Cadmium-113 Shielding Tensors in Cadmium-Substituted Metalloproteins

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Abstract: Solid-state [113]Cd NMR CP/MAS spectra of two [113]Cd-substituted lyophylized metalloproteins, parvalbumin and concanavalin A, were obtained. Line widths of the [113]Cd MAS spectra demonstrate a considerable degree of local disorder at the metal binding region of both proteins in the lyophylized powder. Hydration of the proteins by vapor diffusion with D_2O as solvent narrows the [113]Cd line by approximately a factor of 4, demonstrating restoration of considerable local structure that is lost upon lyophylization. The important consequence of the rehydration is a corresponding increase in signal/noise ratio making the acquisition of solid-state spectra of [113]Cd-substituted proteins practical in reasonable times (4-8 h). Simplex optimization of calculated spinning side band intensities to those obtained experimentally provides estimates of the principal values of the [113]Cd chemical shielding tensor for the unresolved calcium-binding sites in parvalbumin and for the resolved manganese (S1) and calcium (S2) sites in concanavalin A. These data are discussed in terms of previous information and correlations concerning cadmium tensors in general and in terms of the utility of solid state NMR for studying metal-protein interaction in metalloproteins involving zinc and calcium. The dramatic changes in the spectra of both proteins demonstrate that the hydration level of the protein affects the local metal site geometry significantly and that a distribution of states is required to account for the failure of MAS to narrow the [113]Cd NMR lines.

[113]Cd NMR has been used extensively in recent years to study the metal binding properties and conformational states of a number of zinc- and calcium-dependent proteins.[1-3] The facility with which the divalent cadmium cation can replace zinc and calcium in biological systems, the relatively large chemical shift range (800 ppm) for cadmium in coordination with various oxygen, nitrogen, and sulfur containing ligands, and the relatively favorable receptivity of the cadmium nuclide are some of the major advantages. Since [113]Cd spectra of adequate signal/noise ratio are readily obtained in moderately short (4-8 h) times on protein solutions containing cadmium in the concentration range of 2-4 mM, high-resolution [113]Cd NMR has become an attractive method for elucidating the physical and biological properties of a growing number of biological systems.

In contrast to the utility of [113]Cd NMR, however, potential complications can arise due to dynamical effects such as exchange averaging of cadmium chemical shifts in biological systems. In some cases, these effects have precluded the observation of specific groups of resonances in the [113]Cd NMR spectra of protein systems.[4-6] There are two apparent solutions to problems associated with dynamics in such systems. One may be to supercool the solution, as demonstrated by the Ackermans[7] and by others,[8] the consequence of which is to drive the system into the slow exchange regime while maintaining the aqueous environment. Although such an approach appears to be promising, for providing resolution of individual [113]Cd lines, it has not yet been successfully applied to the [113]Cd NMR spectroscopy of metalloproteins. The second approach to circumventing the uncertain dynamics of cadmium in metalloprotein systems is to employ the solid-state NMR techniques which have already been successfully employed with a number of other biological systems.[9,10]

Despite the low sensitivity and resolution problems inherent in [113]Cd CP/MAS experiments on metalloprotein systems, we have attempted to observe [113]Cd solid-state spectra in such systems. We have chosen two proteins, parvalbumin and concanavalin A, because these are representative of zinc- and calcium-dependent proteins and also because of their availability in sufficient quantities (150-300 mg) for solid-state NMR experiments. Furthermore, we have succeeded in obtaining CP/MAS spectra of cadmium in these proteins in a moderately short time interval (4-8 h). From the CP/MAS spectra obtained, estimates of the principal values of the cadmium chemical shielding tensors for

cadmium bound to the protein metal binding site(s) have been obtained. These results are compared with previous information on [113]Cd-shielding tensors obtained from simpler systems.

Experimental Section

Parvalbumin (component 3, ref 11) was prepared from fresh carp muscle obtained locally by scale-up of the previously described method. The protein was determined to be homogeneous by electrophoresis in acrylamide gels and by comparing the results of amino acid analyses to the known amino acid composition of the carp protein. The purified protein was dialyzed extensively at 4 °C against several changes of 10 mM Tris buffer at pH 8.5 containing 0.1 M potassium chloride and 10 mM ethylenediaminetetraacetic acid. The protein was subsequently dialyzed exhaustively against deionized water, and the resulting calcium-free protein was lyophylized and stored in a desiccator at -20 °C.

The [113]Cd-substituted parvalbumin (100-500 mg) was prepared by dissolving the apoparvalbumin in 5-20 mL of deionized water at 4 °C and adding 1 equiv of 95.3% isotopically pure [113]Cd (U.S. Services, Inc.) in the form of the chloride. The protein was subsequently lyophylized and stored as the dry powder at -20 °C.

Concanavalin A (from the Jack Bean) was purchased from Sigma Chemical Co. (product No. C-2631). Demetalized protein was prepared by adaptation of previous methods.[12,13] One gram of protein containing 5 g of sodium chloride was stirred with 50 mL of deionized water. The pH was adjusted to 1.3 with 2 M HCl. After 45 min at room temperature, the sample was centrifuged and the insoluble material was discarded. The clear supernatant representing 54% of the original protein was lyophylized after adjusting the pH to 5.5 with 0.01 M NaOH. The

