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Manganese(II) Electron Spin Resonance and Cadmium-113 Nuclear Magnetic Resonance Evidence for the Nature of the Calcium Binding Site in α-Lactalbumins†

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ABSTRACT: Bovine and goat α-lactalbumins were substituted with $^{113}Cd(II)$ or Mn(II) at the strong calcium site [Murakami, K., Andree, P. J., & Berliner, L. J. (1982) *Biochemistry 21*, 5488–5494] and studied by ^{113}Cd NMR and electron spin resonance. The ^{113}Cd chemical shifts were in the −80 to −85 ppm range vs. $Cd(ClO_4)_2$, which was almost identical with that found for several nearly octahedral (oxygen-coordinated) calcium binding proteins such as calmodulin, parvalbumin, and troponin C. The electron spin resonance spectra of bound Mn(II)–α-lactalbumin complexes at 9 or 35 GHz were also confirmatory of a highly symmetric (cubic) environment around the Mn(II) with only slight distortions. The near identity of this site in α-lactalbumin to those of calcium binding proteins containing an "EF hand domain" was remarkable despite the absence of such a domain sequence in the α-lactalbumin structure.

The α-lactalbumins, which are "modifier" proteins in the biosynthesis of lactose in milk, have been found to have a very strong affinity for Ca(II) at a unique strong binding site. Murakami et al. (1982) have measured the equilibria between several α-lactalbumin species with a variety of di- and trivalent cations, all of which cause the same characteristic fluorescence changes upon binding to a specific α-lactalbumin (e.g., bovine, human, goat, guinea pig). These experiments above as well as more recent evidence verified a competition between Ca(II) and these other cations, for example, Mn(II) or Cd(II), for this site (Murakami & Berliner, 1983).

The substitution of $^{113}Cd(II)$ in several calcium binding proteins of known three-dimensional structure has been shown to be an excellent aid in predicting the coordination nature of the Ca(II) site. In particular, the ^{113}Cd NMR chemical shift is an accurate indicator of the liganding environment of the metal ion (Armitage & Otvos, 1982). Similarly, the success in predicting ligand nature and geometry by ESR[1] with Mn(II)-substituted proteins has been demonstrated by Reed and co-workers (Reed & Markham, 1984).

We present here the ^{113}Cd NMR and Mn(II) ESR of the respective metal–α-lactalbumin complexes as probes of the chemical structure of the α-lactalbumin calcium binding site.

Materials and Methods

Proteins. Electrophoretically pure bovine α-lactalbumin (lot 50F8105) was from Sigma Chemical Co. Other α-lactalbumin species were obtained or isolated as noted earlier (Berliner & Kaptein, 1981). Apo bovine α-lactalbumin was prepared by the procedures noted in our earlier paper (Murakami et al., 1982).

Chemicals. Ultrapure cation salts were from either Aldrich Chemical Co. or Alfa Products. ^{113}Cd metal was purchased from Prochem Isotopes and converted to the chloride salt. All other reagents were as reported earlier (Murakami et al., 1982).

Methods. ESR measurements were made in quartz capillaries or sealed Pasteur dispo-pipets on a Varian E-4 spectrometer at liquid nitrogen and room temperature (Berliner, 1977). Special care was taken with controls in the 77 K spectra by freezing aquomanganese(II) samples in the presence of Sephadex G-25 to avoid artifacts due to dipolar and exchange phenomena as noted by Leigh & Reed (1971). The spectra were processed on a Varian E-935 data system. ^{113}Cd NMR spectra were measured at the South Carolina Magnetic Resonance Laboratory on a Bruker WP-400 operating at 88.756 MHz. Spectral parameters were as follows: sweep width, 41 667 Hz; pulse width, 10 μs; line broadening, 100 Hz. The fractions of free and bound cations were calculated exactly from the single-site dissociation constants for bovine α-lactalbumin: $K_{Cd(II)} = 2.5$ μM; $K_{Mn(II)} = 31.7$ μM (Murakami et al., 1982). In the case of the exceptionally strong calcium dissociation constant ($K_{Ca(II)} = 0.2$ nM), the fraction of calcium-bound α-lactalbumin was estimated precisely by fluorescence (Murakami et al., 1982).

