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Proton and Deuteron Nuclear Magnetic Relaxation Dispersion Studies of Ca^{2+}–Mn^{2+}–Concanavalin A: Evidence for Two Classes of Exchanging Water Molecules†

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ABSTRACT: We have measured the magnetic field dependence of the nuclear magnetic relaxation rates (NMRD profiles) of solvent protons and deuterons in solutions of Ca^{2+}–Mn^{2+}–concanavalin A (Con A) with and without saccharide present. Data were obtained over the range −8 to 35 °C; the extension to the lowest temperature was made possible by the presence of 5 M salt. Since previous theoretical analyses, using accepted relaxation theories of ^1H NMRD profiles alone, led to unsatisfactory conclusions, we have attempted to take advantage of the fact that the residence lifetime of a water ligand of the metal ions can influence the relaxation behavior of protons and deuterons differently. From a comparison of the present proton and deuteron results, we find that Ca^{2+}–Mn^{2+}–Con A has two classes of binding sites: one, associated with the inner coordiation sphere of the Mn^{2+} ions, having a resident lifetime for solvent water of $\sim 10^{-5}$ s that is reduced by the presence of saccharide and another having a lifetime of $\sim 5 \times 10^{-9}$ s, located with the protons of the bound waters ~4.4 Å from the Mn^{2+} ions (assuming two equivalent water molecules in this class), which is well beyond the coordination environment of the Mn^{2+} ions. The relaxation contribution of these more distant sites is unaffected by saccharide. The conclusions are corroborated by measurements of the temperature dependences of the proton NMRD profiles, which show quite clearly that the profiles are composite, containing two contributions with opposite dependences on temperature. The more slowly exchanging water molecules dominate proton relaxation above about 25 °C and dominate deuteron relaxation throughout. The more rapidly exchanging water molecules dominate proton relaxation at lower temperatures but make no detectable contribution to the deuteron data under these conditions. These results are the first in which it can be said with some confidence that exchanging water molecules liganded in two disparate classes of sites, with different exchange rates and different contributions to the NMRD profiles, have been identified.

Measurements of NMRD[1] profiles of solvent protons in solutions of Con A and its complexes with Mn^{2+}, Ca^{2+}, and saccharides have yielded a wealth of biochemical information about the complexes (Brown et al., 1977; Koenig et al., 1978; Brewer et al., 1983a,b). In these investigations, observed NMRD profiles were used as indicators of the chemical state of Mn^{2+} and of the conformation of the protein and its change with time. The conclusions reached depended only on complex-specific contributions to the observed NMRD profiles derived from the interactions of solvent protons with the (paramagnetic) Mn^{2+} ion in its many complexed forms. The details of relaxation theory played little role in the interpretation of the biochemistry of Con A, and indeed, an accurate theory of relaxation from which, for example, one could deduce the number q of exchanging solvent mlecules liganded to Mn^{2+} ions complexed with protein did not exist at the time [cf.

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[1] Abbreviations: NMRD, nuclear magnetic relaxation dispersion, the magnetic field dependence of the solvent nuclear magnetic relaxation rates (in protein solutions); Con A, concanavalin A with unspecified metal ion content; MP, the Mn^{2+}–Con A complex in the unlocked conformation; CMPL, Con A with 1 equiv per monomer of bound Mn^{2+} and Ca^{2+} in the locked conformation; SCMPL, CMPL complexed with saccharide; CZPL, the analogous Zn^{2+} complex; α-MDM, methyl α-D-mannopyranoside.

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Koenig (1978) and Koenig & Brown (1980)].

