



Title	Conformational ensemble of a multidomain protein explored by Gd <sup>3+</sup> electron paramagnetic resonance
Author(s)	Saio, Tomohide; Hiramatsu, Soya; Asada, Mizue; Nakagawa, Hiroshi; Shimizu, Kazumi; Kumeta, Hiroyuki; Nakamura, Toshikazu; Ishimori, Koichiro
Citation	Biophysical journal, 120(15), 2943-2951 <a href="https://doi.org/10.1016/j.bpj.2021.06.033">https://doi.org/10.1016/j.bpj.2021.06.033</a>
Issue Date	2021-08-03
Doc URL	<a href="http://hdl.handle.net/2115/86494">http://hdl.handle.net/2115/86494</a>
Rights	©2021. This manuscript version is made available under the CC-BY-NC-ND 4.0 license <a href="http://creativecommons.org/licenses/by-nc-nd/4.0/">http://creativecommons.org/licenses/by-nc-nd/4.0/</a>
Rights(URL)	<a href="http://creativecommons.org/licenses/by-nc-nd/4.0/">http://creativecommons.org/licenses/by-nc-nd/4.0/</a>
Type	article (author version)
File Information	Biophys. J.120-15_2943-2951.pdf



[Instructions for use](#)

1 **Conformational ensemble of a multidomain protein explored by Gd<sup>3+</sup> electron**  
2 **paramagnetic resonance**

3

4 Tomohide Saio,<sup>1†\*</sup> Soya Hiramatsu,<sup>2†</sup> Mizue Asada,<sup>3</sup> Hiroshi Nakagawa,<sup>4,5</sup> Kazumi Shimizu,<sup>6</sup> Hiroyuki  
5 Kumeta,<sup>7</sup> Toshikazu Nakamura,<sup>3</sup> and Koichiro Ishimori<sup>2,8\*</sup>

6

7 <sup>1</sup>*Division of Molecular Life Science, Institute of Advanced Medical Sciences, Tokushima University,*  
8 *Tokushima, Japan*

9 <sup>2</sup>*Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo, 060-8628,*  
10 *Japan*

11 <sup>3</sup>*Instrument Center, Institute for Molecular Science, Okazaki 444-8585, Japan.*

12 <sup>4</sup>*Materials Sciences Research Center, Japan Atomic Energy Agency, Tokai, Ibaraki 319-1195, Japan*

13 <sup>5</sup>*J-PARC Center, Japan Atomic Energy Agency, Tokai, Ibaraki 319-1195, Japan*

14 <sup>6</sup>*Faculty of Education and Integrated Arts and Sciences, Waseda University, Tokyo 169-8050, Japan*

15 <sup>7</sup>*Faculty of Advanced Life Science, Hokkaido University, Sapporo, 011-0021, Japan*

16 <sup>8</sup>*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: [saio@tokushima-u.ac.jp](mailto:saio@tokushima-u.ac.jp), [koichiro@sci.hokudai.ac.jp](mailto:koichiro@sci.hokudai.ac.jp)

22

23 Running title: Structural variation of a protein enzyme

24

25 Keywords: Conformational ensemble, integrated structural analysis, DEER EPR, NMR,  
26 lanthanide ion

27

28

1 **ABSTRACT**

2           Despite their importance in function, the conformational state of proteins and its  
3 changes are often poorly understood mainly because of the lack of an efficient tool. MurD, a 47-  
4 kDa protein enzyme responsible for peptidoglycan biosynthesis, is one of those proteins whose  
5 conformational states and changes during their catalytic cycle are not well understood. Although  
6 it has been considered that MurD takes a single conformational state in solution as shown by a  
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  
13 developed for paramagnetic nuclear magnetic resonance (NMR). The combined use of NMR and  
14 EPR enables accurate interpretation of the DEER distance information to the structural  
15 information of MurD. The DEER distance measurement for apo MurD shows a broad distance  
16 distribution, whereas the presence of the inhibitor narrows the distance distribution. The results  
17 suggest 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  
19 conformational states of MurD were previously implied by NMR experiments, but our DEER  
20 data provided structural characterization of the conformational variety of MurD.

21

22 **STATEMENT OF SIGNIFICANCE (<120)**

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

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

2 Proteins, especially protein enzymes, exert their activities through dynamic structural  
3 changes that are often poorly understood. Even after the determination of their high-resolution  
4 structure, the lack of information about these dynamic structural changes obscures the  
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  
12 extensively studied as a potent drug target among the Mur ligases (5, 6, 7).

13 MurD consists of three domains, and previous crystallographic studies have identified  
14 that domain 3 undergoes drastic orientational changes with respect to domain 2, so that MurD  
15 changes its conformation from the open to the closed state upon binding to ligands or inhibitors  
16 (1, 2, 5, 6, 8, 9). More recently, a nuclear magnetic resonance (NMR) study using paramagnetic  
17 lanthanide ions showed that drastic conformational changes of MurD regulate the order of the  
18 ligand binding in the enzymatic reaction (10). Interestingly, the NMR study also suggested that  
19 MurD exists in multiple conformational states exchanging in millisecond to microsecond (ms–  
20  $\mu$ s) time scale in the absence of ligands (10), whereas it had been believed that apo MurD exists  
21 in the open conformation as seen in the crystal structure (10). However, these conformational  
22 variations of MurD are not fully understood. The conformational equilibrium can be hindered  
23 upon crystal formation, or in the solution NMR experiments, the conformational exchange  
24 averages or broadens out the resonances, which obscures the structural information for each of  
25 the states. Molecular dynamics (MD) simulations have been performed to investigate the  
26 conformational dynamics of MurD *in silico* (11, 12), but limited simulation time has, so far,  
27 impeded the tracking of the relatively large size as well as slow conformational dynamics (ms– $\mu$ s

1 time scale) of MurD.

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

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

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.

3

4

## 1 MATERIALS AND METHODS

### 2 Preparation of CLaNP-5

3 CLaNP-5 was synthesized, purified, and chelated to Lu<sup>3+</sup>, Yb<sup>3+</sup>, and Gd<sup>3+</sup> according to  
4 the procedure described in previous reports (10, 21, 25).

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  
9 described in a previous report (10). For PCS observation, isotopically labeled MurD domain 1–2  
10 Q170C/V172C (D12<sub>170</sub>) and MurD domain 1–2 M145C/D148C/C151A (D12<sub>145</sub>), both having a  
11 single lanthanide ion, were prepared using the same procedure as reported previously for MurD  
12 domain 1–2 E260C/K262C (10). The proteins were prepared in a buffer containing 20 mM Tris–  
13 HCl (pH 7.2) and 100 mM NaCl. The CLaNP-5 tag was attached to the protein by mixing the  
14 protein and the tag at a 1:1.1 ratio for 15 minutes on ice, followed by gel filtration to remove  
15 unreacted tag. For EPR distance measurement, MurD domain 1–2 Q170C/V172C/E260C/K262C  
16 (D12<sub>170–260</sub>), MurD domain 1–2 M145C/D148C/C151A/E260C/K262C (D12<sub>145–260</sub>), and MurD  
17 E260C/K262C/N360C/D362C (MurD<sub>260–360</sub>) were prepared using a previously described  
18 procedure (10). Note that D12<sub>145–260</sub> 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  
20 the protein and the tag at a 1:2.2 ratio for 15 minutes on ice, followed by gel filtration. The  
21 attachment of the tag was confirmed by NMR (Figure S1–S2). The data showed that the tags  
22 were attached to the specific position of MurD at a high efficiency. Although MurD has seven  
23 cysteine residues (C20, C99, C151, C208, C227, C368, and C413), all cysteine residues have a  
24 thiol group buried in the protein and did not react to the CLaNP-5 tag (10). MurD domain 1–2  
25 Q170C/V172C/E260C/K262C (D12<sub>170–260</sub>) was prepared in 20 mM Tris-*d*<sub>11</sub>-HCl (pD 7.2), 100  
26 mM NaCl in 70% D<sub>2</sub>O/30% glycerol-*d*<sub>8</sub>. Full-length MurD E260C/K262C/N360C/D362C



1 (MurD<sub>260–360</sub>) and full-length MurD E260C/K262C/L396C/R400C (MurD<sub>260–396</sub>) were prepared  
2 in 20 mM Tris-*d*<sub>11</sub>-HCl (pD 7.2), 200 mM NaCl in 70% D<sub>2</sub>O/30% glycerol-*d*<sub>8</sub>.

3

#### 4 **Determination of the metal position by PCS analysis**

5 The positions of the metal fixed at Q170C/V172C and M145C/D148C were estimated  
6 via analysis of PCSs (31, 32). The diamagnetic lanthanide ion Lu<sup>3+</sup> as a diamagnetic reference or  
7 the paramagnetic lanthanide ion Yb<sup>3+</sup> was attached to MurD domain 1–2 Q170C/V172C (D12<sub>170</sub>)  
8 or MurD domain 1–2 M145C/D148C/C151A (D12<sub>145</sub>) by CLaNP-5. PCSs were measured as a  
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  
11 assignments, amino acid-selective <sup>15</sup>N-labeled samples were prepared as previously described  
12 (10). <sup>1</sup>H–<sup>15</sup>N heteronuclear single quantum coherence (HSQC) spectra were recorded on Bruker  
13 Avance III 600 and 800 MHz NMR spectrometers (Bruker Corp., Billerica, MA, USA). The  
14 spectra were recorded at 25°C. Spectra were then processed using the NMRPipe program (33),  
15 and data analysis was performed on the Olivia program (Yokochi et al.;  
16 <https://github.com/yokochi47/Olivia>).  $\Delta\chi$ -tensor and the position of the lanthanide ion were  
17 calculated from the PCS values and the crystal structure of MurD (PDB code 3uag) (2), using the  
18 Numbat program (34) with equation Eq. (1),

$$19 \quad \Delta\delta^{\text{PCS}} = \frac{1}{12\pi r^3} \left[ \Delta\chi_{\text{ax}}(3\cos^2\theta - 1) + \frac{3}{2}\Delta\chi_{\text{rh}}\sin^2\theta\cos 2\phi \right], \quad (1)$$

20 where  $\Delta\delta^{\text{PCS}}$  is the PCS;  $r$ ,  $\theta$ , and  $\phi$  are the polar coordinates of the nucleus with respect to the  
21 principal axes of the magnetic susceptibility tensor; and  $\Delta\chi_{\text{ax}}$  and  $\Delta\chi_{\text{rh}}$  are the axial and rhombic  
22 components, respectively, of the anisotropic magnetic susceptibility tensor. The tensor  
23 calculations were performed using 63 and 31 PCSs induced by Yb<sup>3+</sup> and Tm<sup>3+</sup>, respectively, for  
24 D12<sub>145</sub> (Table S1) and using 87 PCSs induced by Yb<sup>3+</sup> for D12<sub>170</sub> (Table S2). Only PCSs  
25 observed from the amide proton located on the rigid region of the protein were used in the fitting.

