2+} for the Gd^{3+} site on the Con A-Zn^{2+}-Ca^{2+} complex (Figure 4) strongly suggests that the single tight binding site for Gd^{3+} per monomer (S3) is at the position of the major Pb^{2+} binding site determined crystallographically. This site is on the surface of the molecule associated with the side-chain carboxyls of Glu-87 of one monomer and Asp-136 of the other monomer in the dimeric structure (Edelman et al., 1972). The other Pb^{2+} binding site is locat-

ed between the side-chain carboxyls of Asp-80 and Asp-83 (Becker et al., 1975).

Hardman and Ainsworth (personal communication) have examined $SmCl_3$ and $GdCl_3$ derivatives of Con A at 6-Å resolution. Although, only two of their sites correspond to sites observed by Edelman et al., and although the relative occupancies for these sites differ from those found by Edelman et al., they do find Gd^{3+} and Sm^{3+} binding to occur in one of the positions in the crystal in which Edelman et al. found Pb^{2+} to bind—Glu-87–Asp-136. They do not find lanthanide binding at the second Pb^{2+} site but they do find that the Gd^{3+} and Sm^{3+} ions bind with the same relative occupancies in the same three sites in their derivatives of Con A. Although it is not possible to completely rule out the Asp-80–Asp-83 site as being S3, the low relative occupancy (0.13 vs. 0.50 for the Glu-87–Asp-136 site) by Sm^{3+} and Gd^{3+} (Becker et al., 1975), together with the failure of Hardman and Ainsworth to observe lanthanide binding at Asp-80–Asp-83 in their derivatives makes such a possibility unlikely.

The location of site S3 on the monomeric unit provides a valuable reference point with which to exploit the paramagnetic mapping capabilities of Gd^{3+} (and also Eu^{3+}) in high resolution NMR experiments (Morallee et al., 1970). In conjunction with the similar potential for the use of paramagnetic ions (e.g., Mn^{2+}, Co^{2+}, Ni^{2+}) in site S1, Con A offers a unique opportunity to expand and develop the capacity to assign and locate residues both on the protein and for small molecules in exchange with sites on the protein, using high resolution paramagnetic "mapping" techniques (Butchard et al., 1972; Fung et al., 1973; Brewer et al., 1973). Such experiments are presently in progress in our laboratory and the specific influence of Gd^{3+} on the 220-MHz proton magnetic resonance spectrum has been discussed elsewhere (J. P. Carver, B. H. Barber, and B. J. Fuhr, manuscript submitted for publication).

In order to more fully define the solvent PRE characteristics of the Con A bound Gd^{3+}, we have investigated the frequency dependence of the relaxation properties of the complex. The results indicate that there is a marked frequency dependence of the enhancement parameter ϵ_{b1} in the region of 5–60 MHz, resulting principally from a frequency dependence in the relaxation properties of the Con A bound Gd^{3+} (B. H. Barber and J. P. Carver, unpublished results). We are presently attempting to fit the experimental results using the conventional Solomon–Bloembergen expressions (Solomon, 1955; Bloembergen, 1957), however, the complex nature of the frequency dependence of τ_s (Reuben, 1971b; Hudson and Lewis, 1970), the electron spin relaxation time, makes the treatment of these data more difficult than the corresponding analysis for a Mn^{2+} complex (Dwek, 1972). Potentially, the achievement of a satisfactory fit to the experimental data would provide an assessment of the relative contributions to the dipolar correlation time τ_c, at any particular magnetic field strength (Dwek, 1972), and, therefore, would be of value in the use of Gd^{3+} as a reference point on Con A for absolute distance calculations (B. J. Fuhr, B. H. Barber and J. P. Carver, manuscript in preparation).

The final point of discussion for the Con A–Gd^{3+} complex concerns the use of the mixed enhancement PRE experiment to confirm the independence of sites S1 and S3 and to possibly detect any influence of binding at one site on the other. The question of whether or not the occupation of S3 by Gd^{3+} alters the nature of the Ca^{2+} binding influence on site S1, as detected previously by PRE methods (Barber and Carver, 1975), can also be approached. Interpretation of the experimental results in terms of the ϵ_{b1} enhancement parameter has been discussed in the Theoretical Treatment section and the results of the calculations are presented in Table II. The good agreement shown in Table II between the calculated and determined ϵ^{**}_1 values indicates, in support of previous evidence, that sites S1 and S3 can be mutually occupied by Mn^{2+} and Gd^{3+}, respectively. This conclusion is based on the fact that the mixed enhancement parameter ϵ^{**}_1 can be adequately predicted by the additive combination of the PRE effects for Gd^{3+} and Mn^{2+} bound to Con A when determined separately. It also appears that the Ca^{2+} effect upon the PRE properties of Mn^{2+} in S1 (Barber and Carver, 1975) is completely intact in the presence of Gd^{3+} at S3, as evidenced by the predicted decrease in $\epsilon^{**}_{1\,calcd}$. This also clearly demonstrates, in support of the previous competition data, the independence of sites S2 and S3. The decrease in the affinity of site S1 for Mn^{2+} upon the addition of Gd^{3+} (Figure 5) observed by ESR, would be expected to reduce the fraction of Mn^{2+} bound in the mixed enhancement experiments. The effect is not large, and in fact allowance for the change in K_D changes ϵ^{**}_1 calculated for the Mn^{2+} plus Gd^{3+} case to 4.0 instead of the value of 4.2 obtained when this effect is ignored and results in better agreement with the observed value (Table I). In the presence of Ca^{2+}, Mn^{2+} remains tightly bound so no correction is required.

In summary, we have utilized the PRE effect of Gd^{3+} bound to Con A to ascertain that there is a single tight binding site for Gd^{3+} per 27,000 mol wt Con A monomer. By reference to heavy metal ion derivatives reported for Con A and competition experiments in solution, it has been possible to locate the Gd^{3+} binding site as one of the sites previously reported for Pb^{2+}, most probably the site involving the side chains of Glu-87 and Asp-136. This site, which we have proposed to refer to as S3, is clearly distinct from the previously described sites S1 and S2 as determined by a variety of competition experiments and the mixed PRE probe technique. The complex of Gd^{3+} with Con A promises both to provide an excellent system with which to develop the understanding of Gd^{3+} as a PRE probe and perhaps even more importantly to serve as a further defined paramagnetic reference point for future high resolution experiments with Con A in solution.

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