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Determination of the Lithium Binding Site in Inositol Monophosphatase, the Putative Target for Lithium Therapy, by Magic-Angle-Spinning Solid-State NMR

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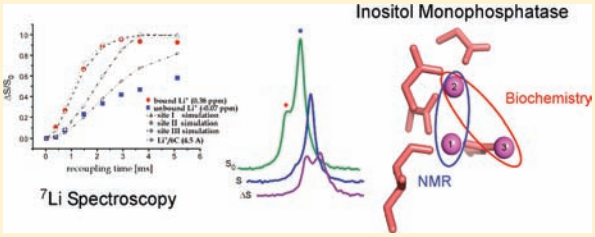
Supporting Information

ABSTRACT: Inositol monophosphatase (IMPase) catalyzes the hydrolysis of inositol monophosphate to inorganic phosphate and inositol. For this catalytic process to occur, Mg^{2+} cations must exist in the active site. According to the inositol depletion hypothesis, IMPase activity is assumed to be higher than normal in patients suffering from bipolar disorder. Treatment with Li^+, an inhibitor of IMPase, reduces its activity, but the mechanism by which lithium exerts its therapeutic effects is still at a stage of conjecture. The *Escherichia coli* SuhB gene product possesses IMPase activity, which is also strongly inhibited by Li^+. It has significant sequence similarity to human IMPase and has most of its key active-site residues. Here we show that by using ^{7}Li magic-angle-spinning solid-state NMR spectroscopy, including $\{^{13}C\}^7Li$ dipolar recoupling experiments, the bound form of lithium in the active site of wild-type *E. coli* SuhB can be unambiguously detected, and on the basis of our data and other biochemical data, lithium binds to site II, coupled to aspartate residues 84, 87, and 212.



INTRODUCTION

Ever since the discovery of lithium's therapeutic action toward manic effects[1] it has been the most prominent treatment for mania in bipolar disorder. In patients suffering from bipolar disorder, inositol monophosphatase (IMPase) activity in the phosphatidylinositol signaling pathway is assumed to be higher than normal, thereby increasing inositol levels. The "inositol depletion hypothesis"[2−4] suggests that lithium affects signal transduction by decreasing the levels of inositol and consequently of phosphatidylinositol. It does so selectively where there are overactive receptors, accounting for its therapeutic action. The most accepted mechanism by which lithium affects signal transduction is the inhibition of IMPase,[3,5−7] which is responsible for the regeneration of inositol from inositol monophosphate. It has also been shown that LiCl selectively decreases the macrophage load in rabbit atherosclerotic plaques via IMPase inhibition without affecting the viability or functionality of smooth muscle cells and endothelial cells.[8] Unfortunately, lithium has a very narrow therapeutic window and can cause adverse effects,[9] so many attempts have been made to understand its inhibition mechanism for the benefit of finding a safer treatment.

IMPase requires several Mg^{2+} cations as activating cofactors and can also be activated to a lower extent by Zn^{2+} or Mn^{2+}. IMPase is inhibited by Li^+ and can be partially inhibited by other divalent and trivalent cations. Mg^{2+} serves as a noncompetitive activator and, at high concentrations, an uncompetitive inhibitor.[10,11] Crystallographic studies of the human enzyme with Gd^{3+} and with Mn^{2+} were carried out,[10,12,13] revealing a single Gd^{3+} binding site that is ligated by the carboxylic groups of residues Glu70, Asp90, and Ile92; a sulfate or phosphate group (the latter coming from the enzyme substrate); and two water molecules. Partial occupancy of this site by Gd^{3+} in the presence of lithium and absence of Mg^{2+} indicated that Li^+ potentially binds to this site. The structure of human IMPase complexed with Mn^{2+} revealed three binding sites of the metal ion. Site I, as in the case of Gd^{3+}, is ligated by the three carboxylic groups and a water molecule. The second Mn^{2+} site is ligated by residues Asp90, Asp93, and Asp220 and either a Cl^- or a phosphate ion and appears to have a slightly distorted tetrahedral or trigonal-bipyramidal geometry. The third Mn^{2+} site is ligated by residue Glu70 and three water molecules. Structural studies on bovine IMPase (95% sequence similarity to human IMPase) in complex with Mg^{2+}, the biological activator,[14] reported a very similar binding to Mn^{2+} in terms of the functional groups, but with a larger coordination number: Mg^{2+} in site I binds the three ligands and three water molecules; Mg^{2+} in site II is also ligated by three additional water molecules, one shared with Mg^{2+} in site I; and Mg^{2+} in site III is ligated by Glu70 and five water molecules, one shared with Mg^{2+} in site I. In archaeal IMPase, isothermal titration calorimetry measurements showed the possible binding of a fourth metal ion.[15] The proposed catalytic mechanism first involved a two-metal-ion[16−18] and later a three-metal-ion[14,19] catalysis, and two main hypotheses regarding the Li^+ mode of inhibition were suggested.[10,11,15,19,20]

