Supporting information

for

Probing the Interaction of Cisplatin with Calmodulin and its Effect upon Binding to Myosin Light-Chain Kinase

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Contents

Experimental details

Protein Expression and Purification: The CaM gene (*Homo sapiens*), incorporated in the bacterial expression vector pET22b containing an N-terminal His_6-tag, was transfected into *Escherichia coli ER2566* cells grown at 37°C in LB medium. The protein expression was induced with 0.4 mM isopropyl thio-β-D-galactosidase (IPTG) at 37°C for 5 h at OD_{600} = 0.8. Then cells were harvested by centrifugation and the cell pellets were resuspended and lysed by sonication for 10 min. The insoluble portion was removed by centrifugation at 16000 rpm for 30 min at 4 °C; then the supernatant was loaded on the Ni-NTA column pre-equilibrated with buffer A (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$ at pH 8.0). The fusion protein was eluted with 20 mL elution buffer (buffer A containing 250 mM imidazole). The His_6-tag was removed by TEV digestion at 16°C for 12 h. The protein was further purified via gel filtration and NaCl was removed by dialysis. The protein was concentrated through an ultrafiltration (3 kDa cutoff) and the concentration was determined by the absorbance at 280 nm.

For the expression of ^{19}F-Trp labeled MLCK, the pET28a plasmid encoding MLCK and an N-terminal His_6-sumo-tag was transfected into *E. coli* BL21 (DE3) cells. Cells were grown in M9 minimal medium at 37 °C until OD_{600} = 0.8, then 5-F-indole (Aladdin, 50 mg L^{-1}) was added to the medium. After cells growing at 37°C for 0.5 h, the protein expression was induced by IPTG (0.4 mM) at 25 °C for 10 h. Cells were harvested by centrifugation and the pellets were resuspended. Following cell lysis by sonication, the protein was purified through a Ni-NTA column. The fusion protein was digested by sumo protease at 16°C for 12 h to remove the His_6-sumo-tag. The proteins were further purified by gel filtration.

For the expression of ^{19}F-Trp CaM-MLCK fusion protein, the pET22b plasmid encoding the CaM-MLCK fusion protein was transfected into *E. coli* BL21 (DE3) cells. Cells were grown in M9 minimal medium at 37 °C until OD_{600} = 0.8, then 5-F-indole (Aladdin, 50 mg L^{-1}) was added to the M9 minimal medium. After cells growing at 37°C for 0.5 h, the protein expression was induced by IPTG (0.4 mM) at 37 °C for 5 h. Cells were harvested by centrifugation and the pellets were resuspended. Following cell lysis by sonication, the protein was purified through an anion exchange column (HiTrap Q XL) (eluents: A: 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0; B: eluent A with 1 M NaCl). The CaM-MLCK fusion protein was further purified by gel filtration and NaCl was removed by equilibrium dialysis. Then the protein was concentrated through an ultrafiltration (3 kDa cutoff) and the concentration was determined by the absorbance at 280 nm.

For the expression of seleno-L-methionine-labeled CaM and CaM-MLCK fusion proteins, the pET22b plasmid encoding the CaM or CaM-MLCK protein was transfected into *E. coli* BL21 (DE3) cells. Cells were grown in M9 minimal medium at 37 °C until OD_{600} = 0.8, then seleno-L-methionine (SeMet, 0.4 g/L) was added to the M9 minimal medium, cell growing protracted for additional 0.5 h and, finally, protein expression was induced by IPTG (0.4 mM) at 37 °C for 5 h. After harvesting the cells by centrifugation, the pellet was resuspended, cells lysed by sonication, and the seleno-L-methionine-labeled CaM or CaM-MLCK purified by the procedure already described for CaM and ^{19}F-Trp CaM-MLCK fusion proteins. The purified proteins were characterized with tricine-SDS-PAGE and high-resolution electrospray mass spectrometry (ESI-MS) (Figure S15).

NMR spectroscopy: NMR spectra were recorded on a Bruker 600 MHz spectrometer equipped with an H/F/(C, N) BBFO CryoProbe. 2D HSQC Spectra were recorded on ^{15}N-labeled or $^{13}CH_3$-Met labeled proteins. The spectral widths were set as 13 ppm for ^{1}H, 50 ppm centered at 25 ppm for ^{13}C and 32 ppm centered at 118 ppm for ^{15}N. Data were processed and analyzed with Topspin 4.0.5. For monitoring the cisplatin reaction in vitro by ^{1}H-^{15}N HSQC NMR, the samples were prepared by

incubating ^{15}N-labeled CaM or CaM/MLCK complex with cisplatin at 37°C in 50 mM phosphate buffer (pH 7.4). For the ^{1}H-^{13}C HSQC experiments, samples were prepared by incubating $^{13}CH_3$-Met labeled CaM or CaM/MLCK complex with cisplatin at 37°C in 20 mM HEPES buffer (pH 7.0).

1D ^{19}F spectra were acquired with spectral width of 100 ppm with a duty cycle delay of 1.0 s. Chemical shifts were referenced to the signal of TFA in water (-75.5 ppm). Data were processed and analyzed with Topspin 4.0.5. For monitoring the Ca^{2+}-induced formation of the CaM/MLCK complex, 0.2 mM ^{19}F-Trp labeled MLCK was incubated with equimolar amount of apo-CaM at 37°C for 10 min in 50 mM phosphate buffer (pH 7.4). Then $CaCl_2$ was added to the mixture. For monitoring the interaction of MLCK with the platinated CaM adducts, the adducts were prepared by incubating CaM with 3 molar equivalents of cisplatin at 37°C for 12 h in 50 mM phosphate buffer (pH 7.4). Then 1.0 mM $CaCl_2$ was added to the mixture and the effect of extra Ca^{2+} on the formation of protein complex detected by 1D ^{19}F NMR.

