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Author(s)	Saio, Tomohide; Hiramatsu, Soya; Asada, Mizue; Nakagawa, Hiroshi; Shimizu, Kazumi; Kumeta, Hiroyuki; Nakamura, Toshikazu; Ishimori, Koichiro				
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1	Conformational ensemble of a multidomain protein explored by Gd ³⁺ electron
2	paramagnetic resonance
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4	Tomohide Saio, ^{1†*} Soya Hiramatsu, ^{2†} Mizue Asada, ³ Hiroshi Nakagawa, ^{4,5} Kazumi Shimizu, ⁶ Hiroyuki
5	Kumeta, ⁷ Toshikazu Nakamura, ³ and Koichiro Ishimori ^{2,8} *
6	
7	¹ Division of Molecular Life Science, Institute of Advanced Medical Sciences, Tokushima University,
8	Tokushima, Japan
9	² Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo, 060-8628,
10	Japan
11	³ Instrument Center, Institute for Molecular Science, Okazaki 444-8585, Japan.
12	⁴ Materials Sciences Research Center, Japan Atomic Energy Agency, Tokai, Ibaraki 319-1195, Japan
13	⁵ J-PARC Center, Japan Atomic Energy Agency, Tokai, Ibaraki 319-1195, Japan
14	⁶ Faculty of Education and Integrated Arts and Sciences, Waseda University, Tokyo 169-8050, Japan
15	⁷ Faculty of Advanced Life Science, Hokkaido University, Sapporo, 011-0021, Japan
16	⁸ Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo, 060-0810, Japan
17	
18	[†] These authors contributed equally to this work.
19	
20	
21	*Corresponding author E-mail: <u>saio@tokushima-u.ac.jp</u> , <u>koichiro@sci.hokudai.ac.jp</u>
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1 ABSTRACT

 $\mathbf{2}$ Despite their importance in function, the conformational state of proteins and its changes are often poorly understood mainly because of the lack of an efficient tool. MurD, a 47-3 kDa protein enzyme responsible for peptidoglycan biosynthesis, is one of those proteins whose 4 5 conformational states and changes during their catalytic cycle are not well understood. Although it has been considered that MurD takes a single conformational state in solution as shown by a 6 7 crystal structure, the solution NMR study suggested the existence of multiple conformational 8 state of apo MurD in solution. However, the conformational distribution has not been evaluated. 9 In this work we investigate the conformational states of MurD by the use of electron 10 paramagnetic resonance (EPR), especially inter-gadolinium distance measurement using double 11 electron-electron resonance (DEER) measurement. The gadolinium ions are fixed on specific 12 positions on MurD via a rigid double-arm paramagnetic lanthanide tag that has been originally developed for paramagnetic nuclear magnetic resonance (NMR). The combined use of NMR and 1314EPR enables accurate interpretation of the DEER distance information to the structural information of MurD. The DEER distance measurement for apo MurD shows a broad distance 1516 distribution, whereas the presence of the inhibitor narrows the distance distribution. The results 17suggest that MurD exists in a wide variety of conformational states in the absence of ligands, 18 whereas binding of the inhibitor eliminates variation in conformational states. The multiple conformational states of MurD were previously implied by NMR experiments, but our DEER 19 20data provided structural characterization of the conformational variety of MurD.

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22 STATEMENT OF SIGNIFICANCE (<120)

A rigid double-arm lanthanide tag was utilized in electron paramagnetic resonance spectroscopy to measure the distance between two specific points on a protein enzyme MurD, and the conformational states and distribution of MurD were investigated. Although previous crystallographic and NMR studies have reported the three distinct conformational states of MurD, our data showed that the protein exists in a much wider variety of conformational states

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- 1 in the absence of ligands. Given the fact that MurD is one of the potent drug targets for infectious
- 2 diseases, it is projected that the findings in this study will provide an important structural basis
- 3 for drug development.
- 4

1 INTRODUCTION

 $\mathbf{2}$ Proteins, especially protein enzymes, exert their activities through dynamic structural changes that are often poorly understood. Even after the determination of their high-resolution 3 4 structure, the lack of information about these dynamic structural changes obscures the $\mathbf{5}$ understanding of the mechanism. MurD, a 47-kDa protein enzyme responsible for peptidoglycan 6 biosynthesis, is one of those proteins whose conformational states and changes are critical for 7 regulating its catalytic process (1). MurD is a member of Mur ligase family that is responsible 8 for peptidoglycan biosynthesis, and catalyzes the ligation of D-Glu to UDP-N-acetylmuramoyl-L-9 alanine (UMA) coupled with adenosine triphosphate (ATP) hydrolysis, in which ATP, UMA, and 10 D-Glu need to bind sequentially to MurD (2). Given the high stereospecificity toward D-Glu (3) 11 and conserved D-Glu-binding site across different bacterial species (4), MurD has been 12extensively studied as a potent drug target among the Mur ligases (5, 6, 7).

MurD consists of three domains, and previous crystallographic studies have identified 1314that domain 3 undergoes drastic orientational changes with respect to domain 2, so that MurD changes its conformation from the open to the closed state upon binding to ligands or inhibitors 1516 (1, 2, 5, 6, 8, 9). More recently, a nuclear magnetic resonance (NMR) study using paramagnetic lanthanide ions showed that drastic conformational changes of MurD regulate the order of the 17ligand binding in the enzymatic reaction (10). Interestingly, the NMR study also suggested that 1819MurD exists in multiple conformational states exchanging in millisecond to microsecond (ms-20µs) time scale in the absence of ligands (10), whereas it had been believed that apo MurD exists 21in the open conformation as seen in the crystal structure (10). However, these conformational 22variations of MurD are not fully understood. The conformational equilibrium can be hindered 23upon crystal formation, or in the solution NMR experiments, the conformational exchange 24averages or broadens out the resonances, which obscures the structural information for each of the states. Molecular dynamics (MD) simulations have been performed to investigate the 2526conformational dynamics of MurD in silico (11, 12), but limited simulation time has, so far, 27impeded the tracking of the relatively large size as well as slow conformational dynamics (ms-µs

1 time scale) of MurD.

 $\mathbf{2}$ In this study, to obtain information about the conformational ensemble of MurD, we used electron paramagnetic resonance (EPR) utilizing double electron-electron resonance (DEER). 3 4 DEER distance measurement provides nanometer-scale distance and population for the paramagnetic centers introduced at specific positions of the protein, and thus potentially resolves $\mathbf{5}$ the multiple conformational states in the equilibrium (13-16). Gd^{3+} ion as a paramagnetic center 6 7 can be introduced to the protein using lanthanide-binding tags (17, 18). Among several 8 paramagnetic centers, including nitroxide spin label (19) and copper ion (20), one of the advantages of Gd³⁺ ion in DEER is availability of plenty of lanthanide-binding tags that have 9 10 been extensively developed originally for use in protein NMR (17, 18). Especially, the 11 availability of the double-arm tag is advantageous because the double-arm tags reduce the 12mobility of the tag with respect to the protein (21-23), and are thus expected to provide higher resolution of structural information. The advantages of double-arm tags have been initially 1314demonstrated in the field of paramagnetic NMR (17, 18), but more recently in DEER measurements in EPR as well (24). In this study we use the double-armed lanthanide tag, Caged 15Lanthanide NMR Probe 5 (CLaNP-5) (21, 25), which is one of the most extensively used tags in 16 17protein NMR experiments (10, 26-28) but has not been used in the DEER distance measurement. We here demonstrate the use of CLaNP-5 tag in DEER EPR for accurate nanometer-scale 18 19 distance measurements.