Studies of bovine IMPase using mutagenesis, kinetics, and fluorescence[10,11,21,22] revealed that Li^+ is an uncompetitive inhibitor with respect to substrate and directly coordinates to

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one of the phosphate oxygens by replacing Mg^{2+}. It was proposed that there exists a high-affinity activating metal ion site I and a low-affinity site II; hence, the lithium cation binds to the low-affinity site. In addition, ^{7}Li solution NMR spectroscopy on human IMPase in the apo form[23] and bovine IMPase circular dichroism measurements[24] also proposed Li^+ binding to site II. It is still unclear whether Li^+ itself promotes hydrolysis prior to inhibition or displaces the Mg^{2+} ion after the cleavage. However, it was found that Li^+ inhibition is a postcatalytic event occurring after the phosphoester bond cleavage.[14,25] A second hypothesis proposed for the Li^+ inhibition mechanism on the basis of archaeal IMPase studies suggested a direct correlation of a catalytic mobile loop, whose conformation varies in different members of the IMPase family.[15] The mobile loop is significant for localization and binding of Mg^{2+} in the third site, and Li^+ replaces this metal ion.

The various suggestions regarding the lithium binding site and inhibition mechanism result from the inability to observe lithium directly via either crystallographic studies[12,13,18] or ^{7}Li solution NMR measurements, which were based on the observation of exchange between free and bound Li^+.[23]

In this work, we used magic-angle-spinning (MAS) solid-state NMR spectroscopy to detect lithium directly in SuhB IMPase, a structural and functional analogue of human IMPase, and to determine its binding site by correlating it to nearby spins via their mutual heteronuclear dipolar interaction, which is a reporter of their internuclear distance.

EXPERIMENTAL SECTION

Expression and Purification of SuhB IMPase. Recombinant *Escherichia coli* SuhB plasmid obtained from Prof. Mary F. Roberts (Boston College) was amplified using Nova Blue competent cells (Novagen) and sequenced. Protein expression followed standard procedures.[26] The plasmid was transformed into BL21(DE3)pLysS competent cells (Novagen), incubated with rapid shaking at 37 °C for 1 h, and then spread over LB-agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The plates were incubated at 37 °C overnight, and a single colony was picked and grown in a 10 mL LB to stationary phase. An aliquot was used to inoculate 1 L of fresh LB medium that was grown to log phase. The cells were harvested by centrifugation. To obtain the fully $^{13}C/^{15}N$ isotopically labeled protein, the cells were washed with M9 medium and resuspended in 250 mL of minimal medium[27] with ^{13}C-glucose and $^{15}NH_4Cl$ as the sole sources of carbon and nitrogen, respectively. Overexpression was induced after 1 h by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM followed by incubation at 16 °C for 20 h with rapid shaking. The cells were harvested by centrifugation, and overexpression was monitored by SDS-PAGE.

For purification, the cells were suspended in buffer A (50 mM Tris-HCl, 1 mM EDTA, pH 8), 2 mM $MgCl_2$, and 0.1% (v/v) DNase and then homogenized and subjected to lysis using a microfluidizer (M-110L from Microfluidics). The supernatant was separated from the cell debris by centrifugation at 14 000 rpm at 4 °C and loaded onto Q-sepharose fast-flow columns. The columns were prewashed with buffer A containing 50 mM KCl during and after the sample loading, and protein fractions were eluted with a linear gradient of 0.05−0.5 M KCl in buffer A. Fractions and washes were subjected to SDS-PAGE (Figure S1 in the Supporting Information), which revealed that along with clean and unclean fractions, the prewash (after sample loading) contained significant amounts of protein. The prewash containing most of the protein was centrifuged at 14 000 rpm at 4 °C to remove the cell debris. The prewash and all of the clean fractions were dialyzed against buffer A with 200 mM KCl and concentrated by ultrafiltration (Vivaspin) to 4.5−5 mg/mL according to the bicinchoninic acid (BCA) protein assay. The sample was then subjected to desalting columns packed with Bio-Gel P-6DG gel and eluted with EDTA-free buffer A containing 100 mM KCl. After this step, the maximum absorption in the UV spectrum shifted to 280 nm (Figure S1), corresponding to a final concentration of ∼4 mg/mL (extinction coefficient of 0.786 mL mg^{-1} cm^{-1}) and a total yield of ∼20 mg per 1 L of culture. For MAS NMR experiments, after the addition of 6 mM $MgCl_2$ and 2 mM LiCl, the sample was lyophilized (−60 °C, ∼30 mtorr).

