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Calcium-43 NMR Studies of Calcium-Binding Lysozymes and α-Lactalbumins†

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ABSTRACT: The calcium-binding properties of equine and pigeon lysozyme as well as those of bovine and human α-lactalbumin were investigated by ^{43}Ca NMR spectroscopy. All proteins were found to contain one high-affinity calcium-binding site. The chemical shifts, line widths, relaxation times (T_1 and T_2), and quadrupole coupling constants for the respective ^{43}Ca NMR signals were quite similar; this is indicative of a high degree of homology between the strong calcium-binding sites of these four proteins. The measured chemical shifts ($\delta \approx -3$ to -7 ppm) and quadrupole coupling constants ($\chi \approx 0.7$–0.8 MHz) are quite distinct from those observed for typical EF-hand calcium-binding proteins, suggesting a different geometry for the calcium-binding loops. The correlation times for bound calcium ions in these proteins were on the order of 4–8 ns, indicating that the flexibilities of these binding sites are limited. The apparent pK_a values for the high-affinity sites ranged from 3.4 to 4.7, confirming the participation of carboxylate-containing residues in the coordination of the calcium ion. Competition experiments with EDTA showed that the affinities of these proteins for calcium follow the series bovine α-lactalbumin ≈ human α-lactalbumin > pigeon lysozyme > equine lysozyme ($K_D \approx 5 \times 10^{-8}$ to 10^{-6} M). Evidence for the existence of a second weak calcium-binding site ($K_D = 3 \times 10^{-3}$ M) was obtained for bovine α-lactalbumin, but not for the other proteins studied. The involvement of a histidine residue(s) at this second site was inferred from the unusual pH dependence ($pK_a' = 6.3$) of the line width of its ^{43}Ca NMR signal. The ^{43}Ca NMR findings presented in this paper provide direct spectroscopic evidence in support of an evolutionary link between the functionally distinct lysozymes and α-lactalbumins.

α-Lactalbumins (α-LA)[1] are an important class of mammalian milk proteins which play a critical role in the biosynthesis of the milk sugar lactose. By binding to the enzyme galactosyltransferase, an enzyme complex, known as lactose synthase, is formed which catalyzes the production of lactose from UDP–galactose and glucose. In contrast, chicken-type lysozymes (LZ) are ubiquitous lytic enzymes which catalyze the degradation of peptidoglycan, a major constituent of bacterial cell walls. From the high degree of homology in (1) the amino acid sequences of α-lactalbumins and lysozymes (Brew et al., 1970), (2) the intron–exon arrangements of their genes (Qasba & Safaya, 1984), and (3) the structures of chicken LZ and bovine α-LA obtained from X-ray and modeling studies (Warme et al., 1974), it has been postulated for a number of years that these functionally different proteins have evolved from a common ancestral molecule.

An interesting facet of this evolutionary hypothesis is the calcium-binding properties of these proteins. Beginning with an initial report on bovine α-LA (Hiraoka et al., 1980), it is now recognized that all known α-lactalbumins possess one high-affinity calcium-binding site. The recent X-ray crystal structures of baboon and human α-LA (Acharya et al., 1989, 1991) revealed that the calcium-binding site in α-lactalbumins is formed by the side-chain carboxylates of three aspartate residues (Asp-82, -87, and -88), which are conserved in all species studied to date, plus the backbone carbonyl oxygens of two other highly conserved residues, Lys-79 and Asp-84. The coordination sphere of the metal ion is completed by two water molecules, resulting in a slightly distorted pentagonal bipyramidal arrangement of oxygen-containing ligands surrounding the calcium (Figure 1). The entire calcium-binding site, which is composed of two helices linked by a tight loop, has been referred to in the literature as the "α-lactalbumin elbow", to indicate that it is different from the more commonly observed "EF-hand" calcium-binding sites (Stuart et al., 1986; Acharya et al., 1989).

The common ability of α-lactalbumins to bind calcium raised some questions about their evolutionary link with lysozymes since the latter were not thought to be metalloproteins. However, lysozymes from two species, namely, horse and pigeon, contain the identical three Asp residues required for calcium binding in α-lactalbumins at virtually the same positions (Asp-85, -90, and -91) in the respective amino acid sequences (see Table I). Recently, calcium-binding properties were established in both lysozymes (Nitta et al., 1987, 1988; Sugai et al., 1989), leading to the proposal that α-lactalbumins evolved in mammals from calcium-binding lysozymes (Nitta & Sugai, 1989). While most lysozymes contain different residues in the relevant portion of the sequence, human lysozyme contains one of the key Asp residues. The introduction of the two missing aspartates by site-directed mutagenesis resulted in the creation of a functional calcium-

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[1] Abbreviations: NMR, nuclear magnetic resonance; LA, α-lactalbumin; LZ, lysozyme; UDP, uridine 5′-diphosphate; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay.

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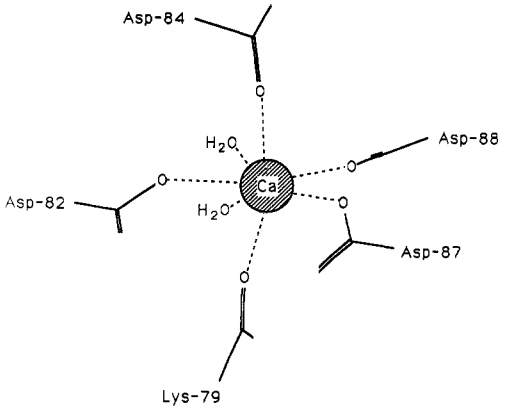

FIGURE 1: Schematic model of the calcium-binding site of baboon α-lactalbumin based on its X-ray structure (Acharya et al., 1989). Only the immediate coordination sphere of the calcium is shown. The metal ion is bound to seven oxygen atoms (five furnished by the protein plus two solvent molecules) in a slightly distorted pentagonal bipyramidal geometry, with the backbone carbonyls positioned at the apices of the pyramids. Atomic coordinates for the baboon α-LA structure were obtained from the Protein Data Bank, Brookhaven National Laboratories, Upton, NY.