A well-developed theory of relaxation that is in good quantitative agreement with observations on aqueous solutions of a wide variety of paramagnetic metal ions [cf. Koenig & Brown (1984)] does exist, and this theory was applied at an early date to the first, limited measurements of solvent proton relaxation in solutions of paramagnetic metalloproteins. With improvements in the range and precision of relaxation data, it has become increasingly clear that the quantitation possible with results on solutions of aquo ions is still not attainable for solutions of these same ions complexed with protein (Burton et al., 1979; Koenig & Brown, 1983, 1985; Koenig et al., 1981). Thus, a question of long interest to biochemists, concerning details of solvent ligand exchange at metal ions complexed with protein, could be answered neither reliably nor quantitatively. The problem is not with the theory itself but with uncertainties in approximations that have to be made. The problem is often expressed as an ambiguity in the value of the residence lifetime τ_M of exchanging water ligands of the protein-bound metal ions: whether it is either comparable to, or very much less than, the relaxation time T_{1M} of a proton in the ligand environment (cf. eq 1 below). This translates into either a severalfold uncertainty in the number q of solvent ligands, assumed equivalent, of the metal ion or an unreasonably small value for the ion–proton distance, assuming a single solvent ligand.

Deuterons, because of their smaller magnetic moment, are relaxed by paramagnetic ions 40-fold less efficiently than are protons; thus, the ratio τ_M/T_{1M} for protons and deuterons on the same exchanging waters differs substantially. It has been suggested, accordingly, that measurements of relaxation rates of both deuterons and protons in a sample with isotopically mixed solvent could remove the fundamental impediments to the theoretical quantitation of relaxation measurements (Burton et al., 1977; Reuben, 1975; Kushnir & Navon, 1984). This protocol is intrinsically difficult, however, because of the weak effects that paramagnetic ions have on deuteron relaxation, and little progress has been reported, as yet. The most complete analysis for any protein has been for the binary Mn^{2+}–Con A complex (Koenig & Brown, 1985). A major result of that work is that theory (and the concomitant approximations required) must still be guided by biochemical information obtained independently. Even then, there is a fundamental assumption not always articulated in discussions of these protocols: proton and deuteron relaxation rates can be used to help quantitate relaxation data only if one class of liganded solvent molecules exists, so that all the bound molecules make identical contributions to the relaxation rates. This need not be the case in general and does not hold in the present investigations.

In this paper we report proton and deuteron NMRD profiles of solutions of Ca^{2+}–Mn^{2+}–Con A (CMPL) and its complex with α-MDM (SCMPL) at several temperatures. A qualitative comparison of proton and deuteron results for CMPL shows that two classes of sites for exchanging water molecules must contribute to the observed relaxation rates: one at the Mn^{2+} ion site and in relatively slow exchange (long τ_M); the other relatively remote from the Mn^{2+} ion and in relatively rapid exchange (short τ_M). The parameters are such that the two classes of sites contribute comparably to proton relaxation, whereas the deuteron relaxation is dominated by the more slowly exchanging water molecules liganded to the Mn^{2+} ions.

Additional evidence for the existence of two classes of solvent exchange sites in CMPL comes from the temperature dependence of the proton NMRD profiles in the range −8 to 35 °C, for both CMPL and SCMPL. There is a change in sign of the temperature dependence that occurs near 5 °C: the relatively remote, rapidly exchanging class of water molecules has a contribution to the NMRD profiles that increases with decreasing temperature and dominates the data at −8 °C; by contrast, the slowly exchanging class of molecules has a thermally activated contribution to the data (Koenig et al., 1973) and dominates the results above 25 °C. A comparison of theory with the −8 °C data gives 4.4 Å for the distance between the Mn^{2+} ions and the protons of the remote water molecules, assuming two waters. The profiles of the saccharide complex show that only the contribution of the slowly exchanging waters is altered by saccharide binding. Similar measurements are reported for the two related Ca^{2+}–Mn^{2+} proteins pea lectin and lentil lectin in the following paper in this issue (Bhattacharyya et al., 1985). These results allow assignment of the location of the remote solvent exchange sites in CMPL, a source of solvent relaxation common to CMPL and the pea and lentil lectins.

Materials and Methods

Material Preparation. Con A, obtained from Miles-Yeda, and its complexes were prepared and characterized as described in detail previously (Brown et al., 1977). Metal ions from calibrated stock solutions were added to solutions of demetallized protein at sufficiently high pH (6.4) so that corrections for unbound Mn^{2+} were minimal. Salts of the different metal ions, of the highest purity available, were from either Mallinckrodt or Fisher Scientific Co.