20The EPR data show that MurD in the absence of the ligand exists as an ensemble of multiple conformational states, whereas the ligand binding induces a drastic conformational 2122change that focuses the conformational ensemble toward the closed state. Well-defined metal 23positions with respect to MurD domains owing to the PCS-NMR analysis as well as the rigid 24architecture of the CLaNP-5 tag enabled us to obtain detailed structural information on MurD in the absence and presence of the inhibitor. Our data indicate that MurD can take a much larger 2526number of conformational states than previously thought on the basis of crystallographic and NMR studies (1, 2, 5, 8-10, 29). Given the usefulness of the CLaNP-5 tag in protein NMR study, 27

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- 1 it was expected that application of the tag in EPR will further accelerate the combined use of
- 2 EPR and paramagnetic NMR in protein structural and dynamics study.

1 MATERIALS AND METHODS

2 **Preparation of CLaNP-5**

- 3 CLaNP-5 was synthesized, purified, and chelated to Lu^{3+} , Yb^{3+} , and Gd^{3+} according to 4 the procedure described in previous reports (10, 21, 25).
- $\mathbf{5}$

6 **Protein sample preparation**

7 Escherichia coli MurD full-length (1–437), domain 1–2 (1–302), and domain 3 (301– 8 437) were cloned into pGBHPS (30), expressed in E. coli strain BL21 (DE3), and purified as described in a previous report (10). For PCS observation, isotopically labeled MurD domain 1-2 9 10 Q170C/V172C (D12₁₇₀) and MurD domain 1–2 M145C/D148C/C151A (D12₁₄₅), both having a single lanthanide ion, were prepared using the same procedure as reported previously for MurD 11 12domain 1–2 E260C/K262C (10). The proteins were prepared in a buffer containing 20 mM Tris-HCl (pH 7.2) and 100 mM NaCl. The CLaNP-5 tag was attached to the protein by mixing the 1314protein and the tag at a 1:1.1 ratio for 15 minutes on ice, followed by gel filtration to remove 15unreacted tag. For EPR distance measurement, MurD domain 1-2 Q170C/V172C/E260C/K262C 16 (D12170-260), MurD domain 1-2 M145C/D148C/C151A/E260C/K262C (D12145-260), and MurD E260C/K262C/N360C/D362C (MurD₂₆₀₋₃₆₀) were prepared using a previously described 1718 procedure (10). Note that D12₁₄₅₋₂₆₀ has a mutation in C151A aiming to avoid intramolecular 19 disulfide bond formation with D148C. The CLaNP-5 tag was attached to the protein by mixing 20the protein and the tag at a 1:2.2 ratio for 15 minutes on ice, followed by gel filtration. The 21attachment of the tag was confirmed by NMR (Figure S1–S2). The data showed that the tags were attached to the specific position of MurD at a high efficiency. Although MurD has seven 2223cysteine residues (C20, C99, C151, C208, C227, C368, and C413), all cysteine residues have a thiol group buried in the protein and did not react to the CLaNP-5 tag (10). MurD domain 1-224Q170C/V172C/E260C/K262C (D12₁₇₀₋₂₆₀) was prepared in 20 mM Tris-d₁₁-HCl (pD 7.2), 100 2526mM NaCl in 70% D₂O/30% glycerol-*d*₈. Full-length MurD E260C/K262C/N360C/D362C

(MurD₂₆₀₋₃₆₀) and full-length MurD E260C/K262C/L396C/R400C (MurD₂₆₀₋₃₉₆) were prepared
 in 20 mM Tris-*d*₁₁-HCl (pD 7.2), 200 mM NaCl in 70% D₂O/30% glycerol-*d*₈.

3

4 Determination of the metal position by PCS analysis

The positions of the metal fixed at Q170C/V172C and M145C/D148C were estimated $\mathbf{5}$ via analysis of PCSs (31, 32). The diamagnetic lanthanide ion Lu^{3+} as a diamagnetic reference or 6 the paramagnetic lanthanide ion Yb^{3+} was attached to MurD domain 1–2 O170C/V172C (D12₁₇₀) 7 or MurD domain 1-2 M145C/D148C/C151A (D12145) by CLaNP-5. PCSs were measured as a 8 9 chemical shift difference between the backbone amide resonances from paramagnetic and 10 diamagnetic samples. To reduce the number of resonances in the spectra for more reliable PCS assignments, amino acid-selective ¹⁵N-labeled samples were prepared as previously described 11 (10). ¹H–¹⁵N heteronuclear single quantum coherence (HSQC) spectra were recorded on Bruker 12Avance III 600 and 800 MHz NMR spectrometers (Bruker Corp., Billerica, MA, USA). The 13spectra were recorded at 25°C. Spectra were then processed using the NMRPipe program (33), 14and data analysis was performed on the Olivia program (Yokochi et al.; 1516 https://github.com/yokochi47/Olivia). $\Delta \chi$ -tensor and the position of the lanthanide ion were calculated from the PCS values and the crystal structure of MurD (PDB code 3uag) (2), using the 1718 Numbat program (34) with equation Eq. (1),

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$$\Delta \delta^{\text{PCS}} = \frac{1}{12\pi r^3} \Big[\Delta \chi_{\text{ax}} (3\cos^2\theta - 1) + \frac{3}{2} \Delta \chi_{\text{rh}} \sin^2\theta \cos 2\phi \Big], \tag{1}$$

where $\Delta \delta^{PCS}$ is the PCS; r, θ , and φ are the polar coordinates of the nucleus with respect to the principal axes of the magnetic susceptibility tensor; and $\Delta \chi_{ax}$ and $\Delta \chi_{rh}$ are the axial and rhombic components, respectively, of the anisotropic magnetic susceptibility tensor. The tensor calculations were performed using 63 and 31 PCSs induced by Yb³⁺ and Tm³⁺, respectively, for D12₁₄₅ (Table S1) and using 87 PCSs induced by Yb³⁺ for D12₁₇₀ (Table S2). Only PCSs observed from the amide proton located on the rigid region of the protein were used in the fitting. Errors were estimated with Monte Carlo analysis using the 100 partial PCS data sets in which
 30% of the input data were randomly deleted.

