Perturbation of the PMR Spectrum of Lysozyme by Co^{+2}

by

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Characteristics of proton magnetic resonance (PMR) spectra of native proteins in aqueous solution are strongly influenced by protein tertiary structure. Consequently, detailed information can be obtained on protein conformation or on interactions that change conformation if one can resolve individual resonances, assign them to particular protein protons, and rationalize the resonance positions in terms of protein structure. Until recently, the usefulness of this spectroscopic technique was severely limited by insufficient resolution. However, with the development of spectrometers operating at higher frequencies (100 MHz and 220 MHz) certain spectral regions of the PMR spectra of small native proteins are sufficiently well resolved to be attractive for detailed analysis.[1,2] Even in these regions resonances frequently overlap and techniques are needed to dissect out the components of partially resolved groups of resonances. We report here one such technique we have found useful for this purpose - the perturbation of protein PMR spectra by addition of paramagnetic Co^{+2} ion to the protein solution. New assignments of proton magnetic resonances of lysozyme made possible by this technique are reported.

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PMR spectra of hen egg white (HEW) lysozyme are presented in Fig. 1 to illustrate the use of this technique. Spectrum (a) shows the "high-field" spectral region of HEW lysozyme (Worthington, 2X crystallized, salt free) obtained

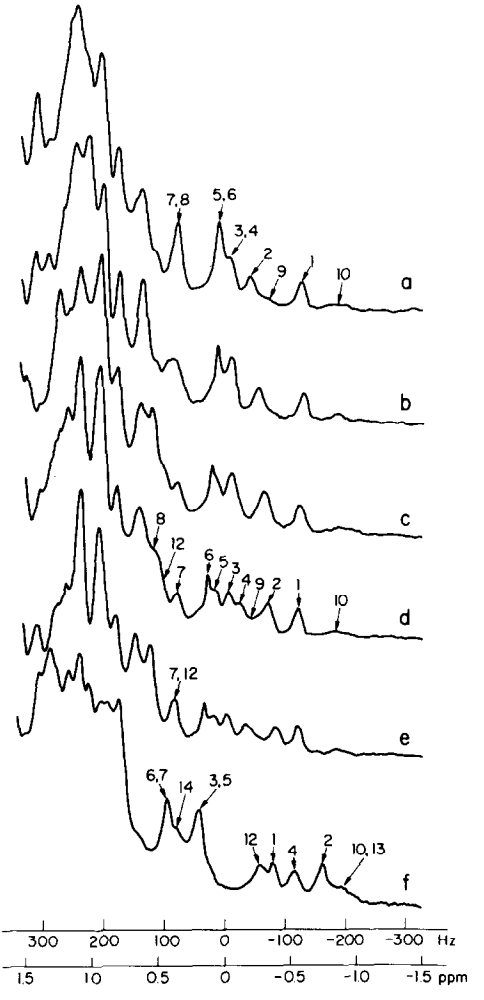

Fig. 1. Perturbation of the high field region of the PMR spectrum of HEW lysozyme by Co^{+2}. Lysozyme: 7×10^{-3} M in D_2O, pD 5.5 at 55°C. Co^{+2} concentrations: (a) 0, (b) 1.75×10^{-3} M, (c) 3.50×10^{-3} M, (d) 7.00×10^{-3} M, (e) 1.05×10^{-2} M, and (f) 1.54×10^{-1} M.

with a Varian 220 MHz PMR spectrometer. Resonance positions are referred to the position of the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) used as an internal reference; positive shifts are to low field. The signal-to-noise ratio was improved by use of a Varian Associates C-1024 computer of average transients. Similar experiments were done with various amounts of $CoCl_2 \cdot 6(D_2O)$ added to the protein solution. Spectra (b)-(f) are representative of the spectral changes induced by Co^{+2}. For these experiments, tetramethylammonium ion was used as a secondary internal reference (its methyl proton resonance occurs at 709 Hz relative to DSS).

