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In situ NMR measurement of macromolecule-bound metal ion concentrations

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Abstract Many nucleic acids and proteins require divalent metal ions such as Mg^{2+} and Ca^{2+} for folding and function. The lipophilic alignment media frequently used as membrane mimetics also bind these divalent metals. Here we demonstrate the use of ^{31}P NMR spectrum of a metal ion chelator (deoxycytidine diphosphate) to measure the bound $[Mg^{2+}]$ and $[Ca^{2+}]$ in situ for several biological model systems at relatively high divalent ion concentrations (1–10 mM). This method represents a general approach to measuring divalent metal ion binding in NMR samples where the amount and type of metal ion added to the system is known.

Keywords Divalent ion concentration · ^{31}P NMR · dCDP · Bicelles · Dickerson dodecamer · $\beta\gamma$ -Crystallin · Ca^{2+} dependent protein

Mg^{2+} and Ca^{2+} play pivotal roles in biomolecular structure and function. Mg^{2+} stabilizes nucleic acid structures and directly participates in the catalytic activity of many

ribozymes and in protein enzymes such as kinases (Sreedhara and Cowan 2002; Black et al. 1994; Riccitelli et al. 2014). Ca^{2+} is involved in cell signaling pathways via its interactions with calcium-binding proteins (Clapham 1995). Membrane mimetics composed of mixtures of long-chain and short-chain phosphatidylcholine lipids (bicelles) also bind Mg^{2+} and Ca^{2+} , which in turn modulate their liquid crystalline phases (Brindley and Martin 2012; Arnold et al. 2002) in ways that are useful for both crystallographic (Faham and Bowie 2002) and NMR protein structural studies. In such complex multi-component samples, the concentration of bound divalent ions can be quite different from the total amount added: several competing binders may be present and all of the relevant equilibria can vary with temperature and pH. Understanding the chemical processes taking place in biomolecular NMR samples requires in situ measurement of the bound divalent ion concentrations. Whereas equilibrium dialysis and size-exclusion chromatography can be used to establish predetermined concentrations of free metal ions, some samples, e.g. the highly concentrated lipid and surfactant mixtures used for residual dipolar coupling (RDC) measurements in biomolecular structure determination, are difficult to prepare using these methods. A known amount of divalent metal ions is added to these samples upon preparation; however the fraction of the ions bound to the macromolecules is usually unknown.

Although fluorescent dyes can be used to measure $[Ca^{2+}]$ in vivo, where the concentration range is in the low μM range, biomolecular NMR samples often contain mM concentrations of the divalent ion and thus require alternative measurement techniques. For example, proteins such as phospholambans (Oxenoid and Chou 2005; Traaseth et al. 2009), annexins (Turnay et al. 2002; Pathuri et al. 2008) and some $\beta\gamma$ -crystallins (Shimeld et al. 2005;

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Barnwal et al. 2009) display Ca^{2+} -dependent conformational changes where the full investigation of their behavior requires stoichiometric or higher concentrations of Ca^{2+} . ^{31}P NMR has previously been used to determine the cytosolic $[\text{Mg}^{2+}]$ in vivo in mM ranges using endogenous probes such as ATP and phosphocreatine (London 1991; Gupta et al. 1978; Gupta and Yushok 1980). Free $[\text{Mg}^{2+}]$ in skeletal and cardiac muscles was found to be 0.6–0.8 mM (Gupta and Moore 1980; Garfinkel and Garfinkel 1984; Alvarez-Leefmans et al. 1987). Shulman et al. (1965) have used ^{31}P NMR to measure the binding of divalent cations with AMP and RNA and discovered that the phosphate groups were involved in this interaction. Changes in the ^{31}P chemical shift of the chelator binding site (e.g. the β - and γ -phosphates in ATP and the α - and β -phosphates in ADP) (Son et al. 1975; Cohn and Hughes 1962) are straightforwardly related to the binding of divalent ions. This relationship enables the ^{31}P spectrum to serve as a sensitive reporter of the amount of divalent ions bound to macromolecules when the total ion concentration in the NMR sample is known.

Here we present a minimally invasive and generally applicable in situ approach for determining the concentration of macromolecule-bound Mg^{2+} or Ca^{2+} given a known amount of a single divalent species added to the sample. We chose deoxycytidine diphosphate (dCDP) as a metal ion chelator over the triphosphate form because its binding constant for divalent ions (i.e. $\log K$ of CDP for Mg^{2+} is 3.22, vs $\log K$ of 4.03 for CTP) (Frey and Stuehr 1972) allows concentration measurements in the low millimolar range (Khan and Martell 1966, 1967). Furthermore, dCDP is more stable than dCTP over the timescales and temperatures used in biomolecular NMR. Additionally, the characteristic doublet of H5-H6 in the ^1H spectrum of dCDP serves as an internal standard for RDC measurements in the bicelle samples used for structural studies. By monitoring the α -phosphate ^{31}P chemical shift of dCDP as a function of free metal ion concentration in solution, the concentration of Mg^{2+} or Ca^{2+} bound to the biomolecule(s) of interest may be calculated using standard curves such as these shown in Fig. 1 and Eqs. 1, 2 and 3. Because pH and temperature can affect the affinity of divalent ions for biomolecules as well as the phosphate chemical shifts, these variables are considered in constructing the standard curves. In this case, low temperatures were considered because the exchange rate of exchangeable protons in nucleic acids is slowed, facilitating NMR structure determination.