3

4 Determination of the metal position by modeling

 $\mathbf{5}$ The position of the metal fixed via N360C/D362C was estimated by docking calculation using Xplor-NIH (35). The coordinates of CLaNP-5 were prepared as described 6 7 previously (31, 36), by using PRODRG2 server (37) and VEGA ZZ (38). In the Xplor calculation, the position and orientation of CLaNP-5 were first randomized around the 8 9 coordinate of MurD (PDB ID: 3uag) and then docked onto MurD with distance restraints 10 between the sulfur atoms in the double arm of CLaNP-5 and cysteine residues at N360 and D362 of MurD. A total of 100 docking calculations were performed, and the 50 lowest-energy 11 12coordinates were selected, in which the positions of the metal were averaged.

13

14 **EPR DEER distance measurement**

EPR spectra were recorded using a Bruker E680 spectrometer (Bruker Corp.). All EPR 15signals were detected at 10 K with a CF935 liquid helium cryostat (Oxford Instruments, Oxford, 16 UK) using Q band (34.2 GHz). For echo-detected (ED) EPR measurement, we used a $\pi/2-\tau-\pi$ 1718sequence with a time interval τ of 200 ns with pulse lengths of 16 and 32 ns. Repetition time was 500 µs. For DEER measurement, a four-pulse sequence $\pi/2(v_{obs})-\tau_1-\pi(v_{obs})-t-\pi(v_{pump})-(\tau_1+\tau_2-\tau_2)$ 19 20t)- $\pi(v_{obs})$ - τ_2 -echo with a time interval τ_1 of 400 ns and τ_2 of 4000 ns were used. Pulse lengths of 21the observation $\pi/2$ and π pulse were 16 and 32 ns, respectively, and the length of the pumping pulse was 20 ns. Repetition time was 500 µs. Artifact signals arising from multiple pulses were 2223removed using an eight-step phase cycling. The microwave frequencies for observation and 24pumping pulses for DEER with a difference of 100 MHz were set to resonate with the magnetic fields indicated by arrows in Figure S4. The distance distributions were estimated using 25

1 DeerAnalysis 2016 software (39).

2 **RESULTS**

3 Gadolinium tagging of a multidomain protein MurD

To investigate the conformational states of a multidomain protein MurD, we aimed to 4 measure the distance between the two gadolinium ions fixed on the domains 2 and 3 by DEER $\mathbf{5}$ experiments. Gd³⁺ ion was fixed on MurD through the CLaNP-5 tag (21, 25) that was attached to 6 the protein by two disulfide bridges (Figure 1). For reliable DEER distance measurements, the 7 tagging points were designed to be located within ~8 nm from each other and located on the 8 9 structured region of MurD, with the reference of the crystal structure of MurD (PDB ID: 3uag) 10 (2). The amino acid residues located on the long loop or disordered regions were excluded from the candidates. Finally, four positions of MurD-three in domain 2 and one in domain 3-were 11 12selected for gadolinium tagging (Figure 1a-c). Following the procedures described in previous reports (10, 25), the pair of surface-exposed residues whose C_{β} atoms are located in a distance of 138–10 Å were selected and mutated to cysteine residues for attachment of CLaNP-5 (Figure 1e). 14Three MurD variants were constructed for DEER measurement: MurD domain 1–2 1516 M145C/D148C/C151A/E260C/K262C (D12145-260), MurD domain 1-2 Q170C/V172C/E260C/K262C (D12170-260), and full-length MurD E260C/K262C/N360C/D362C 17 $(MurD_{260-360})$ (Figure 1a-c). Because D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀ have two Gd³⁺ ions both on 18 domain 2, the distance distributions for D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀ are expected to reflect local 19 conformational variation around the tag. On the other hand, the distance distribution for 20MurD₂₆₀₋₃₆₀, having Gd^{3+} ions on domains 2 and 3, is expected to reflect the conformational 2122variations of domain 3 with respect to domain 2, in addition to the local conformational 23variations.

24

25 Determination of metal positions with respect to MurD domains

1	Before the DEER experiments, the positions of each of the metal ions with respect to
2	the domain holding the CLaNP-5 tag were determined. The precise metal positions with respect
3	to the domains enables the interpretation of the DEER distance data to the detailed structural
4	information of MurD. The position of the metal fixed at E260C/K262C was previously
5	determined by PCSs observed by NMR (10), whereas the positions of the metal fixed at
6	M145C/D148C and Q170C/V172C were determined by PCSs in this study. For PCS
7	measurements, instead of Gd ³⁺ , an anisotropic paramagnetic lanthanide ion Yb ³⁺ or diamagnetic
8	lanthanide ion Lu ³⁺ was attached to MurD domain 1–2 M145C/D148C/C151A (D12 ₁₄₅) and
9	MurD domain 1–2 Q170C/V172C (D12170) by CLaNP-5, and the PCSs were observed as
10	chemical shift differences between paramagnetic and diamagnetic resonances (Figures S1 and
11	S2). Significant PCSs were observed from the resonances of the amide groups (Tables S1 and
12	S2). The parameters of the anisotropic magnetic susceptibility tensor ($\Delta \chi$ -tensor), including the
13	metal position, were determined using Numbat program (34) (Table 1 and Figure S3). The values
14	of $\Delta \chi_{ax}$ and $\Delta \chi_{rh}$ are close to those determined for Yb ³⁺ attached at another position of MurD, at
15	E260C/K262C (10), and to another protein (25). Furthermore, the position of the lanthanide ion
16	was well defined, indicating that the tag was rigidly fixed on MurD. Given the position of the
17	metal fixed via E260C/K262C that was previously determined with the same procedure using the
18	same coordinates of MurD (PDB code: 3uag) (10), the distances between the lanthanide ions
19	fixed at the two positions, M145C/D148C and E260C/K262C, and Q170C/V172C and
20	E260C/K262C, were estimated as 3.58 ± 0.12 and 3.86 ± 0.11 nm, respectively (Figure 1a and
21	b). The metal position for N360C/D362C was estimated by docking calculation using Xplo-NIH
22	(35) (Figure 1e), because of the limited solubility of the isolated MurD domain 3 N360C/D362C
23	(see Materials and methods).

25 Gd³⁺-Gd³⁺ DEER measurement for D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀

The distance distribution obtained from DEER measurement should provide information about the conformational variety of the protein given that the different