For in-cell NMR experiments, *E. coli* BL21(DE3) cells harboring a pET22b-CaM-MLCK plasmid were first grown in 25 mL LB medium until OD = 0.8. The cells were collected and resuspensed in 25 mL of minimal M9 medium supplemented with 0.4 g/L $^{13}CH_3$-labeled methionine and 5 mM $CaCl_2$. After incubation at 37°C for 0.5 h, 0.4 mM IPTG and 0.5 mM cisplatin were added into the medium and cells were further incubated for 4 h. Then the cells were harvested, quickly washed with minimal M9 medium for 3 times, and resuspended in 0.5 ml of minimal M9 medium for in-cell NMR analyses.

ICP-MS analysis: To determine whether Pt binds to CaM in living *E. coli* cells, a gel-based assay integrated with quantitative elemental analysis *via* ICP-MS was applied. The pET22b plasmid encoding CaM was transfected into *E. coli ER2566* cells that were grown in LB medium until OD = 0.8. Then, the cells were harvested and resuspended in minimal M9 medium supplemented with 0.4 g/L SeMet and 5 mM $CaCl_2$. After incubation at 37°C for 0.5 h, 0.4 mM IPTG and 0.5 mM cisplatin were added into the medium and the cells were further incubated for 4 h. Then the cells were harvested, washed three times with M9 medium to remove excess cisplatin and SeMet, resuspended, and sonicated. The supernatant of the cell lysate was loaded on Tricine-SDS PAGE gel. and, after electrophoresis, the gel was stained with Coomassie R250 Brilliant Blue and the band corresponding to CaM was excised and digested with aqua regia. The concentrations of Pt and Se associated to the gel slice were subsequently quantified using a Thermo Fisher Scientific iCAP RQ ICP mass spectrometer.

To determine whether Pt binds to CaM or CaM-MLCK fusion protein in human HepG2 cells, approximately 2.5×10^6 cells were digested with trypsin/EDTA (0.05 % / 0.02 %) and centrifuged at 300 g for 5 min, followed by two washes with pre-warmed PBS. Seleno-L-methionine-labeled holo-CaM or CaM-MLCK fusion protein was added to the cell pellet to a final concentration of 600 µM. The cells, mixed with the SeMet-labeled proteins, were then transferred into cuvettes and electroporated using a NEPA21 Porator. After electroporation, cells were centrifuged and washed three times with pre-warmed DMEM to remove uninternalized proteins. Then cells were resuspended and cultured in DMEM medium supplemented with 10% fetal bovine serum at 37°C for 1 h. Subsequently, 20 µM cisplatin was added to the medium, and the cells were further incubated for either 4 or 8 hours. After harvesting, the cells were washed three times with PBS to remove excess cisplatin, resuspended, and sonicated. The supernatant of the cell lysate was loaded on Tricine-SDS PAGE gel and, after electrophoresis, the gel was stained with Coomassie R250 Brilliant Blue and the band corresponding to CaM or CaM-MLCK was excised and digested with aqua regia. The concentration of Pt and Se associated to the gel slice were quantified using a

Thermo Fisher iCAP RQ ICP mass spectrometer.

ESI-MS analysis. The mass spectrometric analysis was performed on an Orbitrap Exactive Plus (Thermo Fisher Scientific, CA, USA) mass spectrometer. For analysis of the reaction of CaM with cisplatin, 100 μM CaM was incubated with 1.2 or 3 molar equivalents of cisplatin at 37°C for 12 h in 50 mM phosphate buffer (pH 7.4). Then the salts and unreacted cisplatin were removed by ultrafiltration (cutoff of 3 kDa). Samples were directly injected into the mass spectrometer with the nanospray source option. Data were collected in the positive mode and were processed using XCalibur software (version 2.0, Thermo Finnigan).

Anion exchange chromatography: Anion exchange chromatographic analyses were performed with the ÄKTA Purifier system equipped with a Source 15Q column. Samples were directly injected followed by linear gradient elution (eluents: A: 20 mM Tris-HCl, pH 8.0; B: eluent A with 1 M NaCl). The elution profiles were recorded by UV detection at 280 nm. For monitoring the reactivity of cisplatin toward CaM or CaM/MLCK, 0.1 mM protein was incubated with different molar equivalents of cisplatin in 20 mM HEPES buffer (pH 7.0) at 37°C for 24 h. For time-dependent measurements, 0.1 mM CaM or CaM/MLCK complex was incubated with cisplatin (0.3 mM cisplatin for CaM and 0.5 mM cisplatin for CaM/MLCK complex) in 20 mM HEPES buffer (pH 7.0) at 37°C.

Size exclusion chromatography (SEC): SEC analyses were performed with the ÄKTA Purifier system equipped with Superdex 75 10/300 GL column. The column was pre-equilibrated with PBS buffer. The elution profiles were recorded by UV detection at 280 nm. CaM or CaM/MLCK was incubated with 3 molar equivalents of cisplatin at 37°C for 24 h in 50 mM phosphate buffer (pH 7.4).