The solvent, either 10% protons and 90% deuterons or 100% protons, contained 0.1 M KOAc and 0.9 M KCl adjusted to pH 6.4 (uncorrected meter reading), unless otherwise indicated. The deuterium oxide, 99.8% deuterons, was from Stohler Chemicals. Protein concentration, measured spectrophotometrically by using an absorbance $A_{280nm}^{1\%,1cm} = 21.4$ at pH 5.6 (Yariv et al., 1968), is given in monomeric units throughout (though the protein is dimeric).

Relaxation Measurements. Proton data up to 50 MHz[2] were taken on the automated "field-cycling" instrumentation referenced previously (Brown et al., 1977; Koenig & Brown, 1985). Deuteron data at a Larmor frequency equal to that of protons require a 6-fold greater magnetic field because of the lower gyromagnetic ratio of the deuteron. Because of this, the deuteron data obtained by using the field-cycling apparatus were supplemented by data obtained in the more traditional manner with a Bruker SXP console and a superconducting nonpersistent magnet that could reach 270 MHz (proton frequency).

Because of the electric quadrupolar contribution to the relaxation rate of deuterons in ^2H_2O, the contribution of the demetallized protein to the deuteron relaxation is about 10-fold greater than for protons in 100% ^1H_2O. Coupled with the smaller moment, the relative paramagnetic effects for deuterons, particularly at low fields, are reduced 400-fold and become difficult to measure with high accuracy.

Results

^2H NMRD Profiles. Figure 1 shows ^2H NMRD profiles of Ca^{2+}–Mn^{2+}–Con A (CMPL), of Ca^{2+}–Zn^{2+}–Con A (CZPL), used as a diamagnetic control, and of the 90% deuterated solvent, which is the background "base line". The protein samples, 0.85 and 0.80 mM, respectively, were satu-

[2] The magnetic field is expressed throughout in units of the proton Larmor precession frequency at that field.

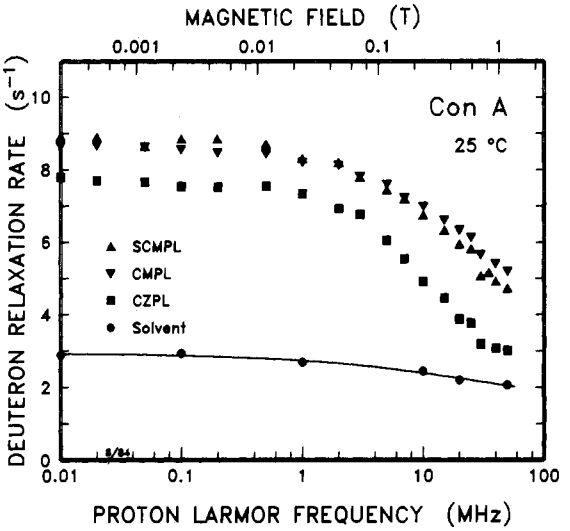

FIGURE 1: Spin–lattice magnetic relaxation rates of solvent deuterons as a function of magnetic field strength (NMRD profiles) of Ca^{2+}–Mn^{2+}–Con A (▼), the same sample with 100 mM α-MDM added (▲), Ca^{2+}–Zn^{2+}–Con A as a diamagnetic control (■), and protein-free solvent (●); all at 25 °C. The protein concentrations were 0.85 mM for the paramagnetic sample and 0.80 mM for the diamagnetic one (in monomeric units), and the solvent was 90% ^2H and 10% ^1H with acetate buffer, pH 6.4 (see text).

