Supporting Information

Determination of Pseudocontact Shifts of Low-Populated Excited States by NMR Chemical Exchange Saturation Transfer

R. S. Ma,a Q. F. Li,b A. D. Wang,a J. H. Zhang,a Z. J. Liu,c J. H. Wu,a X. C. Su*,b and K. Ruan*a

a Hefei National Laboratory for Physical Science at the Microscale, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China. Email: kruan@ustc.edu.cn
b State Key Laboratory of Elemento-Organic Chemistry, Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071 China.
c National Center for Protein Science Shanghai, Shanghai 201210, China

Supplementary Note

Supporting Figures S1 – S7
Supplementary Note

Dimethyl 4-(hydroxymethyl)pyridine-2, 6-dicarboxylate (3)
Dimethyl 4-(hydroxymethyl)pyridine-2, 6-dicarboxylate was synthesized starting from dipicolinic acid (1), as previously reported.\textsuperscript{1} \textsuperscript{1}H-NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) ppm: 8.34 (2H, s), 4.93 (2H, s), 4.05 (6H, s). \textsuperscript{13}C-NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) ppm: 165.2, 153.6, 148.2, 125.4, 67.7, 62.8, 53.3.

Dimethyl 4-((trityloxy)methyl)pyridine-2, 6-dicarboxylate (4)
Similar to a published protocol,\textsuperscript{2} 3 (4.0 g, 17.8 mmol), triphenylchloromethane (15.0 g, 53.8 mmol), N, N-diisopropylethylamine (16 mL, 97.0 mmol), 4-dimethylaminopyridine (15 mg, 0.12 mmol) were dissolved in DMF (80 mL). The above solution was stirred at 65 °C for about 10 h, and then cooled down to room temperature. The solution was diluted with distilled water (400 mL), and the resulting mixture was extracted with ethyl acetate (100 mL). The organic phase was washed with brine, dried with anhydrous Na\textsubscript{2}SO\textsubscript{4}. After filtration, the solvent was removed under reduced pressure, resulting yellowish oil. The residue was purified by column chromatography on silica gel using ethyl acetate:petroleum ether (1:3 by volume) to afford the title compound as white solid (5.8 g, 69.9 %). \textsuperscript{1}H-NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) ppm: 8.27 (2H, s), 7.52-7.24 (15H, m), 4.39 (2H, s), 4.02 (6H, s). \textsuperscript{13}C-NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) ppm: 165.2, 153.6, 148.2, 125.4, 67.7, 62.8, 53.3.
Following the established reduction protocol, 4 (3.0 g, 6.4 mmol), LiCl (1.1 g, 26.2 mmol) were dissolved in absolute ethyl alcohol (50 mL), NaBH₄ (1.0 g, 26.4 mmol) was added to above solution under an ice-water bath. The mixture was stirred at 60 °C for about 5 h. The solvent was removed under reduced pressure, and the residue was diluted with distilled water (50 mL). The solution was extracted with ethyl acetate (100 mL), and the combined organic phase was washed with brine, dried with anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure to give compound 5 as a white solid (2.2 g, 84.6 %).

1H-NMR (400 MHz, CDCl₃) δ ppm: 7.52-7.24 (17H, m), 4.76 (4H, s), 4.23 (2H, s).

13C-NMR (100 MHz, CDCl₃) δ ppm: 158.6, 150.2, 143.6, 128.6, 128.0, 127.3, 117.0, 87.6, 64.8, 64.8. HSQC (C: 64.8, H, 4.23), (C: 64.8, H: 4.76).

2,6-bis(bromomethyl)-4-((trityloxy)methyl)pyridine (6)

Methanesulfonyl anhydride (2.6 g, 14.9 mmol) in 6 mL CH₂Cl₂ was added stepwise to the solution of 5 (2.0 g, 4.9 mmol) and N, N-diisopropylethylamine (5 mL, 30.3 mmol) in 20 mL CH₂Cl₂ under an ice-water bath. The resulting mixture was stirred for about 12 h and then mixed with the saturated NaHCO₃ solution. The organic phase was washed with brine, dried with anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure to afford a yellow residue. The resulting residue was mixed with LiBr (1.5 g, 17.3 mmol) and acetone (20 mL), and the mixture was stirred at room temperature for about 4 h. The solution was filtered and the filtrate was concentrated. The resulting residue was purified by column chromatography on silica gel using ethyl acetate:petroleum ether (1:3 by volume) as eluent to yield a yellowish oil (1.4 g, 53.8 %).

1H-NMR (400 MHz, CDCl₃) δ ppm: 7.55-7.28 (17H, m), 4.55 (4H, s), 4.27 (2H, s).

