In the orthorhombic material, this distance is slightly shortened to 2.350 (14) Å. Recently published high-temperature neutron diffraction work[10] reports a 2.543 (11) Å Cu–O distance at 600 °C. This may account for the distance observed in the XANES data, especially if, during measurement using the intense synchrotron source, localized heating took place and converted some of the material to the tetragonal form.

The other possibility for the observation of a disorder may be related to this material's sensitivity to its thermal history. The sample used for the XANES work may have accidentally been prepared with an anomalous composition. However, on the basis of our data, any disorder between Y and Cu is not routinely observed in either ceramic or melt-grown material.

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^{43}Ca Nuclear Magnetic Resonance Spectra of Ca^{2+}–S100 Protein Solutions

Sir:

Dramatic increase of the line width of ^{43}Ca ($I = {}^{7}/_{2}$) NMR for Ca^{2+}–protein solution has hampered the detection of ^{43}Ca NMR for Ca^{2+}–protein (1:1 per binding site in the protein) complex by using a conventional NMR spectrometer equipped with conventional probe.[1,2]

S100 proteins are nerve specific proteins and belong to a family of EF-loop Ca^{2+}-binding proteins such as calmodulin or troponin C.[3-5] $S100_b$ protein and $S100_{a0}$ protein are homodimer proteins consisting of two α subunits (M_R = 10400) and of two β subunits (M_R = 10500), respectively, while $S100_a$ protein is a heterodimer protein consisting of one α and one β subunit.[3]

We show herein that ^{43}Ca NMR spectra of Ca^{2+}–$S100_b$ protein and Ca^{2+}–$S100_{a0}$ protein solutions consist of two distinguishable resonances that correspond to Ca^{2+} ions bound to different sites of the $S100_b$ and $S100_{a0}$ proteins. This is the first report of ^{43}Ca NMR signals ascribable to two discernible slowly exchanging Ca^{2+} ion binding sites.

$S100_b$, $S100_{a0}$, and $S100_a$ proteins were purified to gel-electrophoretical homogeneity by the method described previously.[6-10]

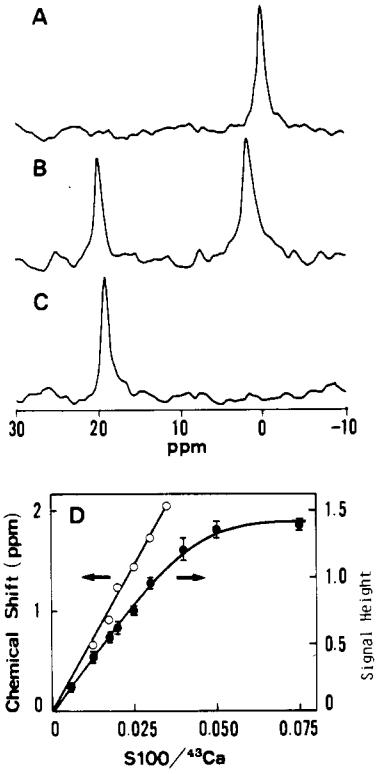

Figure 1. ^{43}Ca NMR spectra of 1.45 mM $^{43}Ca^{2+}$ (A), 1.45 mM $^{43}Ca^{2+}$–0.02 mM $S100_b$ protein (B), and 1.45 mM $^{43}Ca^{2+}$–0.11 mM $S100_b$ protein (C) in 0.15 M HEPES–K^+ buffer (pH 7.2). Signal heights were normalized to that of 1.45 mM $^{43}Ca^{2+}$. A total of 5×10^4 scans were acquired, and exponential line broadening of 20 Hz was applied. Part D shows changes in height of the signal at 19.2 ppm and of the chemical shift of the signal around 0.0 ppm caused by adding $S100_b$ protein. In part D, values along the x axis give the concentration ratio of [$S100_b$ protein]/[$^{43}Ca^{2+}$] and those along the y axis are signal height expressed in arbitrary units.

^{43}Ca NMR spectra of $^{43}Ca^{2+}$ (49.1%)–protein complexes were obtained as described previously[2] in 0.15 M *N*-(2-hydroxyethyl)piperazine-*N*'-ethanesulfonic acid (HEPES)–K^+ buffer (pH 7.2).