conformational states of the protein have distinct distances between paramagnetic centers. 1 $\mathbf{2}$ However, it should be noted that the residual mobility of the tag can also contribute to the distance distribution. Although the CLaNP-5 tag is rigidly attached to the protein via two arms as 3 shown by previous NMR studies (10, 21, 25), there was no information about the actual spatial 4 distribution of the metal ion attached on the protein via CLaNP-5 tag. To assess the distribution $\mathbf{5}$ 6 of paramagnetic centers imposed by the residual local mobility of the CLaNP-5 tag, we 7 performed DEER measurement on D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀, in which CLaNP-5 tags containing Gd³⁺ were attached to the rigid regions of domain 2 (Figure 1a and b). The EPR experiments 8 were performed on D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀ prepared in 80% 2 H₂O/20% glycerol- d_{8} solution at 9 10 10 K on a Q-band spectrometer (resonant frequency, 34 Hz) (Figure S4). The central transition 11 peak in the ED EPR spectrum was similar to the previously reported data recorded on a Q-band 12spectrometer (resonant frequency, 34 Hz) (40). Four-pulse DEER experiments were performed 13on D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀, and distance distributions were obtained using DeerAnalysis 2016 (39) (Figure 2). The data were recorded with dipolar evolution time as long as 3.9 µs. The DEER 1415trace exhibited clear dipolar modulation (Figure 2a–d), indicating the existence of the defined inter-metal distance. Tikhonov regularization and Gaussian fit for D12₁₄₅₋₂₆₀ showed the most 1617intense peaks at 3.64 and 3.68 nm, with full-width at half-maximum (FWHM) of 0.77 and 0.76 nm, respectively (Figure 2e). The fitting for $D12_{170-260}$ showed the most intense peaks at 3.96 nm 18(Tikhonov regularization) and 3.90 nm (Gaussian fit), with FWHM of 0.75 and 0.67 nm, 1920respectively (Figure 2f). In both cases, Tikhonov regularization and Gaussian fit showed 21essentially the same distance distribution, supporting the robustness of the analysis. Thus, 22FWHM of 0.7-0.8 nm are considered to be imposed by local conformational variations of the lanthanide tag. Assuming that the two CLaNP-5 tags have similar conformational distributions, 23the DEER distance distribution indicated that each CLaNP-5 tag has conformational distribution 24of 0.4 nm, which should be mainly accounted for local conformational variation of side chains of 2526the cysteine residues holding the tag. These DEER-derived distances match the distances estimated by $\Delta \gamma$ tensor analysis using PCSs and the crystal structure of MurD, 3.58 ± 0.12 nm 2728for D12₁₄₅₋₂₆₀, and 3.86 ± 0.11 nm for D12₁₇₀₋₂₆₀ (Figure 1a and b), supporting the reliability of

1 the Gd^{3+} – Gd^{3+} distance measurement by DEER.

 $\mathbf{2}$

3 Gd³⁺–Gd³⁺ DEER for MurD_{260–360} identified a variety of conformational states of MurD

The Gd^{3+} – Gd^{3+} distance for MurD_{260–360} was measured to monitor the conformational 4 states of MurD in the absence and presence of the inhibitor, $N-(\{3-[(\{4-[(Z)-(2,4-dioxo-1,3 \mathbf{5}$ thiazolidin-5-ylidene)methyl]phenyl}amino)methyl]phenyl}carbonyl)-D-glutamic acid (5). 6 $\overline{7}$ Because the two gadolinium ions were attached to domains 2 and 3, the inter-gadolinium 8 distance distribution reflects the position and orientation of domain 3 with respect to domain 2 9 (Figure 1c). The distance measurement was performed using four-pulse DEER experiments on a O-band spectrometer, with a dipolar evolution time as long as 3.9 μ s (Figure 3). Unlike D12₁₄₅₋ 10 $_{260}$ or D12₁₇₀₋₂₆₀, which have a defined distance between Gd³⁺ ions (Figure 2c and d), DEER 11 traces for MurD_{260–360}, especially in the absence of the inhibitor, showed that the oscillation 12disappears after ~0.4 μ s (Figure 3a and c), which implies the existence of multiple Gd³⁺–Gd³⁺ 13distances and thus multiple conformational states of $MurD_{260-360}$ in the absence of the inhibitor. 1415The distance distribution for $MurD_{260-360}$ in the absence of the inhibitor indeed showed a broad major peak whose top was located at 3.56 nm (Figure 3e). Although the position of the peak top 16is close to that expected from the crystal structure of MurD in the open conformation (PDB code: 1718 1e0d.pdb, 3.21 ± 0.20 nm) (8) (Figure S5a), there was a small but significant gap between the two distances. This indicates that the major conformational state in solution is somewhat 1920different from that observed in the crystal structure (8).

To obtain more detailed information about the conformational state of apo MurD in solution, the DEER distance distribution was compared with that obtained using MD simulation (41). Although gadolinium ion and CLaNP-5 tag were not included in the simulation, the positions of the gadolinium ion were estimated by superimposing the coordinates of domains 2 and 3 containing the coordinates of the lanthanide ion so that the inter-gadolinium distances can be estimated for each of the coordinates. The trajectory of the inter-gadolinium distance of the MD simulation showed that each of the conformational states of MurD has a distinct inter-

gadolinium distance (Figure 4), indicating that inter-gadolinium distances obtained by DEER 1 $\mathbf{2}$ measurement clearly reflected differences among the conformational states of MurD. Importantly, the distances estimated for the coordinates from MD simulations are all within the 3 4 range of the DEER distance distribution (Figure 4), which highlights the consistency and reliability of the two data sets. The MD trajectory showed that the major conformational state has $\mathbf{5}$ 6 an inter-gadolinium distance of \sim 3.6 nm (Figure 4), which coincides with the position of the peak 7 top of the DEER distance distribution for $MurD_{260-360}$ in the absence of the ligand (3.56 nm) (Figure 3e). The major conformational states in MD simulation show that domain 3 tilted from 8 that in the crystal structure in the open conformation by $\sim 20^{\circ}$ (Figure 4). Thus, both MD 9 10 simulation and DEER distance measurement highlighted the major conformational state of apo MurD in solution. 11

MD simulation also highlighted a variety of conformational states of MurD (Figure 4), 12which well explains the broad distance distribution obtained from DEER experiments for MurD 13in the absence of the inhibitor. The broad distance distribution indicates that MurD exists as an 14ensemble of a variety of conformational states in solution that have not observed in crystal 1516 structures. The FWHM for the major peak in the absence of the inhibitor was estimated as 1.28 17nm, which is much wider than that observed for $D12_{145-260}$ and $D12_{170-260}$ (0.7-0.8 nm)(Figures 18 2e, f and 3e). Note that the wide distance distribution for $MurD_{260-360}$ was also supported by the theoretical DEER traces (Figure S6), in which the distance distribution for $\sigma = 1.0$ nm indicated 19 20the DEER traces very similar to that observed for MurD₂₆₀₋₃₆₀ in the absence of the ligand (Figure 3c). The distance distribution also covers the distance expected from the closed 2122conformation as seen in the crystal structure of MurD in complex with the ligands or the 23inhibitor (Figure S5b), implying that the conformational ensemble of MurD includes the closed 24conformation even in the absence of the inhibitor.