Fluorescence measurements: Fluorescence spectra were recorded on a Hitachi F-4600 fluorescence spectrophotometer using a 1 cm path length cuvette at 25°C. For the reaction of cisplatin with CaM, samples were prepared by incubation of 10 μM CaM with cisplatin in 50 mM phosphate buffer (pH 7.4) at 37°C for 12 h. For the interaction of MLCK with platinated CaM, the cisplatin/CaM adducts were prepared by incubating CaM with different molar equivalents (1, 2, 4, 6) of cisplatin at 37°C for 12 h in 50 mM phosphate buffer (pH 7.4). Then 10 μM MLCK was incubated with equimolar cisplatin/CaM adduct at 37°C for 10 min subjected to fluorescence measurement.

Calcium release assay: For the reaction of CaM with cisplatin, 5 μM CaM was incubated with different molar equivalents of cisplatin at 37°C for 12 h. Then 100 μM arsenazo-III was added and incubated for 10 min before the UV measurement. For the reaction of CaM/MLCK with cisplatin, 10 μM CaM/MLCK was incubated with different molar equivalents of cisplatin at 37°C for 12 h. After addition of 200 μM arsenazo-III and 10 min incubation, the UV spectrum was detected. UV-vis spectra were recorded on an Agilent 8453 spectrophotometer equipped with temperature controller. Samples were measured in a capped glass cuvette with 1 cm path length.

Circular dichroism (CD): CD spectra were recorded on a Jasco J-7100 spectrometer. Far-UV CD spectra were collected using a 1.0 mm quartz cuvette under nitrogen atmosphere. Spectra were recorded from 280 to 190 nm with a scan speed of 100 nm min^{-1}. All measurements were repeated three times. Samples were prepared by incubating 30 μM CaM with different molar equivalents of cisplatin at 37°C for 12 h in 10 mM phosphate buffer (pH 7.0).

(A) CaM:

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                  20                  40                  60
MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG

                  80                 100                 120
NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE

                 140
EVDEMIREADIDGDGQVNYEEFVQMMTAK
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(B) MLCK:

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566                             591
KRRWKKNFIAVSAANRFKKISSSGAL
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Scheme S1. Primary sequence of proteins used in this work. (A) CaM (*Homo sapiens*). The methionine residues for $^{13}CH_3$ selective labeling are highlighted in red. (B) CaM binding motif of MLCK (aa 566 - 591). The tryptophan residue for ^{19}F labeling is highlighted in red.

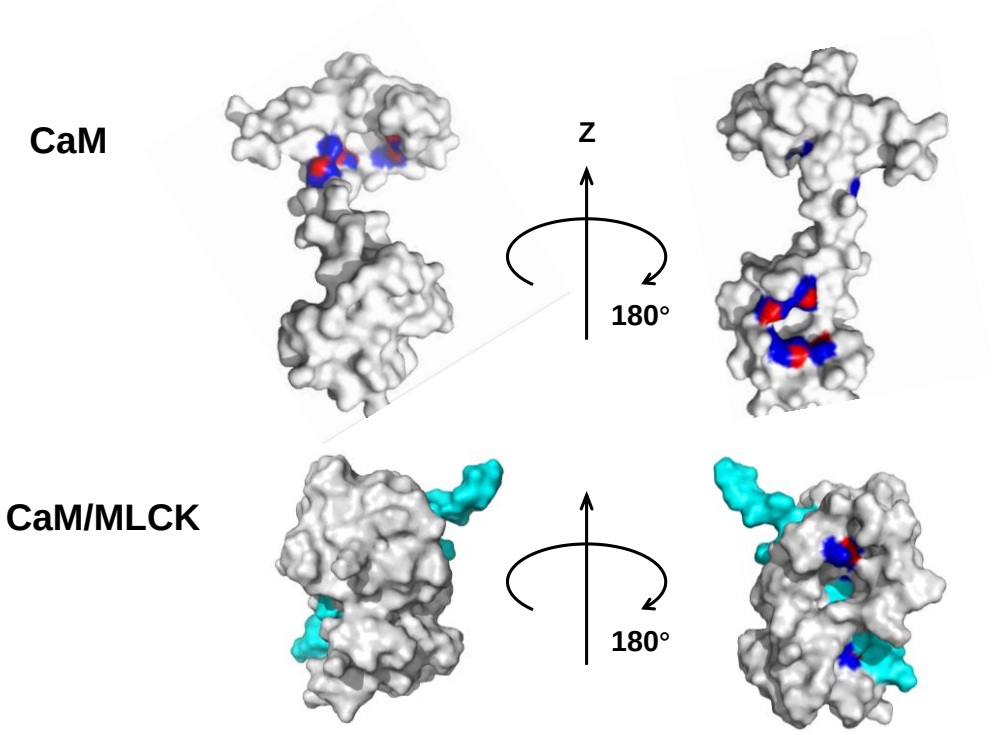

Figure S1. Illustration of solvent exposure of methionine sulfur in holo-CaM and in the CaM/MLCK complex. The protein structures were generated by Pymol with PDB data of 1CLL (CaM) and 2LV6 (CaM/MLCK). The surfaces of the proteins are depicted in gray (CaM) and cyan (MLCK). Methionine (Met) residues are highlighted in blue, and their sulfur atoms are indicated in red.