binding site in human lysozyme (Kuroki et al., 1989), thus accentuating the importance of these Asp residues in calcium binding. X-ray crystallographic studies of this mutated enzyme revealed that the calcium is coordinated by seven oxygen ligands in a geometry analogous to that shown for baboon and human α-LA (Inaka et al., 1991). However, the inability to locate calcium ions in electron density maps from crystals of equine and pigeon lysozyme (Tsuge et al., 1992; Yao et al., 1992) has precluded direct insight into the structural features of the calcium-binding sites of these proteins. Hence, we embarked on a comparative study of the high-affinity calcium-binding sites in these lysozymes and α-lactalbumins by ^{43}Ca NMR.

Calcium-43 has a spin $I = {}^7/_2$ nucleus and is the only spectroscopically observable isotope of this biologically important element. In spite of its very low resonance frequency and low natural abundance, ^{43}Ca NMR has provided considerable insight into the structural and motional characteristics of calcium-binding sites in a number of calcium-binding proteins [for a review, see Vogel and Forsén (1987)].

EXPERIMENTAL PROCEDURES

Materials. Equine and pigeon lysozymes were purified from the pooled milk of Mongolian mares and pigeon egg white, respectively, following published procedures (Bell et al., 1981; Gavilanes et al., 1982). Bovine apo-α-lactalbumin and human Ca(II)-α-lactalbumin were purchased from Sigma Chemical Co. and used without further purification; identical results for the high-affinity calcium-binding sites of these proteins were obtained with samples purified once by hydrophobic-interaction chromatography as previously described (Lindahl & Vogel, 1984). The lysozymes were rendered free of calcium by chromatography on a Bio-Gel P-4 column equilibrated with 0.01 M HCl, pH 2.1, followed by lyophilization. Partially decalcified human α-LA was obtained by applying the holoprotein to a Chelex-100 column equilibrated with 0.05 M ammonium bicarbonate, pH 8–9 at 4 °C; fractions containing the apoprotein were subsequently pooled and lyophilized. The residual calcium content in each apoprotein, determined by atomic absorption[2] and confirmed by ^{1}H NMR, was as follows: equine and pigeon LZ, <0.02 mol equiv; bovine α-LA, 0.23 mol equiv; human α-LA, 0.45 mol equiv. All proteins were stored at −20 °C prior to use.

Ultrapure potassium chloride and calcium chloride were purchased from Aldrich Chemical Co. Reagent-grade EDTA was obtained from Sigma Chemical Co. Isotopically enriched $^{43}CaCO_3$ (≈80%) was purchased from Oak Ridge National Laboratories. The $^{43}Ca^{2+}$ stock solution used in this study was prepared by dissolving 12.5 mg of the carbonate in 1 M HCl and adjusting to neutral pH by the addition of 1 M NaOH. The final concentration of this stock solution was also established by atomic absorption (252 mM).

Samples for NMR analysis were prepared by dissolving each protein in 5.0–5.5 mL of doubly glass-distilled H_2O containing 0.1 M KCl. The concentrations of all protein solutions prepared in this manner were determined by the absorption at λ = 280 nm on a Cary 1 UV–visible spectrophotometer using the following extinction coefficients, $E^{1\%}_{280}$ (cm^{-1}): equine LZ, 23.5; pigeon LZ, 20.8;[3] bovine α-LA, 20.1 (Kuwajima & Sugai, 1978); human α-LA, 18.4 (Nozaka et al., 1978). For each sample, the pH was adjusted to the desired value by microliter additions of 1.0 M KOD and 1.0 M DCl. All pH values recorded were measured with a Fisher Accumet 805MP pH meter. In all cases, sample preparation and manipulation were performed with acid-washed glassware and plasticware.

NMR Spectroscopy. ^{43}Ca NMR measurements were performed using a wide-bore Bruker AM-400 spectrometer operating at a resonance frequency of 26.9 MHz. A horizontal solenoid probe was employed throughout this study. This probe is capable of detecting nuclei with resonance frequencies up to 50 MHz at a field strength of 9.4 T. For work with very low frequency nuclei such as ^{43}Ca, we, as well as other laboratories (Drakenberg & Forsén, 1983), have found that a horizontal coil arrangement is superior to the traditional saddle-shape Helmholtz coil in terms of sensitivity and minimization of acoustic ringing. This system, however, necessitates the removal of the probe each time the sample must be manipulated in some way (i.e., titration with $^{43}Ca^{2+}$). Cylindrical sample tubes were constructed from Pyrex glass and designed to fit snugly in the entire coil region (17-mm o.d. and ≈4 cm in length). The tubes, capable of holding from 4.8 to 5.5 mL of material, were also fitted with a small neck to allow facile insertion and removal of sample. ^{43}Ca NMR spectra were typically acquired with the following parameters: a 40-μs ($\pi/2$) pulse length; a sweep width of 10 000–20 000 Hz; and a repetition time ranging from 60 to 250 ms. In addition, a spectrometer dead time of 260 μs was used; this value was sufficient to result in flat spectral base lines with no observable loss of signal intensity, compared to spectra acquired with much shorter dead times. Values for the longitudinal relaxation time, T_1, were determined by the inversion recovery method [π–τ–($\pi/2$)–FID] using standard Bruker software. All data were zero-filled once prior to processing, and an exponential multiplication resulting in a line broadening of 30–50 Hz was applied. For spectra containing overlapping resonances, the simulation program

[2] Atomic absorption spectrometry was performed at Hokkaido University on a Hitachi 170-10 instrument and at the University of Calgary on a Perkin-Elmer 500 atomic absorption spectrometer.