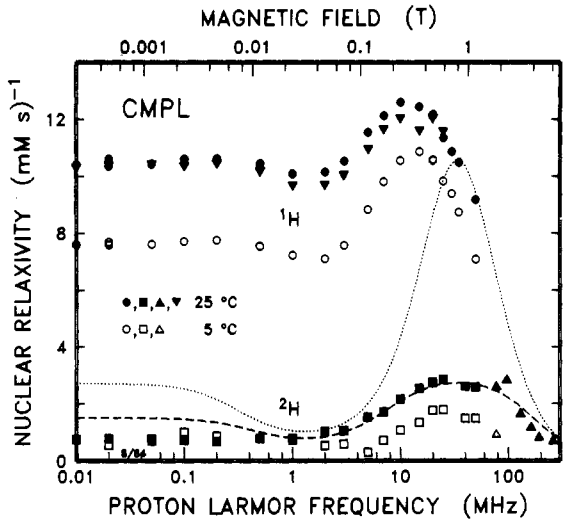

FIGURE 2: Paramagnetic contributions to the NMRD profiles for the samples of Figure 1, and for other samples of Ca^{2+}–Mn^{2+}–Con A, in similar buffer for both protons and deuterons at 5 and 25 °C (open and solid symbols, respectively). The results are expressed as relaxivity, the incremental paramagnetic contribution to the relaxation rates per millimolar bound Mn^{2+} ions. For protons, results are shown for protein in 100% ^1H_2O (●, ○), for which the accuracy is greater than for 10% ^1H (▼). The dotted curve represents the deuteron NMRD profile for Mn^{2+}–Con A for both 5 and 25 °C (Koenig & Brown, 1985). The dashed curved through the 25 °C deuteron profile for Ca^{2+}–Mn^{2+}–Con A is derived from the dotted curve, by the theory, by increasing the residence time τ_M of the exchanging water molecules 30-fold, from 3.0×10^{-7} to 1.0×10^{-5} s.

rated with metal ions. Also shown is the NMRD profile of SCMPL, CMPL with saturating amounts (100 mM) of α-MDM. On addition of saccharide, the maximum change in the paramagnetic component of the relaxivity, which occurs at high fields, is ~25% (from Figure 1 and other data), somewhat greater than the ~18% effect observed for protons [see Figure 3; see also Koenig et al. (1973) and Brewer & Brown (1979)].

Comparison of ^1H and ^2H Profiles. Figure 2 shows the paramagnetic contributions to the ^1H NMRD profiles of CMPL, at 5 and 25 °C, for a sample of 1 mM Ca^{2+}, 0.33 mM

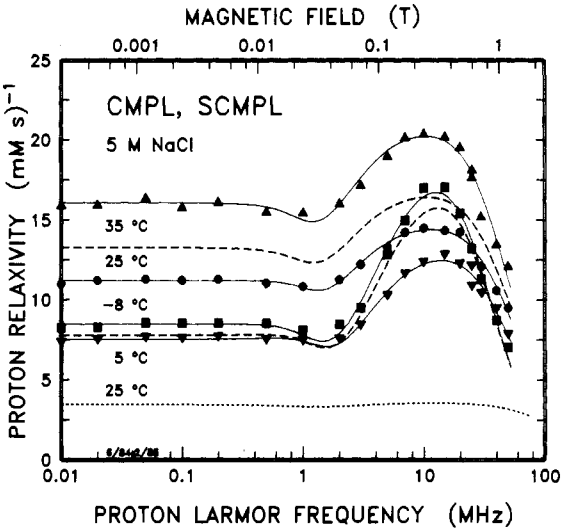

FIGURE 3: Paramagnetic contributions to the proton NMRD profiles of Ca^{2+}–Mn^{2+}–Con A at four temperatures and with 100 mM α-MDM at the two extremes of temperature (dashed curves). The protein concentration was 0.56 mM, and the bound Mn^{2+} and Ca^{2+} concentrations were each 0.48 mM. NaCl (5 M) was added to 100% ^1H buffer to prevent freezing at −8 °C. The solid lines through the data points are meant to serve as visual guides and have no other significance. The dotted curve is the approximate contribution of the slowly exchanging class of water molecules to the 25 °C data, computed for protons from the deuteron CMPL profile, Figure 2.

Mn^{2+}, and 0.39 mM protein in 100% ^1H_2O. Also shown are analogous ^1H data, at 25 °C, for the sample used for the data of Figure 1, demonstrating the absence of any isotope effect on the ^1H NMRD profiles. The results are expressed as relaxivity, the paramagnetic contribution per millimolar bound Mn^{2+} ions. The ^2H contribution of CMPL is also shown, plus data from other samples (Koenig & Brown, 1985) that extend the magnetic field range at the high end.