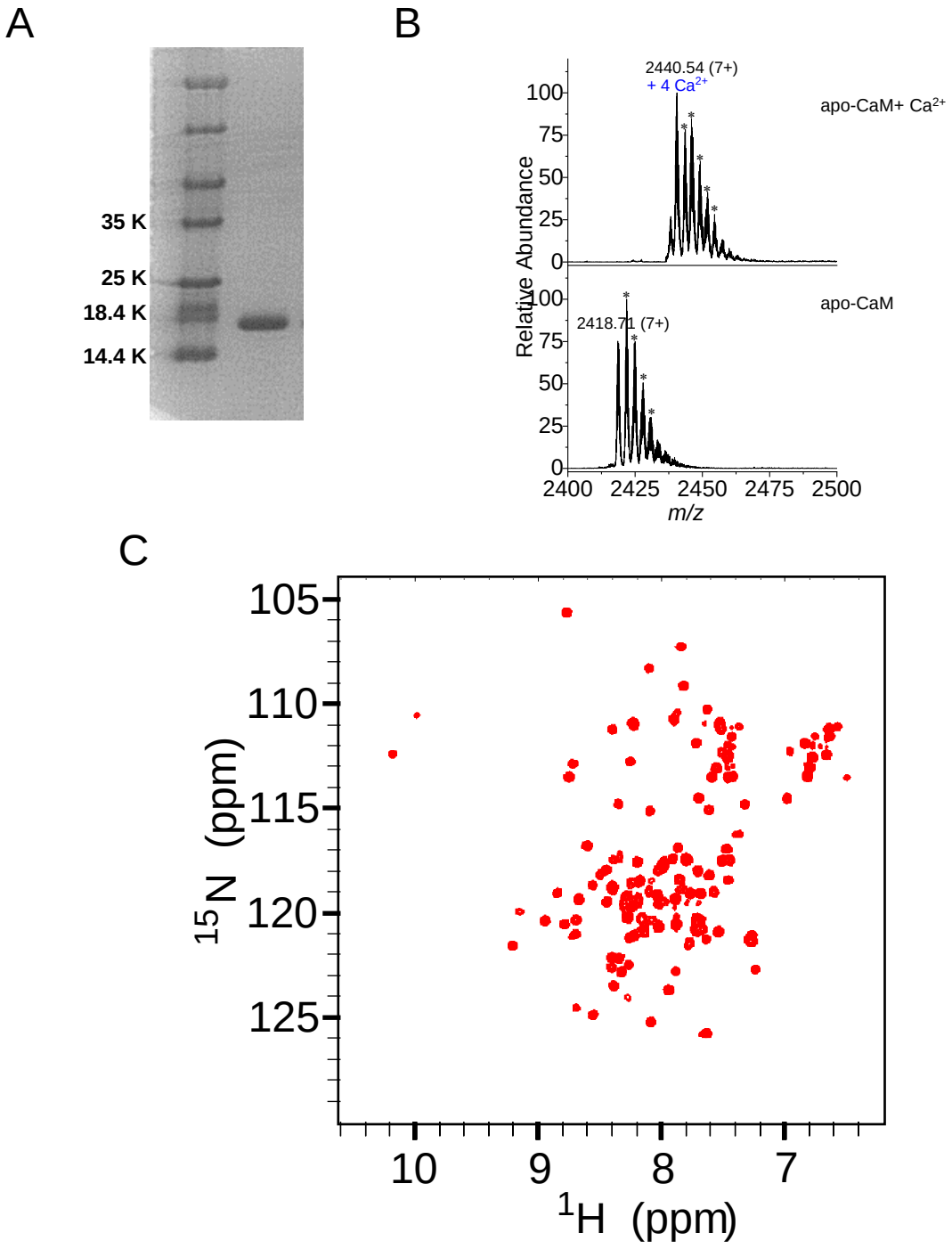

Figure S2. Characterization of CaM. (A) Electrophoretic analysis of CaM with tricine-SDS-PAGE. (B) ESI-MS characterization of apo-CaM (bottom) and holo-CaM (prepared by the addition of 4 molar equivalents of Ca^{2+} ions to apo-CaM) (top). Asterisks (*) denote the adsorption of Na^{+} ions on the protein. (C) 2D ^{1}H-^{15}N HSQC spectra of ^{15}N-labeled apo-CaM.