[3] The values of $E^{1\%}_{280}$ given in the text for the lysozymes were calculated from the amino acid sequences of the respective proteins (equine LZ, McKenzie & Shaw, 1985; pigeon LZ, Rodríguez et al., 1985) based on a published prediction method (Wetlaufer, 1962).

Table I: Partial Amino Acid Sequences of Selected α-Lactalbumins and Lysozymes [a]

					80										90	
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bovine α-lactalbumin	C	D	K	F	L	D	D	D	L	T	D	D	I	M	C	
human α-lactalbumin	C	D	K	F	L	D	D	D	I	T	D	D	I	M	C	
baboon α-lactalbumin	C	D	K	F	L	D	D	D	I	T	D	D	I	M	C	
goat α-lactalbumin	C	D	K	F	L	D	D	D	L	T	D	D	I	V	C	
equine lysozyme	C	S	K	L	L	D	E	N	I	D	D	D	I	S	C	
pigeon lysozyme	C	S	K	L	R	D	D	N	I	A	D	D	I	Q	C	
human lysozyme	C	S	A	L	L	Q	D	N	I	A	D	A	V	A	C	
chicken lysozyme	C	S	A	L	L	S	S	D	I	T	A	S	V	N	C	
	80										90					

[a] The sequence data shown were obtained from the following sources: bovine α-LA, Brew et al., 1970; human α-LA, Hall et al., 1982; goat α-LA, Kumagai et al., 1987; equine LZ, McKenzie & Shaw, 1985; pigeon LZ, Rodríguez et al., 1985; human LZ, Jollès & Jollès 1971; chicken LZ, Jollès et al., 1979. The partial sequence of baboon α-LA shown in this table was elucidated by the X-ray structure of this protein (Acharya et al., 1989). Numbers above and below the table correspond to the amino acid sequence positions for the α-lactalbumins and the lysozymes, respectively. Residues involved in calcium binding via side-chain carboxylates are represented by (●), while those which coordinate the metal ion through backbone carbonyl oxygens are shown by (O).

LINESIM (written by P. Barron, Bruker Australia) was used to extract the salient features (δ, $\Delta\nu_{1/2}$, peak area, and intensity) of each signal in the spectrum. A fitting program using the Simplex algorithm (Caceci & Cacheris, 1984) was employed to calculate pK_a' values and Hill coefficients from pH titration data. ^{43}Ca spectra were acquired unlocked and are referenced to external 1.0 M $CaCl_2$ in H_2O. All spectra were run at room temperature (21 ± 1 °C) unless otherwise indicated.

THEORY

A brief summary of the theory and equations relevant to this study is presented in this section. For a more rigorous treatment of quadrupolar relaxation theory with particular emphasis on the application of NMR to the study of biological systems, the reader is referred to the following papers: Vogel & Forsén, 1987; Drakenberg & Forsén, 1983; Andersson, et al., 1982.

For quadrupolar nuclei (nuclear spin, $I > 1/2$), magnetic relaxation is normally dominated by the quadrupolar relaxation mechanism, a process which involves an electric interaction between the nuclear quadrupole moment and fluctuating electric field gradients at the nucleus. In general, this relaxation phenomenon is quite complex; the return of magnetization to equilibrium conditions following a perturbation (i.e., rf pulse) is characterized by multiexponential behavior. In the case of nuclei with $I = 7/2$, like ^{43}Ca, and $I = 5/2$, the following expressions for the longitudinal (R_1) and transverse (R_2) relaxation rates in the limit of "nearly exponential" relaxation have been derived (Halle & Wennerström, 1981):

$$R_1 = \frac{1}{T_1} = \frac{3\pi^2}{100}\chi^2\frac{2I+3}{I^2(2I-1)}\left[\frac{2\tau_c}{1+(\omega_0\tau_c)^2} + \frac{8\tau_c}{1+4(\omega_0\tau_c)^2}\right] \quad (1)$$

$$R_2 = \frac{1}{T_2} = \frac{3\pi^2}{100}\chi^2\frac{2I+3}{I^2(2I-1)}\left[3\tau_c + \frac{5\tau_c}{1+(\omega_0\tau_c)^2} + \frac{2\tau_c}{1+4(\omega_0\tau_c)^2}\right] \quad (2)$$

where ω_0 is the Larmor frequency of the nucleus (in radians per second), τ_c is the correlation time (in seconds), and χ is the quadrupole coupling constant (in megahertz).[4] These equations are considered valid in the limit $\omega_0\tau_c \leq 1.5$. Information regarding the environment of the quadrupolar nucleus may be gleaned from the important parameters χ and τ_c. With respect to the interaction of ^{43}Ca with proteins, χ provides a measure of the symmetry of the metal-binding site, while τ_c describes the motion of the metal ion in the site. In the limit of slow exchange between protein-bound and free $^{43}Ca^{2+}$ (i.e., $k_{off} \ll R_1, R_2$) and strong binding ($K_D < 10^{-4}$ M), then χ and τ_c may easily be determined from eq 1 and 2. By using values of T_1, obtained from the inversion recovery method, and T_2, derived from spectral line widths ($\Delta\nu_{1/2}$) using the simple relation given in eq 3, one can compute τ_c from the ratio of T_2 to T_1 (eq 4) and insert this value back

$$T_2 = 1/\pi\Delta\nu_{1/2} \quad (3)$$

$$\frac{T_2}{T_1} = \frac{2/[1+(\omega_0\tau_c)^2] + 8/[1+4(\omega_0\tau_c)^2]}{3 + 5/[1+(\omega_0\tau_c)^2] + 2/[1+4(\omega_0\tau_c)^2]} \quad (4)$$

into either eq 1 or eq 2 to elucidate χ. For fast-exchange situations (i.e., $k_{off} \gg R_1, R_2$), the observed line shape is a weighted average of the signals corresponding to free and bound $^{43}Ca^{2+}$ ions. For a single fast-exchanging site, the predicted line width is given by eq 5 where p_B is the fraction

$$\Delta\nu_{1/2} = \frac{(1-p_B)R_2^F + p_B R_2^B}{\pi} \quad (5)$$

of bound $^{43}Ca^{2+}$ and R_2^F and R_2^B represent the transverse relaxation rates of free and bound metal ion, respectively. The dependence of $\Delta\nu_{1/2}$ on [Ca^{2+}]/[protein] may then be used to calculate the binding constant and the quadrupole coupling constant if the correlation time can be estimated from another experiment. For the intermediate exchange situation (i.e., $k_{off} \approx R_1, R_2$), one has to resort to a total band-shape analysis (Drakenberg et al., 1983) to obtain the kinetic and thermodynamic parameters.