25 On the other hand, the DEER measurement for MurD_{260–360} in the presence of the 26 inhibitor showed a narrower distance distribution (Figure 3f). In the presence of the inhibitor, the 27 distance distribution obtained from Tikhonov regularization showed that the FWHM for the

major peak decreased from 1.28 nm to 1.14 nm, and the peak top shifted from 3.56 nm to 4.10 1 nm (Figure 3e and f), which corresponds to the distance expected for the crystal structure of $\mathbf{2}$ MurD in the closed conformation $(4.35 \pm 0.25 \text{ nm})$ (Figure S5b) (5). Although the distance 3 distribution of MurD₂₆₀₋₃₆₀ became narrower in the presence of the inhibitor, the FWHM of 1.14 4 nm for $MurD_{260-360}$ in complex with the inhibitor is still wider than that observed for $D12_{145-260}$ $\mathbf{5}$ or D12₁₇₀₋₂₆₀ (0.7-0.8 nm) (Figures 2 and 3). As the binding is expected to be saturated given the 6 7affinity and concentrations of MurD and the inhibitor (10), the wider distance distribution for MurD₂₆₀₋₃₆₀ in complex with the inhibitor suggests residual conformational variety of MurD 8 even in complex with the inhibitor. Note that single Gaussian fitting showed that the overall 9 10 average distance shifted from 3.78 to 4.21 nm after addition of the inhibitor (Figure S7), supporting the inhibitor-induced conformational change of MurD. Thus, the results showed that 11 MurD in solution exists in an ensemble of a variety of conformational states, and the binding of 1213the inhibitor shifts the population toward the closed conformation.

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1 **DISCUSSION**

 $\mathbf{2}$ MurD, a protein enzyme consisting of three domains, is known to undergo drastic 3 conformational changes during its catalytic process. Previous crystallographic studies have concluded that in the absence of ligands, MurD exists in an open conformation (8), whereas in 4 the presence of ligands or inhibitors it exists in a closed conformation (1, 2, 5, 9). Although it has 5 been believed that MurD takes a specific conformational state as seen in the crystal structure, the 6 $\overline{7}$ DEER distance measurement carried out for MurD in this study has shown wide distance 8 distribution for the apo state (Figure 3e), indicating that apo MurD exists in a wide variety of conformational states in solution. DEER distance distribution for MurD₂₆₀₋₃₆₀ shows that the 9 10 FWHM for the major peak in the absence of the inhibitor is 1.28 nm, which is much wider than that obtained for $D12_{145-260}$ and $D12_{170-260}$ (0.7-0.8 nm), in which the two gadolinium ions are 11 12fixed on the same domain (Figures 2e, f and 3e). The increased distance distribution of apo 13MurD_{260–360} indicates the conformational variation of MurD, especially variation in position 14and/or orientation of domain 3 with respect to domain 2.

The distance distribution for $MurD_{260-360}$ in the absence of the ligand covers not only the 15distance expected for the open conformation but also that for the closed conformation (Figure 3 16and Figure S5), and thus suggests that MurD can also exist in a closed conformation even in the 1718 absence of the ligand. The existence of the closed conformation in the absence of the ligands was initially implied by the previous NMR observation: The resonances derived from the interface 1920between the domains 2 and 3 in the closed conformation show significant line broadening in the 21absence of the ligand (10). The resonance broadening in NMR indicates the exchange among 22multiple states in the μ s-ms time scale. Furthermore, our experimental data from neutron spin echo (NSE) and small-angle X-ray scattering (SAXS) as well as MD simulation (41) also 2324indicated that conformational ensemble and amplitude of domain motion are larger in the apo state than in the ligand-bound state. Collectively, our NMR, EPR, NSE, SAXS, and MD data all 25suggest that MurD at apo state in solution is much more dynamic than previously thought and 2627exists in multiple conformational states in exchange, including open and closed conformational

states. The details about the conformational variation of apo MurD is further discussed based on
all possible experimental and computational data in our other paper (41).

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It should be also noted that the DEER distance distribution for MurD_{260–360} in the absence 3 4 of the ligand showed a major peak with a maximum height of 3.56 nm, which is different from $\mathbf{5}$ the inter-gadolinium distance expected from the crystal structure of apo MurD (1e0d), but 6 coincides with the distance estimated for the major conformational states of MurD in the MD 7 simulation (~3.6 nm) (Figure 4) (41). The typical MD coordinates of MurD having an inter-8 gadolinium distance of \sim 3.6 nm show domain 3 rotated from the crystal open structure by \sim 20° 9 (Figure 4), which results in a wide, open cavity for ATP binding (Figure S8). Thus, the crystal 10 structure of apo MurD has the orientation of the domain 3 hindered by crystal packing. Given the 11 fact that the cavity for nucleotide binding is more widely exposed in this major open conformation, this conformation can be beneficial for capturing ATP molecules. 12

In contrast to the wide conformational distribution of apo MurD, the presence of the inhibitor narrows down the population toward the closed conformation (Figure 3f). The peak top of the DEER distance distribution of MurD₂₆₀₋₃₆₀ (4.10 nm) roughly matches to the distance expected from the crystal structure in the closed conformation $(4.35 \pm 0.25 \text{ nm})$ (5). Thus, the data indicate that MurD in solution exists in a wide variety of conformational states in the absence of the ligand, but the binding of the ligand causes MurD to settle down in a specific conformational state.

20In this study, we used DEER distance measurements for a multidomain protein attached with a rigid double-arm gadolinium-chelating tag to characterize the conformational states of the 2122protein in the absence and presence of the inhibitor. Given the fact that the gadolinium ion can be 23attached to specific positions of a protein by the use of the tag, this strategy can be applied to 24other proteins as well. Our results also demonstrate that the combination of inter-gadolinium distance measurement by DEER EPR and MD simulation visualizes the conformational 25ensemble of a multi-domain protein and its conformational change upon binding to the inhibitor. 26Simple but reliable distance information obtained from DEER distance measurement is 27

corroborated by MD simulation. The DEER distance measurement is often performed for several 1 $\mathbf{2}$ different sets of the protein samples having the paramagnetic centers at different sites, in order to obtain detailed structural information of the protein. However, design of the multiple mutations 3 for the introduction of the paramagnetic centers within appropriate distances (< -8 nm) is not 4 always straightforward. Instead of acquiring multiple sets of the DEER distance distribution, our $\mathbf{5}$ 6 study has demonstrated the combined use of MD simulation and DEER distance measurement to 7 obtain experimentally guided, detailed structural information for investigation of protein structure as an ensemble. 8

9 This strategy will be efficient not only in structural studies, but also in the evaluation of 10 drug candidates in drug design. Conformational states and changes of a protein in the absence and presence of the drug candidates should be a good probe for drug validation. Thus, the 11 information about the conformational states in solution is essential in drug design and evaluation. 12Furthermore, given the usefulness of the CLaNP-5 tag in protein NMR study, different sets of 13structural information can, in principle, be added: Attachment of the lanthanide ion that induces 14anisotropic paramagnetic effects enables the observation of a variety of paramagnetic effects in 1516 NMR that contain long-range quantitative structural information. The integrated structural 17analysis that includes these NMR-derived paramagnetic effects will further expand the ability of 18 the strategy to explore protein structure and dynamics in solution.