1 Errors were estimated with Monte Carlo analysis using the 100 partial PCS data sets in which  
2 30% of the input data were randomly deleted.

3

#### 4 **Determination of the metal position by modeling**

5 The position of the metal fixed via N360C/D362C was estimated by docking  
6 calculation using Xplor-NIH (35). The coordinates of CLaNP-5 were prepared as described  
7 previously (31, 36), by using PRODRG2 server (37) and VEGA ZZ (38). In the Xplor  
8 calculation, the position and orientation of CLaNP-5 were first randomized around the  
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  
11 of MurD. A total of 100 docking calculations were performed, and the 50 lowest-energy  
12 coordinates were selected, in which the positions of the metal were averaged.

13

#### 14 **EPR DEER distance measurement**

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

1 DeerAnalysis 2016 software (39).

## 2 **RESULTS**

### 3 **Gadolinium tagging of a multidomain protein MurD**

4 To investigate the conformational states of a multidomain protein MurD, we aimed to  
5 measure the distance between the two gadolinium ions fixed on the domains 2 and 3 by DEER  
6 experiments. Gd<sup>3+</sup> ion was fixed on MurD through the CLaNP-5 tag (21, 25) that was attached to  
7 the protein by two disulfide bridges (Figure 1). For reliable DEER distance measurements, the  
8 tagging points were designed to be located within ~8 nm from each other and located on the  
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  
11 the candidates. Finally, four positions of MurD—three in domain 2 and one in domain 3—were  
12 selected for gadolinium tagging (Figure 1a–c). Following the procedures described in previous  
13 reports (10, 25), the pair of surface-exposed residues whose C<sub>β</sub> atoms are located in a distance of  
14 8–10 Å were selected and mutated to cysteine residues for attachment of CLaNP-5 (Figure 1e).  
15 Three MurD variants were constructed for DEER measurement: MurD domain 1–2  
16 M145C/D148C/C151A/E260C/K262C (D12<sub>145–260</sub>), MurD domain 1–2  
17 Q170C/V172C/E260C/K262C (D12<sub>170–260</sub>), and full-length MurD E260C/K262C/N360C/D362C  
18 (MurD<sub>260–360</sub>) (Figure 1a–c). Because D12<sub>145–260</sub> and D12<sub>170–260</sub> have two Gd<sup>3+</sup> ions both on  
19 domain 2, the distance distributions for D12<sub>145–260</sub> and D12<sub>170–260</sub> are expected to reflect local  
20 conformational variation around the tag. On the other hand, the distance distribution for  
21 MurD<sub>260–360</sub>, having Gd<sup>3+</sup> ions on domains 2 and 3, is expected to reflect the conformational  
22 variations of domain 3 with respect to domain 2, in addition to the local conformational  
23 variations.

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<sub>145</sub>) and  
9 MurD domain 1–2 Q170C/V172C (D12<sub>170</sub>) 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  $3.58 \pm 0.12$  and  $3.86 \pm 0.11$  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).

24

## 25 **$Gd^{3+}$ – $Gd^{3+}$ DEER measurement for D12<sub>145–260</sub> and D12<sub>170–260</sub>**

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

1 conformational states of the protein have distinct distances between paramagnetic centers.  
2 However, it should be noted that the residual mobility of the tag can also contribute to the  
3 distance distribution. Although the CLaNP-5 tag is rigidly attached to the protein via two arms as  
4 shown by previous NMR studies (10, 21, 25), there was no information about the actual spatial  
5 distribution of the metal ion attached on the protein via CLaNP-5 tag. To assess the distribution  
6 of paramagnetic centers imposed by the residual local mobility of the CLaNP-5 tag, we  
7 performed DEER measurement on D12<sub>145-260</sub> and D12<sub>170-260</sub>, in which CLaNP-5 tags containing  
8 Gd<sup>3+</sup> were attached to the rigid regions of domain 2 (Figure 1a and b). The EPR experiments  
9 were performed on D12<sub>145-260</sub> and D12<sub>170-260</sub> prepared in 80% <sup>2</sup>H<sub>2</sub>O/20% glycerol-*d*<sub>8</sub> solution at  
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  
12 spectrometer (resonant frequency, 34 Hz) (40). Four-pulse DEER experiments were performed  
13 on D12<sub>145-260</sub> and D12<sub>170-260</sub>, and distance distributions were obtained using DeerAnalysis 2016  
14 (39) (Figure 2). The data were recorded with dipolar evolution time as long as 3.9 μs. The DEER  
15 trace exhibited clear dipolar modulation (Figure 2a-d), indicating the existence of the defined  
16 inter-metal distance. Tikhonov regularization and Gaussian fit for D12<sub>145-260</sub> showed the most  
17 intense peaks at 3.64 and 3.68 nm, with full-width at half-maximum (FWHM) of 0.77 and 0.76  
18 nm, respectively (Figure 2e). The fitting for D12<sub>170-260</sub> showed the most intense peaks at 3.96 nm  
19 (Tikhonov regularization) and 3.90 nm (Gaussian fit), with FWHM of 0.75 and 0.67 nm,  
20 respectively (Figure 2f). In both cases, Tikhonov regularization and Gaussian fit showed  
21 essentially the same distance distribution, supporting the robustness of the analysis. Thus,  
22 FWHM of 0.7-0.8 nm are considered to be imposed by local conformational variations of the  
23 lanthanide tag. Assuming that the two CLaNP-5 tags have similar conformational distributions,  
24 the DEER distance distribution indicated that each CLaNP-5 tag has conformational distribution  
25 of 0.4 nm, which should be mainly accounted for local conformational variation of side chains of  
26 the cysteine residues holding the tag. These DEER-derived distances match the distances  
27 estimated by Δχ tensor analysis using PCSs and the crystal structure of MurD, 3.58 ± 0.12 nm  
28 for D12<sub>145-260</sub>, and 3.86 ± 0.11 nm for D12<sub>170-260</sub> (Figure 1a and b), supporting the reliability of

1 the Gd<sup>3+</sup>–Gd<sup>3+</sup> distance measurement by DEER.

2

### 3 **Gd<sup>3+</sup>–Gd<sup>3+</sup> DEER for MurD<sub>260–360</sub> identified a variety of conformational states of MurD**

4           The Gd<sup>3+</sup>–Gd<sup>3+</sup> distance for MurD<sub>260–360</sub> was measured to monitor the conformational  
5 states of MurD in the absence and presence of the inhibitor, *N*-({3-[(4-[(*Z*)- (2,4-dioxo-1,3-  
6 thiazolidin-5-ylidene)methyl]phenyl}amino)methyl]phenyl}carbonyl)-*D*-glutamic acid (5).  
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  
10 Q-band spectrometer, with a dipolar evolution time as long as 3.9 μs (Figure 3). Unlike D12<sub>145–</sub>  
11 <sub>260</sub> or D12<sub>170–260</sub>, which have a defined distance between Gd<sup>3+</sup> ions (Figure 2c and d), DEER  
12 traces for MurD<sub>260–360</sub>, especially in the absence of the inhibitor, showed that the oscillation  
13 disappears after ~0.4 μs (Figure 3a and c), which implies the existence of multiple Gd<sup>3+</sup>–Gd<sup>3+</sup>  
14 distances and thus multiple conformational states of MurD<sub>260–360</sub> in the absence of the inhibitor.  
15 The distance distribution for MurD<sub>260–360</sub> in the absence of the inhibitor indeed showed a broad  
16 major peak whose top was located at 3.56 nm (Figure 3e). Although the position of the peak top  
17 is close to that expected from the crystal structure of MurD in the open conformation (PDB code:  
18 1e0d.pdb, 3.21 ± 0.20 nm) (8) (Figure S5a), there was a small but significant gap between the  
19 two distances. This indicates that the major conformational state in solution is somewhat  
20 different from that observed in the crystal structure (8).

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

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

12 MD simulation also highlighted a variety of conformational states of MurD (Figure 4),  
13 which well explains the broad distance distribution obtained from DEER experiments for MurD  
14 in the absence of the inhibitor. The broad distance distribution indicates that MurD exists as an  
15 ensemble of a variety of conformational states in solution that have not observed in crystal  
16 structures. The FWHM for the major peak in the absence of the inhibitor was estimated as 1.28  
17 nm, which is much wider than that observed for D12<sub>145-260</sub> and D12<sub>170-260</sub> (0.7-0.8 nm)(Figures  
18 2e, f and 3e). Note that the wide distance distribution for MurD<sub>260-360</sub> was also supported by the  
19 theoretical DEER traces (Figure S6), in which the distance distribution for  $\sigma = 1.0$  nm indicated  
20 the DEER traces very similar to that observed for MurD<sub>260-360</sub> in the absence of the ligand  
21 (Figure 3c). The distance distribution also covers the distance expected from the closed  
22 conformation as seen in the crystal structure of MurD in complex with the ligands or the  
23 inhibitor (Figure S5b), implying that the conformational ensemble of MurD includes the closed  
24 conformation even in the absence of the inhibitor.