RESULTS

Titration of Apo-LZ and Apo-α-LA with $^{43}Ca^{2+}$. The titration of equine apolysozyme with $^{43}Ca^{2+}$ monitored by ^{43}Ca

[4] Here we define the quadrupole coupling constant, χ, by the expression: $\chi = e^2qQ/h$, where e is the charge on the electron, q is the electric field gradient at the nucleus, Q is the nuclear quadrupole moment, and h is Planck's constant. In many derivations, $h/2\pi$ is used (Abragam, 1961); this alters the leading coefficient in eq 1 and 2 to 3/400.

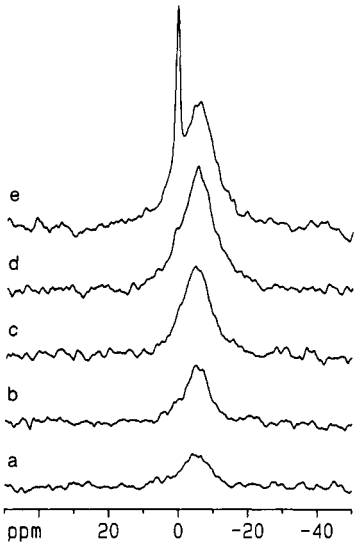

FIGURE 2: ^{43}Ca NMR spectra of the titration of 0.33 mM equine apolysozyme with $^{43}Ca^{2+}$ at pH 6.0. A total of 200 000 scans were collected in each experiment. (a) 0.23 equiv of $^{43}Ca^{2+}$; (b) 0.46 equiv of $^{43}Ca^{2+}$; (c) 0.69 equiv of $^{43}Ca^{2+}$; (d) 0.92 equiv of $^{43}Ca^{2+}$; (e) 1.15 equiv of $^{43}Ca^{2+}$.

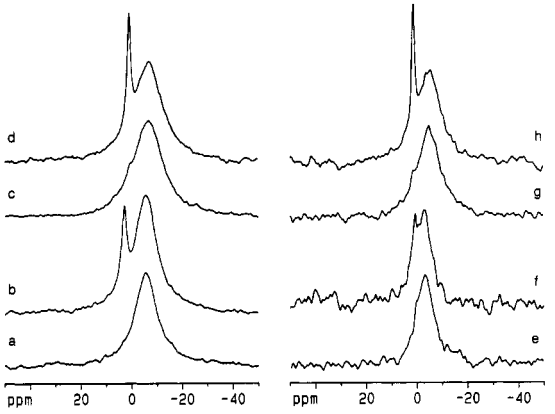

FIGURE 3: ^{43}Ca NMR spectra of bovine and human α-lactalbumin and pigeon and equine lysozyme in the presence of saturating and excess amounts of $^{43}Ca^{2+}$. (a and b) 2.49 mM bovine α-LA plus 0.89 and 1.11 equiv of Ca^{2+} (total), pH 7.0, 50 000 scans; (c and d) 1.52 mM human α-LA plus 1.00 and 1.15 equiv of Ca^{2+} (total), pH 7.0, 350 000 scans; (e and f) 0.094 mM pigeon LZ plus 1.00 and 1.10 equiv of $^{43}Ca^{2+}$, pH 6.0, 1 500 000 and 550 000 scans, respectively; (g and h) 0.33 mM equine LZ plus 0.92 and 1.15 equiv of $^{43}Ca^{2+}$, pH 6.0, 200 000 scans.

NMR is shown in Figure 2. Upon addition of the isotopically enriched calcium, a broad peak ($\Delta\nu_{1/2}$ = 253 Hz) appears at −5.3 ppm, and the signal increases linearly in intensity up to 1 equiv of metal ion. This resonance corresponds to calcium bound to the single high-affinity calcium site in this metalloprotein. In the presence of excess metal ion, a second sharp signal ($\Delta\nu_{1/2}$ = 10 Hz) is observed at ≈1 ppm, in the vicinity of free calcium. Bovine α-LA, human α-LA, and pigeon LZ behave in a similar fashion, and give virtually identical ^{43}Ca NMR spectra (Figure 3). The protein-bound $^{43}Ca^{2+}$ signals in each of the four proteins appear in a very narrow chemical shift window upfield of free Ca^{2+} (−3.2 to −6.7 ppm). The line widths of these resonances are also quite comparable. From the T_1 and T_2 relaxation times, obtained from inversion recovery and spectral line widths, respectively, values for the quadrupole coupling constant (χ) and correlation time (τ_c) of bound calcium in each protein were computed (Table II). In every case, τ_c is on the order of nanoseconds, which for proteins of this size (M_r ≈14.5K) is in the range expected for the entire molecule on the basis of the Debye–Stokes–Einstein equation (Andersson et al., 1982). In addition, $\omega_0\tau_c$ is below 1.5 for each protein studied, which does not violate the condition under which eq 1 and 2 may be used. The values of χ for ^{43}Ca in the strong calcium-binding sites of these proteins are remarkably similar, ranging from χ ≈0.7 to 0.8. A substantial degree of homology between the high-affinity sites of the α-lactalbumins and calcium-binding lysozymes is strongly suggested by the overall agreement in the ^{43}Ca NMR parameters for these proteins.