In spectrum (a) resonances to high field from 180 Hz are attributed the C<u>H</u> protons (principally methyl protons) of amino acid side chains that in the folded native protein are close to the faces of conjugated rings of aromatic side chains and are therefore subject to high-field shifts from their normal resonance positions induced by the ring-current fields of the conjugated structures.[2] Relatively few protons contribute to the region above 100 Hz and it is therefore particularly attractive for detailed analysis. However, even with the resolution available with a 220 MHz spectrometer, several resonances overlap; only resonances designated 1, 10, and 11 are completely resolved. Resonance 1 with intensity of 3 protons is assumed to arise from a methyl group; resonances 10 and 11 arise from single protons (11 at -435 Hz is not shown in Fig. 1). When Co^{+2} is added to the protein solution, positions of resonances throughout the entire PMR spectrum are perturbed. Here we will consider only changes in the region from 100 Hz to -200 Hz. In the presence of Co^{+2} some resonances are shifted to high field, some to low field, and the magnitudes of the shifts are quite different for different resonances. The Co^{+2}-induced shifts increase regularly as Co^{+2} concentration is increased over a considerable concentration range. Consequently, the relative positions of individual

resonances change gradually as Co^{+2} concentration is changed and one can dissect out the components of initially incompletely resolved groups. In this way, varying Co^{+2} concentration from 0 to 0.15 M we have obtained evidence for 10 individual resonances contributing to the spectral region from 100 to -200 Hz in spectrum (a). In particular, the resonance group initially between 30 and -30 Hz can be seen in spectra (b) to (e) to separate into four resonances (designated 3, 4, 5, and 6). Resonances 1 through 7 appear to correspond in intensity to 3 protons each and are therefore attributed to methyl groups. Resonances 12, 13, and 14 are to low field of 100 Hz until Co^{+2} is added to the protein solution.

The dependences of resonance positions on Co^{+2} concentration are shown in Fig. 2. For clarity, data points are included only for resonances 2, 3, 4, and 6. These are representative of the precision of the data of resonances plotted as solid lines. Measurements for several broader

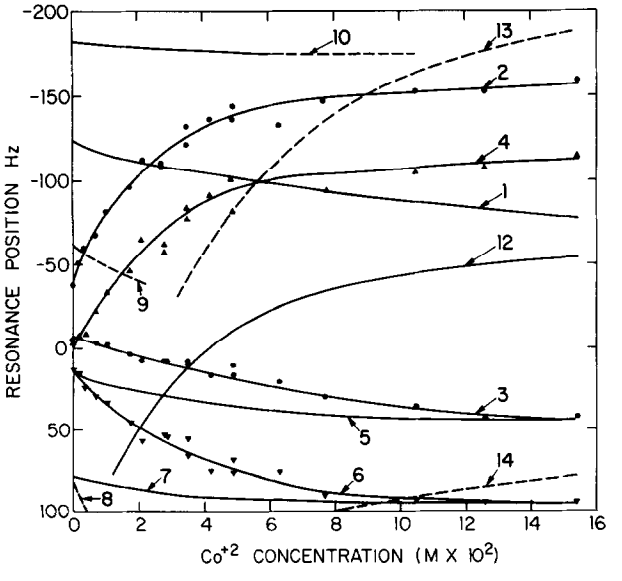

Fig. 2. Resonance positions as a function of Co^{+2} concentration. Lysozyme 7 x 10^{-3} M in D_2O, pD 5.5 at 55°C.

resonances plotted as dashed lines were less precise. The resonance positions are plotted only through the field regions where they could be clearly detected.

The Co^{+2}-induced shifts are presumed to result from binding of Co^{+2} to lysozyme. Such shifts could arise from a conformational change of lysozyme, from the paramagnetism of bound Co^{+2}, or from a combination of these mechanisms. Studies from another laboratory* indicate, however, that the conformation of lysozyme is not affected by Co^{+2}, and we conclude, therefore, that the Co^{+2}-induced shifts are entirely a consequence of Co^{+2} paramagnetism. Clearly, exchange between free protein and the protein/Co^{+2} complex is fast (on a msec. time scale) since time-averaged spectra are observed rather than separate spectra for the individual species. The curves for resonances 2, 4, and 6 in Fig. 2 indicate that the Co^{+2}-induced shift is almost complete at 0.15 M Co^{+2} for these resonances. If we designate the complete shift of a proton resonance as Δ_T and assume a one-to-one binding of lysozyme and Co^{+2}, we can compute the expected fractional shift Δ/Δ_T as a function of the Co^{+2} added to the lysozyme solution. Such a computed curve for an association constant of 57 liters $mole^{-1}$ is shown in Fig. 3. The experimental data for resonances 2, 4, and 6 replotted on this basis are in good agreement with this model, and we conclude that perturbation of these resonances arises from binding of Co^{+2} at a single site on lysozyme. The