(1) (a) Ellis, P. D. In "The Multinuclear Approach to NMR Spectroscopy"; Lamberg, J. B., Riddell, F. B., Eds.; D. Reidel: Boston, 1983; p 457. (b) Ellis, P. D. *Science (Washington, D.C.)* **1983**, *221*, 4616.
(2) Vogel, H. J.; Drakenberg, T.; Forsen, S. In "NMR of Newly Accessible Nuclei"; Laszlo, P., Ed.; Academic Press: New York, 1983; Vol. 1, p 157.
(3) Armitage, I. M.; Boulanger, Y. In "NMR of Newly Accessible Nuclei"; Laszlo, P., Ed.; Academic Press: New York, 1983; Vol. 2, p 337.
(4) Ellis, P. D.; Strang, P.; Potter, J. D. *J. Biol. Chem.* **1984**, *259*, 10348.
(5) Armitage, I. M.; Schoot-Uiterkamp, A. J. M.; Chlebowski, J. F.; Coleman, J. E. *J. Magn. Reson.* **1978**, *29*, 374.
(6) Armitage, I. M.; Pajer, R. T.; Schoot-Ueterkamp, A. J. M.; Chlebowski, J. F.; Coleman, J. E. *J. Am. Chem. Soc.* **1976**, *98*, 5710.
(7) Ackerman, M. J. B; Ackerman, J. J. H. *J. Phys. Chem.* **1980**, *84*, 3151.
(8) Jakobsen, H. J.; Ellis, P. D. *J. Phys. Chem.* **1981**, *85*, 3367.
(9) Opella, S. J. *Annu. Rev. Phys. Chem.* **1982**, *33*, 533.
(10) Torchia, D. A.; VanderHart, D. L. In "Topics in Carbon-13 NMR Spectroscopy"; Levy, G. C., Ed.; Wiley: New York, 1979; Vol. 3, p 325.
(11) Pechère, J. F.; Demaille, J.; Capony, J. P. *Biochim. Biophys. Acta* **1971**, *236*, 391.
(12) Brown, R. D.; Brewer, C. F.; Koenig, S. H. *Biochemistry* **1977**, *16*, 3883.
(13) Palmer, A. R.; Bailey, D. B.; Behnke, W. D.; Cardin, A. D.; Yang, P. P.; Ellis, P. D. *Biochemistry* **1980**, *19*, 5063.

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8192 *J. Am. Chem. Soc., Vol. 107, No. 26, 1985*

Marchetti et al.

lyophylized protein was dissolved in 8 mL of deionized water and the pH was adjusted to 6.0. Subsequently, 2.5 equiv of 95.3% isotopically pure ^{113}Cd was added as the chloride. The solution was allowed to stand for 36 h at room temperature and was then lyophylized. The lyophylized protein was stored desiccated at -20 °C. Concanavalin A exists in two forms,[12,13] locked and unlocked. Since only the locked conformation gives rise to cadmium NMR signals from protein-bound cadmium ions, and since sufficient time was allowed for the locking dynamics, this is the form which was generated as described above and is the form used in the present study.

Lyophylized protein samples were equilibrated with D_2O (Aldrich, 99.8% D) by vapor diffusion. The lyophylized (dehydrated) protein sample (120–250 mg) was placed in a small (3 × 3 cm) plastic weighing boat which was then placed in a 100 mL evaporating dish along with a small beaker containing a few millilters of D_2O. A glass plate was sealed to the top of the evaporating dish with vacuum grease. The equilibration was carried out at 4 °C for several days until the desired extent of D_2O binding was attained. The extent of D_2O binding to the protein was determined from the weight difference of the sample before and after equilibration using the initial weight of the lyophylized protein.

The extent of D_2O binding to protein samples is expressed in all cases as grams of D_2O per gram of protein. Initial plots of the extent of solvent binding with time at 4 °C and at room temperature typically gave exponential profiles which leveled off after several days. D_2O contents beyond the extent of 0.6 g of D_2O/g of protein required excessive equilibration times, on the order of many weeks. Such samples, although still solid, were tacky. This made packing and unpacking rotors difficult. No dramatic improvement in spectral resolution (line width) was achieved with these samples, compared to the more easily handled free-flowing powders having D_2O contents in the range of 0.25–0.40 g of D_2O/g of protein.

Data acquisition for CP/MAS experiments was according to a standard Hartmann–Hahn spin-locked cross-polarization pulse sequence.[14] All experiments were performed on a modified wide-bore WP-200 spectrometer.[15] Typically, the sample was packed into a 7 mm o.d. aluminum oxide rotor and was spun at speeds ranging from 1 to 3 kHz with use of a Doty designed MAS probe (Doty Scientific, Inc.). A typical contact time was 2 ms along with a 2-s recycle time.

The principal elements of the cadmium-shielding tensors were determined from the ^{113}Cd MAS spectra by Simplex optimization of computed spinning sideband intensities to those obtained experimentally.[16,17] Good fits to the experimental intensities were obtained in all cases. The optimization procedure involved a two-parameter fit which resulted in best estimates for the anisotropy (δ) and for the asymmetry parameter (η). These two parameters, along with the rotor spinning speed and the spectrometer frequency, determine the relative sideband intensities in the MAS spectra.[16] From these optimized parameters, the best estimates of the principal values of the cadmium-shielding tensors were obtained.

Results and Discussion

The ^{113}Cd CP/MAS spectrum of the ^{113}Cd-substituted parvalbumin (mol wt 12000) obtained at 44.40 MHz with a rotor speed of 2.6 kHz is presented in Figure 1. The negative anisotropy of the parvalbumin cadmium tensor is apparent from the relative intensities of the spinning sidebands. The line width is 1300 Hz and the isotropic cadmium chemical shift corresponds to the peak at -90.2 ppm relative to cadmium perchlorate which was used as a reference standard.

The result (Figure 1) of our initial attempt to characterize the ^{113}Cd-shielding tensor in solid parvalbumin is disappointing when the spectrum is compared to those obtained on a number of simple cadmium complexes. The protein MAS spectrum is broad but in qualitative agreement, however, with the MAS spectrum of ^{113}Cd-substituted parvalbumin first reported by Armitage (Figure 2, ref 3). The spectrum reported by Armitage was taken at a 4.2 kHz rotor spinning speed and gave an isotropic cadmium shift of -120 ppm and a line width in excess of 1000 Hz. The origin of this difference in isotropic chemical shift values reported here and by Armitage is unknown at present. The large line widths
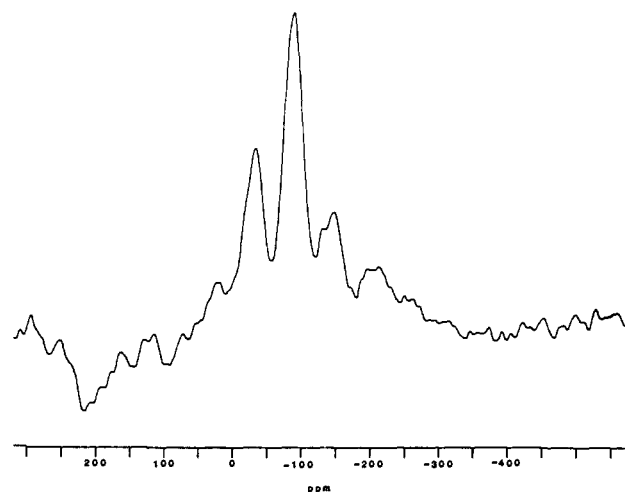