^1H NMRD Profiles of CMPL and SCMPL from −8 to 35 °C. The paramagnetic contribution to the NMRD profiles of CMPL and SCMPL, in 100% ^1H_2O buffer containing 0.1 M KOAc and 5 M NaCl adjusted to pH 6.4, is shown in Figure 3. The high salt concentration allowed extension of the temperature range to −8 °C. NMRD profiles of CZPL in the same buffer (not shown) were used for the diamagnetic correction. The effect of the high salt concentration on the NMRD profiles was slight, as may be seen from comparing these results with those for CMPL at 5 and 25 °C in 0.9 M KCl buffer, pH 6.4 (Brown et al., 1977), and with those in Figure 2, above. Additionally, no change in the NMRD profiles was observed in the range 0.01–1 M of either NaCl or KCl.

Of note in Figure 3 is that there is a reversal of the sign of the temperature dependence of the NMRD profiles near the lower end of the range and that the shape of the profiles changes as well, exhibiting the greatest variation over its field range at the lowest temperature. In addition, the effect of saccharide decreases with temperature and is almost nil at −8 °C.

Evidence for Two Classes of Solvent Binding Sites in CMPL. A qualitative comparison of the data for protons and deuterons, Figure 2, shows that deuteron relaxation must arise from water molecules bound at a different class of sites than that which dominates the proton relaxation. The argument, which depends on eq 1, follows.

The theory of relaxation, with approximations most appropriate to describing NMRD profiles of solutions of Mn^{2+} complexes of Con A, and Mn^{2+}–protein complexes more

generally, has been critically reviewed recently (Koenig & Brown, 1985). The only equation that bears directly on the present study is one that relates τ_M to the paramagnetic contribution to the observed relaxation rate ($1/T_{1para}$) of a particular class of exchanging water molecules:

$$1/T_{1para} = pq/(T_{1M} + \tau_M) \quad (1)$$

where p is the molar fraction of paramagnetic ions. T_{1M} and therefore T_{1para}, but not τ_M, vary with the strength of the applied magnetic field.

The ^{2}H NMRD profile for the binary Mn^{2+}–Con A complex (MP) is the same at 5 and 25 °C (Koenig & Brown, 1985), indicating that τ_M is appropriately short. This profile, shown by the dotted line in Figure 2, peaks at about 12 mM^{-1} s^{-1}. Addition of Ca^{2+} to MP to form CMPL is now seen to lower the deuteron relaxivities substantially, suggesting an increase in τ_M, consistent with earlier proton data (Brown et al., 1977). The decrease of the peak ^{2}H relaxivity for CMPL, from 3 mM^{-1} s^{-1} at 25 °C to about 1.8 mM^{-1} s^{-1} at 5 °C, can be attributed to a progressive increase in τ_M, from a value $\ll T_{1M}$ for deuterons to something comparable to T_{1M}. The dashed curve through the 25 °C ^{2}H profile was derived from the curve for the deuteron MP data, Figure 2, simply by increasing τ_M (from 3×10^{-7} to 1.0×10^{-5} s); it displays the essence of the 25 °C CMPL data. Thus, at 5 °C, CMPL must be very much in the slow exchange regime with τ_M limiting the deuteron data. But, as is evident from eq 1, slow exchange for deuterons implies the slow exchange limit for protons as well; therefore, one expects the same relaxivities in this limit for deuterons and protons if there is only one class of water binding sites. As is seen in Figure 2, this is far from true, and as implied by the ^{1}H profiles, the disparity cannot be ascribed to an isotope effect.

The biochemical implications of the foregoing are that the proton NMRD profiles of CMPL must be composite. One contribution must derive from the slowly exchanging water molecules at the same class of sites that generates the deuteron data. These molecules are directly coordinated to the Mn^{2+} ions, and changes in their τ_M are responsible for the decrease in the relaxivity of both protons and deuterons in going from MP to CMPL. The other contribution must be from water molecules in a class of sites from which they exchange rapidly with solvent. These latter molecules would have a contribution to the deuteron relaxation rates that would be about 40-fold less than that for protons, or ≤ 0.25 mM^{-1} s^{-1}, thereby contributing only a small fraction of the observed deuteron relaxivities. [This contribution, as well as outer-sphere corrections (Koenig & Brown, 1985), would have to be included to improve the fit of the dashed curve in Figure 2.] Because these rapidly exchanging waters produce as little ^{1}H relaxivity as is observed in CMPL, they must bind well beyond the inner coordination sphere of the bound Mn^{2+} ions, as is readily seen by comparison with the results for Mn^{2+}–Con A (Brown et al., 1977).