By addition of $S100_b$ protein to a 1.45 mM $^{43}Ca^{2+}$ solution, the ^{43}Ca NMR signal of the free $^{43}Ca^{2+}$ moved downfield by 2.0 ppm with concomitant increase of the line width (measured at half-height) from 0.5 Hz to more than 100 Hz (Figure 1A,B,D).[11] Simultaneously a new signal appeared at 19.2 ppm downfield from the signal of free $^{43}Ca^{2+}$ (Figure 1B). The increase of the peak height of the 19.2 ppm resonance saturated at the concentration ratio [$S100_b$]/[Ca^{2+}] = 0.050 (Figure 1D). For the $^{43}Ca^{2+}$ (1.45 mM)–$S100_b$ protein (0.11 mM) solution, only the signal at 19.2 ppm was observed (Figure 1C). Since Mg^{2+} is known to bind to S100 proteins,[3] excess Mg^{2+} was added to the Ca^{2+}–$S100_b$ solution to determine whether the Ca^{2+}- and Mg^{2+}-binding sites are the same. The signal at 19.2 ppm decreased by half, and a new signal appeared at 2.1 ppm upon addition of 50 mM Mg^{2+} to the Ca^{2+} (1.45 mM)–$S100_b$ (0.11 mM) solution.

When $S100_{a0}$ protein was added to the $^{43}Ca^{2+}$ (1.45 mM) solution, a new signal appeared at 19.3 ppm downfield from the free $^{43}Ca^{2+}$ signal (at 0.0 ppm) (Figure 2A,B). The appearance of the new signal at 19.3 ppm was concomitant with the shift of the resonance from 0.0 to 5.8 ppm and increased broadening of the original signal (now at 5.8 ppm).[11] The peak height of the

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(11) In Figures 1 and 2, the signal/noise ratio was uniformly improved by exponential multiplication that introduced 20-Hz line broadening. This purposive line broadening outwardly decreases the difference of the line widths between A and B in Figures 1 and 2.

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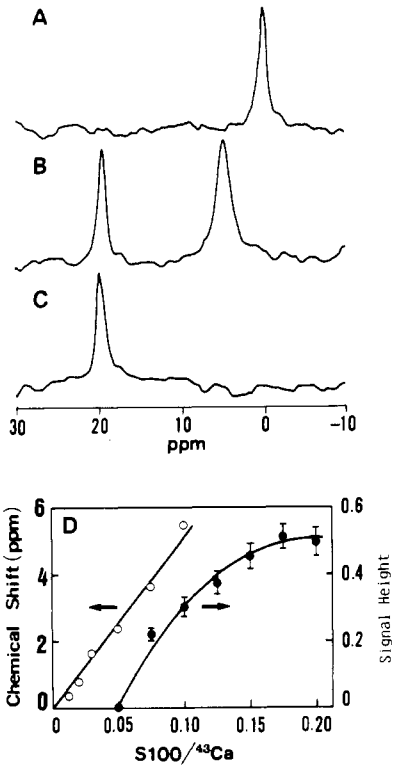

Figure 2. ^{43}Ca NMR spectra of 1.45 mM $^{43}Ca^{2+}$ (A), 1.45 mM $^{43}Ca^{2+}$–0.095 mM $S100_{a0}$ protein (B) and 1.45 mM $^{43}Ca^{2+}$–0.29 mM $S100_{a0}$ protein (C) in 0.15 M HEPES–K^+ buffer (pH 7.2). Signal heights were normalized to that of 1.45 mM $^{43}Ca^{2+}$. A total of 5×10^4 scans were acquired, and exponential line broadening of 20 Hz was applied. Part D shows changes in height of the signal at 19.3 ppm and of the chemical shift of the signal around 0.0 ppm caused by adding $S100_{a0}$ protein. In part D, values along the x axis give the concentration ratio of [$S100_{a0}$ protein]/[$^{43}Ca^{2+}$] and those along the y axis are the signal height expressed in arbitrary units.

resonance at 19.3 ppm saturated at the concentration ratio $[S100_{a0}]/[Ca^{2+}] = 0.15$ (Figure 2D). For the $^{43}Ca^{2+}$ (1.45 mM)–$S100_{a0}$ (0.29 mM) solution, only the peak at 19.3 ppm was observed (Figure 2C). Addition of 40 mM Mg^{2+} to the $^{43}Ca^{2+}$ (1.45 mM)–$S100_{a0}$ (0.29 mM) solution decreased the band at 19.3 ppm by half and gave rise to a band around 5.8 ppm.

Upon addition of $S100_a$ protein to 1.45 mM $^{43}Ca^{2+}$ solution, the free $^{43}Ca^{2+}$ NMR resonance moved downfield by nearly 5 ppm concomitant with an increase of line width of 0.5 Hz to 80 Hz (Figure 3A,B). These spectral changes saturated at 0.145 mM $S100_a$ (Figure 3C). Addition of 50 mM Mg^{2+} to the $^{43}Ca^{2+}$ (1.45 mM)–$S100_a$ protein (0.145 mM) solution caused a decrease in the line width from 80 to 40 Hz (not shown).