Figure 1. The ^{113}Cd CP/MAS spectrum of ^{113}Cd-substituted lyophylized parvalbumin. The sample was prepared without equilibration with solvent. The total number of scans accumulated was 35600 at a 2.6 kHz rotor spinning rate; 2 ms contact time; 4.0 s recycle delay. The applied line broadening was 400 Hz.

observed in the parvalbumin MAS spectra referred to above most likely arise from the same source which may be due to structural heterogeneity or dynamical effects of motions close to the rotor frequency. The native properties of proteins are dependent upon maintenance of a native conformation which depends upon a number of solvent–protein interactions affecting both the structure and dynamics of the protein.[18-23] The hydration of the protein is maintained upon crystallization, but it is obviously lost upon lyophylization. In a number of cases, the dehydration effects are reversible and the activity is fully restored on rehydrating the lyophylized powder. Rehydration thus restores native conformation.

The addition of water may complicate the cross-polarization spin dynamics, affecting T_{IS}, T_1^H, and $T_{1\rho}^H$. In the last two cases, proton magnetization equilibration or spin diffusion may lead to a major sacrifice in signal to noise. These potential problems may be minimized, though not eliminated, by using D_2O as solvent in rehydrating protein samples.

The effect on the ^{113}Cd spectrum of equilibrating the lyophylized protein with D_2O to a level of 0.29 g of D_2O/g of protein is shown in Figure 2. The spectra in parts a and b of Figure 2, taken at rotor speeds of 1.0 and 1.6 kHz, respectively, indicate that there is significant line narrowing as a result of D_2O binding to the protein in the solid state. The resulting line width was 360 Hz at the 1.6 kHz spinning speed compared to the 1300 Hz line width observed for the unequilibrated parvalbumin sample (Figure 1). The spectra in parts a and b of Figure 2 were each obtained in 3.8 h, a substantial improvement in signal/noise ratio over the spectrum obtained from the unequilibrated sample. The ^{113}Cd CP/MAS spectrum presented in Figure 2c represents 0.8 h of accumulation at a rotor speed of 2.9 kHz and clearly shows an isotropic cadmium chemical shift of -83.8 ppm.

Figure 3 is the ^{113}Cd static powder spectrum taken under conditions which allow cross polarization for a sample of ^{113}Cd-substituted parvalbumin whose D_2O content was 0.43 g of D_2O/g of protein. This spectrum represents 15.4 h of accumulation, and although the edges of the powder spectrum are less distinct than would be anticipated for an ideal powder spectrum, it does demonstrate the nonaxial nature of the parvalbumin-shielding tensor.

(14) Pines, A.; Gibby, M E.; Waugh, J. S. *J. Chem. Phys.* **1973**, *59*, 569.

(15) Inners, R. R.; Doty, F. D.; Garber, A. R.; Ellis, P. D. *J. Magn. Reson.* **1981**, *45*, 503.

(16) (a) Maricq, M. M.; Waugh, J. S. *J. Chem. Phys.* **1979**, *70*, 3300. (b) Herzfeld, J.; Burger, A. E. *J. Chem. Phys.* **1980**, *73*, 602.

(17) Box, G. E. P.; Hunter, W. G.; Hunter, J. S. "Statistics for Experiments. An Introduction to Design, Data Analysis, and Model Building"; John Wiley & Sons, Inc.: New York, New York, 1978.

(18) Hinz, H. J *Annu. Rev. Biophys. Bioeng.* **1983**, *12*, 285.

(19) Jaenicke, R. *Annu. Rev. Biophys. Bioeng.* **1981**, *10*, 1.

(20) Richards, F. M. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 151.

(21) Maikinen, M. W.; Fink, A. L. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 301.

(22) Berendsen, H. J. C. In "Water, A Comprehensive Treatise"; Franks, F., Ed.; Plenum Press: New York, 1975; Vol. 5, p 293.

(23) Finney, J. L. In "Water, A comprehensive Treatise"; Franks, F., Ed.; Plenum Press: New York, 1979; Vol. 6, p 47.
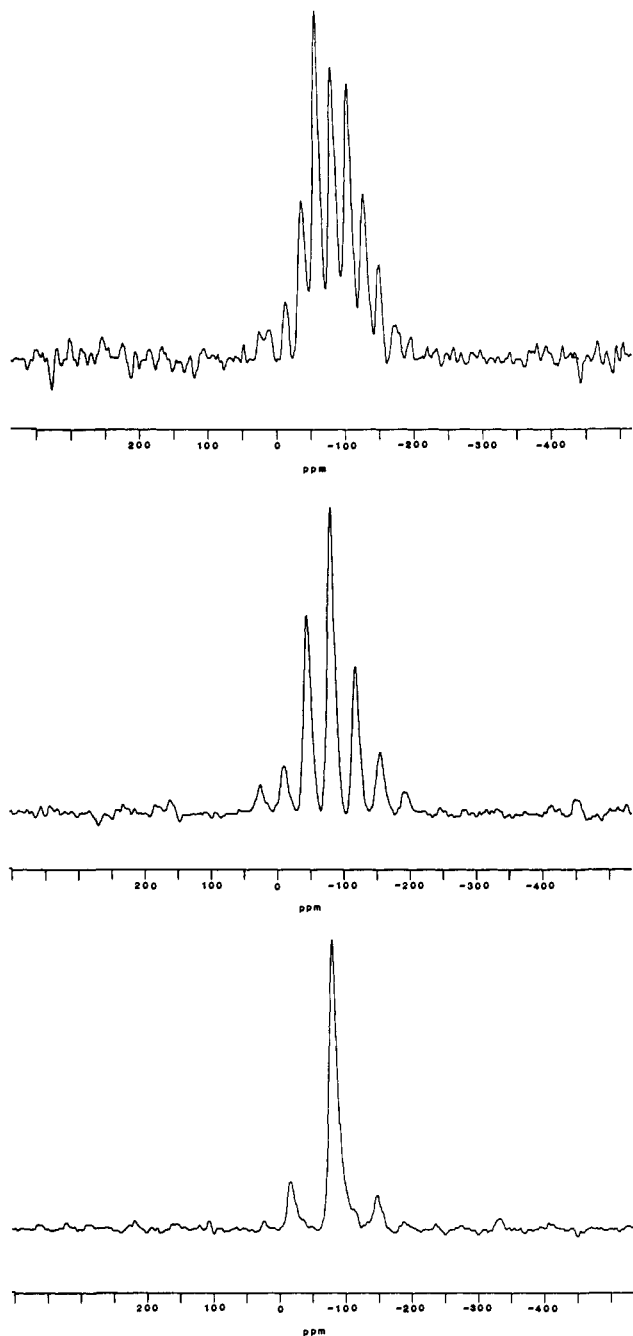
¹¹³Cd Shielding Tensors in Cd-Substituted Metalloproteins