25 On the other hand, the DEER measurement for MurD<sub>260-360</sub> 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

1 major peak decreased from 1.28 nm to 1.14 nm, and the peak top shifted from 3.56 nm to 4.10  
2 nm (Figure 3e and f), which corresponds to the distance expected for the crystal structure of  
3 MurD in the closed conformation ( $4.35 \pm 0.25$  nm) (Figure S5b) (5). Although the distance  
4 distribution of MurD<sub>260-360</sub> became narrower in the presence of the inhibitor, the FWHM of 1.14  
5 nm for MurD<sub>260-360</sub> in complex with the inhibitor is still wider than that observed for D12<sub>145-260</sub>  
6 or D12<sub>170-260</sub> (0.7-0.8 nm) (Figures 2 and 3). As the binding is expected to be saturated given the  
7 affinity and concentrations of MurD and the inhibitor (10), the wider distance distribution for  
8 MurD<sub>260-360</sub> in complex with the inhibitor suggests residual conformational variety of MurD  
9 even in complex with the inhibitor. Note that single Gaussian fitting showed that the overall  
10 average distance shifted from 3.78 to 4.21 nm after addition of the inhibitor (Figure S7),  
11 supporting the inhibitor-induced conformational change of MurD. Thus, the results showed that  
12 MurD in solution exists in an ensemble of a variety of conformational states, and the binding of  
13 the inhibitor shifts the population toward the closed conformation.

14

15



## 1 DISCUSSION

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  
4 concluded that in the absence of ligands, MurD exists in an open conformation (8), whereas in  
5 the presence of ligands or inhibitors it exists in a closed conformation (1, 2, 5, 9). Although it has  
6 been believed that MurD takes a specific conformational state as seen in the crystal structure, the  
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  
9 conformational states in solution. DEER distance distribution for MurD<sub>260-360</sub> shows that the  
10 FWHM for the major peak in the absence of the inhibitor is 1.28 nm, which is much wider than  
11 that obtained for D12<sub>145-260</sub> and D12<sub>170-260</sub> (0.7-0.8 nm), in which the two gadolinium ions are  
12 fixed on the same domain (Figures 2e, f and 3e). The increased distance distribution of apo  
13 MurD<sub>260-360</sub> indicates the conformational variation of MurD, especially variation in position  
14 and/or orientation of domain 3 with respect to domain 2.

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

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

3         It should be also noted that the DEER distance distribution for MurD<sub>260-360</sub> in the absence  
4 of the ligand showed a major peak with a maximum height of 3.56 nm, which is different from  
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 ~3.6 nm show domain 3 rotated from the crystal open structure by ~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  
12 conformation, this conformation can be beneficial for capturing ATP molecules.

13         In contrast to the wide conformational distribution of apo MurD, the presence of the  
14 inhibitor narrows down the population toward the closed conformation (Figure 3f). The peak top  
15 of the DEER distance distribution of MurD<sub>260-360</sub> (4.10 nm) roughly matches to the distance  
16 expected from the crystal structure in the closed conformation ( $4.35 \pm 0.25$  nm) (5). Thus, the  
17 data indicate that MurD in solution exists in a wide variety of conformational states in the  
18 absence of the ligand, but the binding of the ligand causes MurD to settle down in a specific  
19 conformational state.

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

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

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  
11 and presence of the drug candidates should be a good probe for drug validation. Thus, the  
12 information about the conformational states in solution is essential in drug design and evaluation.  
13 Furthermore, given the usefulness of the CLaNP-5 tag in protein NMR study, different sets of  
14 structural information can, in principle, be added: Attachment of the lanthanide ion that induces  
15 anisotropic paramagnetic effects enables the observation of a variety of paramagnetic effects in  
16 NMR that contain long-range quantitative structural information. The integrated structural  
17 analysis that includes these NMR-derived paramagnetic effects will further expand the ability of  
18 the strategy to explore protein structure and dynamics in solution.

19

20

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**

12 We thank T. Kanaba and H. Hara (Bruker) for fruitful discussions and suggestions  
13 during the early phase of the project. This work was partially supported by JSPS KAKENHI  
14 (17H05657, 17H05867, 18H05229, and 19K06504 to T.S. and 19H05769 to K.I.), JST PRESTO  
15 Program to T.S., JST FOREST Program to T.S., AMED Practical Research Project for  
16 Rare/Intractable Diseases (JP21ek0109437) to T.S. This work was also partially supported by  
17 Toyota Riken Scholar, Takeda Science Foundation Grant, The Sumitomo Foundation, Astellas  
18 Foundation for Research on Metabolic Disorders, Senri Life Science Foundation, The Nakajima  
19 Foundation, The Asahi Glass Foundation, Akiyama Life Science Foundation Grants-in-Aid, and  
20 Northern Advancement Center for Science and Technology Grants-in-Aid to T.S. The EPR  
21 measurements were supported by Nanotechnology Platform Program <Molecule and Material  
22 Synthesis> (JPMXP09S18MS0006, JPMXP09S17MS0002, JPMXP09S16MS0040) of the  
23 Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The NMR  
24 experiments were performed at Hokkaido University Advanced NMR Facility, a member of  
25 NMR Platform.

26

27

## 1 REFERENCES

- 2 1. Smith, C.A. 2006. Structure, Function and Dynamics in the mur Family of Bacterial Cell  
3 Wall Ligases. *Journal of Molecular Biology*. 362: 640–655.
- 4 2. Bertrand, J.A., G. Auger, L. Martin, E. Fanchon, D. Blanot, D. Le Beller, J. van  
5 Heijenoort, and O. Dideberg. 1999. Determination of the MurD mechanism through  
6 crystallographic analysis of enzyme complexes. *Journal of Molecular Biology*. 289: 579–  
7 590.
- 8 3. Pratiel-Sosa, F., F. Acher, F.O. Trigalo, D. Blanot, R. Azerad, and J. Heijenoort. 1994.  
9 Effect of various analogues of D-glutamic acid on the D-glutamate-adding enzyme from  
10 *Escherichia coli*. *FEMS Microbiology Letters*. 115: 223–228.
- 11 4. Bouhss, A., S. Dementin, C. Parquet, D. Mengin-Lecreulx, J.A. Bertrand, D. Le Beller, O.  
12 Dideberg, J. van Heijenoort, and D. Blanot. 1999. Role of the Ortholog and Paralog  
13 Amino Acid Invariants in the Active Site of the UDP-MurNAc- l-alanine: d-glutamate  
14 Ligase (MurD). *Biochemistry*. 38: 12240–12247.
- 15 5. Zidar, N., T. Tomašić, R. Šink, V. Rupnik, A. Kovač, S. Turk, D. Patin, D. Blanot, C.  
16 Contreras-Martel, A. Dessen, M. Müller Premru, A. Zega, S. Gobec, L. Peterlin Mašič,  
17 and D. Kikelj. 2010. Discovery of Novel 5-Benzylidenerhodanine and 5-  
18 Benzylidenethiazolidine-2,4-dione Inhibitors of MurD Ligase. *J. Med. Chem.* 53: 6584–  
19 6594.
- 20 6. Tomašić, T., R. Šink, N. Zidar, A. Fic, C. Contreras-Martel, A. Dessen, D. Patin, D.  
21 Blanot, M. Müller Premru, S. Gobec, A. Zega, D. Kikelj, and L.P. Mašič. 2012. Dual  
22 Inhibitor of MurD and MurE Ligases from *Escherichia coli* and *Staphylococcus aureus*.  
23 *ACS Med. Chem. Lett.* 3: 626–630.
- 24 7. Perdih, A., M. Hrast, H. Barreteau, S. Gobec, G. Wolber, and T. Solmajer. 2014. Inhibitor  
25 Design Strategy Based on an Enzyme Structural Flexibility: A Case of Bacterial MurD  
26 Ligase. *J. Chem. Inf. Model.* 54: 1451–1466.

- 1 8. Bertrand, J.A., E. Fanchon, L. Martin, L. Chantalat, G. Auger, D. Blanot, J. van  
2 Heijenoort, and O. Dideberg. 2000. “Open” structures of MurD: domain movements and  
3 structural similarities with foyllypolyglutamate synthetase. *Journal of Molecular Biology*.  
4 301: 1257–1266.
- 5 9. Kotnik, M., J. Humljan, C. Contreras-Martel, M. Oblak, K. Kristan, M. Hervé, D. Blanot,  
6 U. Urleb, S. Gobec, A. Dessen, and T. Solmajer. 2007. Structural and Functional  
7 Characterization of Enantiomeric Glutamic Acid Derivatives as Potential Transition State  
8 Analogue Inhibitors of MurD Ligase. *Journal of Molecular Biology*. 370: 107–115.
- 9 10. Saio, T., K. Ogura, H. Kumeta, Y. Kobashigawa, K. Shimizu, M. Yokochi, K. Kodama,  
10 H. Yamaguchi, H. Tsujishita, and F. Inagaki. 2015. Ligand-driven conformational changes  
11 of MurD visualized by paramagnetic NMR. *Sci. Rep.* 5: 16685.
- 12 11. Perdih, A., M. Kotnik, M. Hodoscek, and T. Solmajer. 2007. Targeted molecular  
13 dynamics simulation studies of binding and conformational changes in *E. coli* MurD.  
14 *Proteins: Structure, Function, and Bioinformatics*. 68: 243–254.
- 15 12. Perdih, A., and T. Solmajer. 2012. MurD ligase from *Escherichia coli*: C-terminal domain  
16 closing motion. *Computational and Theoretical Chemistry*. 979: 73–81.
- 17 13. Puljung, M.C., H.A. DeBerg, W.N. Zagotta, and S. Stoll. 2014. Double electron-electron  
18 resonance reveals cAMP-induced conformational change in HCN channels. *Proc. Natl.*  
19 *Acad. Sci. U.S.A.* 111: 9816–9821.
- 20 14. Abdelkader, E.H., A. Feintuch, X. Yao, L.A. Adams, L. Aurelio, B. Graham, D. Goldfarb,  
21 and G. Otting. 2015. Protein conformation by EPR spectroscopy using gadolinium tags  
22 clicked to genetically encoded p-azido-L-phenylalanine. *Chem. Commun.* 51: 15898–  
23 15901.
- 24 15. Manglik, A., T.H. Kim, M. Masureel, C. Altenbach, Z. Yang, D. Hilger, M.T. Lerch, T.S.  
25 Kobilka, F.S. Thian, W.L. Hubbell, R.S. Prosser, and B.K. Kobilka. 2015. Structural  
26 Insights into the Dynamic Process of  $\beta$ 2-Adrenergic Receptor Signaling. *Cell*. 161: 1101–  
27 1111.