In each case, the existence of signals due to protein-bound and free $^{43}Ca^{2+}$ when the metal ion is present in excess is indicative of slow-exchange binding of calcium. This behavior is further confirmed by the relatively flat temperature dependence of the line widths of the bound ^{43}Ca signals for the proteins studied up to ≈50 °C (data not shown). In the case of bovine α-LA, the sharp peak occurs slightly downfield of free calcium. This signal is attributable to $^{43}Ca^{2+}$ in intermediate to fast exchange with a second weak calcium-binding site in this protein (vide infra); the other three proteins do not appear to have a second calcium-binding site.

Determination of Apparent pK_a Values for the Strong Ca^{2+} Sites. The apparent pK_a's of the strong Ca^{2+}-binding sites of bovine and human α-LA and equine and pigeon LZ were estimated by ^{43}Ca NMR. For each protein, the decrease in the relative area of the protein-bound ^{43}Ca signal with decreasing pH was monitored (Figure 4). The data for three of the proteins were fit using a Simplex routine (Caceci & Cacheris, 1984) to obtain the apparent pK_a's and Hill coefficients. For pigeon LZ, a severe shortage in material prevented the completion of a pK_a' curve; instead, an approximate value was obtained by determining the pH where the amounts of free and bound $^{43}Ca^{2+}$ are identical. In all cases, a substantial drop in the total (free + bound) ^{43}Ca area was observed at very low pH values (≤3.5). Relaxation studies on bovine α-LA established that this drop in area was due solely to saturation of the free calcium signal (data not shown); all low-pH data were treated with an appropriate correction factor to account for this effect. Also, a significant amount of precipitation occurs for the α-lactalbumins between pH 4.5 and 3.5, in the vicinity of the isoelectric points of these proteins. However, in both cases, we found that the total amount of detectable ^{43}Ca remains constant in this range, suggesting that only the apoprotein comes out of solution.

The apparent pK_a's and Hill coefficients (n) for each protein are shown in Table III. The pK_a' values for the four proteins range from 3.4 to 4.7, indicative of the presence of carboxylate ligands in the coordination sphere of the metal ion in the strong Ca^{2+} sites of these molecules. However, a difference exists between the Hill coefficients for the lysozymes compared to the α-lactalbumins. For equine LZ, the drop in protein-bound calcium is quite gradual, corresponding to a Hill coefficient of nearly 1. From the partial titration curve of pigeon LZ, it appears that this protein exhibits a similar behavior. In contrast, for the α-lactalbumins, the peak area drops over a much smaller pH range, translating into substantially higher values for the Hill coefficients.

Determination of K_D Values for the Strong Ca^{2+} Sites. The dissociation constants (K_D) for the high-affinity Ca^{2+}-binding sites of bovine and human α-LA and equine and pigeon LZ were determined by competition experiments with EDTA. Equimolar mixtures of Ca^{2+}, EDTA, and each protein were examined by ^{43}Ca NMR (Figure 5). Spectra were obtained at several pH values until the integrated areas of the ^{43}Ca–EDTA (+20 ppm) and ^{43}Ca–protein signals were the same.

Table II: ^{43}Ca NMR Data for Bovine α-LA, Human α-LA, Equine LZ, and Pigeon LZ[a]

protein	chemical shift (ppm)	$\Delta\nu_{1/2}$ (Hz)	T_1 (ms)	T_2 (ms)	τ_c (ns)	χ (MHz)
bovine α-lactalbumin	−5.8	256	3.99	1.24	8.6	0.70
human α-lactalbumin	−6.7	335	2.84	0.95	8.0	0.81
equine lysozyme	−5.3	253	2.89	1.26	6.0	0.75
pigeon lysozyme	−3.2	186	3.12	1.71	4.4	0.69

[a] For each protein, T_1 was determined by the inversion recovery method under the following conditions: (i) 2.94 mM $Ca_{1.00}$–bovine α-LA, pH 7.00, 30 000 scans per τ value; (ii) 1.52 mM $Ca_{1.00}$–human α-LA, pH 6.50, 60 000 scans each; (iii) 0.33 mM $Ca_{0.92}$–equine LZ, pH 6.00, 200 000 scans each; (iv) 0.094 mM $Ca_{1.00}$–pigeon LZ, pH 6.00, 450 000 scans each. All other parameters listed in this table were obtained from the spectra shown in Figure 3 as described in the text.

Table III: pK_a' and K_D Data for Bovine α-LA, Human α-LA, Equine LZ, and Pigeon LZ

protein	pK_a' [a]	n[a]	K_D (M)[b]
bovine α-lactalbumin	3.8	2.6	5×10^{-8}
human α-lactalbumin	4.7	4.0	5×10^{-8}
equine lysozyme	3.8	1.1	1×10^{-6}
pigeon lysozyme	3.4		1×10^{-7}

[a] Calculated from the pH dependence of the signal due to the strong calcium-binding site in each protein (Figure 4). [b] Determined by competition experiments with EDTA (Figures 5 and 6).