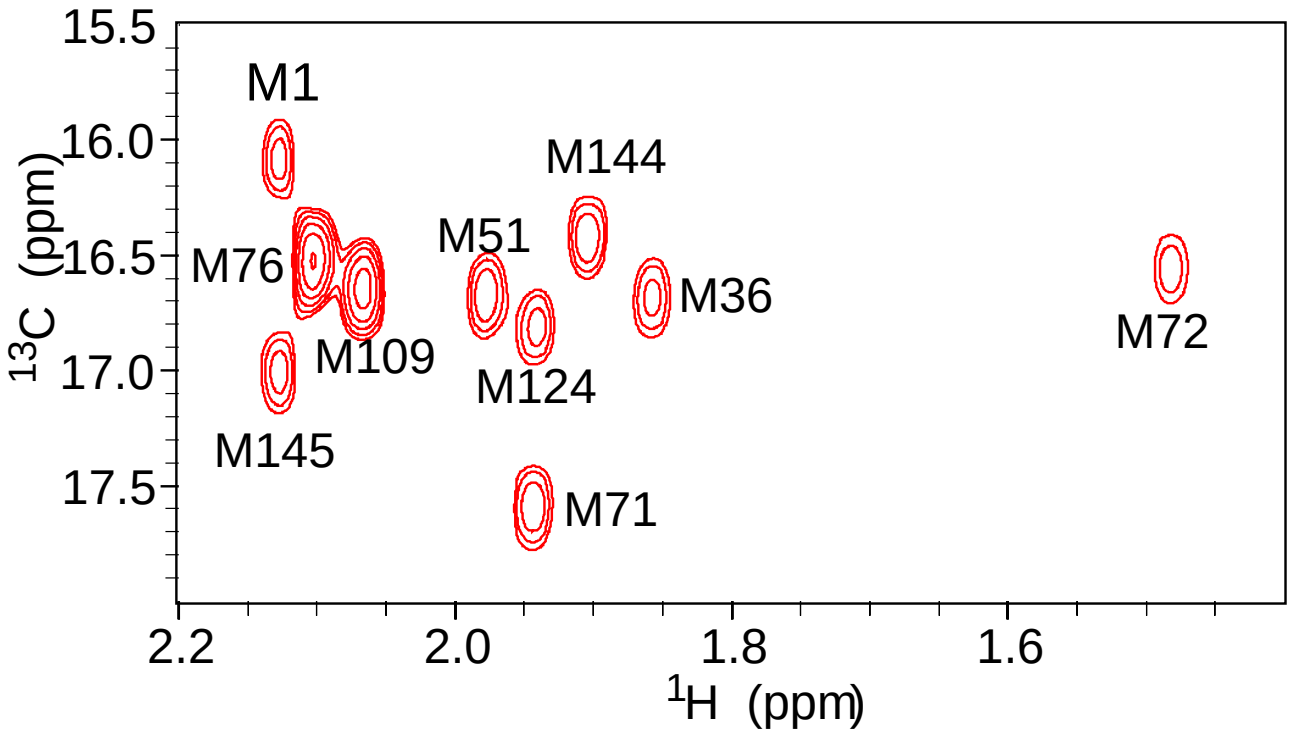

Figure S3. 2D ^{1}H-^{13}C HSQC spectrum of apo-CaM labeled with $^{13}CH_3$-Met in 20 mM HEPES buffer (pH 7.0).