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*J. A. Rupley and J. D. Sakura, Department of Chemistry, University of Arizona, have advised us, in a private communication that circular dichroism spectra of lysozyme in aqueous solution at pH 5.5 show no evidence of a lysozyme conformational change on addition of Co^{+2} up to a concentration of 0.1 M. They find, however, that Co^{+2} does reduce the activity of lysozyme and that Zn^{+2} is an even more effective inhibitor of lysozyme activity.

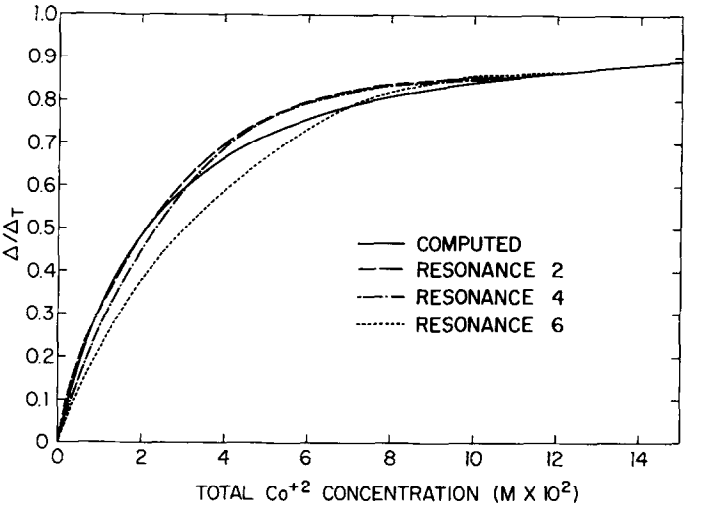

Fig. 3. Computed fractional resonance shifts as a function of Co^{+2} concentration.

curves of resonances 5, 12, and 13 also appear to be in accordance with this binding. The Co^{+2}-induced shifts of resonances 1 and 3 may still be changing at the highest Co^{+2} concentrations studied; they are too small to permit a firm conclusion. If so, these resonances may also be perturbed by Co^{+2} binding at a second site. Thus, although there may be several binding sites for Co^{+2} on HEW lysozyme with different association constants, at Co^{+2} concentrations up to 0.15 M the PMR spectral changes appear to result almost entirely from binding of Co^{+2} at a single site.

Some of the Co^{+2}-induced shifts are strongly dependent on the pD of the lysozyme/Co^{+2} solution. To illustrate this point, positions of a number of resonances are plotted as a function of pD at a fixed Co^{+2} concentration in Fig. 4. At pD 4, the resonance positions are similar to those in a Co^{+2}-free solution at the same pD (indicated by X's in Fig. 4). Except for resonance 1 which we have noted may be perturbed by Co^{+2} at a second binding site, they shift with increasing pD following an apparent titration curve with a pK of about 5.2. The apparent pK varies from about 5 to 6 depend