- 1 16. Martorana, A., G. Bellapadrona, A. Feintuch, E. Di Gregorio, S. Aime, and D. Goldfarb.  
2 2014. Probing Protein Conformation in Cells by EPR Distance Measurements using Gd<sup>3+</sup>  
3 Spin Labeling. *J. Am. Chem. Soc.* 136: 13458–13465.
- 4 17. Saio, T., and K. Ishimori. 2019. Accelerating structural life science by paramagnetic  
5 lanthanide probe methods. *Biochimica et Biophysica Acta (BBA) - General Subjects*.
- 6 18. Nitsche, C., and G. Otting. 2017. Pseudocontact shifts in biomolecular NMR using  
7 paramagnetic metal tags. *Progress in Nuclear Magnetic Resonance Spectroscopy*. 98-99:  
8 20–49.
- 9 19. Fielding, A., M. Concilio, G. Heaven, and M. Hollas. 2014. New Developments in Spin  
10 Labels for Pulsed Dipolar EPR. *Molecules* 2014, Vol. 19, Pages 16998-17025. 19: 16998–  
11 17025.
- 12 20. Cunningham, T.F., M.R. Putterman, A. Desai, W.S. Horne, and S. Saxena. 2015. The  
13 Double-Histidine Cu<sup>2+</sup>-Binding Motif: A Highly Rigid, Site-Specific Spin Probe for  
14 Electron Spin Resonance Distance Measurements. *Angewandte Chemie*. 127: 6428–6432.
- 15 21. Keizers, P.H.J., J.F. Desreux, M. Overhand, and M. Ubbink. 2007. Increased  
16 Paramagnetic Effect of a Lanthanide Protein Probe by Two-Point Attachment. *J. Am.*  
17 *Chem. Soc.* 129: 9292–9293.
- 18 22. Saio, T., K. Ogura, M. Yokochi, Y. Kobashigawa, and F. Inagaki. 2009. Two-point  
19 anchoring of a lanthanide-binding peptide to a target protein enhances the paramagnetic  
20 anisotropic effect. *J Biomol NMR*. 44: 157–166.
- 21 23. Barthelmes, K., A.M. Reynolds, E. Peisach, H.R.A. Jonker, N.J. DeNunzio, K.N. Allen,  
22 B. Imperiali, and H. Schwalbe. 2010. Engineering Encodable Lanthanide-Binding Tags  
23 into Loop Regions of Proteins. *J. Am. Chem. Soc.* 133: 808–819.
- 24 24. Welegedara, A.P., Y. Yang, M.D. Lee, J.D. Swarbrick, T. Huber, B. Graham, D.  
25 Goldfarb, and G. Otting. 2017. Double-Arm Lanthanide Tags Deliver Narrow Gd<sup>3+</sup>–Gd<sup>3+</sup>  
26 Distance Distributions in Double Electron–Electron Resonance (DEER) Measurements.  
27 *Chemistry - A European Journal*. 23: 11694–11702.

- 1 25. Keizers, P.H.J., A. Saragliadis, Y. Hiruma, M. Overhand, and M. Ubbink. 2008. Design,  
2 Synthesis, and Evaluation of a Lanthanide Chelating Protein Probe: CLaNP-5 Yields  
3 Predictable Paramagnetic Effects Independent of Environment. *J. Am. Chem. Soc.* 130:  
4 14802–14812.
- 5 26. Lescanne, M., S.P. Skinner, A. Blok, M. Timmer, L. Cerofolini, M. Fragai, C. Luchinat,  
6 and M. Ubbink. 2017. Methyl group assignment using pseudocontact shifts with  
7 PARAssign. *J Biomol NMR.* 69: 183–195.
- 8 27. Guan, J.-Y., P.H.J. Keizers, W.-M. Liu, F. Löhr, S.P. Skinner, E.A. Heeneman, H.  
9 Schwalbe, M. Ubbink, and G. Siegal. 2013. Small-Molecule Binding Sites on Proteins  
10 Established by Paramagnetic NMR Spectroscopy. *J. Am. Chem. Soc.* 135: 5859–5868.
- 11 28. Keizers, P.H.J., B. Mersinli, W. Reinle, J. Donauer, Y. Hiruma, F. Hannemann, M.  
12 Overhand, R. Bernhardt, and M. Ubbink. 2010. A Solution Model of the Complex Formed  
13 by Adrenodoxin and Adrenodoxin Reductase Determined by Paramagnetic NMR  
14 Spectroscopy. *Biochemistry.* 49: 6846–6855.
- 15 29. Šink, R., M. Kotnik, A. Zega, H. Barreteau, S. Gobec, D. Blanot, A. Dessen, and C.  
16 Contreras-Martel. 2016. Crystallographic Study of Peptidoglycan Biosynthesis Enzyme  
17 MurD: Domain Movement Revisited. *PLoS ONE.* 11: e0152075.
- 18 30. Kobashigawa, Y., H. Kumeta, K. Ogura, and F. Inagaki. 2009. Attachment of an NMR-  
19 invisible solubility enhancement tag using a sortase-mediated protein ligation method. *J*  
20 *Biomol NMR.* 43: 145–150.
- 21 31. Saio, T., and F. Inagaki. 2016. NMR Structural Biology Using Paramagnetic Lanthanide  
22 Probe. In: *Advanced Methods in Structural Biology.* Tokyo: Springer Japan. pp. 315–340.
- 23 32. Saio, T., and F. Inagaki. 2018. Structural Study of Proteins by Paramagnetic Lanthanide  
24 Probe Methods. In: *Experimental Approaches of NMR Spectroscopy.* Singapore:  
25 Springer, Singapore. pp. 227–252.



- 1 33. Delaglio, F., S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, and A. Bax. 1995. NMRPipe: a  
2 multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 6:  
3 277–293.
- 4 34. Schmitz, C., M.J. Stanton-Cook, X.-C. Su, G. Otting, and T. Huber. 2008. Numbat: an  
5 interactive software tool for fitting  $\Delta\chi$ -tensors to molecular coordinates using  
6 pseudocontact shifts. *J Biomol NMR*. 41: 179–189.
- 7 35. Schwieters, C.D., J.J. Kuszewski, and M. Clore. 2006. Using Xplor–NIH for NMR  
8 molecular structure determination. *Progress in Nuclear Magnetic Resonance*  
9 *Spectroscopy*. 48: 47–62.
- 10 36. Saio, T., K. Ogura, K. Shimizu, M. Yokochi, T.R. Burke Jr, and F. Inagaki. 2011. An  
11 NMR strategy for fragment-based ligand screening utilizing a paramagnetic lanthanide  
12 probe. *J Biomol NMR*. 51: 395–408.
- 13 37. Schüttelkopf, A.W., and D.M.F. van Aalten. 2004. PRODRG: a tool for high-throughput  
14 crystallography of protein–ligand complexes. *Acta Crystallogr D Biol Crystallogr*. 60:  
15 1355–1363.
- 16 38. Pedretti, A., L. Villa, and G. Vistoli. 2002. VEGA: a versatile program to convert, handle  
17 and visualize molecular structure on Windows-based PCs. *Journal of Molecular Graphics*  
18 *and Modelling*. 21: 47–49.
- 19 39. Jeschke, G., V. Chechik, P. Ionita, A. Godt, H. Zimmermann, J. Banham, C.R. Timmel,  
20 D. Hilger, and H. Jung. 2006. DeerAnalysis2006—a comprehensive software package for  
21 analyzing pulsed ELDOR data. *Appl. Magn. Reson*. 30: 473–498.
- 22 40. Dalaloyan, A., M. Qi, S. Ruthstein, S. Vega, A. Godt, A. Feintuch, and D. Goldfarb. 2015.  
23 Gd(III)–Gd(III) EPR distance measurements – the range of accessible distances and the  
24 impact of zero field splitting. *Phys. Chem. Chem. Phys*. 17: 18464–18476.
- 25 41. Nakagawa, H., Saio, T., Nagao, M., Inoue, R., Sugiyama, M., Ajito, S., Tominaga, T., Kawakita,  
26 Y. Conformational dynamics of a multi-domain protein explored by neutron scattering and  
27 computational analysis. *submitted*.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13

**Table 1 Magnetic susceptibility tensors of Yb<sup>3+</sup> attached to D12<sub>145</sub>, D12<sub>170</sub>, and D12<sub>260</sub>.**

Position	Lanthanide ion	$\Delta\chi_{ax}^a$	$\Delta\chi_{rh}^a$	$\alpha^b$	$\beta^b$	$\gamma^b$	$x^c$	$y^c$	$z^c$
M145C/D148C	Yb <sup>3+</sup>	7.3 ± 0.3	1.6 ± 0.5	80	70	172	46.9 ± 0.3	9.8 ± 0.3	36.1 ± 1.3
	Tm <sup>3+</sup>	43.7 ± 1.3	11.4 ± 2.0	80	71	171	0.3	0.3	1.3
Q170C/V172C	Yb <sup>3+</sup>	5.7 ± 0.1	1.9 ± 0.1	24	42	151	51.8 ± 0.2	-13.2 ± 0.3	41.7 ± 0.3
	Yb <sup>3+</sup>	8.4 ± 0.5	0.6 ± 0.7	137	69	22	14.3 ± 0.5	-4.7 ± 0.5	38.4 ± 1.3
E260C/K262C <sup>d</sup>	Tm <sup>3+</sup>	47.0 ± 2.3	8.5 ± 5.2	137	72	59	0.5	0.5	1.3

<sup>a</sup> $\Delta\chi_{ax}$  and  $\Delta\chi_{rh}$  values are in 10<sup>-32</sup> (m<sup>3</sup>), and error estimates were obtained by Monte Carlo protocol using 100 partial pseudo-contact shift (PCS) data sets, in which 30% of the input data were randomly deleted. <sup>b</sup>Euler angles ( $\alpha, \beta, \gamma$ ) are represented in ZYZ convention in degrees. <sup>c</sup>Position of the lanthanide ion is expressed in angstrom (Å) as a coordinate in the crystal structure of MurD (PDB ID: 3uag). <sup>d</sup>Parameters determined in a previous study (10).