Activity and Inhibition. The activity of IMPase was qualitatively monitored by the malachite green (MG) colorimetric phosphate assay. IMPase was active in the presence of 6 mM $MgCl_2$ and substrate, and its activity was significantly reduced at high concentrations of Mg^{2+} or upon addition of 2 mM LiCl. Control samples with Mg^{2+} and no substrate or with lithium and substrate showed a lack of activity.

MAS Solid-State NMR Experiments. All NMR experiments were performed on an active sample inhibited by lithium using a Bruker Avance-III NMR spectrometer operating at a magnetic field of 14.1 T (^{1}H Larmor frequency of 600.2 MHz) using a wide-bore 4 mm probe operating in $^{1}H-^{7}Li-^{13}C$ triple-resonance mode with ^{7}Li and ^{13}C Larmor frequencies of 233.2 and 150.9 MHz, respectively. ^{7}Li spectra were referenced to a 1 M solution of $^{7}LiCl$ in purified H_2O (18.2 MΩ) at 0 ppm.

During single-pulse-excitation (SPE), cross-polarization (CP), and two-dimensional (2D) triple-quantum/single-quantum (TQ/SQ) experiments, a spinning frequency of 11 kHz was employed, and proton decouplings of 70, 80, and 60 kHz, respectively, were applied using the SW_f-TPPM scheme.[28,29] The TQ/SQ spectrum was recorded (States[30] acquisition) using a two-90°-pulse excitation and reconversion scheme with a 22.7 μs interpulse delay, acquisition times of 2.18 ms (12 points) and 28 ms (5590 points), and processing with line broadenings of 10 and 20 Hz for t_1 and t_2, respectively. $\{^{13}C\}^{7}Li$ rotational-echo double resonance (REDOR)[31,32] experiments were acquired using alternating pulses following the XY8 scheme[33] and using ^{7}Li π pulses of 70 kHz and ^{13}C pulses of 50 kHz. Every REDOR point was acquired using 1600 scans and a CP contact time of 0.5 ms. The carrier frequency on the ^{13}C channel was centered in the carbonyl region. For SPE- and CP-based experiments, relaxation delays of 20 and 5 s were used, respectively. All of the NMR data were acquired and processed using Topspin 2.1. The signal intensities of the experimental REDOR points were obtained by deconvolution of the peaks using the DMfit software.[34] REDOR simulations were carried out using the Spinevolution software[35] taking into account all of the interactions, including ^{13}C homonuclear couplings and isotropic shifts.

RESULTS AND DISCUSSION

Separation of Bound, Unbound, and Nonspecifically Bound Lithium Species. Various examples of direct metal detection in enzymes using MAS NMR spectroscopy have been demonstrated,[36,37] in which the quadrupolar tensor parameters were observed and related to structure using chemical calculations. Here we observed lithium species in uniformly ^{13}C- and ^{15}N-enriched SuhB IMPase prepared with activating Mg^{2+} and inhibitory Li^+. The results of SPE and $^{1}H-^{7}Li$ CP[38] MAS NMR experiments are shown in Figure 1. The SPE experiment (Figure 1b) revealed a single lithium peak resonating at −0.07 ppm, with a broadened baseline (in different preparations, this number varied between 0.0 and −0.07 ppm, and LiCl resonates at 0 ppm). This large signal arose from large amounts of unbound or weakly bound lithium species, from LiCl in the lyophilized sample, or both. Because the unbound lithium species can be very mobile, a $^{1}H-^{7}Li$ CPMAS experiment was performed to select for the rigid lithium species. In the CPMAS spectrum (Figure 1a), a downfield peak was revealed at 0.38 ppm, suggesting it to be bound lithium. To obtain better spectral separation between the lithium species, a 2D TQ/SQ correlation experiment was performed (Figure 1c). As expected, a narrow peak