J. Am. Chem. Soc., Vol. 107, No. 26, 1985 8193

Figure 3. ¹¹³Cd static powder spectrum of ¹¹³Cd-substituted lyophylized parvalbumin. The sample was equilibrated with D_2O (0.43 g of D_2O/g of protein). A total of 55 600 scans were taken with a 1.0 s recycle delay; 2 ms contact time; and applied line broadening of 400 Hz.

Figure 4. ¹¹³Cd line widths vs. D_2O content (w/w) taken from CP/MAS spectra of ¹¹³Cd-substituted lyophylized parvalbumin samples having differing D_2O contents.

Figure 2. ¹¹³Cd CP/MAS spectrum of ¹¹³Cd-substituted lyophylized parvalbumin. The protein was equilibrated with D_2O to the extent of 0.29 g of D_2O/g of protein. Instrumental parameters are the following: 2 ms contact time; 2.0 s recycle delay; 200 Hz applied line broadening. The rotor speed and number of scans were (a, top) 1.0 kHz, 6916; (b, middle) 1.6 kHz, 6916; (c, bottom) 2.9 kHz, 1400.

D_2O binding to the lyophylized (dehydrated) protein results in a significant reordering of the cadmium coordination environment which appears to be substantially disordered in the dehydrated protein. The degree of disorder is reflected in the observed line width as a function of D_2O content as shown in Figure 4 for parvalbumin. The lower limit on the line widths taken from CP/MAS spectra of parvalbumin samples having differing D_2O contents is approximately 200 Hz (4.5 ppm) and occurs at a D_2O/protein ratio above 0.6.

The cadmium-coordination sites in parvalbumin have been well characterized for the carp muscle protein with X-ray diffraction techniques.[24-33] The two cadmium binding sites in parvalbumin
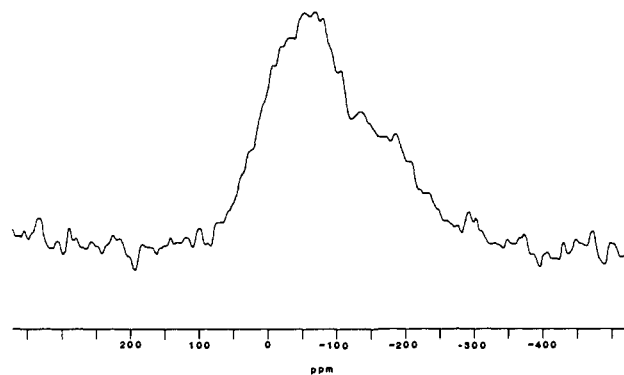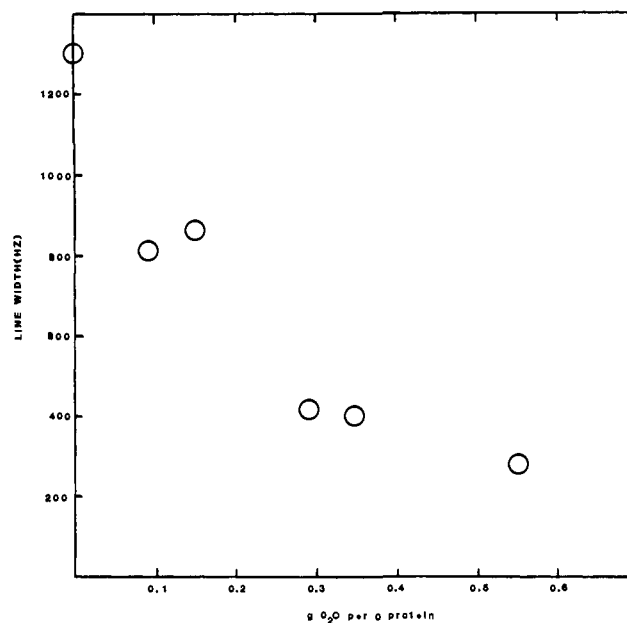
chelate cadmium with high affinity ($K_d = 1 \times 10^{-8}$ M).[34] The X-ray structure shows the presence of six α-helical domains in the protein structure, usually labeled A through F. The loops between the CD and EF helices constitute two metal-binding sites having approximate octahedral symmetry. The section of polypeptide chain between the two helices that constitute each site loops around the metal atom to position the side chains of amino acids such as aspartate and glutamate residues and possibly a water molecule in the cadmium-coordination sphere. For parvalbumin, only oxo ligands are coordinated to cadmium.

High-resolution NMR studies on ¹¹³Cd-substituted parvalbumin from carp muscle yield two sharp resonances in the cadmium NMR spectrum at -93.8 and -97.5 ppm relative to cadmium perchlorate.[35] The two cadmium-coordination environments are

(24) Kretsinger, R. H.; Dangelat, D.; Bryan, R. F. *J. Mol. Biol.* **1971,** *59*, 213.