Since the ^{43}Ca ($I = {}^7/_2$) NMR resonance of free $^{43}Ca^{2+}$ is dramatically broadened upon addition of ligands or Ca^{2+}-binding proteins due to fast exchange of the Ca^{2+} ion,[1] detection of ^{43}Ca NMR spectra of a Ca^{2+}–Ca^{2+}-binding protein (1:1 per binding site) has been very difficult with conventional NMR spectrometers and probes. In addition, up to now, there has been no report of the detection of two slowly exchanging ^{43}Ca NMR signals in a Ca^{2+}–protein solution. In this paper, we report the first detection of two slowly exchanging ^{43}Ca NMR signals for $^{43}Ca^{2+}$–$S100_b$ and $^{43}Ca^{2+}$–$S100_{a0}$ protein solutions using a conventional NMR probe.

The appearance of a ^{43}Ca resonance at 19.2 ppm (Figure 1D) observed in the $^{43}Ca^{2+}$–$S100_b$ solution suggests that $S100_b$ protein has at least 20 Ca^{2+}-binding sites. The signal at 19.2 ppm may reflect the slowly exchanging ($k_{off} \simeq 3 \times 10^{-3}$ s^{-1})[12] Ca^{2+} ions bound to specific sites[13] in $S100_b$ while the broadened signal at

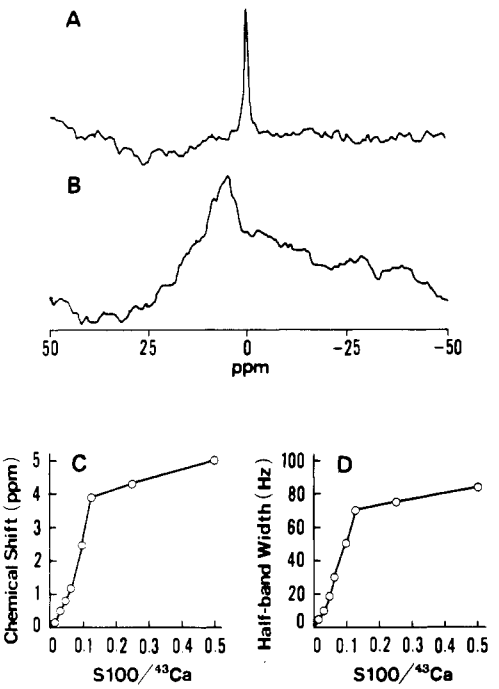

Figure 3. ^{43}Ca NMR spectra of 1.45 mM $^{43}Ca^{2+}$ (A) and 1.45 $^{43}Ca^{2+}$–0.73 mM $S100_a$ protein (B) in 0.15 M HEPES–K^+ buffer (pH 7.2). A total of 1×10^5 scans (A) and 4×10^5 scans (B) were acquired and exponential line broadening of 20 Hz was applied. Parts C and D show changes of chemical shift and line width, respectively, of ^{43}Ca NMR of 1.45 mM $^{43}Ca^{2+}$ caused by adding $S100_a$ protein. In parts C and D, values along the x axis give the concentration ratio [$S100_a$]/[$^{43}Ca^{2+}$].

2.0 ppm may reflect the more rapidly exchanging ($k_{off} \geq 10^2$ s^{-1})[12] Ca^{2+} ions bound to other specific sites of $S100_b$.

The ^{43}Ca resonance at 19.3 ppm (Figure 2D) observed in the $^{43}Ca^{2+}$–$S100_{a0}$ solution suggests that $S100_{a0}$ protein has at least 7 Ca^{2+}-binding sites. Signals at 19.3 and 5.8 ppm for the $^{43}Ca^{2+}$–$S100_{a0}$ solution may reflect the slowly exchanging ($k_{off} \simeq 3 \times 10^{-3}$ s^{-1})[12] Ca^{2+} ions bound to specific sites[13] and the rapidly exchaning ($k_{off} \simeq 10^2$ s^{-1})[2] Ca^{2+} ions bound to other specific sites in $S100_{a0}$, respectively.