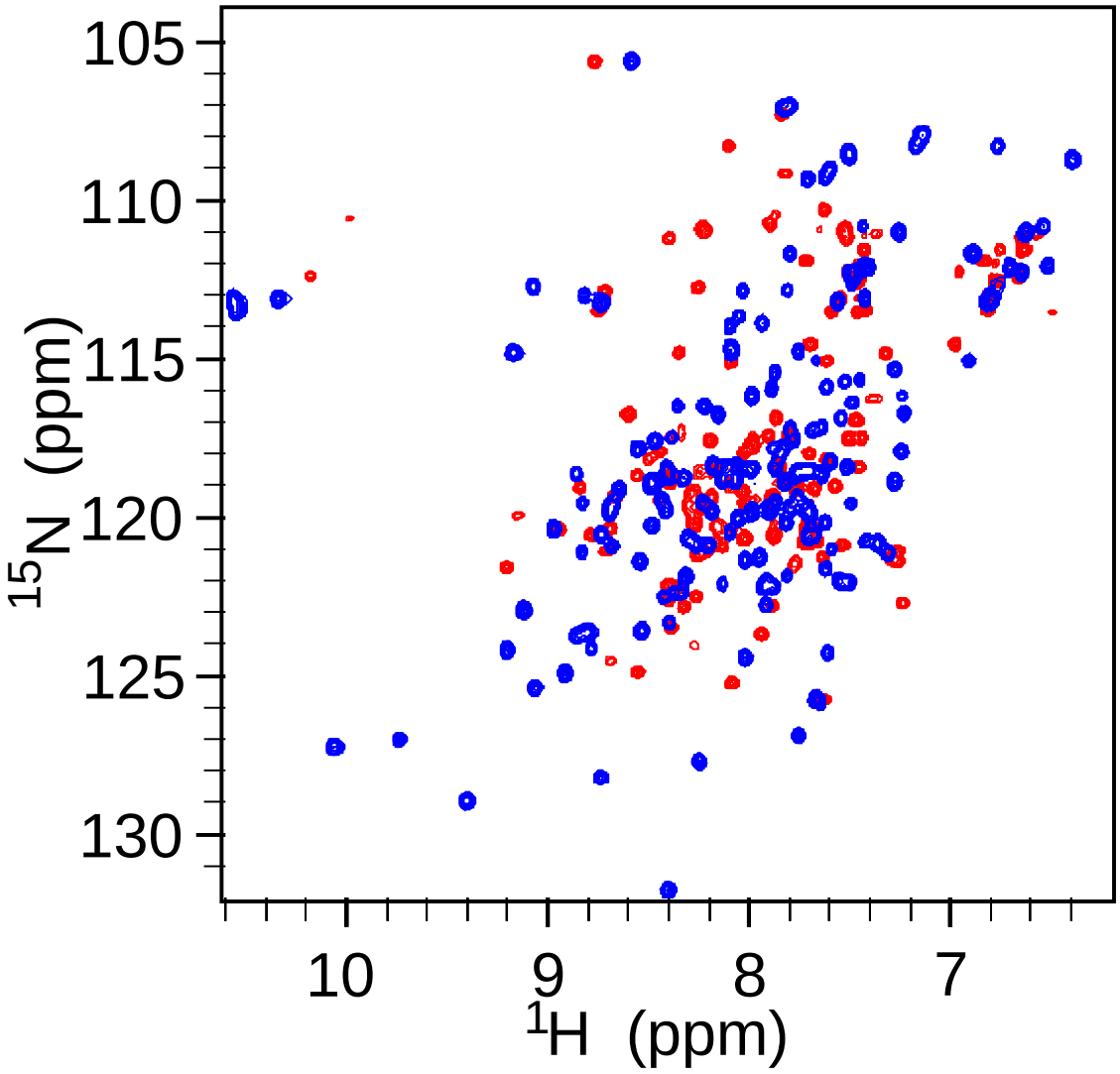

Figure S4. 2D ^{1}H-^{15}N HSQC spectra of apo-CaM before (red) and after the addition of $CaCl_2$ (blue, holo-CaM). Apo-CaM (0.3 mM) was incubated with 4 molar equivalents of Ca^{2+} in 50 mM phosphate buffer (pH 7.4) at 37°C for 10 minutes.

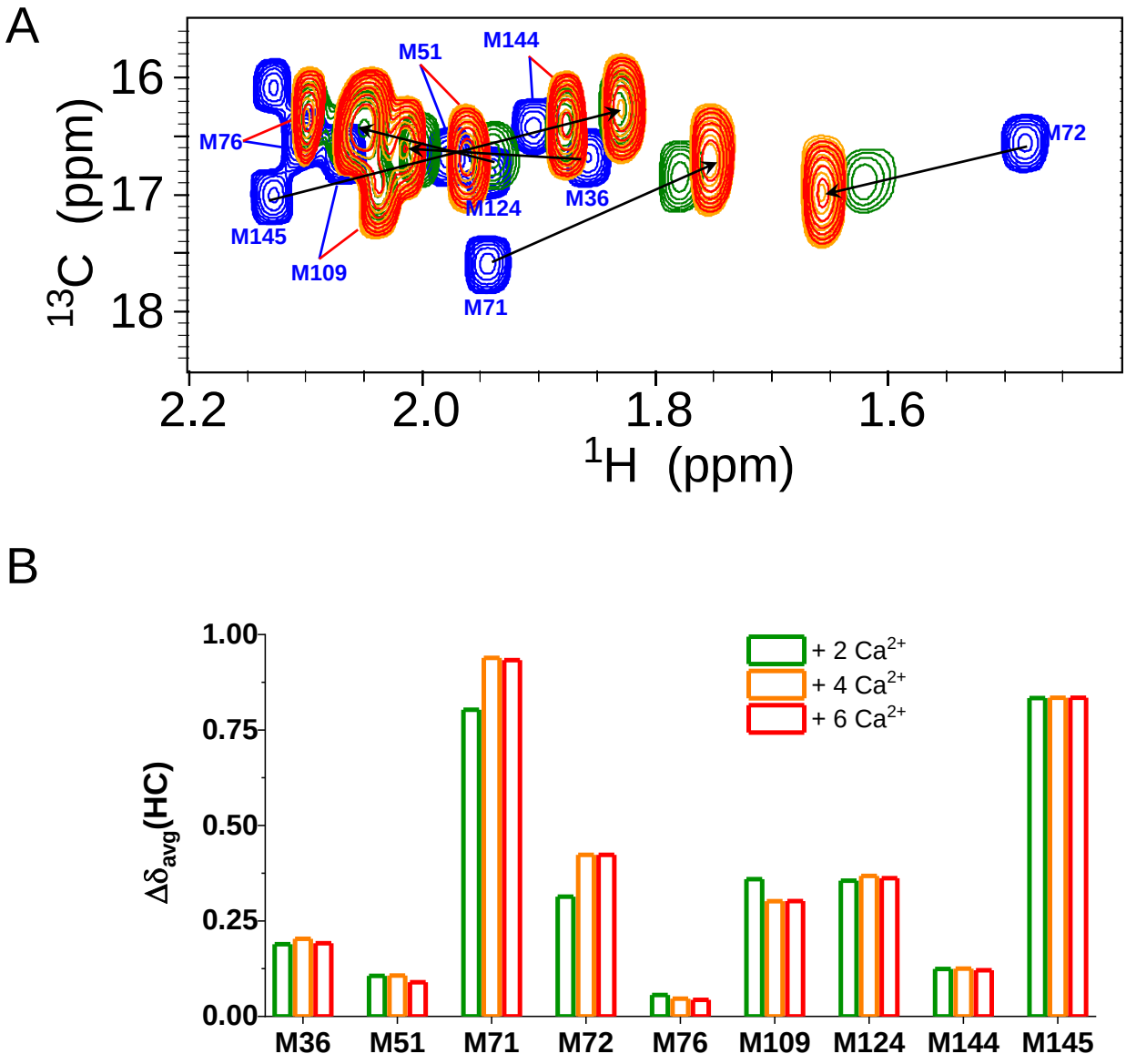

Figure S5. Characterization of Ca^{2+} binding to apo-CaM. (A) 2D ^{1}H-^{13}C HSQC spectra of apo-CaM labeled with $^{13}CH_3$-Met (blue) upon the addition of 2 (green), 4 (orange), or 6 (red) molar equivalents of Ca^{2+} in 20 mM HEPES buffer (pH 7.0). (B) Weighted average chemical shift differences ($\Delta\delta_{avg}(HC) = [(\Delta\delta_H)^2 + (\Delta\delta_C)^2]^{1/2}$) of CaM in the presence of different molar equivalents of Ca^{2+}.