## Figure legends

**Figure 1 Gadolinium tagging of MurD.** The positions of metals in MurD domain 1–2 M145C/D148C/C151A/E260C/K262C (D12<sub>145–260</sub>) (a), MurD domain 1–2 Q170C/V172C/E260C/K262C (D12<sub>170–260</sub>) (b), and MurD E260C/K262C/N360C/D362C (MurD<sub>260–360</sub>) (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<sub>β</sub> atoms are indicated.

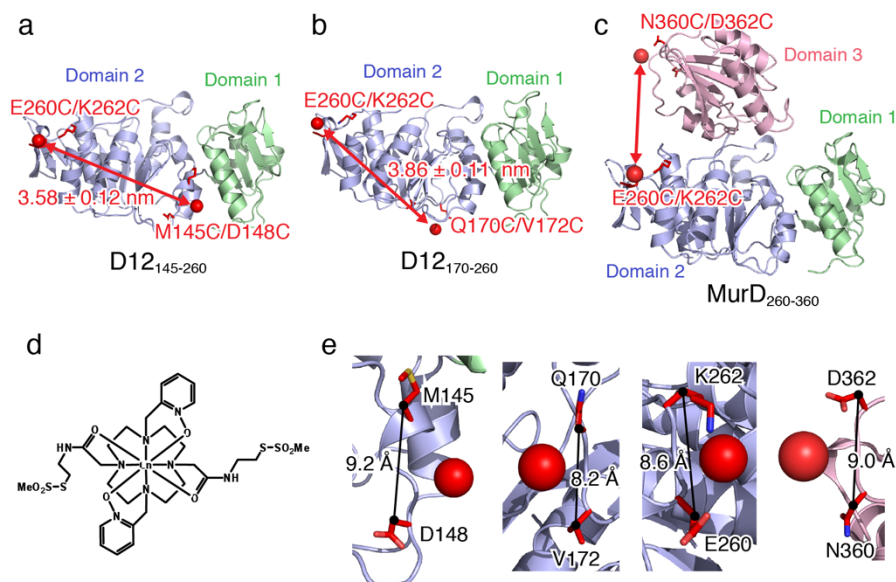
**Figure 2 Four-pulse DEER distance measurement for D12<sub>145–260</sub> and D12<sub>170–260</sub>.** The experiment was performed on ~30 μL of 200 μM frozen solution of D12<sub>145–260</sub> (a, c, and e) or D12<sub>170–260</sub> (b, d, and f) in 70% D<sub>2</sub>O/30% glycerol-*d*<sub>8</sub> at 10 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 MurD<sub>260–360</sub>.** The experiment was performed on ~30 μL of 150 μM frozen solution of MurD<sub>260–360</sub> in the absence (a, c, and e) and presence (b, d, and f) of the inhibitor in 70% D<sub>2</sub>O/30% glycerol-*d*<sub>8</sub> 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.

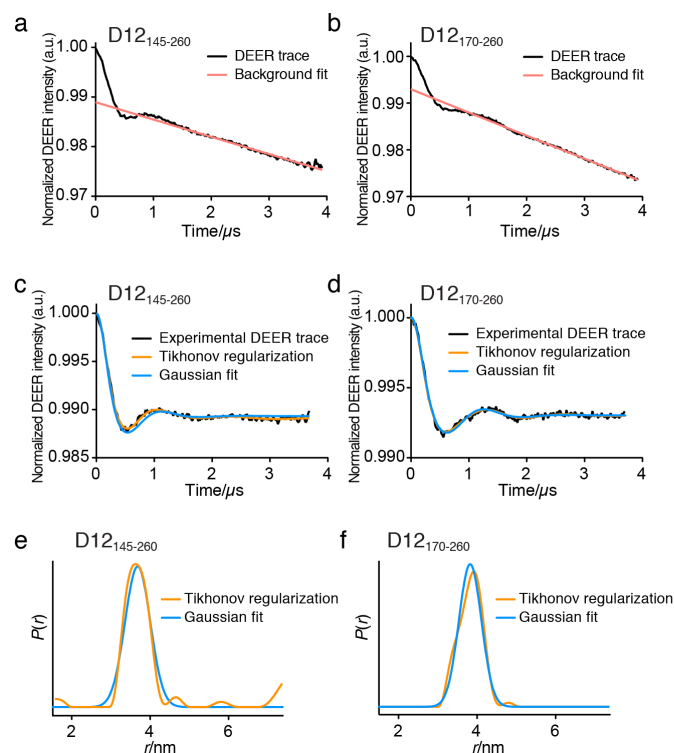
**Figure 4 Comparison between DEER distance measurement and molecular dynamics (MD) simulation for apo MurD.** The representative coordinates observed in MD simulations are shown with corresponding positions in the trajectories of the inter-gadolinium distance. The coordinates from MD calculation are 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<sub>260-360</sub> 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.

Figure 1



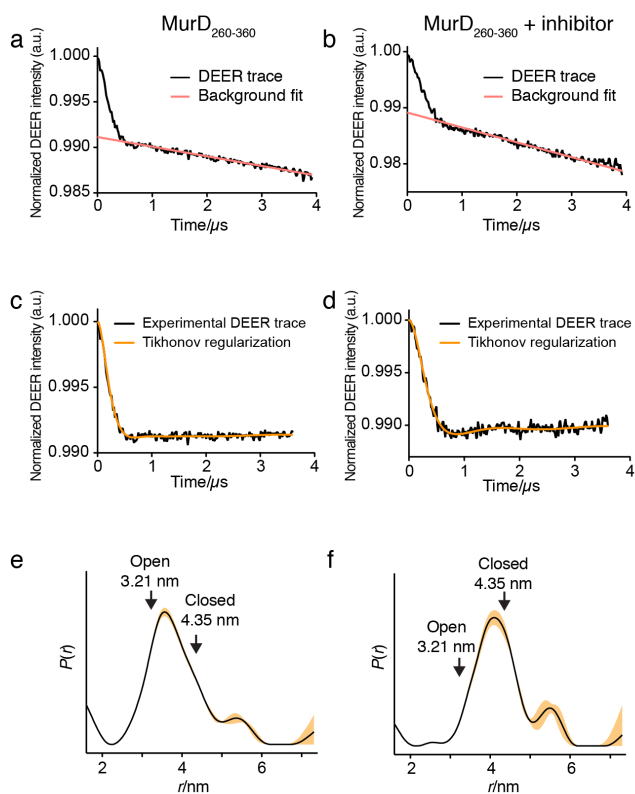
**Figure 1 Gadolinium tagging of MurD.** The positions of metals in MurD domain 1–2 M145C/D148C/C151A/E260C/K262C (D12<sub>145–260</sub>) (a), MurD domain 1–2 Q170C/V172C/E260C/K262C (D12<sub>170–260</sub>) (b), and MurD E260C/K262C/N360C/D362C (MurD<sub>260–360</sub>) (c) are shown as red spheres in 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<sub>β</sub> atoms are indicated.

Figure 2

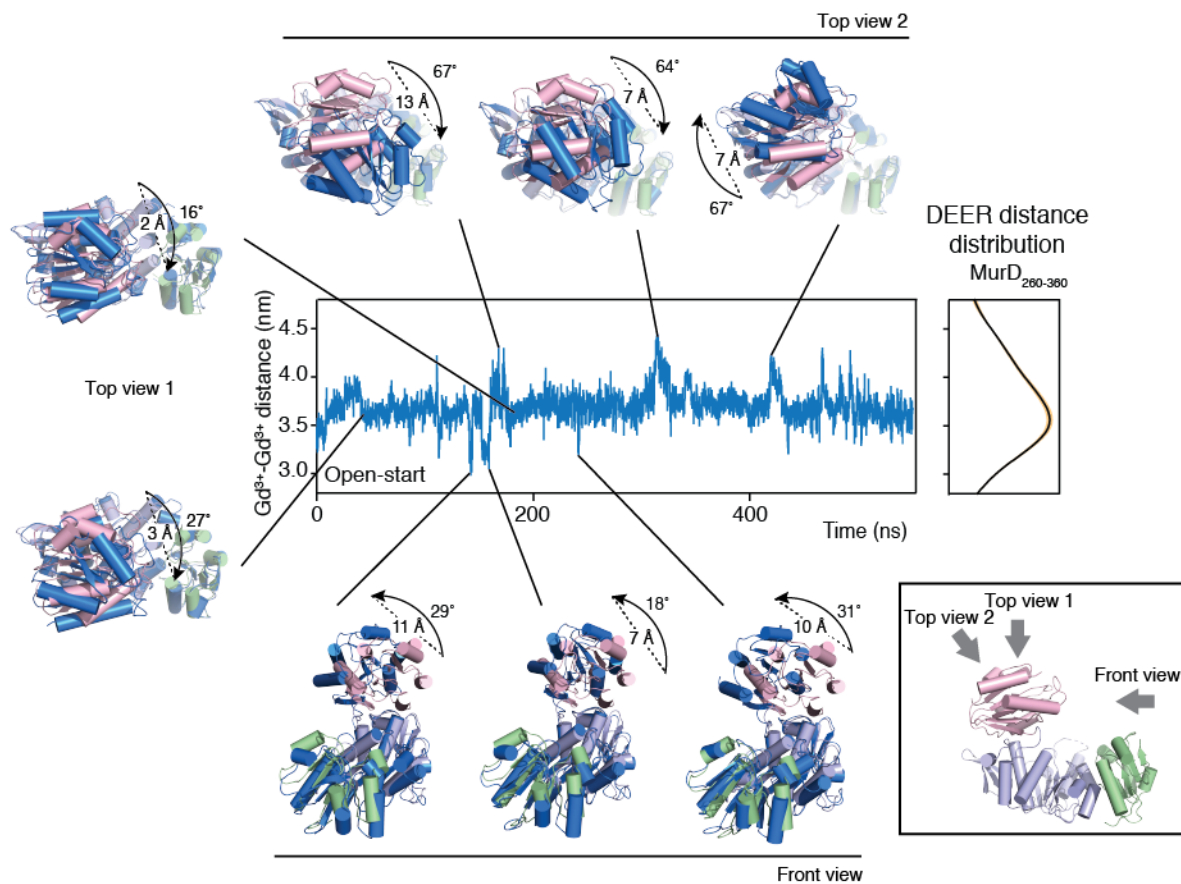


**Figure 2 Four-pulse DEER distance measurement for D12<sub>145-260</sub> and D12<sub>170-260</sub>.** The experiment was performed on  $\sim 30 \mu\text{L}$  of  $200 \mu\text{M}$  frozen solution of D12<sub>145-260</sub> (a, c, and e) or D12<sub>170-260</sub> (b, d, and f) in 70% D<sub>2</sub>O/30% glycerol-*d*<sub>8</sub> 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 MurD<sub>260-360</sub>.** The experiment was performed on  $\sim 30 \mu\text{L}$  of  $150 \mu\text{M}$  frozen solution of MurD<sub>260-360</sub> in the absence (a, c, and e) and presence (b, d, and f) of the inhibitor in 70% D<sub>2</sub>O/30% glycerol-*d*<sub>8</sub> 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<sub>260-360</sub> 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<sup>3+</sup> electron  
paramagnetic resonance**