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1 SUPPORTING MATERIAL

2	Supporting Material can be found online at https://doi.org/
3	
4	AUTHOR CONTRIBUTIONS
5	T.S. designed the research project. T.S. and S.H. mainly carried out the research. M.A.,
6	S.H., T.S., and T.N. performed EPR measurement and analysis. S.H., T.S., and H.K. performed
7	NMR measurement and analysis. S.H. and K.S. conducted preparation of the lanthanide tags and
8	protein samples. T.S., H.N., and K.I. discussed the results and wrote the manuscript. All authors
9	read and approved the final manuscript.
10	
11	ACKNOWLEDGMENTS

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2 Table 1 Magnetic susceptibility tensors of Yb³⁺ attached to D12₁₄₅, D12₁₇₀, and

3 **D12₂₆₀.**

1

Position	Lanthanide ion	$\Delta \chi_{\mathrm{ax}} ^{\mathrm{a}}$	$\Delta \chi_{\rm rh} ^{a}$	α^{b}	β ^b	γ^{b}	X ^c	У ^с	Z^{C}
	Vh3+	$7.3 \pm$	$1.6\pm$	80	70	172			
M145C/D149C	100	0.3	0.5				$46.9\pm$	$9.8\pm$	$36.1\pm$
M143C/D146C	Tm ³⁺	$43.7\pm$	$11.4\pm$	80	71	171	0.3	0.3	1.3
		1.3	2.0						
01706/W1726	Yb ³⁺	$5.7 \pm$	$1.9\pm$	24	42	151	$51.8\pm$	$\textbf{-13.2}\pm$	$41.7\pm$
Q170C/V172C		0.1	0.1				0.2	0.3	0.3
	Vh3+	$8.4~\pm$	$0.6\pm$	137	69	22			
F260C/K262Cd	10*	0.5	0.7				$14.3\pm$	$-4.7\pm$	$38.4\pm$
E2000/ K2020*	Tm ³⁺	$47.0~\pm$	$8.5 \pm$	137	72	59	0.5	0.5	1.3
		2.3	5.2						

⁴ ${}^{a}\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ values are in 10⁻³² (m³), and error estimates were obtained by Monte Carlo ⁵ protocol using 100 partial pseudo-contact shift (PCS) data sets, in which 30% of the input ⁶ data were randomly deleted. ^bEuler angles (α , β , γ) are represented in ZYZ convention in ⁷ degrees. ^cPosition of the lanthanide ion is expressed in angstrom (Å) as a coordinate in the ⁸ crystal structure of MurD (PDB ID: 3uag). ^d Parameters determined in a previous study ⁹ (10).

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Figure legends

Figure 1 Gadolinium tagging of MurD. The positions of metals in MurD domain 1–2 M145C/D148C/C151A/E260C/K262C (D12_{145–260}) (a), MurD domain 1–2 Q170C/V172C/E260C/K262C (D12_{170–260}) (b), and MurD E260C/K262C/N360C/D362C (MurD_{260–360}) (c) are shown as red spheres in the crystal structure of MurD [Protein Data Bank (PDB) ID: 3uag]. Domains 1, 2, and 3 are presented in green, blue, and pink, respectively. The distance between lanthanoid ions are indicated by a red arrow. (d) The chemical structure of Caged Lanthanide NMR Probe 5 (CLaNP-5) tag. (e) Close-up views of the positions of lanthanoid ions fixed on MurD. The residues that were mutated to cysteine residue for ligation with the CLaNP-5 tag are shown as red stick model. The distances between C_β atoms are indicated.

Figure 2 Four-pulse DEER distance measurement for D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀. The experiment was performed on ~30 μ L of 200 μ M frozen solution of D12₁₄₅₋₂₆₀ (a, c, and e) or D12₁₇₀₋₂₆₀ (b, d, and f) in 70% D₂O/30% glycerol-*d*₈ at 10 K. (a and b) Normalized DEER traces fitted with appropriate background decay (in red). (c and d) Same DEER trace after background removal along with the fits obtained either by Tikhonov regularization (orange) or fitting to single Gaussian (blue). (e and f) Distance distribution obtained by the two different fits shown in panels c and d. The data were analyzed using the program DeerAnalysis 2016. DEER, double electron–electron resonance.

Figure 3 Four-pulse DEER distance measurement for MurD₂₆₀₋₃₆₀. The experiment was performed on ~30 μ L of 150 μ M frozen solution of MurD₂₆₀₋₃₆₀ in the absence (a, c, and e) and presence (b, d, and f) of the inhibitor in 70% D₂O/30% glycerol-*d*₈ at 10 K. (a and b) Normalized DEER traces fitted with appropriate background decay (in red). (c and d) DEER traces after background removal along with the fits obtained using Tikhonov regularization. (e and f) Distance distributions obtained by the fit shown in panels c and d. The shaded region shows an estimate of the range of distance distributions that arise from varying background subtraction. The arrows indicate the distance between the gadolinium ions expected from the crystal structures of MurD in open (PDB code 1e0d, 3.21 nm) and closed (PDB code 3uag, 4.35 nm) conformations. The data were analyzed using the program DeerAnalysis 2016. DEER, double electron–electron

resonance.

Figure 4 Comparison between DEER distance measurement and molecular dynamics (MD) simulation for apo MurD. The representative coordinates observed in MD simulations are shown with corresponding positions in the trajectories of the inter-gadolinium distance. The coordinates from MD calculation are colored blue and superimposed on domain 2 of the crystal structure of MurD in the open conformation (PDB code: 1e0d), in which domains 1, 2, and 3 are presented in green, blue, and pink, respectively. The changes in rotation and translation of domain 3 are indicated. The DEER distance distributions for $MurD_{260-360}$ in the absence of the inhibitor are shown on the right, in which horizontal and vertical axes represent relative population and intergadolinium distance, respectively. The shaded region in orange shows an estimate of the range of distance distributions that arise from varying background subtraction. DEER, double electron–electron resonance.



Figure 1 Gadolinium tagging of MurD. The positions of metals in MurD domain 1–2 M145C/D148C/C151A/E260C/K262C (D12_{145–260}) (a), MurD domain 1–2 Q170C/V172C/E260C/K262C (D12_{170–260}) (b), and MurD E260C/K262C/N360C/D362C (MurD_{260–360}) (c) are shown as red spheres in the crystal structure of MurD [Protein Data Bank (PDB) ID: 3uag]. Domains 1, 2, and 3 are presented in green, blue, and pink, respectively. The distance between lanthanoid ions are indicated by a red arrow. (d) The chemical structure of Caged Lanthanide NMR Probe 5 (CLaNP-5) tag. (e) Close-up views of the positions of lanthanoid ions fixed on MurD. The residues that were mutated to cysteine residue for ligation with the CLaNP-5 tag are shown as red stick model. The distances between C_β atoms are indicated.

Figure 2



Figure 2 Four-pulse DEER distance measurement for D12₁₄₅₋₂₆₀ and D12₁₇₀₋₂₆₀. The experiment was performed on ~30 µL of 200 µM frozen solution of D12₁₄₅₋₂₆₀ (a, c, and e) or D12₁₇₀₋₂₆₀ (b, d, and f) in 70% D₂O/30% glycerol- d_8 at 10 K. (a and b) Normalized DEER traces fitted with appropriate background decay (in red). (c and d) Same DEER trace after background removal along with the fits obtained either by Tikhonov regularization (orange) or fitting to single Gaussian (blue). (e and f) Distance distribution obtained by the two different fits shown in panels c and d. The data were analyzed using the program DeerAnalysis 2016. DEER, double electron–electron resonance.