To construct standard titration curves for Mg^{2+} and Ca^{2+} at different pHs and temperatures, increasing concentrations of MgCl_2 (Fig. 1a) and CaCl_2 (Fig. 1b) (0.01–50 mM) were added to NMR samples containing 0.3 mM of dCDP in either 10 mM piperazine or Tris-HCl buffer. We chose this relatively low concentration of dCDP

Fig. 1 Titration curves of the α -phosphate ^{31}P chemical shift as a function of increasing **a** $[\text{Mg}^{2+}_{\text{free}}]$ and **b** $[\text{Ca}^{2+}_{\text{free}}]$ at different pH values and temperatures relative to 85 % phosphoric acid (external reference). Blue represents titrations at 8 °C and red represents titrations at 25 °C. At 8 °C, the pH increases by about 0.2 units from the pH values given

to minimize interference of the probe with our biological systems. If a different concentration of dCDP is desired, new titration curves must be generated; however, we observed that doubling the concentration of dCDP only minimally changes the free divalent metal concentration and therefore the macromolecule-bound concentration (as shown for 0.6 mM dCDP in Figure S1). The chemical shift values for each of the data points are tabulated in Tables S1–S13. To verify that negligible amounts of divalent metal ions were present in the dCDP samples prior to titration of divalent cations, EDTA was added to a sample of dCDP at pH 7.05 and ^{31}P spectrum was acquired at 25 °C as a control. The acquired α -phosphate chemical shift was within the ± 0.03 ppm error in the chemical shift determination as described in the error analysis section of the Supplemental Information. The resulting titration curves (Fig. 1) were fit using nonlinear regression in Mathematica to a function shown below in Eq. 1, which relates to the Hill equation (Hill 1910) and describes cooperative binding:

$$\delta_c = \delta_\infty + \text{Span} \left(1 - \frac{[M_{\text{Free}}^{2+}]^n}{K_{1/2} + [M_{\text{Free}}^{2+}]^n} \right) \quad (1)$$

where $[M_{\text{Free}}^{2+}]$ is the free metal ion concentration assuming that dCDP is in a 1:1 stoichiometric ratio with the metal ion, δ_∞ represents the chemical shift at binding saturation, δ_c represents the chemical shift corresponding to the $[M_{\text{Free}}^{2+}]$ on the titration curve, *Span* is the range encompassing the initial (δ_0) and final (δ_∞) chemical shifts measured during the titrations, *n* is the polynomial exponent that determines if the binding of metal ions to the dCDP ligand is cooperative and $K_{1/2}$ is the midpoint concentration when the bound dCDP is in equilibrium with the free dCDP. Values acquired via the nonlinear regression for ^{31}P δ_∞ , $K_{1/2}$, and *n* at several pHs and temperatures are tabulated in Table 1 for Mg^{2+} and Table S14 for Ca^{2+} .

Our data in Tables 1 and S14 show that the $K_{1/2}$ values of Ca^{2+} for dCDP were higher than those of Mg^{2+} , representing a tighter binding of dCDP to Mg^{2+} than to Ca^{2+} . The stronger affinity is due to the smaller ionic radius and larger intermolecular interaction energy of Mg^{2+} (Gresh 1980). Because the pK_a of the terminal phosphate is 6.38 (Frey and Stuehr 1972), it is partially protonated throughout the range of pH values studied, affecting the chemical shift of both phosphates. Binding of divalent ions results in displacement of the protons on the phosphate groups causing the ^{31}P chemical shift to change (London 1991). We found that the $K_{1/2}$ values for the binding of divalent ions to the α -phosphate were highly sensitive to pH, which is an important consideration in measuring the bound concentrations in situ because presumably binding for other biomolecules is also pH sensitive. The polynomial exponent (*n*) values were ~ 1 for Ca^{2+} implying the formation of a 1:1 dCDP: metal ion complex, whereas for Mg^{2+} they became >1 upon an increase in pH, indicating a cooperative binding process where binding of dCDP to the metal ions was facilitated by previously bound dCDP (2:1 dCDP: metal ion complex) as observed by Frey and Stuehr (1972).