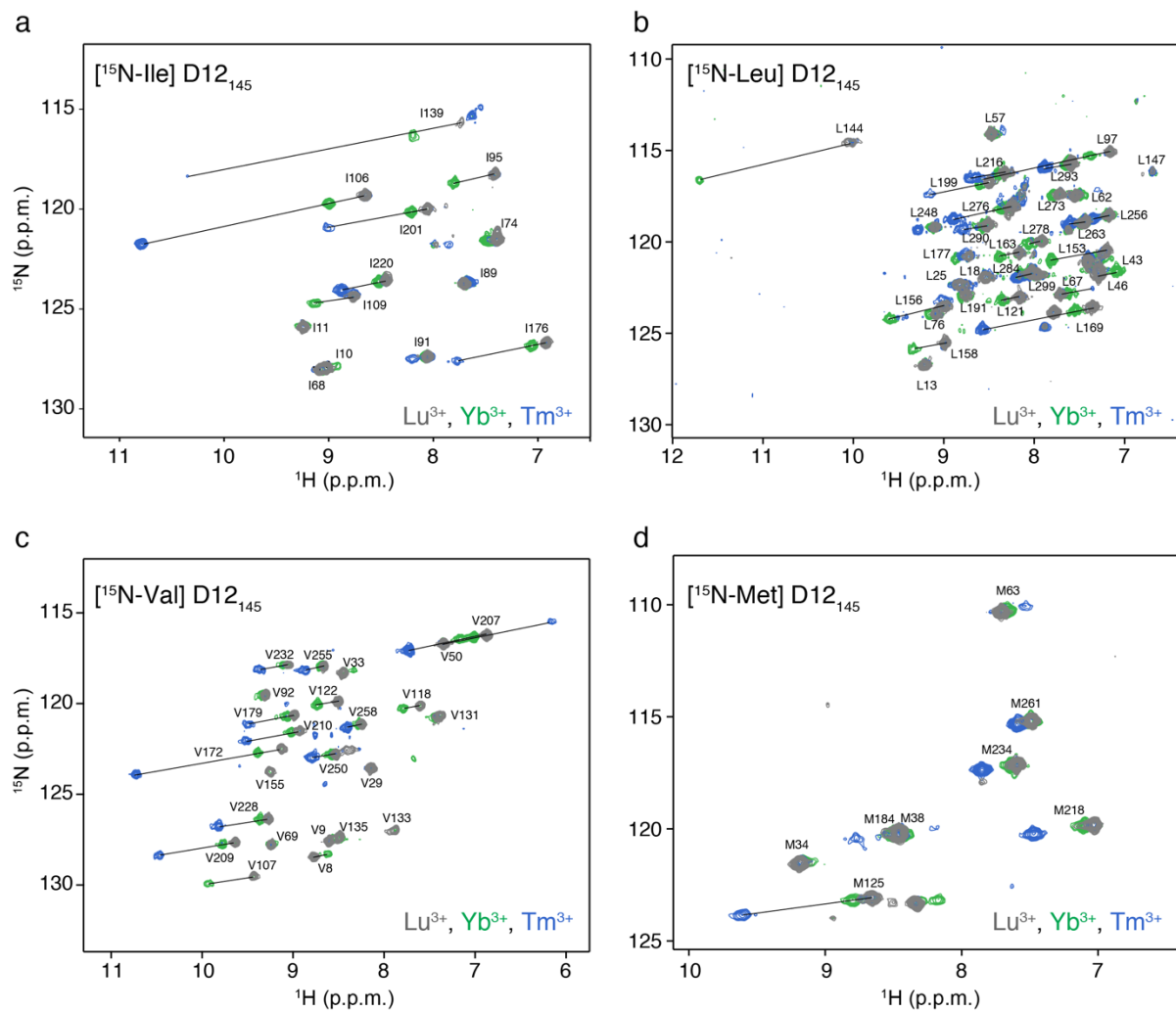
T. Saio, S. Hiramatsu, M. Asada, H. Nakagawa, K. Shimizu, H. Kumeta, T. Nakamura, K. Ishimori

**Table S1 PCS values observed for D12<sub>145</sub> attached with CLaNP-5 containing Yb<sup>3+</sup> and Tm<sup>3+</sup>.**

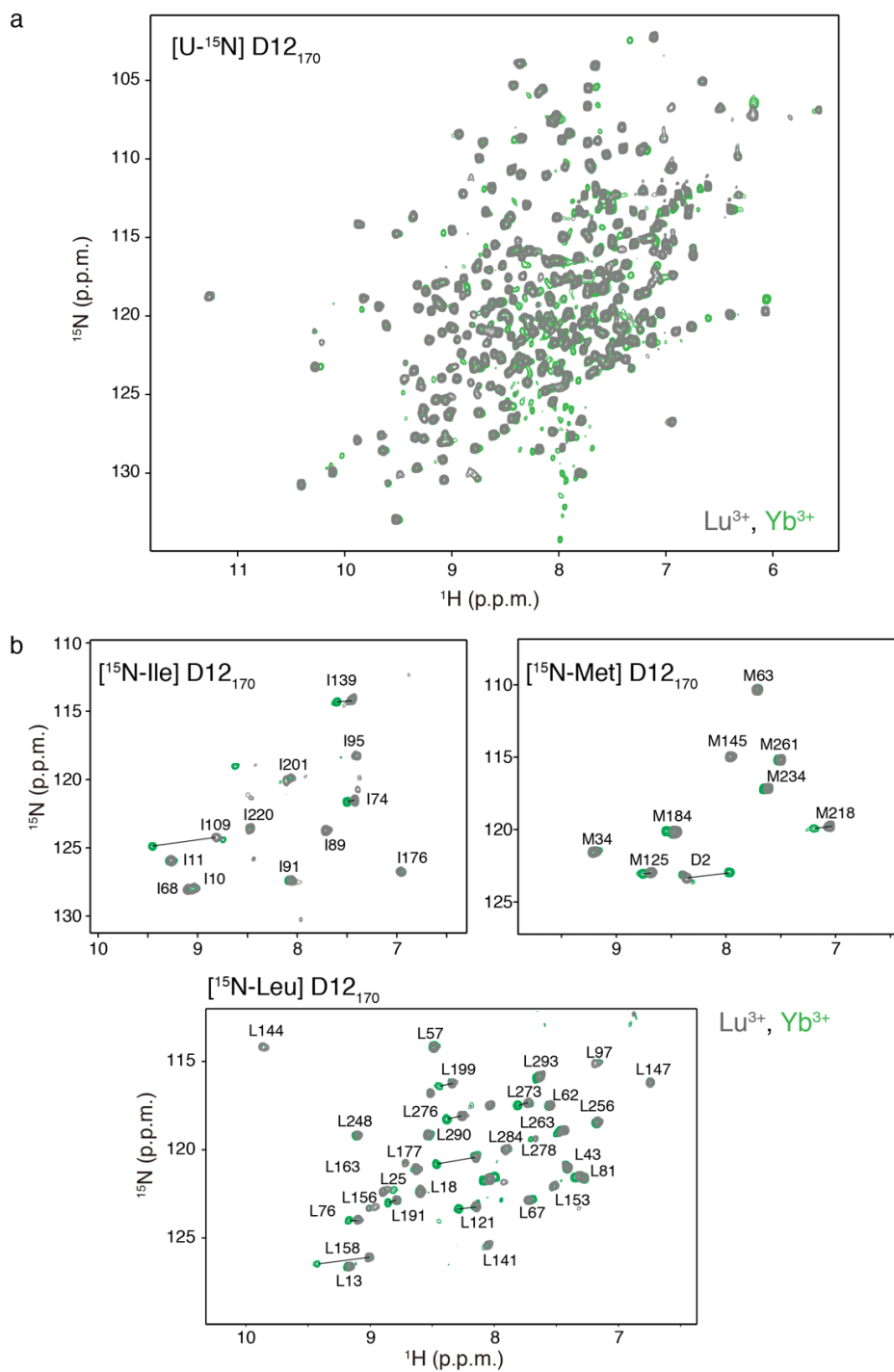
	Yb <sup>3+</sup>		Tm <sup>3+</sup>	
	H <sup>N</sup>	N	H <sup>N</sup>	N
VAL8	-0.153	-0.148		
ILE10	-0.092	-0.092		
ILE11	-0.007	-0.063	-0.005	-0.060
LEU13	0.000	-0.097	-0.005	-0.104
VAL29	-0.460	-0.552		
VAL33	-0.113	-0.174		
LEU46	-0.207	-0.259		
VAL50	-0.189	-0.208		
LEU57	-0.009	-0.009	-0.123	-0.346
LEU62	-0.033	-0.034		
MET63	-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		
ILE74	0.086	0.082	0.460	0.337
LEU76	0.055	0.021		
LEU81	-0.006	0.069		
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
VAL107	0.505	0.450		
ILE109	0.368	0.304		
VAL118	0.183	0.188		
LEU121	0.189	0.197		
VAL122	0.227	0.177		
MET125	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		
LEU163	0.215	0.212	1.263	1.317
LEU169	0.189	0.170	1.212	1.252
VAL172	0.261	0.225		
ILE176	0.139	0.159	0.853	0.946
LEU177	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
VAL207	0.141	0.129		
VAL209	0.138	0.121		
VAL210	0.100	0.077		
LEU216	0.064	0.028	0.377	0.293
MET218	0.073	0.090	0.446	0.424
ILE220	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
VAL258	0.021	0.019		
MET261	0.009	0.028	0.108	0.217
LEU263	0.017	0.005	0.165	0.127
LEU273	0.077	0.075	0.472	0.425
LEU276	0.106	0.119	0.646	0.668
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 S2 PCS values observed for D12<sub>170</sub> attached with CLaNP-5 containing Yb<sup>3+</sup>.**