Results and Discussion

^{113}Cd NMR. Figure 1A shows the ^{113}Cd NMR spectrum of a 1:1 complex of 3.4 mM bovine α-lactalbumin–$^{113}Cd(II)$, pH 6.3, in 25 mM Tris-HCl and 20% D_2O at 25 °C. The

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[1] Abbreviations: ESR, electron spin resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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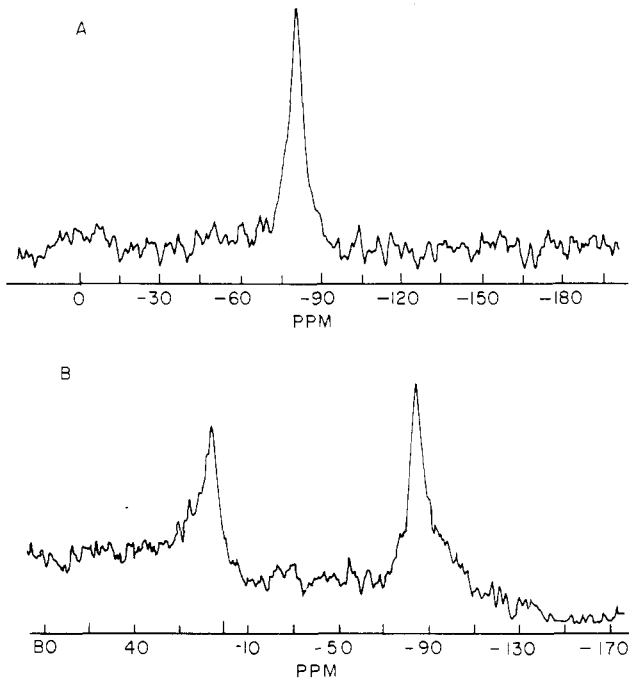

FIGURE 1: ^{113}Cd NMR spectra of α-lactalbumins. (A) 3.4 mM $^{113}Cd(II)$–bovine α-lactalbumin (pH 6.3, 25 mM Tris-HCl, 20% D_2O, 25 °C). This spectrum represents 93 846 accumulations, although satisfactory spectra were obtained in 10 000–15 000 scans where line shape was not critical. (B) 1.5 mM goat α-lactalbumin [which was intially 37% Ca(II) bound], which contains ca. 1.1 mM bound $^{113}Cd(II)$ and ca. 1.2 mM excess free $^{113}CdCl_2$, pH 6.6. The number of scans was 164 000. Upon addition of equimolar Ca(II), the protein-bound line at −85 ppm shifted completely to free $^{113}Cd(II)$ at 5 ppm (not shown). Similar results were obtained with bovine α-lactalbumin in (A) upon Ca(II) addition. All chemical shifts are relative to $Cd(ClO_4)_2$.

chemical shift of −79.5 to −80.5 ppm was observed over several sample concentrations and pH range 6.2–7.8 (not shown). Figure 1B shows the ^{113}Cd NMR spectrum for a mixture of $^{113}Cd(II)$ and 1.5 mM goat α-lactalbumin that was initially 37% Ca(II) bound. The spectrum (Figure 1B) reflects the bound $^{113}Cd(II)$ at −85 ppm and the *excess* $^{113}Cd(II)$ at ca. 5 ppm, which could not displace the strongly bound calcium (Murakami et al., 1982). Upon addition of sufficient $CaCl_2$ to saturate either the bovine or goat samples above, the spectra completely reverted to that of free $^{113}Cd(II)$ (not shown). Note that the chemical shifts observed for both the bovine and goat α-lactalbumins were almost identical and were precisely in the same range found for several *all oxygen coordinated* calcium binding proteins such as calmodulin, parvalbumin, and troponin C (Forsén et al., 1982; Armitage & Otvos, 1982). Of particular note in both spectra (Figure 1) was the unusually large linewidth (ca. 550 Hz), which reflects in part an unusally short T_1 for bound $^{113}Cd(II)$ but must also involve other contributions to the linewidth at this frequency (H. J. Vogel, private communication of unpublished results).