The line shapes observed in the ^{31}P spectra of dCDP are dominated by the exchange process between the bound and unbound forms. At concentrations well below saturation,

the phosphate peak is broad, with substantial narrowing observed near the titration extremes. Even at low temperatures, broad peaks were observed for the bound versus the unbound state of the α phosphate of dCDP similar to Sontheimer et al. (1986) did for ATP.

After acquiring ^{31}P data on a dCDP sample in the presence of a biomolecule and a known concentration of $[\text{Mg}^{2+}]$ or $[\text{Ca}^{2+}]$, the α -phosphate chemical shift is first used (Fig. 2a) in determining the $[M_{\text{Free}}^{2+}]$ by following the appropriate pH and temperature titration curve (Fig. 2b). This concentration along with the $[M_{\text{total}}^{2+}]$ and [dCDP] is then used in Eqs. 2 and 3 to determine the F_b and the $[M_{\text{bound}}^{2+}]$ by the biomolecule of interest:

$$F_b = 1 - \frac{1}{[M_{\text{total}}^{2+}]} \left[[M_{\text{Free}}^{2+}] + \frac{\delta_0 - \delta_c}{\delta_0 - \delta_\infty} \times [\text{dCDP}] \right] \quad (2)$$

$$[M_{\text{bound}}^{2+}] = [M_{\text{total}}^{2+}] \times F_b \quad (3)$$

where F_b is fraction of metal bound by the biomolecule.

Using the titration curves from Fig. 1 and Eqs. 1, 2 and 3, we determined the bound concentration of divalent metal ions in three different systems. To account for divalent metals initially present in the biomolecular samples, EDTA was added to each sample prior to addition of divalent metals and ^{31}P spectra were acquired to compare with spectra in the absence EDTA. As was the case for the dCDP sample, the change in the ^{31}P chemical shift was within experimental error, indicating negligible initial concentrations of divalent cations. If necessary, the amount of divalent metal ions initially present in the sample can be minimized by adding EDTA before protein purification or using EDTA-containing denaturing conditions for nucleic acid purification, followed by dialysis to ensure that no

Table 1 Interaction of the dCDP α -phosphate with free $[\text{Mg}^{2+}]$

pH/Temp (°C)	δ_∞^* (ppm)	$K_{1/2}^*$ (mM)	<i>n</i> *
6.05/25.0	9.33 $\pm$ 0.03	0.84 $\pm$ 0.12	0.77 $\pm$ 0.08
6.25/8.0	9.51 $\pm$ 0.03	0.79 $\pm$ 0.11	1.10 $\pm$ 0.12
7.05/25.0	9.28 $\pm$ 0.03	0.47 $\pm$ 0.07	1.06 $\pm$ 0.11
7.25/8.0	9.48 $\pm$ 0.03	0.43 $\pm$ 0.06	1.51 $\pm$ 0.16
8.05/25.0	9.25 $\pm$ 0.03	0.25 $\pm$ 0.03	1.23 $\pm$ 0.13
8.25/8.0	9.44 $\pm$ 0.03	0.13 $\pm$ 0.02	1.47 $\pm$ 0.16

* ^{31}P δ_∞ , midpoint ($K_{1/2}$) and polynomial exponent (*n*) values with their relative uncertainties at different pH and temperature values

Fig. 2 a ^{31}P spectra of dCDP in the presence of $\beta\gamma$ -crystallin and 7 mM Ca^{2+} showing differences in the α -phosphate chemical shifts at two different temperatures and neutral pH. The differences in the chemical shifts correspond to differences in the free divalent ion concentration in solution as interpolated using the method shown in (b) for 8 °C: e.g. 5.7 $\pm$ 1.0 mM of free plus bound Ca^{2+} to dCDP out of 7 mM added in the protein sample (shown by the arrow) implying that 1.1 $\pm$ 1.0 mM was bound by the protein. Using this method, the DNA bound 2.3 $\pm$ 0.7 mM Mg^{2+} , bicelles bound 7.7 $\pm$ 0.3 mM Mg^{2+} , and the bicelles bound 6.0 $\pm$ 0.7 mM Ca^{2+} at 8 °C

Table 2 Macromolecule- bound and free $[\text{Mg}^{2+}]$ and $[\text{Ca}^{2+}]$ at 25 and 8 °C calculated from the ^{31}P chemical shifts of dCDP