^{43}Ca NMR spectra of the $^{43}Ca^{2+}$–$S100_a$ protein solutions indicate the range of fast exchange in contrast to the $S100_b$ and $S100_{a0}$ solutions.[14] The ^{43}Ca NMR spectral changes (chemical shift and line width) caused by adding $S100_a$ saturated at a concentration ratio of $[S100_a]/[Ca^{2+}] = 1/10$ (Figure 3C). This finding suggests that the ^{43}Ca NMR of the $^{43}Ca^{2+}$–$S100_a$ solution may reflect the environment of the 10 Ca^{2+}-binding sites to which Ca^{2+} exchanges very fast ($k_{off} \geq 10^2$ s^{-1}).[12] Our finding agrees with a previous suggestion that there are 9–11 Ca^{2+}-binding sites in $S100_a$ protein in 100–120 mM Tris buffer.[15]

If the Ca^{2+}-binding sites of the S100 proteins are also the Mg^{2+}-binding sites, addition of excess Mg^{2+} to the Ca^{2+}–protein

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(13) The sharp resonances of the $S100_b$ and $S100_{a0}$ solutions might partly be attributed to a high symmetry of the Ca^{2+}-binding sites. A complex formation of Ca^{2+} with buffer is unlikely, however, to be a cause of the sharp resonances of the $S100_b$ and $S100_{a0}$ proteins since we observed the same NMR spectra in another buffer systems such as tris(hydroxymethyl)aminomethane (Tris) and 3-morpholinopropanesulfonic acid (MOPS) solutions.

(14) $S100_b$ protein and $S100_{a0}$ protein are homodimers, each of which has identical subunits, $\alpha\alpha$ and $\beta\beta$, respectively. Both proteins have sharp resonances of slowly exchanging Ca^{2+}. However, the heterodimer ($\alpha\beta$) protein, $S100_a$ protein, had no sharp ^{43}Ca NMR resonance. Probably the homodimer protein has a special conformation at the Ca^{2+}-binding site which is different from that of the heterodimer protein. Many oligomeric proteins such as hemoglobin are known to be active only when several subunits are bound together. Conformation of the subunit protein is often different from that of the oligomeric protein. Similarly conformation of the homodimer protein ($S100_b$ and $S100_{a0}$) may be different from that of the heterodimer protein ($S100_a$). In those respects, activities of the homodimer proteins ($S100_b$ and $S100_{a0}$) may be fairly different from that of the heterodimer protein ($S100_a$), although functions of these proteins have not been well understood.[3]

solutions should cause band narrowing of the resonance of the rapidly exchanging $^{43}Ca^{2+}$ ions bound to the protein and/or a decrease in NMR intensity of those slowly exchanging bound $^{43}Ca^{2+}$ ion resonances.[2] Since addition of excess Mg^{2+} to the Ca^{2+}–$S100_b$ and Ca^{2+}–$S100_{a0}$ solutions changes both ^{43}Ca NMR signals, the two environmentally different Ca^{2+}-binding sites of $S100_b$ and $S100_{a0}$ appear to be Mg^{2+}-binding sites. Similarly at least 10 Ca^{2+}-binding sites of $S100_a$ may be Mg^{2+}-binding sites since addition of excess Mg^{2+} to the Ca^{2+}–$S100_a$ solution caused ^{43}Ca NMR resonance narrowing. Our ^{43}Ca NMR findings on the Ca^{2+}- and Mg^{2+}-binding sites of the S100 proteins are in accordance with the previous suggestion that Ca^{2+}-binding sites of S100 proteins are Mg^{2+}-binding sites.[15,16]

Using ^{43}Ca NMR studies, we have presented direct evidence that (1) $S100_b$, $S100_{a0}$, and $S100_b$ proteins have at least 20, 7, and 10 Ca^{2+}-binding sites, respectively, and that (2) the Ca^{2+}-binding sites of S100 proteins are also Mg^{2+}-binding sites.

We would like to emphasize the utility of ^{43}Ca NMR in the study of Ca^{2+}-binding proteins using a conventional NMR spectrometer and probe.

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Exploitation of Crystalline Architecture and Solution Data in the Rational Preparation of Novel Mixed-Metal ATP Complexes

Sir:

Metal ions and metalloenzymes are ubiquitous in nucleotide and nucleic acid biochemistry.[1-8] However, crystallization of relevant metal complexes is difficult since nucleotides have three types of moieties (charged phosphate; flexible puckered sugar; planar aromatic bases). Crystallization of nucleoside triphosphate complexes has been particularly difficult due to the high negative charge of species such as ATP^{4-}. Nevertheless, considerable information concerning metal binding modes and complex stability in solution has been gathered.[2,3,5] We now report that we have rationally exploited this solution information, together with the sparse crystallographic information on ATP complexes, to prepare the first X-ray quality mixed-metal ATP crystals. Examples include the first crystalline Cd complex and a new Zn complex.