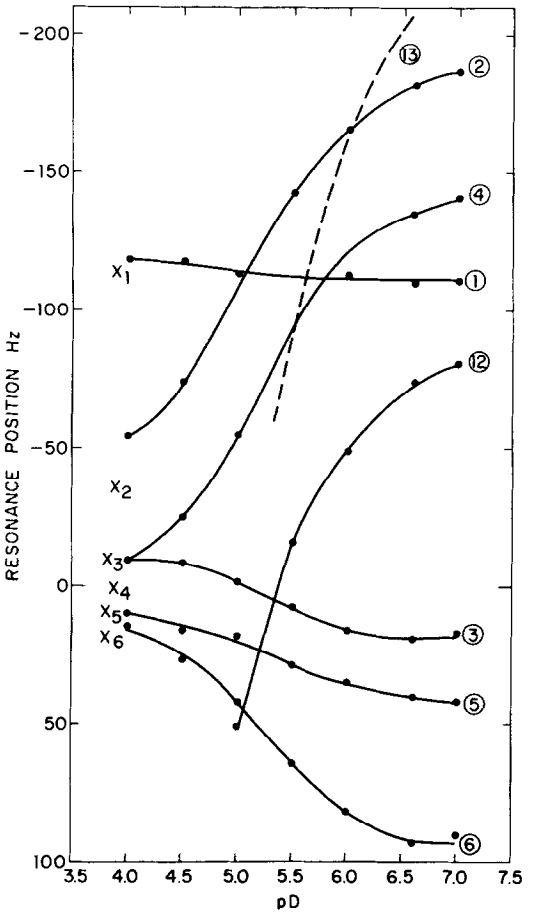

Fig. 4. Resonance positions as a function of pD. Lysozyme 7×10^{-3} M in D_2O at 55°C. Co^{+2}, 4.2×10^{-2} M.

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ing on Co^{+2} concentration, decreasing with increasing Co^{+2}. Without Co^{+2}, changes in these resonance positions from pD 4 to 7 are relatively small. Thus, we conclude that the site that binds the Co^{+2} which perturbs these resonances becomes available by titration of one or more functional groups with a pK around 6 (the apparent pK decreasing with increasing Co^{+2} concentration because of competition between Co^{+2} and H^{+} for the same site). This site does not appear to involve the single histidine residue of lysozyme since the C-2 proton resonance of this residue was not

appreciably shifted by addition of Co^{+2}. We suggest that it probably involves one or more carboxyl groups of aspartic or glutamic acid residues. A weak interaction of Co^{+2} with ionized carboxyl groups was noted previously in studies of binding of Co^{+2} to amino acids.[3] The stronger interaction with lysozyme indicates that more than one functional group is involved. Inspection of the structure of native lysozyme (as determined by X-ray spectroscopy[4]) indicates that the Co^{+2} binding site could be provided by the carboxyl groups of glu-35 and asp-52, which are at the active site of enzyme. Furthermore, although the pK of 6 is high for a carboxyl group, evidence has been presented[5] that the pK of glu-35 is 6.3. The inhibition of lysozyme activity by metal ions noted by Rupley and Sakura (see footnote above) is consistent with this interpretation.

The spectral region from 100 to -200 Hz was examined from 35°C to 65°C for a Co^{+2} concentration of 3.5×10^{-3} M at pD 5.7. The Co^{+2}-induced shifts are influenced by temperature, but the effects are small compared to those of changing pD discussed above. The Co^{+2}-induced shifts of lysozyme resonances were not affected by oxygen. The high field PMR spectra of lysozyme were not detectably perturbed at pD 5.5 by addition of Fe^{+2}, Ni^{+2}, or Zn^{+2} at concentrations up to 3.5×10^{-2} M.

We conclude that the Co^{+2}-induced shifts can be attributed to the rather unique ability of Co^{+2} to produce paramagnetic contact shifts by coordinative binding.[3] The observed shifts are probably pseudocontact or dipolar in origin, although the possibility of their arising from isotropic hyperfine contact interaction cannot be ignored.[6] From information provided by this study and several other lines of investigation that will be described elsewhere, we tentatively assign the methyl group resonances designated 1 to 6 as follows: resonances 1 and 3 to leu-17, resonances 2 and 4 to the δ and γ methyls of ileu-98 respectively, resonance 5 to leu-8, and

resonance 6 to met-105. These residues are in various regions of lysozyme[4] and from 10 to 14 Å from asp-35 where we believe Co^{+2} is bound. Thus, it appears that Co^{+2} bound at a single site on lysozyme can perturb resonance positions of protons throughout most of the protein molecule. Further studies to map this perturbing field are in progress.

We have found that Co^{+2} also causes changes in the PMR spectrum of human lysozyme similar in magnitude to those observed for HEW lysozyme. Shifts of resonances of pancreatic ribonuclease and horse ferrocytochrome-c have also been noted on addition of Co^{+2} to neutral solutions of these proteins. For these latter proteins, the spectral changes, for a particular Co^{+2} concentration, were much smaller than for the lysozymes.

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