Figure 3

Figure 3 Four-pulse DEER distance measurement for MurD₂₆₀₋₃₆₀. The experiment was performed on ~30 μ L of 150 μ M frozen solution of MurD₂₆₀₋₃₆₀ in the absence (a, c, and e) and presence (b, d, and f) of the inhibitor in 70% D₂O/30% glycerol-*d*₈ at 10 K. (a and b) Normalized DEER traces fitted with appropriate background decay (in red). (c and d) DEER traces after background removal along with the fits obtained using Tikhonov regularization. (e and f) Distance distributions obtained by the fit shown in panels c and d. The shaded region shows an estimate of the range of distance distributions that arise from varying background subtraction. The arrows indicate the distance between the gadolinium ions expected from the crystal structures of MurD in open (PDB code 1e0d, 3.21 nm) and closed (PDB code 3uag, 4.35 nm) conformations. The data were analyzed using the program DeerAnalysis 2016. DEER, double electron–electron resonance.



Figure 4 Comparison between DEER distance measurement and molecular dynamics (MD) simulation for apo MurD. The representative coordinates observed in MD simulations are shown with corresponding positions in the trajectories of the inter-gadolinium distance. The coordinates from MD calculation are colored blue and superimposed on domain 2 of the crystal structure of MurD in the open conformation (PDB code: 1e0d), in which domains 1, 2, and 3 are presented in green, blue, and pink, respectively. The changes in rotation and translation of domain 3 are indicated. The DEER distance distributions for MurD_{260–360} in the absence of the inhibitor are shown on the right, in which horizontal and vertical axes represent relative population and inter-gadolinium distance, respectively. The shaded region in orange shows an estimate of the range of distance distributions that arise from varying background subtraction. DEER, double electron–electron resonance.

Supporting information for

Conformational ensemble of a multidomain protein explored by Gd^{3+} electron

paramagnetic resonance

T. Saio, S. Hiramatsu, M. Asada, H. Nakagawa, K. Shimizu, H. Kumeta, T. Nakamura, K. Ishimori

	Yh)3+	Tm ³⁺		
	HN .~	N	HN	N	
	-0.153	_0 1/8			
	-0.155	-0.140			
ILEIU	-0.092	-0.092			
ILE11	-0.007	-0.063	-0.005	-0.060	
LEU13	0.000	-0.097	-0.005	-0.104	
VAL29	-0.460	-0.552			
VAI 33	-0 113	-0 174			
L FU46	-0 207	-0.259			
	0.190	0.200			
VALSU	-0.109	-0.200	0.100	0.040	
LEU57	-0.009	-0.009	-0.123	-0.340	
LEU62	-0.033	-0.034			
ME163	-0.033	-0.035	-0.167	-0.264	
LEU67	-0.078	-0.027	-0.356	-0.289	
ILE68	-0.032	-0.017			
VAL69	-0.008	-0.014			
II F74	0.086	0.082	0 460	0 337	
LEU76	0.055	0.021	01.00	0.001	
	0.000	0.060			
	-0.000	0.009	0.010	0.010	
ILE89	-0.014	-0.010	-0.019	-0.018	
ILE91	0.010	0.006	0.142	0.142	
VAL92	0.025	0.027			
ILE95	0.389	0.431			
LEU97	0.223	0.181	1.466	1.420	
ILE106	0.345	0.399	2,132	2.400	
VAI 107	0.505	0 450			
ILE109	0.368	0 304			
	0.000	0.004			
VALIIO	0.103	0.100			
LEU121	0.189	0.197			
VAL122	0.227	0.177			
ME I 125	0.145	0.104	0.952	0.773	
VAL131	0.046	0.019			
ILE139	0.467	0.652	2.625	2.710	
LEU144	1.631	2.073			
LEU153	0.615	0.535			
LEU156	0.600	0.685			
LEU158	0.352	0.318			
LE0100	0.215	0.212	1 263	1 317	
	0.180	0.170	1.200	1 252	
	0.103	0.170	1.212	1.252	
	0.201	0.225	0.050	0.040	
ILE1/0	0.139	0.159	0.853	0.946	
LEU1//	0.137	0.060	0.930	0.899	
VAL179	0.081	0.074			
MET184	0.036	0.055	0.308	0.243	
LEU191	0.043	0.061	0.269	0.372	
LEU199	0.106	0.069	0.697	0.649	
ILE201	0.153	0.172	0.968	0.893	
VAI 207	0 141	0 129			
VAL 209	0 138	0 121			
VAL 210	0.100	0.077			
	0.064	0.028	0 377	0 203	
METO10	0.004	0.020	0.077	0.233	
	0.073	0.090	0.440	0.424	
	0.069	0.099	0.423	0.528	
VAL228	0.093	0.028			
VAL232	0.050	0.042			
MET234	0.041	0.048	0.257	0.255	
LEU248	0.016	0.007	0.188	0.157	
VAL250	0.037	0.007			
VAL255	0.032	-0.013			
LEU256	0 024	0 046	0 178	0 246	
VAL 258	0.021	0.019			
MFT261	0.009	0.028	0 108	0 217	
I FI 1263	0.017	0.005	0 165	0 127	
	0.017	0.000	0.105	0.121	
	0.077	0.075	0.472	0.420	
LEU2/6	0.106	0.119	0.646	0.008	
LEU278	0.143	0.116	0.853	0.780	
LEU284	0.131	0.091	0.719	0.667	
LEU290	0.044	0.050	0.272	0.254	
LEU293	0.040	0.059	0.292	0.238	
LEU299	0.018	0.016	0.222	0.238	
			-		

Table S1 PCS values observed for $D12_{145}$ attached with CLaNP-5 containing Yb^{3+} and Tm^{3+} .