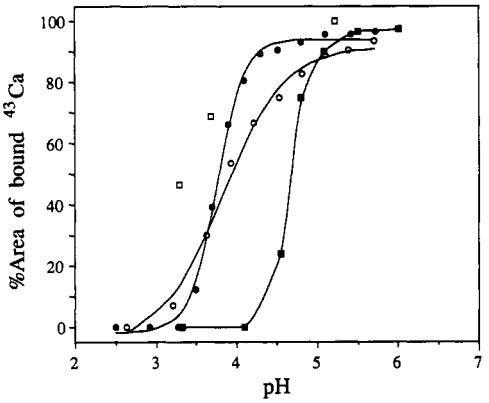

FIGURE 4: pH dependence of the relative area of the ^{43}Ca NMR signal resulting from calcium bound to the high-affinity site in bovine and human α-lactalbumin and equine and pigeon lysozyme. (●) 2.94 mM $Ca_{1.00}$–bovine α-LA, 30 000 scans each; (■) 1.52 mM $Ca_{1.00}$–human α-LA, 50 000 scans each; (○) 0.33 mM $Ca_{1.00}$–equine LZ, 50 000 scans each; (□) 0.094 mM $Ca_{1.00}$–pigeon LZ, 400 000 scans each.

When an equivalent amount of Ca^{2+} is complexed to the protein and the chelator, the K_D can be determined from the effective formation constant of the Ca^{2+}–EDTA complex (K'_{CaEDTA}) at that pH using the calibration curve shown in Figure 6 (Skoog & West, 1976). The K_D values for the α-lactalbumins and pigeon LZ were found to be markedly less than for equine LZ (Table III), and the following affinity series was observed: bovine α-LA ≈ human α-LA > pigeon LZ > equine LZ. In each case, we found that results were accurate to within 0.05 of a pH unit, which translates into an uncertainty of ±10% in the dissociation constants listed in Table III. This method offers a simple approach to the elucidation of dissociation constants for high-affinity Ca^{2+}-binding sites ranging from $10^{-6} > K_D > 10^{-10}$ M. It should be noted that the absolute values of dissociation constants determined in this fashion are dependent on the literature value for the Ca^{2+}–EDTA complex; for comparative studies such as this one, however, relative K_D values would not be altered. Furthermore, this technique hinges on the assumption that the chelator does not bind to the protein and that the dissociation constant for the Ca^{2+}-binding site is independent of pH in the pH interval examined. In the case of bovine α-LA, and

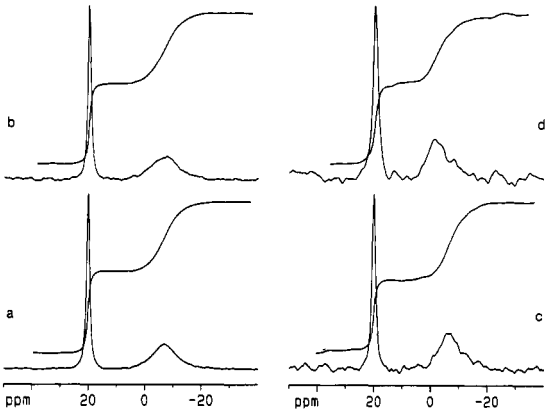

FIGURE 5: ^{43}Ca NMR spectra of bovine and human α-lactalbumin and equine and pigeon lysozyme in the presence of equimolar amounts of both Ca^{2+} and EDTA. (a) 2.94 mM bovine α-LA, pH 7.0, 50 000 scans; (b) 1.52 mM human α-LA, pH 7.0, 100 000 scans; (c) 0.29 mM equine LZ, pH 6.0, 200 000 scans; (d) 0.094 mM pigeon LZ, pH 6.8, 400 000 scans. For each spectrum shown, the time between pulses was 250 ms; a small correction to the area of the ^{43}Ca–EDTA signal was required to account for saturation effects.

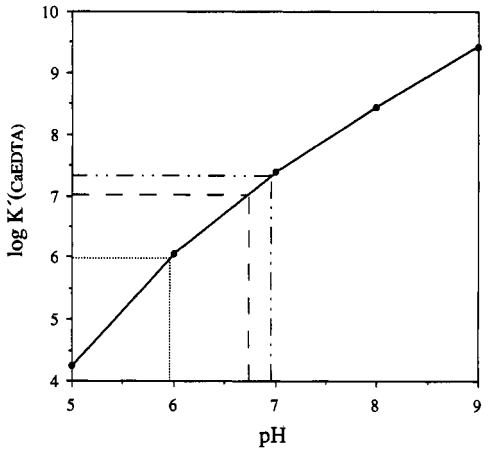

FIGURE 6: Elucidation of the dissociation constants (K_D) for bovine and human α-lactalbumin and equine and pigeon lysozyme using the pH dependence of the affinity of EDTA for Ca^{2+} [data taken from Skoog and West (1976)]. (···) Equine LZ; (– –) pigeon LZ; (– ·· –) bovine and human α-LA.

presumably the human protein, the former condition is known to be satisfied (Permyakov et al., 1987).

Weak Ca^{2+} Site in Bovine α-LA. The existence of a second weak Ca^{2+} site in bovine α-LA could be demonstrated by ^{43}Ca NMR. As discussed above, in the presence of a molar excess of calcium, a second narrow peak downfield of free calcium appears (Figure 3b). Both the line width and chemical shift of this signal are highly dependent on pH and the total amount of calcium present, a phenomenon that is diagnostic of the fast exchange of calcium between a binding site and the bulk solvent. Variable-temperature experiments also indicate the presence of intermediate to fast exchange (data

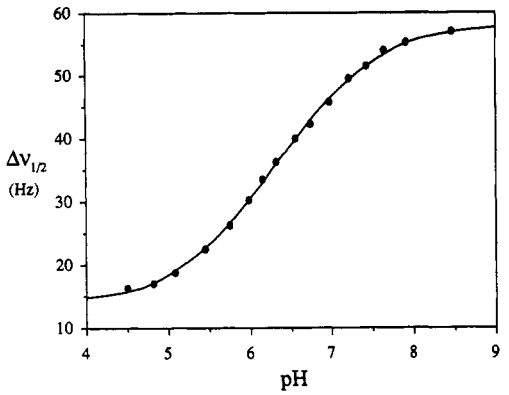

FIGURE 7: pH dependence of the line width of the ^{43}Ca NMR signal due to calcium bound to the weak site of bovine α-lactalbumin; 2.60 mM $Ca_{1.13}$–bovine α-LA, 40 000 scans each.