ESR of Mn(II) Complexes. Figure 2 depicts ESR spectra for Mn(II)–α-lactalbumin complexes at two frequencies and temperatures, respectively. Figure 2A depicts a frozen (77 K) spectrum of bovine α-lactalbumin which contained 92% bound Mn(II) as calculated from the known dissociation constant previously measured by Murakami et al. (1982). The spectrum shown was computer corrected for the 8% unbound Mn(II). Nonetheless, the line shape was quite similar to that of an aquomanganese(II) standard (frozen in Sephadex G-25) with the exception of a slightly more pronounced broad shoulder at the low-field edge of the spectrum. This broad

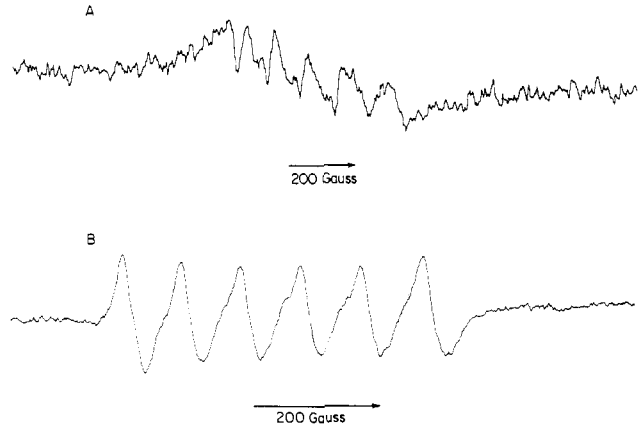

FIGURE 2: ESR spectra of Mn(II)–α-lactalbumin complexes at 9 and 35 GHz, pH 7.4, and 0.02 M Tris-HCl. (A) X-band (9-GHz) ESR spectrum of 0.59 mM Mn(II) and 1.2 mM bovine α-lactalbumin, 77 K. The spectrum was computer corrected for cavity background and unbound Mn(II) by subtracting a Mn(II) standard in frozen Sephadex G-25 buffer under the same conditions. Conditions were as follows: magnetic field, 3200 G; sweep width, 2000 G; power, 20 mW; modulation, 10 G; sweep time, 2 min; response time, 0.064 s. (B) Q-band (35-GHz) ESR spectrum of 0.50 mM Mn(II)–4.0 mM bovine α-lactalbumin at 10 °C. The contribution due to free Mn(II) was 0.8% of the total spectral intensity (Murakami et al., 1982). The spectrum did not narrow when the temperature was increased to ambient temperature. Conditions were as follows: magnetic field, 12 450 G; sweep width, 1000 G; power attenuation, 2 dB; modulation, 6.3 G; response time, 1 s.

shoulder and barely discernible extra weak lines interspersed between the six main hyperfine lines were most likely attributable to a small zero-field splitting, i.e., from slight distortions from octahedral symmetry. The absence of any fine structure features in this spectrum makes it difficult to quantitate the extent of the (quite small) zero-field splitting. We would expect to resolve second-order fine structure features if the value of D were greater than ca. 0.02 cm^{-1} as an upper limit for these spectra. In fact, these spectra were remarkably similar to those for Mn(II)–troponin or –parvalbumin complexes, both of which have been shown to contain nearly octahedral arrays of oxygen donor ligands at the calcium site (Hartshorne & Boucher, 1974). The spectrum at higher frequency (35 GHz) is shown in Figure 2B for a complex which was 99.2% bound Mn(II). The spectrum was almost completely devoid of inhomogeneous broadening contributions from second-order effects due to zero-field splitting interactions, further supporting a relatively highly symmetric (cubic) environment around the Mn(II) with only slight distortions (Buttlaire et al., 1974). The absence of any spectral narrowing with increasing temperature verified that the rather homogeneous line shape was *not* due to free, unbound Mn(II) (Reed & Markham, 1984).