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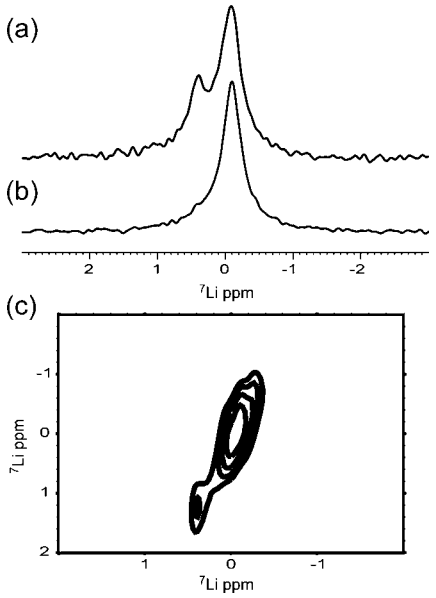

Figure 1. ^{7}Li MAS NMR spectra of SuhB inositol monophosphatase. (a) ^{1}H−^{7}Li CPMAS spectrum (32 scans, 1 ms contact time). (b) SPE MAS spectrum (8 scans). (c) CP-filtered TQ/SQ lithium correlation experiment (120 scans, 0.5 ms contact time). The spectrum was generated using SPARKY.[39]

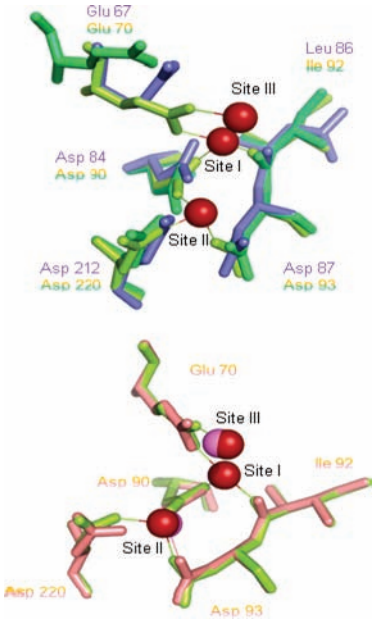

Figure 2. Alignment of four IMPases by the backbones of the binding-site residues generated by PyMOL. Top: SuhB IMPase (violet) aligned with the apo form (green) and the Mn-bound form (yellow) of human IMPase. Bottom: Mg-bound bovine IMPase (pink) aligned with the Mn-bound form of human IMPase (yellow). Mn^{2+} ions are depicted in red and Mg^{2+} ions in magenta. Exact distances are given in the Supporting Information.

corresponding to the bound lithium was found to resonate at approximately a tripled chemical shift value of 1.24 ppm, and the high-field lithium signal showed a broad line shape arising from either increased dynamics or the existence of many nonspecifically bound lithium species.

Direct Correlation of ^{13}C to Lithium Species. Our experimental results were obtained for wild-type *E. coli* SuhB. Nevertheless, a structural overlay of human IMPase in the apo form (1IMF) with the Mn-bound form (1IMC)[18] and the R184A mutant of SuhB (2QFL)[40] (Figure 2 top) shows very close residue and structural similarities. The binding-site residues of SuhB and human IMPase are almost completely identical, the only difference being a leucine in SuhB in place of isoleucine in the human form, while both ligate the metal ion through their backbone carbonyl. The only apparent structural difference between the apo and bound forms of human IMPase is the retraction of the glutamate carboxylic group participating in the binding of sites I and III (the actual distances, are tabulated in Table S1 in the Supporting Information). Mg^{2+} and Li^+ share many similar chemical properties and have very similar ionic radii,[41] reflecting their diagonal relationship in the periodic table. Thus, we also performed an overlay of Mn-bound human IMPase (1IMC) with Mg-bound bovine IMPase (2BJI) (Figure 2 bottom). The binding-site residues are virtually indistinguishable; hence, to fit our experimental data, we used the coordinates of the three known Mg^{2+} binding sites of bovine IMPase.[14]