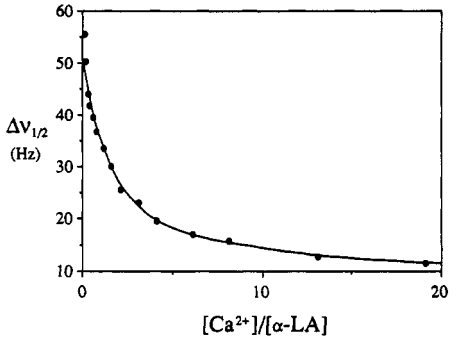

FIGURE 8: Dependence of the ^{43}Ca NMR line width of the signal corresponding to calcium in exchange with the weak site of bovine α-lactalbumin on the molar ratio of Ca^{2+}/α-LA; 2.60 mM $Ca_{1.13}$–bovine α-LA, pH 7.7, 40 000 scans each. The first equivalent of Ca^{2+}, initially in the high-affinity site, has been subtracted from the $[Ca^{2+}]/[\alpha\text{-LA}]$ values. (●) Experimental points. The solid line was calculated using the following parameters: $K_D = 3 \times 10^{-3}$ M; $k_{off} = 220$ s^{-1}; $\chi = 1$ MHz; $\tau_c = 8 \times 10^{-9}$ s.

not shown). The line width of this signal is largest ($\Delta\nu_{1/2}$ = 55 Hz) at high pH values (≥8), and the signal appears at ≈3 ppm. However, as the pH is reduced, the signal becomes quite narrow ($\Delta\nu_{1/2}$ = 10 Hz), and its chemical shift moves upfield (≈1.5 ppm) toward that of the free metal ion. In contrast, none of the other proteins studied exhibited such a trend (i.e., line widths of 10 Hz at all pH values), indicating the absence of this second weak calcium site in these molecules. An apparent pK_a of 6.3 for this site was obtained from a fit of the pH dependence of the line width of this signal (Figure 7; chemical shift data not shown). This value suggests that one or more histidine residues either directly participate in metal ion binding at this site or are sufficiently close to the site to affect the binding of the metal ion electrostatically. The further addition of calcium to a sample of bovine α-LA in the presence of a slight excess of $^{43}Ca^{2+}$ results in a decrease in signal line width as shown in Figure 8. Values for the binding constant and the off-rate of the calcium ion for the second site were obtained by fitting this curve with the assumption of a single weakly bound Ca^{2+} ion; $K_D = 3 \times 10^{-3}$ M, $k_{off} = 220$ s^{-1}. The relatively slow exchange makes it possible to evaluate the quadrupole coupling constant for this site only when assuming a value for the correlation time. An analogous fit of the change in chemical shift of the signal with $[Ca^{2+}]/[\alpha\text{-LA}]$ generated a similar value for K_D (data not shown).

DISCUSSION

The ^{43}Ca NMR studies of the calcium-binding lysozymes and the α-lactalbumins presented in this paper provide direct spectroscopic evidence in support of an evolutionary link between these functionally unique proteins. The ^{43}Ca NMR data for the single high-affinity Ca^{2+}-binding sites in each protein are remarkably similar. From this and the amino acid homology between the calcium-binding loops of all α-lactalbumins and the scarce calcium-binding lysozymes, one can predict that the strong Ca^{2+}-binding sites of all these proteins are analogous to that of baboon α-LA (Figure 1). In the case of human α-LA, this notion has been verified by the very recent X-ray crystal structure of this protein (Acharya et al., 1991). In addition, the engineered calcium-binding site in mutated human lysozyme would be expected to possess virtually identical ^{43}Ca NMR properties. The ^{43}Ca chemical shifts of these sites fall in a very narrow region upfield of free calcium and ≈15 ppm upfield of EF-hand calcium-binding proteins, such as calmodulin (+10 ppm) (Andersson et al., 1982). Despite the relatively narrow chemical shift range displayed for ^{43}Ca (Brevard & Granger, 1981) and the typically large line widths for protein-bound $^{43}Ca^{2+}$, this technique is sensitive to subtle differences in the calcium sites of these separate classes of proteins. Most EF-hand Ca^{2+}-binding proteins contain at least two high-affinity Ca^{2+} sites, and structural studies have revealed that the calcium ions in these sites are coordinated by seven oxygen ligands, as in the α-lactalbumins and most likely the calcium-binding lysozymes [see McPhalen et al. (1991) and references cited therein]. A significant discrepancy between the helix–loop–helix motif and the α-lactalbumin elbow lies in the fact that in the former one of the carboxylate ligands binds in a bidentate fashion to the calcium; this is not observed in the latter. Also, in the α-lactalbumin elbow, all side-chain carboxylates involved in metal binding originate from aspartate residues, whereas in EF-hand proteins the side chains from both Asp and Glu participate in calcium binding. These incongruencies may explain the appreciable difference in the characteristic resonance frequencies of these classes of metalloproteins. In addition, the χ values for the proteins studied here are substantially less than those found for EF-hand Ca^{2+}-binding proteins, such as calmodulin (χ = 1.15), troponin C (χ = 1.05), and parvalbumin (χ = 1.3) (Andersson et al., 1982). This reflects a greater degree of symmetry in the environment of the calcium in the α-lactalbumin elbow compared to the EF-hand site. Other calcium-binding sites which are structurally different from the motifs described above also display unique ^{43}Ca NMR properties. For example, aside from a signal characteristic of an EF-hand Ca^{2+} site (+6 ppm, χ = 0.9), in the case of calbindin D_{9K} one observes a broad resonance at –8 ppm (χ = 1.3) assigned to $^{43}Ca^{2+}$ in the so-called "pseudo-EF-hand site", which deviates from the typical helix–loop–helix motif due to the insertion of two amino acids in the binding loop (Vogel et al., 1985). Also, for the serine protease trypsin, a signal at +13 ppm is found for the lone calcium-binding site, which is formed by a loop flanked by two β-strands (Chiancone et al., 1985). In this protein, the metal ion is complexed by only six oxygen ligands, all monodentate, in an octahedral geometry (McPhalen et al., 1991). This high order of symmetry is demonstrated by the low quadrupole coupling constant (χ = 0.7) found for $^{43}Ca^{2+}$ bound to this site.