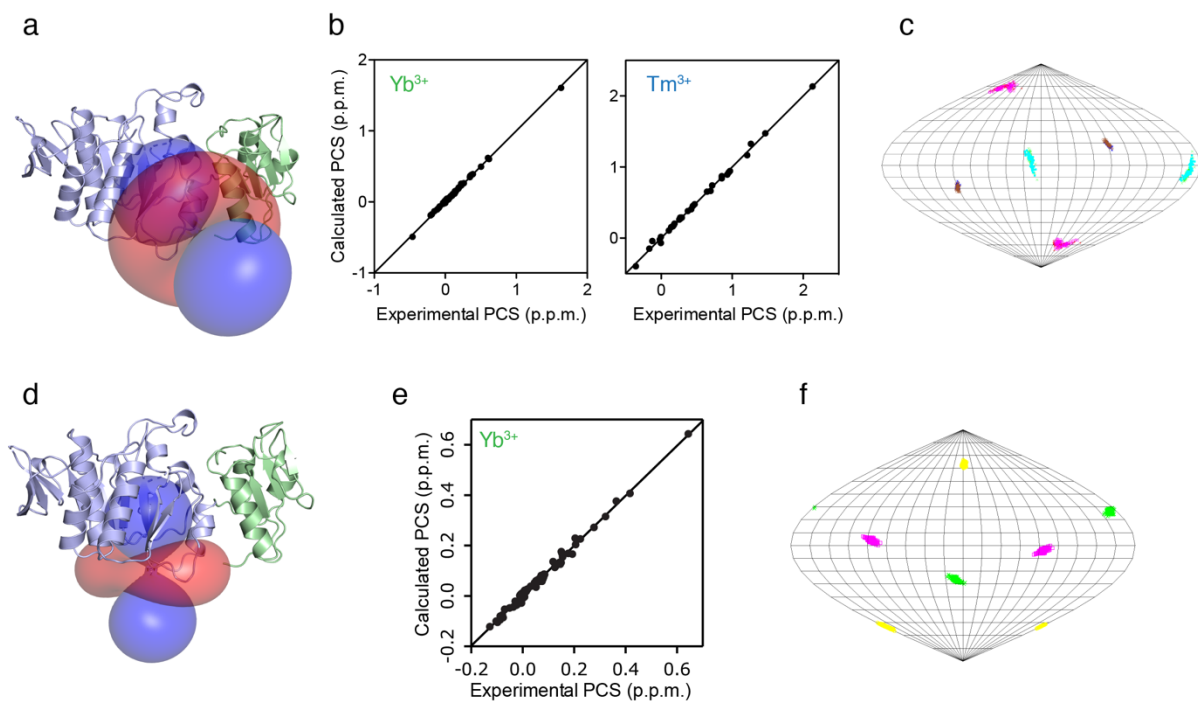
	H <sup>N</sup>	N		H <sup>N</sup>	N
GLY5	-0.079	-0.078	VAL122	0.193	0.217
ASN7	-0.051	-0.054	GLY123	0.118	0.394
VAL8	-0.037	-0.046	MET125	0.075	0.104
LEU13	0.012	0.011	VAL131	-0.103	-0.126
LEU18	-0.003	0.023	ILE139	0.150	0.154
LEU25	-0.087	-0.115	LEU141	0.034	0.080
ARG27	-0.128	-0.071	LEU144	0.002	-0.034
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



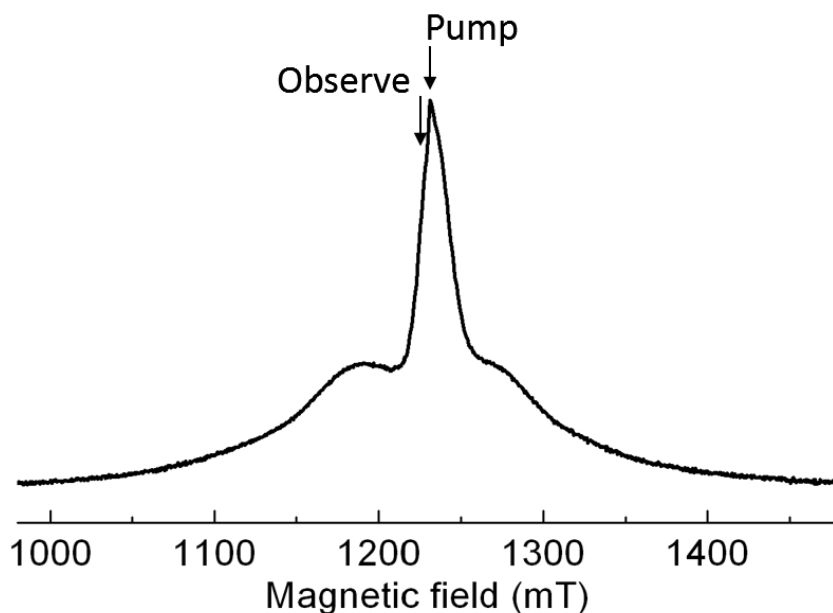
**Figure S1 Observation of pseudo-contact shift for D12<sub>145</sub>.** Overlay of the  $^1\text{H}$ - $^{15}\text{N}$  heteronuclear single quantum coherence (HSQC) spectra for [ $^{15}\text{N}$ -Ile] D12<sub>145</sub> (a), [ $^{15}\text{N}$ -Leu] D12<sub>145</sub> (b), [ $^{15}\text{N}$ -Val] D12<sub>145</sub> (c), and [ $^{15}\text{N}$ -Met] D12<sub>145</sub> (d). The spectra for D12<sub>145</sub> attached with Lu<sup>3+</sup>, Yb<sup>3+</sup>, and Tm<sup>3+</sup> are colored gray, green, and blue, respectively.



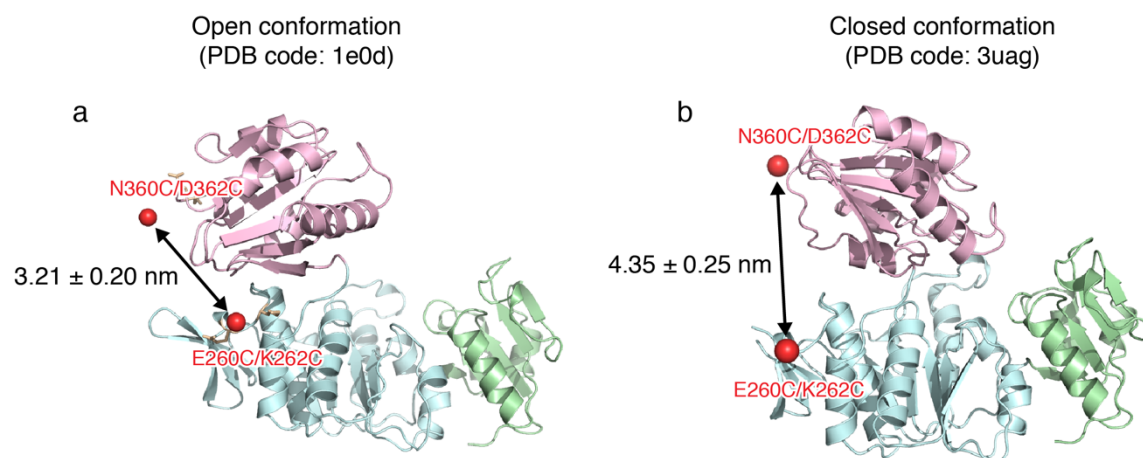
**Figure S2 Observation of pseudo-contact shift for D12<sub>170</sub>.** Overlay of the  $^1\text{H}$ - $^{15}\text{N}$  heteronuclear single quantum coherence (HSQC) spectra for  $[\text{U-}^{15}\text{N}] \text{D12}_{170}$  (a) and amino acid type-selectively  $^{15}\text{N}$  labeled D12<sub>170</sub> (b). The spectra for D12<sub>170</sub> attached with diamagnetic lanthanoid ion  $\text{Lu}^{3+}$  and paramagnetic lanthanoid ion  $\text{Yb}^{3+}$  are colored gray and green, respectively.



**Figure S3  $\Delta\chi$  tensor analysis for D12<sub>145</sub> and D12<sub>170</sub>.** View of the experimentally determined PCS isosurface for D12<sub>145</sub> (a) and D12<sub>170</sub> (d) depicting the surface corresponding to  $\pm 0.7$  ppm induced by  $\text{Yb}^{3+}$ . 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 D12<sub>145</sub> containing  $\text{Yb}^{3+}/\text{Tm}^{3+}$ -CLaNP-5 (b) and D12<sub>170</sub> containing  $\text{Yb}^{3+}$ -CLaNP-5 (e). Orientation of the principal axes of the  $\Delta\chi$  tensors of  $\text{Yb}^{3+}$  and  $\text{Tm}^{3+}$  in complex with CLaNP-5 fixed on D12<sub>145</sub> (c) and that of  $\text{Yb}^{3+}$  in complex with CLaNP-5 fixed on D12<sub>170</sub> (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 5; PCS, pseudo-contact shift.