(25) Coffee, C. J.; Bradshaw, R. A. *J. Biol. Chem.* **1973,** *248*, 3305.

(26) Kretsinger, R. H.; Nockolds, C. E. *J. Biol. Chem.* **1973,** *248*, 3313.

(27) Hendrickson, W. A.; Karle, J. *J. Biol. Chem.* **1973,** *248*, 3327.

(28) Tufty, R. M.; Kretsinger, R. H. *Science* **1975,** *187*, 167.

(29) Moews, P. C.; Kretsinger, R. H. *J. Mol. Biol.* **1975,** *91*, 229.

(30) Kretsinger, R. H.; Barry, C. D. *Biochim. Biophys. Acta* **1975,** *405*, 40.

(31) Moews, P. C.; Kretsinger, R. H. *J. Mol. Biol.* **1975,** *91*, 201.

(32) Sowadski, J.; Cornick, G.; Kretsinger, R. H. *J. Mol. Biol.* **1978,** *124*, 123.

(33) Cavé, A.; Saint-Yves, J.; Sward, M.; Thulin, E.; Lindman, B. *Mol. Cell. Biochem.* **1982,** *44*, 161.

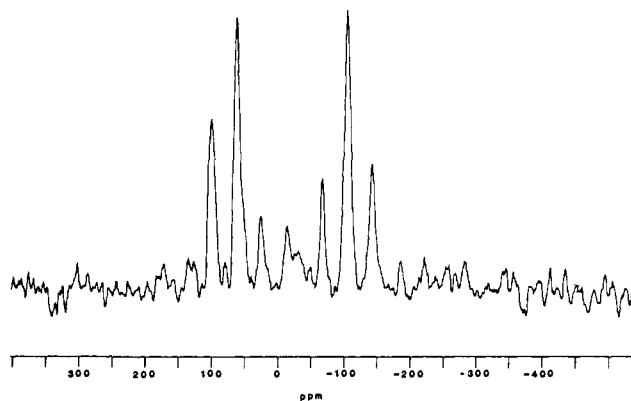(34) Haiech, J.; Derancourt, J.; Pechère, J.; Demaille, J. G. *Biochemistry* **1979,** *18*, 2752.

Figure 5. ^{113}Cd CP/MAS spectrum of ^{113}Cd-substituted lyophilized concanavalin A. The sample was equilibrated with D_2O to the extent of 0.41 g of D_2O/g of protein. The number of scans taken was 13964; 1.7 kHz rotor spinning rate; 2 ms contact time; 2 s recycle delay; and 200 Hz applied line broadening.

Table I. Cadmium Shielding Tensor Data[a] for Parvalbumin and Concanavalin A

protein	$\Delta\sigma$	η	$\bar{\sigma}$	σ_{11}	σ_{22}	σ_{33}
parvalbumin	−127.6	0.71	−83.4	−10.65	−71.02	−168.5
Con A (S1)	−133.2	0.00	59.1	104.0	103.1	−29.65
Con A (S2)	113.1	0.86	−108.4	−178.6	−113.4	−32.96

[a] Parameters are defined as follow: $\Delta\sigma = 3/2\delta = {}^3/_2(\sigma_{33} - \bar{\sigma})$; $\bar{\sigma} = {}^1/_3(\sigma_{11} + \sigma_{22} + \sigma_{33})$; $\eta = (\sigma_{22} - \sigma_{11})/\delta$. According to the above convention, δ is the anisotropy, $\bar{\sigma}$ is the isotropic shift, and η is the asymmetry parameter.

very similar as indicated by the similar cadmium chemical shift values cited above for the two sites and by the structural details of the two sites in terms of the nature of the ligands coordinated to cadmium and in terms of the individual site geometries. It is therefore not unexpected that the two cadmium tensors in parvalbumin are unresolved in the ^{113}Cd CP/MAS spectra presented above.

Experiments similar to those described for parvalbumin were carried out on the ^{113}Cd-substituted concanavalin A (Monomer mol wt 25500). As indicated in Figure 5, two distinct cadmium-shielding tensors are observed for the D_2O-equilibrated protein. For concanavalin A, the effect of hydration with D_2O is even more dramatic than the effect on parvalbumin in that no CP/MAS spectrum could be observed for the unequilibrated protein after 12 h of data acquisition. In contrast to parvalbumin wherein the ligands to cadmium are all oxygen donors or water, concanavalin A has two different types of metal-binding sites, designated S1 and S2. In the native protein, the S1 site is occupied by manganese and the S2 site is occupied by calcium. Both of these sites are known to bind cadmium as has been demonstrated with high-resolution NMR spectroscopy.[13] The S2 site is composed exclusively of oxygen donors in coordination with the metal ion, while the S1 site has in addition to oxo ligands a single imidazole nitrogen coordinated with cadmium. The clear resolution of the two tensors in the CP/MAS spectrum of concanavalin A is expected since nitrogen ligands are known to have a deshielding influence on cadmium chemical shifts.[1,3]

The cadmium-shielding tensors for the spectrum in Figure 5 reflect isotropic chemical shifts of 59.1 and −108 ppm, respectively. These values correspond reasonably to the values of 46 and −125 ppm obtained in the high-resolution NMR spectra of the same system.[13] The spectrum in Figure 5 represents a total accumulation of 7.8 h for a protein sample having a D_2O content of 0.41 g of D_2O/g of protein. The observed line widths in the case of concanavalin A CP/MAS spectra are 280 and 340 Hz (6.3 and 7.6 ppm) for cadmium at the S1 and S2 sites, respectively.

The cadmium-shielding tensor parameters for the metal-binding sites in parvalbumin and concanavalin A are presented in Table I. Some interesting observations may be made. The calcium-binding sites in parvalbumin and the calcium site (S2) in concanavalin A have similar isotropic cadmium chemical shifts of −83.4 and −108.4 ppm, respectively. These shifts lie in the chemical shift range characteristic of the cadmium cation coordinated exclusively with oxygen-containing ligands. The shifts agree fairly well with results from high-resolution NMR experiments. It is also notable that the calcium sites for these two proteins have similar asymmetry parameters of 0.71 and 0.86, respectively. These similarities may well reflect the general sim-

ilarity in ligand type and coordination geometry for the calcium-binding sites under consideration.