Direct measurement of distances using REDOR experiments[31] have been used previously[42−46] to determine ligand−protein distances of ^{31}P spins coupled to ^{13}C and ^{15}N. To determine the lithium binding site in IMPase and to characterize further the lithium signals, we performed CPMAS-filtered {^{13}C}^{7}Li REDOR experiments.[32] In REDOR, the heteronuclear dipolar interaction between the rigid lithium species (selected by the application of CP) and the surrounding carbon atoms attenuates the NMR signal when two alternating π pulses are applied every rotor period (one at each of the spins, using the XY8 phase cycle[33] to compensate for pulse errors), generating the signal, S. A reference signal, S_0, which is obtained by applying pulses only to the detected nucleus ^{7}Li and eliminating all pulses on the ^{13}C channel, generates a spin echo that accounts for relaxation effects. The recoupling signal, $(S_0 - S)/S_0 = \Delta S/S_0$, is obtained by combining two data sets: the dephasing signal S, measured as a function of the number of applied pulses, and the corresponding reference signal S_0. $\Delta S/S_0$ is affected only by the dipolar interaction and hence reports on the internuclear Li−C distance, since the dipolar interaction is proportional to the inverse cube of the distance.

As can be seen in Figures 3 and 4, the two lithium signals of SuhB underwent recoupling. However, only the signal at 0.38 ppm underwent complete recoupling, reaching a $\Delta S/S_0$ value of ∼1.0 at 3 ms. The recoupling of the high-field peak was slower, indicating a larger internuclear distance, and reached a value of only 0.6 after 5 ms. Representative spectra, peak deconvolution, and the calculated REDOR curves are shown in Figure 3. For sites I and II, depicted as empty squares and circles, respectively, the REDOR curves are in very close agreement with the experimental data. The curves were calculated by including the closest carbonyls (Asp Cγ's, Glu Cδ, and Ile C′) and the nearest carbon neighbors (Asp Cβ's, Glu Cγ, and Ile Cα), a total of six carbons, and a single lithium. A simulation of site III, taking into account a single ligand (Glu-70) and the three closest carbon atoms up to a distance of ∼5 Å, is not consistent with our results.

An attempt to fit the recoupling curves to a single carbon atom (Figure 4 top) showed that the high-field peak cannot be reasonably fit with any single distance. Fitting the bound lithium signal (0.38 ppm) to a single carbon required a distance of 2.5−2.7 Å, but even for this fit, either the initial decay or the behavior at longer times was not properly reproduced, and

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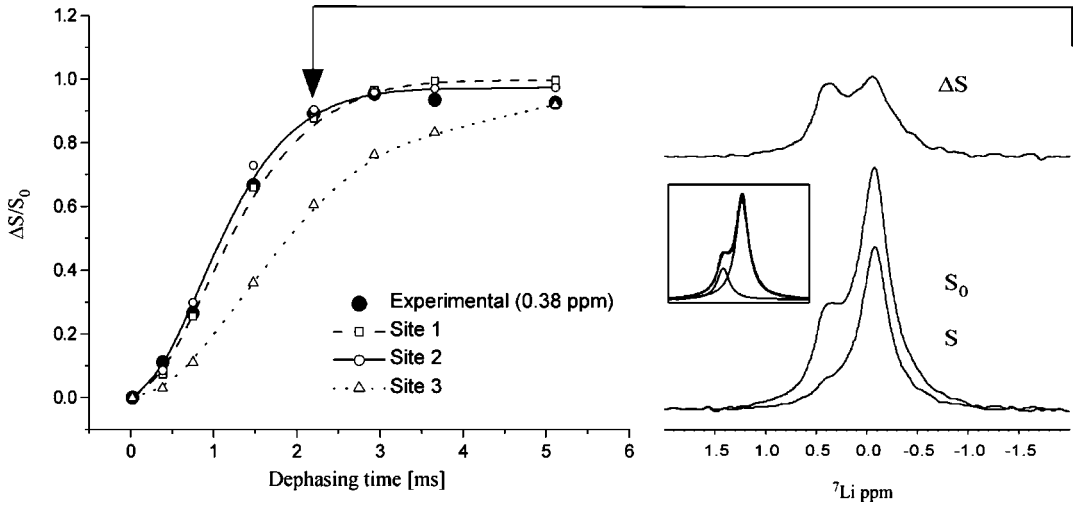

Figure 3. Comparison of experimental CP-filtered, ^{7}Li-detected REDOR data points ($\Delta S/S_0$) of the 0.38 ppm lithium signal from fully labeled SuhB IMPase with Spinevolution[35] simulations of the three Mg^{2+} binding sites from bovine IMPase (PDB entry 2BJI).[14] For sites I and II, three carboxyl carbons and their three nearest carbons were used in a seven-spin simulation. Site I: Glu-70 Cδ and Cγ, Asp-90 Cγ and Cβ, and Ile-92 C′ and Cα. Site II: Asp-90/93/220 Cγ and Cβ. For site III, simulations were performed using all four carbons within 5 Å of this site (Lys-36 Cε, Asp-41 Cγ, Glu-70 Cδ and Cγ). Typical lithium spectra, corresponding to S and S_0 at the time point indicated by the arrow, are shown at the right along with the difference spectrum ΔS; the peak deconvolution of S_0 is shown in the inset.