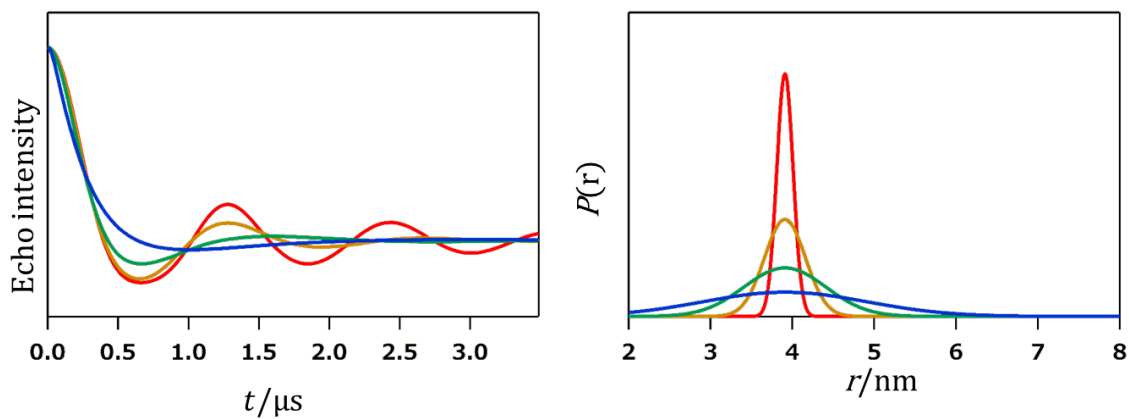
**Figure S4 Echo-detected (ED) EPR spectrum around the central  $|1/2\rangle \leftrightarrow |-1/2\rangle$  transition of D12<sub>145-260</sub> recorded at 10 K.** The measurements were carried out using a two-pulse  $\{\pi/2-\tau-\pi-\tau\}$ -echo sequence with  $t(\pi/2) = 16$  ns,  $t(\pi) = 32$  ns, and  $\tau = 200$  ns. Positions of the pump and observed frequencies for the DEER experiment are indicated. The ED-EPR spectrum of MurD<sub>170-260</sub> and MurD<sub>260-360</sub> look very similar. DEER, double electron-electron resonance; EPR, electron paramagnetic resonance.



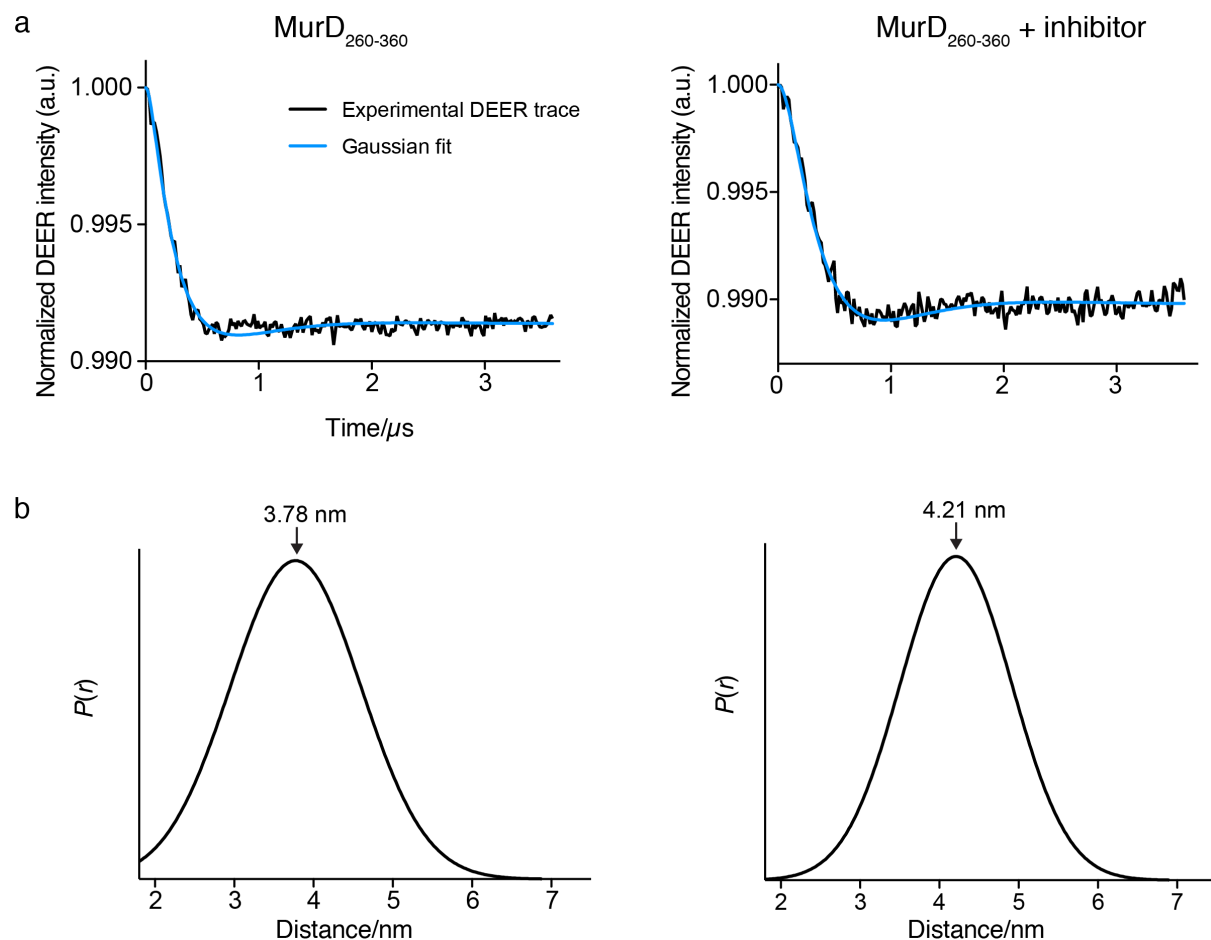
**Figure S5 Inter-gadolinium distances of MurD<sub>260-360</sub> expected from the crystal structures.**

Crystal structures of MurD in the open (a) and closed (b) conformations with expected inter-gadolinium distances for MurD<sub>260-360</sub> are indicated. The metal positions for each site were determined using  $\Delta\chi$  tensor analysis based on pseudo-contact shifts (PCSs) (D12<sub>260</sub>) or Xplor-NIH docking (D3<sub>360</sub>).

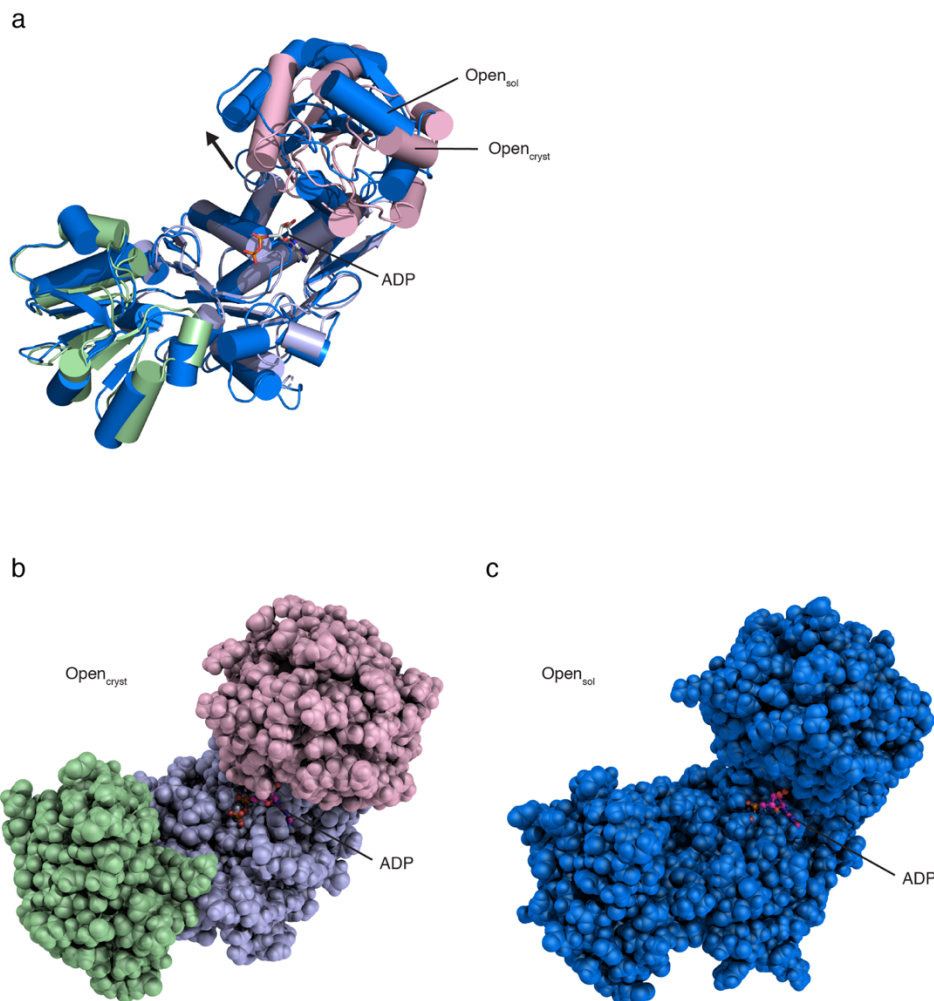




**Figure S6 Theoretical DEER traces for the two paramagnetic centers with varying distributions centered at 3.9 nm.** Superimposition of theoretical DEER traces (left panel) and distance distributions (right panel), with varying standard deviations of the Gaussian distribution:  $\sigma = 0.1$  (red),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



**Figure S7 Single Gaussian fit of the DEER traces of MurD<sub>260-360</sub> in the absence and presence of the inhibitor.** The DEER results shown in Figure 3 were fitted by single Gaussian. The results for MurD<sub>260-360</sub> 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.