This point is supported by X-ray data available for the two proteins. In parvalbumin,[24-33] the two calcium-binding sites may be viewed as approximate octahedra which are related to one another by an approximate twofold rotation axis. This axis relates the C and D helices associated with one metal site to the E and F helices of the second binding site. The CD calcium is 6 coordinate, involving two aspartate carboxylates, a serine hydroxyl group, a phenylalanine peptide backbone carbonyl, and two glutamate carboxylates. The EF calcium site is of very similar coordination geometry but has been reported to be 8 coordinate. It involves three aspartates, one of which is bidentate, a lysine backbone carbonyl, a bidentate glutamic acid, and a water molecule.

By comparison, the metal-binding site of concanavalin A[36-48] may be viewed as a binuclear complex of two polyhedra which share a common edge. The calcium ion in the S2 site of concanavalin A is 7 coordinate, involving a bidentate aspartate, an amide carbonyl from asparagine, a backbone carbonyl from tyrosine, an aspartate which also bridges to the nearby manganese site, and two water molecules. Although the coordination numbers for the calcium-binding sites in parvalbumin and concanavalin A may differ, an overall similarity exists between ligand coordination environments in these two proteins.

Although the anisotropies of the cadmium-shielding tensors corresponding to the calcium-binding sites in the two proteins are of about the same magnitude, their signs are different. The difference in sign is difficult to rationalize without knowledge of the orientation of each tensor in a suitable molecular reference frame, information which is clearly not available from these experiments.

The manganese (S1) site of concanavalin A is 6 coordinate with cadmium coordination involving two aspartate, one glutamate, and two water molecules. In addition, there is a cadmium−nitrogen bond involving the imidazole group of histidine-24 of the protein. For this reason, the coordination environment at the S1 site is distinctly different from that of calcium at the S2 site in concanavalin A.

From the X-ray data, the ligand geometry for cadmium at the S1 site in concanavalin A is described as being more nearly octahedral compared to the calcium (S2) site.[44] The cadmium chemical shielding environment at the nitrogen-containing site

(35) Drakenberg, T.; Lindman, B.; Cavé, A.; Parello, J. *FEBS Lett.* **1978**, 92, 346.

(36) Kalb, A. J.; Levitzki, A. *Biochem. J.* **1968**, 109, 669.

(37) Shoham, M.; Kalb, A. J.; Pecht, I. *Biochemistry* **1973**, 12, 1914.

(38) Hardman, K. D.; Wood, M. K.; Schiffer, M.; Edmundson, A. B.; Ainsworth, C. F. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, 68, 1393.

(39) Weinzierl, J.; Kalb, A. J. *FEBS Lett.* **1971**, 18, 268.

(40) Edelman, G. M.; Cunningham, B. A.; Reeke, G. N.; Becker, J. W.; Waxdal, M. J.; Wang, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, 69, 2580.

(41) Hardman, K. D.; Ainsworth, C F. *Biochemistry* **1972**, 11, 4910.

(42) Hardman, K. D. *Adv. Exp. Med. Biol.* **1973**, 40, 103.

(43) Becker, J. W.; Reeke, G. N.; Wang, J. L.; Cunningham, B. A.; Edelman, G. M. *J. Biol. Chem.* **1975**, 250, 1513.

(44) Reeke, G. N.; Becker, J. W.; Edelman, G. M. *J. Biol. Chem.* **1975**, 250, 1525.

(45) Reeke, G. N.; Becker, J. W.; Edelman, G. M. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, 75, 2286.

(46) Shoham, M.; Yonath, A.; Sussman, J. L.; Moult, J.; Traub, W.; Kalb, A. J. *J. Mol. Biol.* **1979**, 131, 137.

(47) Kalb, A. J.; Stern, E. A.; Heald, S. M. *J. Mol. Biol.* **1979**, 135, 501.

(48) Hardman, K. D.; Agarwal, R. C.; Freiser, M. J. *J. Mol. Biol.* **1982**, 157, 69.

(S1) might be expected to be significantly influenced by the imposition of a single cadmium–nitrogen bonding interaction into what would otherwise be a pure oxo-ligand shielding environment, much like that present at the S2 site. Even though the cadmium C_1 site symmetry imposes no constraints on the orientation of the cadmium shielding tensor at the S2 site in concanavalin A, observation of an axially symmetric tensor must (based upon studies of model systems) reflect the orientation of the tensor in the molecular reference frame. Whether the axial symmetry observed for the cadmium tensor at the S1 site is merely accidental or whether it is indicative of a higher S1 site symmetry compared to that at the S2 site is uncertain. The deshielding influence due to the occurrence of a single cadmium–nitrogen bonding interaction gives rise to an isotropic chemical shift of 59.1 ppm. On the basis of the observations and correlations made through previous extensive cadmium NMR single crystal studies carried out in this laboratory,[49,50] it has been suggested that tensor elements with similar eigenvalues should experience similar orthogonal environments. It may therefore be expected that the unique tensor element in the case of the concanavalin A S1 site be oriented in a direction most nearly parallel to the Cd–N bond direction. According to the correlations derived from model systems, this would correspond to the most shielded direction. The deshielding effect of the Cd–N bond interaction would be most dominant for tensor elements oriented most nearly orthorgonal to the Cd–N vector.

The effect of D_2O hydration of lyophylized parvalbumin and concanavalin A is remarkable in that solvent binding to the proteins results in sufficient line narrowing which makes feasible the observation of [113]Cd CP/MAS spectra of samples containing 1–2% by weight of [113]Cd. The requirement for using D_2O to hydrate the protein is in accord with previous work involving proton NMR of a model protein system. Bryant[52,53] has reported that there is extensive cross relaxation (spin diffusion) between the protein proton and the water proton spin populations in the hydrated lysozyme powder system and in protein crystal systems. In the present study, we have been unable to cross polarize microcrystalline parvalbumin obtained by dialysis of the protein vs. aqueous 85% saturated ammonium sulfate. The D_2O-equilibration procedure therefore minimizes the spin diffusion problem and permits efficient cross polarization of cadmium through the protein proton reservoir. Furthermore, a significant degree of cross polarization may also occur by way of any water molecules which may be tightly bound to cadmium in the lyophylized protein. A sacrifice in magnetization transfer efficiency (T_{IS}) may occur because any metal-coordinated water protons may be replaced by deuterium. We have not attempted to quantitate this contribution.