13C-NMR (100 MHz, CDCl₃) δ ppm: 156.6, 151.0, 143.5, 128.6, 128.0, 127.4, 120.5, 87.5, 64.3, 33.6.

Tetraethyl 2,2',2'',2'''-(((4-((trityloxy)methyl)pyridine-2,6-diyl)bis(methylene))bis(azanetriyl)tetraacetate (7)

6 (1.0 g, 1.9 mmol), diethyl iminodiacetate (0.75 mL, 4.2 mmol), K₂CO₃ (1.25 g, 9.0 mmol), KI (10 mg, 0.06 mmol) were mixed with 20 mL dry acetonitrile, and the reaction mixture was stirred at 60 °C for about 6 h and then cooled to room temperature. The solvent was removed under reduced pressure and the resulting oil was purified by column chromatography on silica gel using ethyl acetate:petroleum ether (1:1 by volume) as eluent to afford the title compound 7 as a yellowish oil (1.2 g, 85.7 %). 1H-NMR (400 MHz, CDCl₃) δ ppm: 7.55-7.28 (17H, m), 4.55 (4H, s), 4.27 (2H, s), 4.05 (8H, q), 3.54 (8H, s), 1.41 (12H, t).

13C-NMR (100 MHz, CDCl₃) δ ppm: 171.1, 158.3, 149.7, 143.8, 128.7, 128.0, 127.1, 119.0, 87.4, 64.8, 60.5, 59.9, 54.9, 14.2.

Tetraethyl 2,2',2'',2'''-(((4-(hydroxymethyl)pyridine-2,6-diyl)bis(methylene))bis(azanetriyl)tetraacetate (8)

7 (1.0 g, 1.3 mmol) in 3 mL CH₂Cl₂ was added stepwise to the solution of trifluoroacetic acid (10 mL), triethylsilane (0.34 mL, 2.3 mmol) in 10 mL CH₂Cl₂ under an ice-water bath. The reaction mixture was then stirred at room temperature for about 12 h. The solvent was removed under reduced pressure. The residue was redissolved in 20 mL ethyl acetate, washed with saturated NaHCO₃ solution, brine, dried with anhydrous Na₂SO₄, and filtered. The organic phase was concentrated and the resulting yellow oil was purified by column chromatography on silica gel using ethyl acetate:petroleum ether (1:3 by volume) as eluent to give the title compound 8 as a yellowish oil (0.42 g, 61.8 %).

1H-NMR (400 MHz, CDCl₃) δ ppm: 7.49 (2H, s), 4.73 (2H, s), 4.16 (8H, q), 4.03 (4H, s), 4.06 (8H, s), 1.26 (12H, t).

13C-NMR (100 MHz, CDCl₃) δ ppm: 171.2, 158.1, 158.1, 119.0, 63.3, 60.5, 59.6, 54.8, 14.2.
0.15 mL PBr₃ in 2 mL CHCl₃ was added stepwise to the mixture of 8 (0.4 g, 0.78 mmol), DMF (one drop) and 5 mL CHCl₃ under an ice-water bath. The resulting reaction mixture was stirred at room temperature for about 12 h. The organic phase was then washed with saturated NaHCO₃ solution, brine, dried with anhydrous Na₂SO₄, filtered, and evaporated under reduced pressure. The resulting residue was purified by column chromatography on silica gel using ethyl acetate:petroleum ether (1:1 by volume) as eluent to give the title compound 9 as a yellowish oil (0.39 g, 86.7 %). ¹H-NMR (400 MHz, CDCl₃) δ ppm: 7.51 (2H, s), 4.40 (2H, s), 4.18 (8H, q), 4.03 (4H, s), 3.60 (8H, s), 1.26 (12H, t). ¹³C-NMR (100 MHz, CDCl₃) δ ppm: 171.2, 158.1, 157.3, 119.0, 60.5, 59.7, 54.8, 30.2, 14.2.

2,2',2'',2'''-(((4-(mercaptomethyl)pyridine-2,6-diyl)bis(methylene))bis(azanetriyl)tetraacetate acid (4-MMPyMTA)

9 (0.35 g, 0.76 mmol) and thiourea (0.07 g, 0.92 mmol) were dissolved in 10 mL CHCl₃ under an argon atmosphere, and the resulting solution was heated to reflux for 6 h. The reaction mixture was cooled to room temperature and the solvent was removed under reduced pressure. The resulting residue was mixed with the 5 mL solution of NaOH (0.13 g, 3.3 mmol) in deoxygenated water. The mixture was refluxed for about 2 h under an argon atmosphere, and then cooled to room temperature. The solution was adjusted to pH 3.0 through a cation exchange resin (H⁺) and the solvent was removed under reduced pressure. The resulting oil was suspended in 15 mL acetone and the precipitated was filtered to give the target compound 4-MMPyMTYA tag as a yellowish powder (0.18 g, 72 %). ¹H-NMR (400 MHz, D₂O, pD>12) δ ppm: 7.22 (2H, s), 3.80 (4H, s), 3.58 (2H, s), 3.20 (8H, s). ¹³C-NMR (100 MHz, D₂O, pD>12) δ ppm: 179.5, 158.7, 157.7, 122.0, 59.1, 58.1, 28.0. MS (ESI) 416.11 (M+1)+.