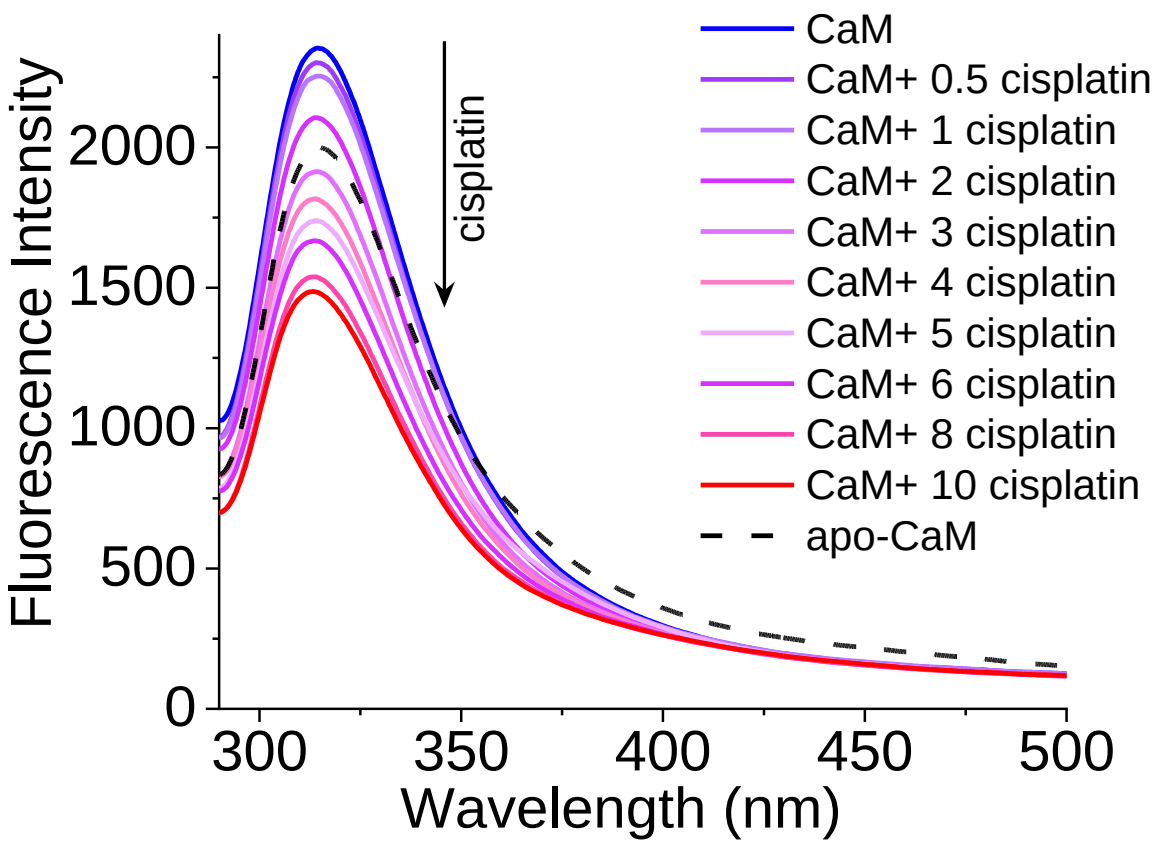

Figure S6. Fluorescence spectra of holo-CaM upon addition of cisplatin. Samples were prepared by treating holo-CaM (10 µM) with various amounts of cisplatin in 50 mM phosphate buffer (pH 7.4) at 37°C and incubating for 12 h. Spectra were recorded at 25°C with excitation at 275 nm.