The lyophylized proteins may be considered to be disordered in two ways: (1) there is no long-range intermolecular arrangement as found in a single crystal; (2) there is a local intramolecular disorder modulated by near-neighbor hydrogen bonds, salt bridges, counterions, and buffer ions that may distort the dehydrated protein from a native conformation. The rehydration process, which significantly decreases sample heterogeneity as sensed by the [113]Cd MAS spectrum, must allow the protein to assume a more native conformation with greatly reduced structural heterogeneity at the metal-binding sites. The resulting decrease in structural disorder at the metal-binding sites clearly aids the practical acquisition of high-resolution MAS spectra, and the shape of the titration data (Figure 4) is similar to other measures of protein–water interactions that are less sensitive to microstructure at the active site.[54-56] The width of the CP/MAS

spectra obtained on the lyophylized proteins is unlikely to be due to motions of large magnitude at the cadmium sites during the rotor period.[61] Thus, the line width reflects a distribution of cadmium environments in the lyophylized protein that produce a considerable distribution of [113]Cd isotropic chemical shifts. These data, therefore, demonstrate clearly that the protein experience considerable but reversible deformation during the dehydration process.

The limiting MAS line width obtained in these experiments is several times larger than those obtained in model polycrystalline systems.[49,50] The width may be due to further structural disorder not eliminated by the rehydration process, or it may be due to motions that occur during the rotor period, which defeat the perfect averaging affected by it in a rigid system. An additional source of broadening may also be the $^2H-^{113}Cd$ dipolar coupling that may be incompletely collapsed by the MAS experiment because the ^2H nuclear electric quadrupole interaction may rotate the deuterium quantization axis slightly away from the Zeeman direction.[57-59]

The question as to how closely the rehydrated protein powders, such as those used in the present study, do in fact represent a system in which the protein is in its native conformation is an important one, since it is desirable to obtain cadmium-shielding tensor data that reflect the metal-coordination environment in these and other proteins in their biologically native state. The water contents attained in the rehydration experiments reported here are high and in the range of 0.3–0.45 g of solvent/g of protein. This range of solvent contents spans the lower part of the range of water contents reported in a survey of a large variety of crystalline proteins.[60] Hydration levels used in the present work are therefore well above the hydration levels associated with satisfying the primary and most secondary hydration sites on the proteins.[51,61,62] We have no apparent evidence which would cause us to believe that the protein conformation in these hydrated powders is far from the native one.

In the previous literature,[51-53] proton NMR studies involving the lysozyme molecule were concerned primarily with monitoring the solvent properties at the protein interface and on the behavior of surface residues (intrinsic or extrinsic) with the extent of protein hydration. From these spectroscopic data and from thermodynamic data, inference was made regarding the conformational properties of the lysozyme system. The present work, however, demonstrates that [113]Cd NMR provides a sensitive and direct means of monitoring the metal-coordination environments of calcium- and zinc-dependent metalloproteins in the solid state. These observations show that the sensitivity of metal ion NMR in the solid state to structural differences is high and thus provide a useful tool for continued investigation of a number of metalloprotein systems including troponin, calmodulin, carboxypeptidase, and thermolysin.

Conclusion

We have presently demonstrated the practicality of obtaining [113]Cd CP/MAS spectra of cadmium in metal ion coordination site(s) in two representative metalloprotein systems, parvalbumin and concanavalin A. Initial problems of low resolution and low signal/noise ratio characteristic of a disordered cadmium coordination environment as well as potential problems of spin diffusion in hydrated solids have been overcome by rehydrating the proteins with D_2O as solvent. Principal elements of the [113]Cd-shielding tensors have been determined from CP/MAS spectra for the calcium-binding sites in parvalbumin and for the manganese (S1)

(49) Honkonen, R. S.; Doty, F. D.; Ellis, P. D. *J. Am. Chem. Soc.* **1983**, *105*, 4163.
(50) Honkonen, R. S.; Ellis, P. D. *J. Am. Chem. Soc.* **1984**, *106*, 5488.
(51) Rupley, J. A.; Yang, P. H.; Tollin, G. *ACS Symp.* **1980**, *127*, 111.
(52) Bryant, R. G.; Shirley, W. M. *ACS Symp.* **1980**, *127*, 147.
(53) Hilton, B. D.; Hsi, E.; Bryant, R. G. *J. Am. Chem. Soc.* **1977**, *99*, 8483.
(54) Kuntz, I. D.; Kauzman, W. *Adv. Protein Chem.* **1974**, *28*, 239.
(55) Edsall, J. T.; McKenzie, H. A. *Adv. Biophys.* **1978**, *10*, 137.

(56) Edsall, J. T.; McKenzie, H. A. *Adv. Biophys.* **1983**, *16*, 53.
(57) Opella, S. J.; Frey, M. H.; Cross, T. A. *J. Am. Chem. Soc.* **1979**, *101*, 5856.
(58) Naito, A.; Ganapathy, S.; McDowell, C. A. *J. Chem. Phys.* **1981**, *74*, 5393.
(59) Naito, A.; Ganapathy, S.; McDowell, C. A. *J. Magn. Reson.* **1982**, *48*, 367.
(60) Matthews, B. W. *J. Mol. Biol.* **1968**, *33*, 491.
(61) Careri, G.; Fasella, P.; Gratton, E. *Annu. Rev. Biophys. Bioeng.* **1979**, *8*, 69.
(62) Careri, G.; Gratton, E.; Yang, P. H.; Rupley, J. A. *Nature (London)* **1980**, *284*, 572.

and calcium (S2) site in concanavalin A. Similarities in isotropic chemical shifts and in shielding tensor parameters for the parvalbumin calcium sites and for the S2 site in concanavalin A reflect similar metal-coordination environments in the two proteins. The significantly different isotropic shift and asymmetry parameter for the cadmium cation in the manganese (S1) site of concanavalin A is consistent with the distinctive coordination environment involving a cadmium–nitrogen bonding interaction at this site. The axially symmetric shielding tensor at the S1 site may be indicative of a higher cadmium site symmetry at the S1 site compared to that at the S2 site. Additional data are required in order to more fully understand cadmium-shielding tensors in metalloproteins. Such data should now be attainable with use of solid-state NMR techniques. With 7-mm rotors at 200 MHz,

it appears that proteins with molecular weights on the order of 100 000 are amenable to this approach.