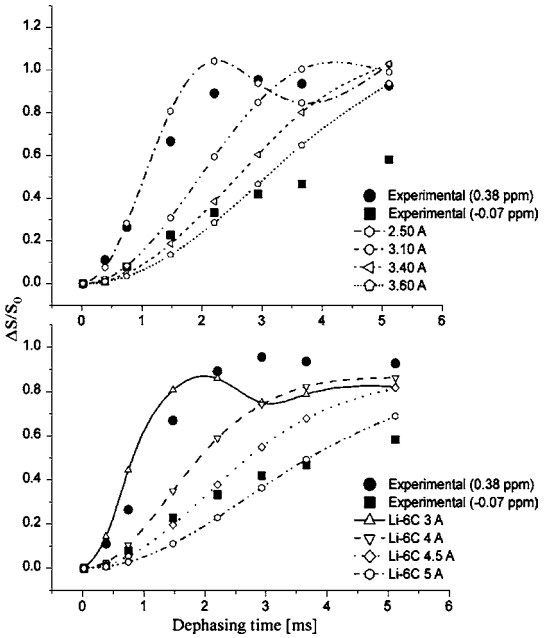

Figure 4. Attempts to fit the experimental SuhB {^{13}C}^{7}Li REDOR signal intensities of the bound (●) and unbound (■) species with simulations of (top) a single Li−C pair at various distances, including the closest fit of the initial rise (2.5 Å), a simulation of site III (3.1 Å), and distances that roughly fit the initial or average rise of the high-field signal, and (bottom) lithium and six equidistant carbons at distances of 3−5 Å.

structurally, such a distance would position the ligating oxygens too close to the metal. An attempt to fit six equidistant carbons (Figure 4 bottom) to the high-field signal (−0.07 ppm) also failed. The closest fit weakly representing our data was obtained using six carbons distanced 4.5−5 Å from the metal ion site and therefore cannot represent a lithium coordination site. Hence, it is more probable that this curve represents a distribution of lithium species that are mobile to some extent on the surface of the protein.

CONCLUSIONS

Magic-angle-spinning NMR spectroscopy has been used for direct observation and characterization of the lithium binding site in a structural and functional analogue of the putative target for lithium therapy, the SuhB IMPase enzyme, with inhibitory Li^{+} and activating Mg^{2+}. The lithium signal belonging to the inhibitory binding site could be identified and resolved from other species using cross-polarization and multiple-quantum-filtered experiments, and its chemical shift was determined to be 0.38 ppm. We have demonstrated for the first time that the REDOR experiment can be utilized to probe metal ion−carbon distances in proteins. We used the known crystallographic structure of the active Mg-bound bovine IMPase, which has a very close identity to SuhB IMPase and a similar structure of the binding sites, to simulate the REDOR experiment. The experimental data are in excellent agreement with lithium binding at sites I or II but not at site III. On the basis of biochemical data that report on a much stronger affinity of site I for Mg^{2+}, we have determined that lithium resides in site II. These results are also in agreement with a recent calculation-based study of the competition between lithium and magnesium,[47] based on their binding free energies, which showed that lithium ions preferentially bind to solvent-exposed magnesium sites with a positive charge density; a calculation performed on the entire binding site of IMPase indicated that lithium preferentially binds to site II as well. Lithium, in the form of lithium carbonate, is not only a common drug for treating bipolar disorder and certain types of depression (synergistically with other drugs[48]) but is also thought to affect other types of diseases such as chronic neurodegenerative diseases because of its role in major metabolic pathways and the possible inhibition of glycogen synthase kinase 3[49,50] among other targets. Our results therefore show that the possibility of resolving bound lithium species and correlating them to their binding residues, even when they exhibit weak binding, may have implications in many other biomedically significant biomolecules.

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■ ASSOCIATED CONTENT

Supporting Information

SDS-PAGE results and full UV spectra of SuhB IMPase; human, bovine, and R184A SuhB IMPase binding-site distances; and control ^{7}Li NMR measurements on ubiquitin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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