Conclusions

While the structural consequences of cation binding at the calcium site of the α-lactalbumins have been characterized by fluorescence (Murakami et al., 1982; Murakami & Berliner, 1983) and ^1H NMR (H. Nishikawa, J. E. Scheffler, and L. J. Berliner, unpublished results), some conclusions about the nature of the calcium site itself can be drawn from these measurements. The ^{113}Cd NMR chemical shifts observed for both the bovine and goat species were precisely in the range found for several calcium binding proteins containing "EF hand" domains (Kretsinger & Nockolds, 1973), all of which contain multidentate oxygen coordination in nearly regular octahedral environments with only slight distortions from idealized (cubic) symmetry. Furthermore, our previous water

proton relaxation studies suggested either that all of the metal coordination sites were occupied by protein ligands or that any first coordination sphere H_2O ligands were hindered from fast proton exchange with the solvent.[2] A high-resolution three-dimensional X-ray structure of any α-lactalbumin species has yet to be published, but it can be predicted that the calcium site will contain all oxygen ligands. This study and several complementary solution conformation techniques can describe protein conformation in much structural detail. The long-awaited X-ray results would also be valuable in comparing the precise three-dimensional structure of the α-lactalbumin site with the quite well understood EF hand domains of the several calcium binding proteins reported to date (Kretsinger, 1980).

Acknowledgments

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[2] This was also suggested from ^1H water proton relaxation studies at 60 MHz with Gd(III)–α-lactalbumin where enhancements slightly less than 1.0 were measured (L. J. Berliner and H. Gilboa, unpublished results).

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Articles

Photolysis of Cholesteryl Diazoacetate in Small Unilamellar Vesicles†

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ABSTRACT: Cholesteryl diazoacetate (**1**), a potential membrane photolabeling reagent, has been incorporated into dipalmitoylphosphatidylcholine (DPPC) small unilamellar vesicles (SUV). Immobilization of the photolabel within the bilayer matrix was demonstrated by ^{13}C and ^1H NMR and was found to be closely analogous to that of cholesterol. SUV composition was verified by integration of proton NMR resonances from $CDCl_3$ solutions of chloroform extracts of vesicle preparations. Photolysis of the label in DPPC SUV resulted in C–H insertion into the choline head group of DPPC, O–H insertion into water, and also production of cholesterol. These intermolecular C–H and O–H insertions indicate that the photogenerated carbene from **1** is situated at the aqueous interface of the membrane, analogously to the known orientation of the OH group of cholesterol. Therefore, by these criteria, **1** appears to behave as a cholesterol analogue in DPPC bilayers and may be a useful membrane photolabeling reagent.

Cholesterol, the major sterol of mammalian cells, regulates membrane fluidity (Schreier-Muccillo et al., 1973; Demel & de Kruijff, 1976; Lindblom et al., 1981), influences enzymatic activity (Klappauf & Schubert, 1977; Klein et al., 1978; Madden et al., 1979), passive transport (Schreier-Muccillo et al., 1973; Benz & Cros, 1978), immune response (Inbar & Shinitzky, 1974; Shinitzky et al., 1979), and phospholipid fatty acyl chain composition (Dahl et al., 1980), complexes with polyene antibiotics (Bittman et al., 1981), cytochrome $P\text{-}450_{scc}$ (Lambeth et al., 1980), and lysophosphatidylcholine (Rammy & Brockerhoff, 1982), and mediates bilayer structure (de Kruijff et al., 1979). Many of these effects seem to be the result of the modulation of phospholipid behavior by cholesterol. According to differential scanning calorimetry data, the preference of cholesterol for different phospholipids is sphingomyelin ≫ phosphatidylserine, phosphatidylglycerol > phosphatidylcholine ≫ phosphatidylethanolamine, except in mixtures of PS–PE,[1] PS–PC, and PC–PG where the lower

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received April 28, 1983.* This research was supported by National Institutes of Health Grants GM-22,647 and NS-16,826. For further details, see Keilbaugh (1983).

[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; SM, sphingomyelin; PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; EtOAc, ethyl acetate; CI, chemical ionization; *hν*, light; IR, infrared spectoscopy; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; MeOH, methanol.

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