	H ^ℕ	N		HN	Ν
GLY5	-0.079	-0.078	VAL122	0.193	0.217
ASN7	-0.051	-0.054	GLY123	0.118	0.394
VAL8	-0.037	-0.046	MET125	0.075	0.104
LEU13	0.012	0.011	VAL131	-0.103	-0.126
LEU18	-0.003	0.023	ILE139	0.150	0.154
LEU25	-0.087	-0.115	LEU141	0.034	0.080
ARG27	-0.128	-0.071	LEU144	0.002	-0.034
GLY28	-0.098	-0.076	LEU158	0.417	0.398
VAL29	-0.088	-0.064	LEU163	0.322	0.345
VAL33	-0.004	0.000	THR165	0.224	0.214
MET34	0.000	-0.039	ILE176	0.364	0.447
GLY42	-0.004	-0.012	LEU177	0.277	0.138
LEU43	0.002	0.006	ASN178	0.206	0.243
LYS45	-0.001	-0.026	VAL179	0.149	0.190
ALA49	-0.005	-0.002	GLY190	0.072	0.089
GLU51	-0.011	-0.006	LEU191	0.065	0.158
HIS53	-0.008	-0.022	GLN192	0.081	0.146
GLY55	0.002	-0.022	LEU199	0.117	0.156
SER56	0.001	0.028	MET218	0.143	0.146
LEU57	0.003	-0.020	ILE220	0.301	0.261
TRP61	-0.006	0.096	GLY231	0.057	0.063
LEU62	0.006	0.011	VAL232	0.046	0.002
MET63	-0.003	-0.001	MET234	0.052	0.110
ALA64	-0.005	-0.019	ASN240	0.007	0.011
ASP66	-0.022	-0.027	LEU248	0.008	0.004
LEU67	-0.026	-0.039	VAL250	0.003	0.070
VAL69	0.004	0.037	GLY252	0.007	-0.074
ALA70	0.008	0.017	VAL255	-0.008	-0.012
LEU76	0.072	-0.002	LEU256	0.008	0.063
HIS78	0.044	0.129	VAL258	0.012	-0.002
ILE89	-0.001	0.004	MET261	0.010	0.027
GLU90	-0.029	0.014	LEU263	0.031	0.034
ILE91	0.014	-0.001	THR270	0.072	0.042
VAL92	0.017	0.014	LEU273	0.083	0.144
GLY93	0.128	0.175	LEU276	0.125	0.184
ILE95	0.153	0.121	LEU278	0.204	0.553
LEU97	-0.041	-0.092	GLY283	-0.076	-0.101
PHE98	-0.006	-0.051	ARG286	-0.070	-0.010
ILE109	0.644	0.659	LEU290	-0.011	-0.006
ASN113	0.155	0.143	LEU293	0.019	0.053
VAL118	0.177	0.193	GLY298	0.044	0.041
LEU121	0.146	0.096	LEU299	0.052	0.063

Table S2 PCS values observed for $D12_{\rm 170}$ attached with CLaNP-5 containing $Yb^{\rm 3+}.$



Figure S1 Observation of pseudo-contact shift for D12₁₄₅. Overlay of the ¹H–¹⁵N heteronuclear single quantum coherence (HSQC) spectra for [¹⁵N-Ile] D12₁₄₅ (a), [¹⁵N-Leu] D12₁₄₅ (b), [¹⁵N-Val] D12₁₄₅ (c), and [¹⁵N-Met] D12₁₄₅ (d). The spectra for D12₁₄₅ attached with Lu³⁺, Yb³⁺, and Tm³⁺ are colored gray, green, and blue, respectively.



Figure S2 Observation of pseudo-contact shift for D12₁₇₀. Overlay of the ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) spectra for $[U^{-15}N]$ D12₁₇₀ (a) and amino acid type-selectively ¹⁵N labeled D12₁₇₀ (b). The spectra for D12₁₇₀ attached with diamagnetic lanthanoid ion Lu³⁺ and paramagnetic lanthanoid ion Yb³⁺ are colored gray and green, respectively.



Figure S3 $\Delta \chi$ tensor analysis for D12₁₄₅ and D12₁₇₀. View of the experimentally determined PCS isosurface for D12₁₄₅ (a) and D12₁₇₀ (d) depicting the surface corresponding to ±0.7 ppm induced by Yb³⁺. Positive and negative PCS values are indicated by blue and red, respectively. Comparison of experimental and back-calculated PCSs of backbone amide protons observed for D12₁₄₅ containing Yb³⁺/Tm³⁺-CLaNP-5 (b) and D12₁₇₀ containing Yb³⁺-CLaNP-5 (e). Orientation of the principal axes of the $\Delta \chi$ tensors of Yb³⁺ and Tm³⁺ in complex with CLaNP-5 fixed on D12₁₄₅ (c) and that of Yb³⁺ in complex with CLaNP-5 fixed on D12₁₇₀ (f), visualized in Sanson–Flamsteed projection. The plots show the points where the principal axes of the $\Delta \chi$ tensor penetrate the sphere. One hundred sets of plots represent the results of the Monte Carlo analysis using the 100 partial PCS data sets in which 30% of the input data were randomly deleted. CLaNP-5, Caged Lanthanide NMR Probe S; PCS, pseudo-contact shift.



Figure S4 Echo-detected (ED) EPR spectrum around the central $|1/2\rangle \langle -\rangle |-1/2\rangle$ transition of D12₁₄₅₋₂₆₀ recorded at 10 K. The measurements were carried out using a two-pulse { $\pi/2-\tau-\pi-\tau-$ echo} sequence with t($\pi/2$) = 16 ns, t(π) = 32 ns, and τ = 200 ns. Positions of the pump and observed frequencies for the DEER experiment are indicated. The ED-EPR spectrum of MurD₁₇₀₋₂₆₀ and MurD₂₆₀₋₃₆₀ look very similar. DEER, double electron–electron resonance; EPR, electron paramagnetic resonance.



Figure S5 Inter-gadolinium distances of MurD₂₆₀₋₃₆₀ expected from the crystal structures. Crystal structures of MurD in the open (a) and closed (b) conformations with expected intergadolinium distances for MurD₂₆₀₋₃₆₀ are indicated. The metal positions for each site were determined using $\Delta \chi$ tensor analysis based on pseudo-contact shifts (PCSs) (D12₂₆₀) or Xplor-NIH docking (D3₃₆₀).



Figure S6 Theoretical DEER traces for the two paramagnetic centers with varying distributions centered at 3.9 nm. Superimposition of theoretical DEER traces (left panel) and distance distributions (right panel), with varying standard deviations of the Gaussian distribution: $\sigma = 0.1$ (red), 0.25 (orange), 0.5 (green), 1 (blue) nm. DEER, double electron–electron resonance.



Figure S7 Single Gaussian fit of the DEER traces of MurD₂₆₀₋₃₆₀ in the absence and **presence of the inhibitor.** The DEER results shown in Figure 3 were fitted by single Gaussian. The results for MurD₂₆₀₋₃₆₀ in the absence (left panels) and presence (right panels) of the inhibitor are shown. (a) DEER traces after background removal along with the fits obtained with single Gaussian fitting. (b) Distance distribution obtained by the fit shown in (a). The standard deviations were estimated as 0.81 nm (left panel) and 0.70 nm (right panel). The data were analyzed using DeerAnalysis2016. DEER, double electron–electron resonance.



Figure S8 Comparison between the crystal structure and Open_{sol} of MurD for ATP-binding site. (a) Superimposition of the crystal structure of MurD and the major conformational state of MurD in MD simulation. The coordinates are superimposed for domain 2. ADP, shown in sticks, is modeled based on the superimposition of the crystal structure of MurD in complex with ADP and UMA (3uag.pdb) for domain 2. (b), (c) The sphere representations of crystal structure (b) and the major MD conformation (c) of MurD. The ATP/ADP-binding site is wide open in the simulated structure. ADP, adenosine diphosphate; ATP, adenosine triphosphate; MD, molecular dynamics; UMA, UDP-*N*-acetylmuramoyl-L-alanine.