The ^{43}Ca NMR results presented here also shed some light on other aspects of the nature of the high-affinity calcium-binding sites in α-LA and LZ. First, calcium is bound in slow exchange to these sites, in agreement with ^{1}H NMR studies of conformational changes resulting from metal ion binding in bovine α-LA (Berliner et al., 1987) and equine LZ (Tsuge

et al., 1991). Second, this slow-exchange behavior is reflected by the high affinities of these sites for calcium. The K_D values obtained by this technique are in good agreement with data obtained using other techniques (equine LZ, pigeon LZ, and bovine α-LA, K_D 4×10^{-7}, 6×10^{-8}, and 3×10^{-8} M, respectively; Sugai et al., 1989; K_D values for bovine, human, and goat α-LA were found to be virtually identical; Segawa & Sugai, 1983), though dissociation constants reported in the literature for bovine α-LA vary over a considerable range (Berliner & Johnson, 1988). Third, the correlation times calculated for bound $^{43}Ca^{2+}$ suggest that the motional freedom of the bound calcium with respect to the protein is negligible in the α-lactalbumins and calcium-binding lysozymes. Finally, the pK_a' data reflect the involvement of carboxylate groups in the ligation of the metal ion in the strong Ca^{2+}-binding sites of all four proteins. In the case of bovine α-LA, the apparent pK_a value obtained by this technique is markedly lower than that from fluorescence studies of the acid conformational change in this protein ($pK_a' = 5.0$; Permyakov et al., 1981, 1985). This group also reported a Hill coefficient on the order of 3, which is in agreement with our results. The authors rationalized this value of n as being due to the replacement of the bound calcium by three H^+ (i.e., acidification of the carboxylate ligands involved in metal binding). However, since it is thought that three carboxylate groups (from the conserved Asp residues) also participate in calcium binding in the lysozymes, this hypothesis cannot account for the much lower Hill coefficients observed for these proteins. It seems, therefore, that the large Hill coefficients for the α-lactalbumins are due to contributions from some other process(es), such as the precipitation which occurs upon the removal of calcium in α-lactalbumins at low pH or the substantially greater conformational changes associated with calcium binding/removal in bovine α-LA compared to equine LZ (Berliner et al., 1987; Desmet et al., 1989; Tsuge et al., 1991).

Fluorescence studies of α-lactalbumins from several species, including bovine and human, indicated the presence of a distinct metal-binding site, commonly called the "zinc site" (Murakami & Berliner, 1983; Musci & Berliner, 1985), with a K_D for zinc in the low micromolar range. The existence of such a site was further suggested by the manner in which numerous metal ions, including Zn^{2+}, affect the binding of α-lactalbumins to hydrophobic matrices such as phenyl-Sepharose (Lindahl & Vogel, 1984). In addition, a secondary site for Ca^{2+} (K_D = 0.4 mM) in bovine α-LA has also been reported (Murakami et al., 1982). Using ^{43}Ca NMR, we directly observed the binding of the latter metal ion to a second site on bovine α-LA, but found no evidence for a second calcium-binding site in the human protein (as well as the lysozymes). Furthermore, we found that the affinity of this site in bovine α-LA for calcium is quite weak (K_D = 3 mM) and that at least one histidine influences metal ion binding ($pK_a' = 6.3$). It is also interesting to note that the Ca^{2+} exchange from this site is not as fast as one could have estimated assuming a diffusion-limited on-rate (i.e., $k_{on} = 6 \times 10^4$ s^{-1} M^{-1} compared to $k_{on} > 10^8$ s^{-1} M^{-1} for a diffusion-controlled process). A similar observation has been made for Ca^{2+} binding to some serine proteases (Chiancone et al., 1985). From ^{1}H NMR studies of the C2 and C4 protons of the three His residues in bovine α-LA, it is known that the apparent pK_a value of His-68 ($pK_a' \approx 6.4$) is closest to the value obtained by ^{43}Ca NMR compared to His-107 ($pK_a' \approx 5.7$) and His-32 ($pK_a' \approx 6.6$) (Bradbury & Norton, 1975; Hiraoki and Vogel, unpublished results). In the case of human α-LA, the residue at position 68 is not a histidine, and neither of the pK'_a values for the other two histidines (His-32 and -107) are in the vicinity of that found for the weak Ca^{2+}-binding site of bovine α-LA (Hiraoki and Vogel, unpublished results). In addition, photo-CIDNP studies of the surface exposure of His residues (in addition to Tyr and Trp) in several α-lactalbumins revealed the presence of one exposed histidine (at position 68) in bovine α-LA, while for the human protein a complete absence of any exposed histidines was observed (Berliner & Kaptein, 1981). Also, the addition of the paramagnetic metal ion Co^{2+}, which is thought to interact with the zinc site in bovine α-LA, seriously alters the aromatic ^{1}H NMR signals of His-68 (Berliner et al., 1987; Hiraoki and Vogel, unpublished results). On the basis of these findings and our ^{43}Ca NMR results, one can propose that the weak second calcium-binding site of bovine α-LA is close to His-68. The lack of a detectable second calcium site in human α-LA suggests either that the putative zinc-binding site in this protein is unable to chelate Ca^{2+} or that the second Ca^{2+} site in bovine α-LA is distinct from the zinc site and simply does not exist in the human protein.

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