Finally, these techniques have provided a sensitive and direct means of observing structural changes that occur at metal-binding regions in metalloproteins when such systems are dehydrated.

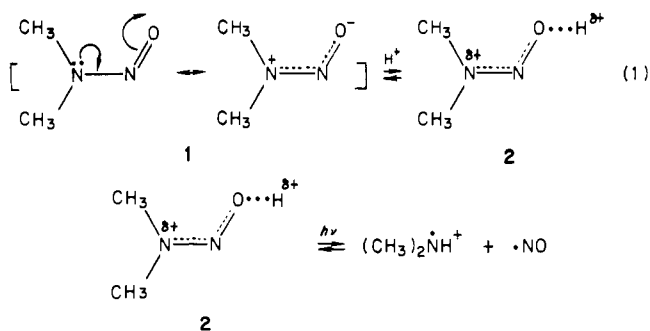# On the Singlet and Triplet Excited States of Nitrosamines

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Contribution from the Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6, and the Division of Chemistry, National Research Council, Ottawa, Ontario, Canada K1P 0R6. Received July 8, 1985

Abstract: With use of absorption and emission spectroscopy, flash photolysis, quenching experiments, and quantum yield determinations, the triplet and singlet excited-state energies of N-nitrosodimethylamine (NND) and N-nitrosopiperidine (NNP) were determined to be E_S = 72–73 and E_T = 58–59 kcal/mol and their chemical reactivities in the excited states were described. While the singlet excited state of nitrosamine–acid complexes rapidly dissociates to give aminium radicals and nitric oxide, with high quantum yields of > 1, their triplet excited state shows no apparent chemical changes. In neutral conditions, nitrosamines photolytically dissociate equally efficiently to give amine radicals and nitric oxide that recombine to give nitrosamines leading to an efficient degradation of photoenergy. The $S_0 \rightarrow T_1(n,\pi^*)$ electronic transition of NND was found in the 450-nm region and phosphorescence excitation spectra revealed the lack of the $S_1 \rightarrow T_1$ intersystem crossing for NND. Low-temperature photolysis of NND has confirmed the reaction pattern and also revealed an intermediate species, assumed to be the nitrosodimethylammonium ion, which underwent irreversible photoreaction at –150 °C and reverted readily to the NND–acid complex at –30 °C.

The nitrosamine group[3-5] is theoretically interesting because of extensive electron delocalization to give a dipolar resonance hybrid with a partial N–N double bond character.[5-7] The association of the nitrosamine group with an acid[8] or metal[7] ion has been shown to occur at the oxygen atom. The association constant of N-nitrosodimethylamine (NND) in dilute sulfuric acid is determined to be[8] 0.18 M^{-1}. In spite of this fact, nitrosamines rapidly photodissociate to aminium radicals and nitric oxides in the presence of an acid.[9-11] The chemistry of aminium radicals has

been well documented.[9-12] Since 1965, the world-wide concern with nitrosamines arises largely from the fact that many nitros-

amines are pernicious *animal carcinogens* (and, therefore, human carcinogens by extrapolation) with an organotropic action mode and that they are naturally formed in the human environmental samples with amazing facility.[13] Undoubtedly, their carcino-

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(3) Smith, P. A. S. "Open Chain Nitrogen Compounds"; W. A. Benjamin: New York, 1966; Chapter 15.

(4) Fridman, A. L.; Mukhametshin, F. M.; Novikov, S. S. *Russ. Chem. Rev.* **1971,** *40,* 34.

(5) Raban, M.; Greenblatt, J. In "Chemistry of Amino, Nitroso and Nitro Compounds, Supplement F"; Part 1; Patai, S., Ed.; John Wiley: New York, 1982; p 53.

(6) (a) Looney, C. E.; Phillips, W. D.; Reilly, E. L. *J. Am. Chem. Soc.* **1957,** *79,* 6136. (b) Karabatsos, G. J.; Taller, R. A. *J. Am. Chem. Soc.* **1964,** *86,* 4373. (c) Chow, Y. L.; Colon, C. J. *Can. J. Chem.* **1968,** *46,* 2827.

(7) (a) Klement, U.; Schmidpeter, A. *Angew. Chem.* **1968,** *80,* 444. (b) Brown, R. D.; Coates, G. E. *J. Chem. Soc.* **1962,** 4723. (c) Rademacher, P.; Stølevik, R. *Acta Chem. Scand.* **1969,** *23,* 660.

(8) Layne, W. S.; Jaffé, H. H.; Zimmer, H. *J. Am. Chem. Soc.* **1963,** *85,* 1816, 435.

(9) Chow, Y. L. *Acc. Chem. Res.* **1973,** *6,* 354.

(10) Chow, Y. L. In "Chemistry of Amino, Nitroso and Nitro Compounds, Supplement F"; Part 1; Patai, S., Ed.; John Wiley & Sons: New York, 1982; p 181.

(11) (a) Chow, Y. L. *Can. J. Chem.* **1967,** *45,* 53. (b) Mojelsky, T.; Chow, Y. L. *J. Am. Chem. Soc.* **1974,** *96,* 4549. (c) Chow, Y. L.; Lau, M. P.; Perry, R. A.; Tam, J. N. S. *Can. J. Chem.* **1972,** *50,* 1044.

(12) Chow, Y. L. In "Reactive Intermediates"; Abramovitch, R. A., Ed.; Plenum: New York, 1980; Vol. 1, p 151.