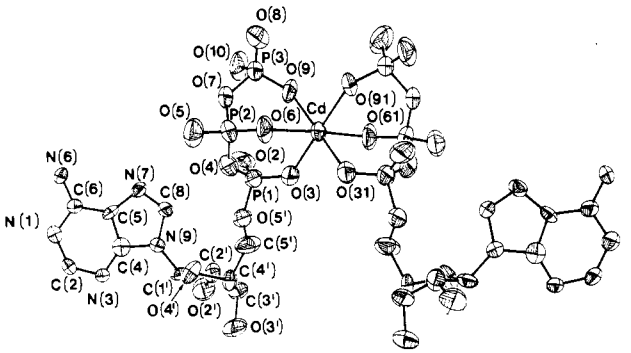

Figure 1. ORTEP drawing showing the $[Cd(HATP)_2]^{4-}$ ion of I with the labeling scheme of the atoms. The view is parallel to the *b* axis. Ellipsoids enclose 30% probability.

Solutions of bis(2-pyridyl)amine (BPA), ATP, and Mg^{2+}, Ca^{2+}, or Mn^{2+} cations at ca. pH 4.5 formed single crystals suitable for X-ray analysis formulated as $[M(H_2O)_6][M'(HATP)_2]\cdot 2HBPA\cdot nH_2O$.[6,7] In previous studies, M = M' and it appears essential that the $[M(H_2O)_6]^{2+}$ cations insulate the highly charged $[M'(HATP)_2]^{4-}$ anions from each other. For such a role, each cation forms four H-bonds to two anions for a total of eight H-bonds. The $HBPA^+$ moieties stack with the adenine, which is protonated at N(1). Those metal species that gave the best crystals (Mg^{2+}, Ca^{2+}, Mn^{2+}) have been characterized by solution studies as having a high preference for O over N and for having a >80% "open" coordination of ATP with little involvement of adenine directly or indirectly (via a bridging H_2O) with the metal.[2,3] Zn^{2+} gives crystals of poor quality, and Fe^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+} have not given similar crystalline materials. Compared to Mg^{2+}, Ca^{2+}, and Mn^{2+}, all these latter metal species either have a greater preference for N or have >30% "closed" coordination by ATP, i.e. involvement of the base either via indirect interaction

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(9) Crystal data for I: $C_{40}H_{76}N_{16}O_{41}P_6CaCd$, mol wt 1775.4, orthorhombic, space group $C222_1$, a = 22.846 (3) Å, b = 10.252 (2) Å, c = 31.914 (6) Å, V = 7475 (1) Å^3, Z = 4, d_{calcd} = 1.58 g/cm^3, μ(Mo Kα) = 5.79 cm^{-1}. Crystal data for II: $C_{40}H_{82}N_{16}O_{44}P_6Mg_{0.72}Zn_{1.28}$, mol wt 1778.2, orthorhombic, space group $C222_1$, a = 22.666 (3) Å, b = 10.131 (2) Å, c = 30.893 (6) Å, V = 7094 (1) Å^3, Z = 4, d_{calcd} = 1.66 g/cm^3, μ(Mo Kα) = 6.25 cm^{-1}. Data collection and structure solution for I and II: Data were collected on a Syntex $P2_1$ diffractometer with crystals measuring about 0.15 × 0.15 × 0.10 mm. A total of 3632 (I) and 2612 (II) independent reflections were measured in the range 3° < 2θ < 50° (I) and 3° < 2θ < 45° (II) at 20 ± 1 °C. The data were corrected for *Lp* effects but not for absorption because of the small absorption coefficients and crystal sizes. Only the 2394 (I) and 2206 (II) observed reflections with $F > 3\sigma(F)$ were used subsequently. The cell parameters and the distribution of reflection intensities indicated that the structures of I and II are essentially isomorphous with reported ternary complexes.[6,7,10] However, the Patterson synthesis was still carried out for I and II. The positions of the Cd^{2+} and Zn^{2+} ions were determined and the structures were completed by a series of three-dimensional difference-Fourier maps. All the metals and the ATP non-hydrogen atoms were refined anisotropically for I and II. The N(1) hydrogen atom, located for II from the Fourier-difference analysis, was included in the refinement. Twelve blocked full-matrix least-squares cycles (SHELXTL program system 1985, implemented on a Data General Eclipse S/140 minicomputer) decreased the $R = (\sum(|F_o| - |F_c|)/\sum|F_o|)$ index to 10.15% (I) and 9.03% (II). The $R_w = ([\sum w(|F_o| - |F_c|)^2/\sum wF_o^2]^{1/2})$ index converged to 10.89% (I) and 9.89% (II). The weights were of the form $w = a/(\sigma^2(F) + bF^2)$. Scattering factors were from SHELXTL.
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