References.

Fig. S1  Site specific labeling of lanthanide ions to the Abp1p SH3 domain. A) Structure of the lanthanide chelating ligand, which can be chemically linked to proteins via the formation of a disulfide bond with a cysteine. B) Superimposed $^1$H-$^{15}$N HSQC spectra of wild-type Abp1p SH3 (red) and S56C mutant with the PCS tag chemically linked (blue). Annotated are the chemical shift assignments. C) The $^1$H-$^{15}$N HSQC spectra of SH3 S56C mutant with PCS tag attached, in the presence (red) or absence (black) of diamagnetic $Y^{3+}$ ions. D) The $^1$H-$^{15}$N HSQC spectra of the above PCS tag modified SH3 domain with 3 mol % Ark1p peptides, in the presence of paramagnetic Tb$^{3+}$ (blue), Tm$^{3+}$ (green) and diamagnetic $Y^{3+}$ (red). The Tb$^{3+}$ and Tm$^{3+}$ were titrated at the molar ratio of about 0.5 with respect to SH3 domain, such that both diamagnetic and paramagnetic peaks were observed simultaneously. Residues showing distinguishable chemical shifts of the minor states are highlighted using red lines. E) Similar to D but in the presence of 1.5 fold excess of Ark1p peptides.
Fig. S2  The 2D CEST profiles for residues of SH3 domain in the presence of 3% Ark1p peptides, under diamagnetic (red) or Tb$^{3+}$ labeled paramagnetic (blue) conditions. Depicted are residues with distinguishable chemical shifts in the minor states with respect to those in the ground state. The minor-state peaks are magnified in the inset, with PCS values of the minor states annotated.
Fig. S3  Cartoon representation of Abp1p SH3 domain in complex with Ark1p peptide. Blue spheres with residue number annotated represent $^{15}$N nuclei with detectable minor states using PCS-CEST, and grey ball denotes the Ln$^{3+}$ chelating site.
Fig. S4  Site specific labeling of lanthanide ions to the A39G FF domain. A) The $^1$H-$^{15}$N HSQC spectra of the FF domain with A39G mutation (red) and additional K26C mutation (blue) at 25 °C. B) The $^1$H-$^{15}$N HSQC spectra of the above A39G/K26C mutant at 25 °C, which was (blue) or was not (red) chemically bonded to the PCS tag. C) The $^1$H-$^{15}$N HSQC spectra of the FF domain A39G and additional K26C mutant at 4 °C with PCS tag attached, in the presence (red) or absence (black) of diamagnetic Y$^{3+}$ ions. D) The $^1$H-$^{15}$N HSQC spectra of the above chemically modified FF domain at 4 °C in the presence of paramagnetic Tb$^{3+}$ (blue), Tm$^{3+}$ (green) and diamagnetic Y$^{3+}$ ions (red). The Tb$^{3+}$ and Tm$^{3+}$ ions were titrated to a molar ratio of ca. 0.5 with respect to the protein for the simultaneous observation of diamagnetic and paramagnetic peaks. Red lines denote residues showing distinguishable chemical shifts for the minor states displayed in Fig. S4.
The 2D CEST Profiles for residues of the FF domain under diamagnetic (red) or Tb$^{3+}$ labeled paramagnetic (blue) conditions. Depicted are residues with distinguishable chemical shifts between the minor states and ground state. Annotated are the PCS values of the ground state retrieved directly from HSQC spectra, and PCS values of the minor states extracted from CEST curve fitting.
Cartoon representation of the FF domain in its natively folded state. Blue spheres with residue number annotated represent $^{15}$N nuclei with detectable minor states using PCS-CEST, and grey ball denotes the Ln$^{3+}$ chelating site.
Fig. S7 The 1D selective CEST spectra for residue R48 of the FF domain, and V32 of the SH3 domain. A) 1D selective CEST spectra of residue R48 with a CEST scanning frequency at 103.5 ppm. B) 1D selective CEST spectra of residue V32, with a CEST scanning frequency at 108 ppm. Inset: peak fitting for signals from V32 and A12. C) 2D CEST profile for residue A12.