Temperature Dependence of the ^{1}H Profiles. The existence of two classes of sites for exchanging water molecules, one partially exchange limited for ^{1}H relaxation and the other not, suggests that the ^{1}H profiles of CMPL should change their form with temperature (since exchange-limited profiles are field independent and thermally activated). That this is indeed the case is seen in Figure 3. The data can be described as the superposition of two contributions to the NMRD profiles: one, with a relatively high peak near 20 MHz compared to its low-field limiting value, that decreases in relaxivity with increasing temperature and is unaffected by saccharide binding, and a second, relatively flat, that is thermally activated, increases with increasing temperature, and is reduced by binding of saccharide. This would explain the reversal of sign of the temperature dependence of the data for both CMPLC and SCMPL.

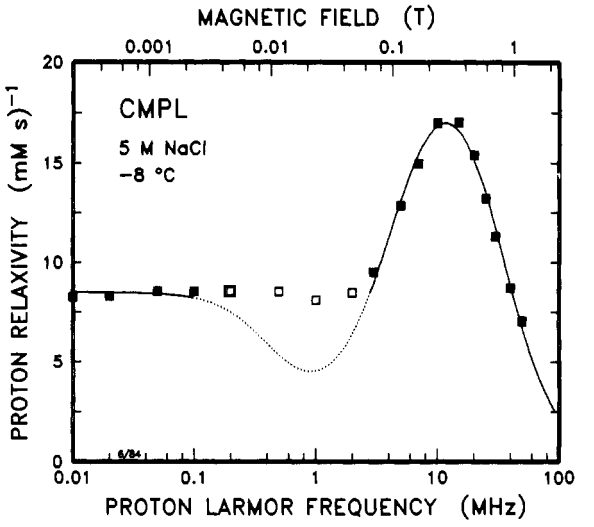

FIGURE 4: Results of a least-squares comparison of the −8 °C data, Figure 3, with theory. The open squares, which were excluded from the comparison, are in a region of field for which the theory, as used, is expected to give a poor representation of the data (Koenig & Brown, 1985). The essence of the fit is that, assuming two equivalent exchanging water molecules, their protons are 4.4 Å from the Mn^{2+} centers and their residence time τ_M is 5.2×10^{-9} s, which is indeed rapid exchange.

The lowest dashed curve in Figure 3 is a rough estimate of the contribution of the exchange-limited sites to the 25 °C proton NMRD profile, computed by using the value for τ_M of 1.0×10^{-5} s, which was obtained from the fitting that gave the curve through the deuteron CMPL profile, Figure 2. A decrease of τ_M of a bit over a factor of 2 can account for the change from 25 to 35 °C. An activation energy consistent with this change would make the contribution of the slowly exchanging class of waters to the −8 °C profile essentially nil; thus, this profile is ostensibly all due to the rapidly exchanging class of water molecules. Accordingly, we performed a least-squares comparison of the −8 °C data with the theory of relaxation, following the protocol suggested recently (Koenig & Brown, 1985). In that work and elsewhere (Koenig & Brown, 1980), it is argued that, for Mn^{2+}–protein complexes for which the ligand field splitting of the electronic levels of Mn^{2+} is very small (and Con A complexes with Mn^{2+} are particularly favorable), the comparison should give good results (for protons) for the parameters of the theory so long as the data in the decade of field centered about 1 MHz are excluded from the fit. The resulting fit is shown in Figure 4; the solid squares represent the data used for the fit, and the solid curve represents the fit. The theory is expected to be inappropriate in the region where the curve is dotted and where the data, open squares, have been excluded. Two equivalent, rapidly exchanging waters were assumed. The fit gave $\tau_M = 5.2 \times 10^{-9}$ s and a distance $r = 4.4$ Å for the Mn^{2+}–proton distance, indicating that the exchanging water molecules are well outside the inner coordination spheres of the Mn^{2+} ions. Values for the other parameters, in the usual notation [cf. Koenig et al. (1971)], are $\tau_V = 1.0 \times 10^{-10}$ s and $\tau_{S10} = \tau_{S20} = 6.5 \times 10^{-10}$ s. The value 4.2×10^{-8} s, computed from Stokes' law, was used for τ_R. [Note that for rapid exchange, as is well-known (Koenig et al., 1971), only the quantity $\tau_M^{-1} + \tau_R^{-1}$ can be obtained from a fit. Therefore, oligomerization of the protein, which increases τ_R, would decrease the derived τ_M value