	Temp (°C)	$\delta^{31}\text{P}$ (ppm)	$[\text{M}_{\text{Free}}^{2+}]$ from $\delta^{31}\text{P}$ of dCDP (mM)	Bound $[\text{M}^{2+}]$ (mM)
12 bp DNA + 6 mM Mg^{2+}	25.0	9.41	3.3 ± 0.7	2.4 ± 0.7
	8.0	9.54	3.4 ± 0.6	2.3 ± 0.6
Bicelles + 10 mM Mg^{2+}	8.0	9.58	2.0 ± 0.3	7.7 ± 0.3
$\beta\gamma$ -crystallin + 7 mM Ca^{2+}	25.0	9.34	4.0 ± 0.7	2.8 ± 0.8
	8.0	9.49	5.7 ± 1.0	1.1 ± 1.0
Bicelles + 10 mM Ca^{2+}	8.0	9.55	3.8 ± 0.7	6.0 ± 0.7

divalent metal ions or EDTA are left in the sample. The Dickerson dodecamer DNA (Drew et al. 1981), $[\text{d}(\text{CGCGAATTCGCG})]_2$, at a concentration of 1.5 mM bound Mg^{2+} at slightly more than a 1:2 stoichiometric ratio (2.3 ± 0.6 mM) at 25 °C, and 2.4 ± 0.6 mM Mg^{2+} at 8 °C. The 1.5 Mg^{2+} ions bound to the dodecamer in solution at 25 °C are likely to bind to the major grooves in agreement with a crystal structure solved at low $[\text{Mg}^{2+}]$ (Shui et al. 1998). As expected, these solution measurements at low salt concentration differ from a crystal structure solved in the presence of higher salt, where 13 Mg^{2+} ions were bound (Minason et al. 1999). A DLPC/DHPC/cholesterol sulfate bicelle mixture (DLPC/DHPC/CS, $q = 3.5$, 15 % w/v) (Yamamoto et al. 2014) bound at 8 °C a large fraction (7.7 ± 0.3 mM) of the 10 mM Mg^{2+} added to the sample. When 10 mM Ca^{2+} was added to a bicelle sample, the sample bound 6.0 ± 0.7 mM Ca^{2+} at 8 °C, slightly less than the amount of Mg^{2+} bound by the same mixture. The differences in binding of DLPC/DHPC/CS to Mg^{2+} and Ca^{2+} at low temperatures are consistent with Mg^{2+} having a tighter binding to phosphates as observed from its lower $K_{1/2}$ value than that of Ca^{2+} in dCDP. A calcium-binding $\beta\gamma$ -crystallin protein from *Ciona intestinalis* (2 mM) bound 2.8 ± 0.8 mM Ca^{2+} in the presence of 7 mM total Ca^{2+} at 25 °C, corresponding to 1.4 divalent metal ions per protein, and at 8 °C 0.6 Ca^{2+} ions bound each protein. The X-ray crystal structure of this tunicate $\beta\gamma$ -crystallin revealed two Ca^{2+} binding sites per protein (Shimeld et al. 2005). Our results indicate that these two binding sites are not fully occupied in solution even at 25 °C at this lower salt concentration. These results are tabulated in Table 2 for Mg^{2+} and Ca^{2+} .

Some of the plots in Fig. 1 plateau at divalent metal ion concentrations >10 mM, indicating saturation. In this regime, the error in the concentration measurements is substantially high, making it difficult to extrapolate the exact concentrations of the bound metal ions. Similarly, large errors are obtained below 1 mM. Given that most in situ NMR studies of biological macromolecules focus on metal ion concentrations in the range of 1–10 mM, this is not problematic for typical applications; however, it is useful to place upper and lower bounds on the utility of

dCDP for this application. Errors for metal concentrations that are the focus of this study are also shown in Table 2. In order to obtain divalent metal ion concentrations below 1 mM, a stronger metal ion chelator must be used such as a fluorescent dye. Similarly a weaker chelating agent could be used above 10 mM if such measurements are desired.

In conclusion, we have demonstrated a method for determining the bound $[\text{Mg}^{2+}]$ and $[\text{Ca}^{2+}]$ in situ within a 1–10 mM concentration range for complex biomolecular NMR samples, including highly concentrated lipids, using ^{31}P NMR of an internal dCDP standard. This method is suitable under conditions where the type and total amount of the metal ion added to the sample is known. We determined the bound $[\text{Mg}^{2+}]$ and $[\text{Ca}^{2+}]$ in a DNA dodecamer, a DLPC/DHPC/CS bicelle mixture, and a *Ci*- $\beta\gamma$ -crystallin protein sample, each containing a known total concentration of their respective divalent ion. From these findings, we conclude that the DNA dodecamer binds at least a single Mg^{2+} per duplex in solution and that the DLPC/DHPC/CS bicelles bind divalent ions at cold temperatures, consistent with the observation that ion binding is temperature-dependent (Brindley and Martin 2012). We further show that at least one of the Ca^{2+} binding sites in the tunicate $\beta\gamma$ -crystallin protein appears to be fully occupied in solution at room temperature. Because this method is minimally invasive and uses standard NMR instrumentation, it is applicable to a wide range of macromolecular samples used for NMR structural studies that require divalent metal ions.

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