somewhat, but the fit would be *identical*, and no other parameter would be altered.]

Discussion

The major finding, which derives from the qualitative aspects of Figure 2 and is corroborated by the data of Figure 3, is that there are two classes of sites for exchanging water molecules in CMPL (and SCMPL): a class with molecules that are in relatively slow exchange ($\tau_M \sim 10^{-5}$ s) and a class with molecules that are in relatively rapid exchange ($\tau_M \sim 5 \times 10^{-9}$ s). Binding of saccharide affects the relaxivity contribution of the two sites differently. The slowly exchanging water molecules are ostensibly those coordinated directly to the Mn^{2+} ions, the same ones that are in relatively rapid exchange on Mn^{2+}–Con A (MP) before addition of Ca^{2+} to form CMPL (Brown et al., 1977). Their exchange rate is lowered by saccharide binding. The location of the rapidly exchanging class of water molecules, the existence of which is established for the first time in this work, and shown to have their protons ~4.4 Å from the Mn^{2+} ion, is considered in the following paper in this issue (Bhattacharyya et al., 1985). Saccharide binding does not influence the relaxivity contribution of these waters.

Though it was initially suggested that a comparison of deuteron with proton relaxation data could help quantitate the proton data by resolving ambiguities relating to the derived value of τ_M, and ultimately q, in a rather straightforward way (Burton et al., 1977; Reuben, 1975; Kushnir & Navon, 1984), it has turned out to be otherwise, both in this report and in a recent investigation of Mn^{2+}–carbonic anhydrase and Mn^{2+}–carboxypeptidase (Kushnir & Navon, 1984). In the present case, the reason is that the phenomena are richer than anticipated. Deuterons and protons undergo their predominant paramagnetic interactions with CMPL in different ligand locations on the protein; solvent molecules bound in these locations have different values of τ_M and thereby contribute to relaxation in different relative proportions for deuterons and protons. For carbonic anhydrase and carboxypeptidase, two different classes of sites for exchanging water molecules were also posited: inner and outer coordination positions on the same Mn^{2+} ion. However, another interpretation possible in this instance is that there is only one class of exchanging molecules and that the theory is more complex for Mn^{2+} in a tetrahedral protein environment, as in carbonic anhydrase and carboxypeptidase (Koenig & Brown, 1985). Our results are the first in which it can be said with some confidence that exchanging water molecules liganded in two disparate classes of sites, with different values of τ_M and different relaxivity contributions, have been observed. The conclusions follow from a qualitative comparison of the proton and deuteron NMRD profiles for CMPL. In retrospect, it may be said that the major conclusion could have been conjectured on the basis of the proton relaxation data in Figure 3 alone. Historically, however, the motivation to extend the temperature range, by using high salt concentrations, arose from the desire to confirm the implications of the comparison of the proton and deuteron results from CMPL, and it was this comparison that led to the discovery discussed here and its corroboration by the results shown in Figure 3.

Finally, these findings are a further example of the continued utility of NMRD measurements for the study of the biochemical structure of metalloproteins and were made possible by recent improvements in the instrumentation that have allowed relatively accurate measurements of the small paramagnetic contributions that characterize deuteron NMRD profiles in solutions of metalloproteins. Further application of these techniques, to pea and lentil lectins, is considered in the following paper in this issue (Bhattacharyya et al., 1985).

Registry No. Ca, 7440-70-2; Mn, 7439-96-5; concanavalin, 11028-71-0.

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