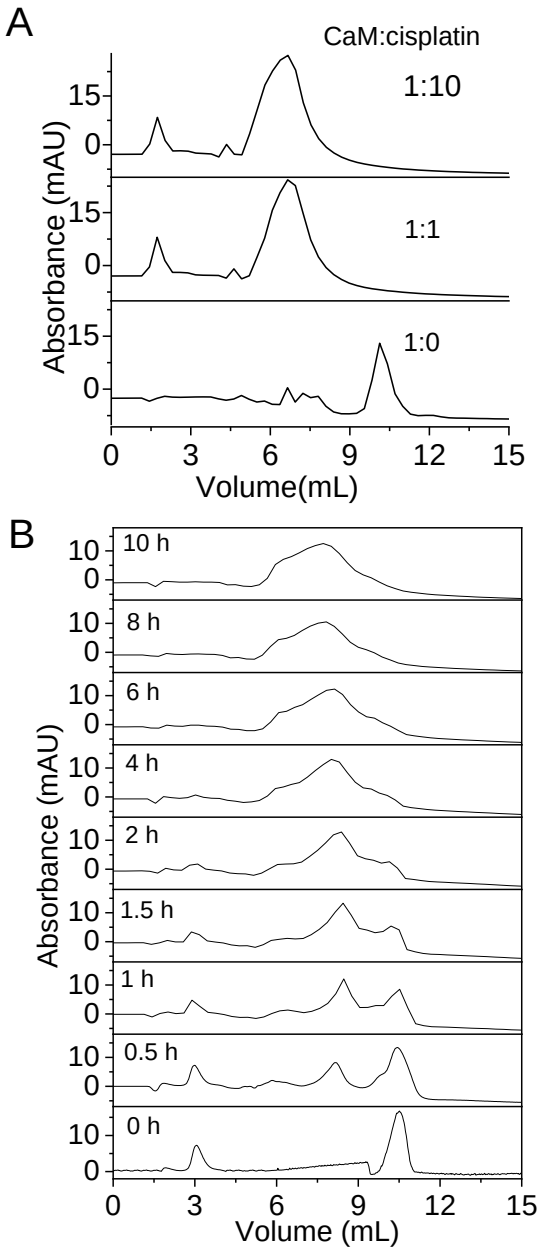

Figure S7. Anion exchange chromatographic analysis of the reaction of cisplatin with holo-CaM. (A) Holo-CaM (0.1 mM) was treated with different molar equivalents of cisplatin in 20 mM HEPES buffer (pH 7.0) at 37°C and incubated for 24 h. (B) Holo-CaM (0.1 mM) was treated with 3 molar equivalents of cisplatin in 20 mM HEPES buffer (pH 7.0) at 37°C, and the reaction monitored as a function of incubation time.

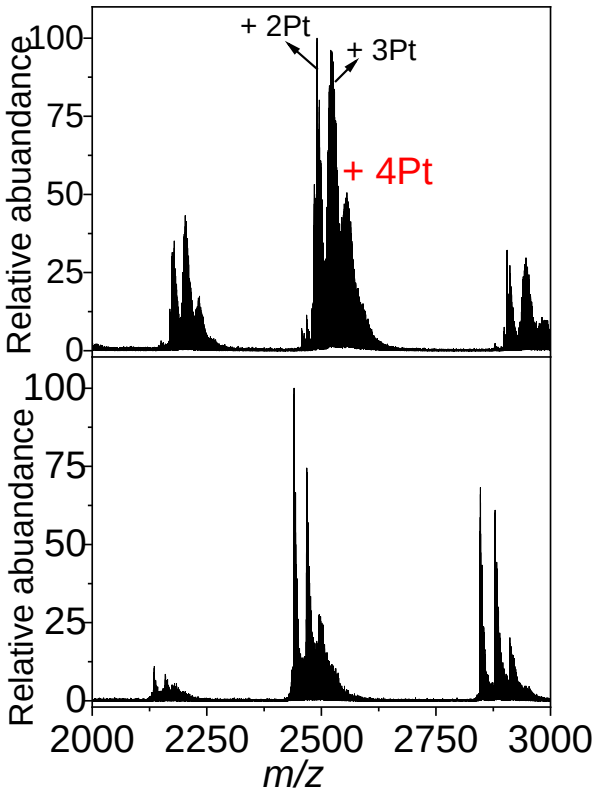

Figure S8. ESI-MS spectra of holo-CaM reacted with 1.2 (bottom) or 3 (top) molar equivalents of cisplatin. Holo-CaM (100 μM) was incubated with 1.2 or 3 molar equivalents of cisplatin at 37°C for 12 h in 50 mM phosphate buffer (pH 7.4).

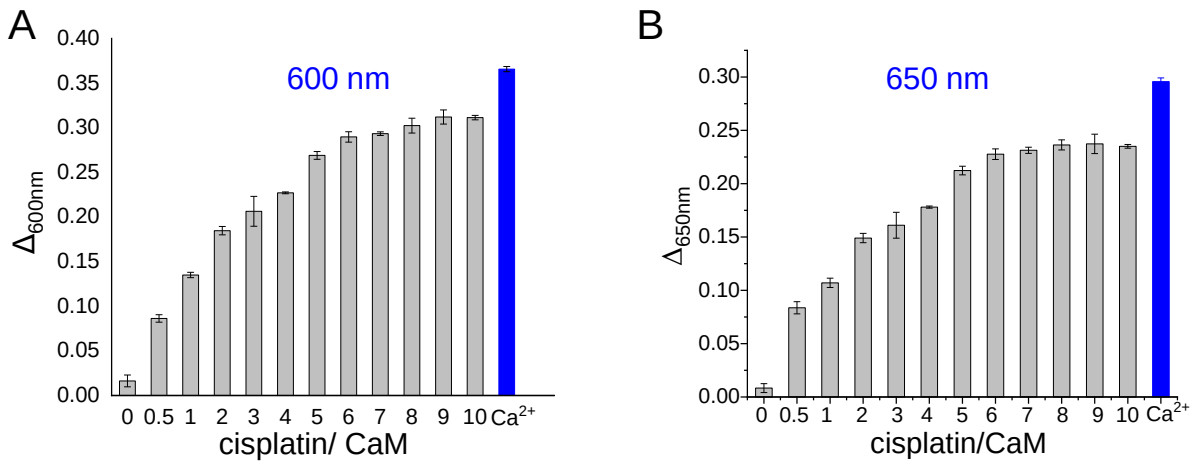

Figure S9. Detection of Ca^{2+}-release from holo-CaM using arsenazo-III. Holo-CaM (5 μM) was incubated with different molar ratios of cisplatin (incubation time 12 h, 37°C) and then treated with the Ca^{2+} chelator arsenazo-III (100 μM). The change in absorbance, as a function of the cisplatin/CaM ratio, is reported for 600 (A) and 650 nm (B). A $CaCl_2$ solution (20 μM) was used as a positive control (blue bar).

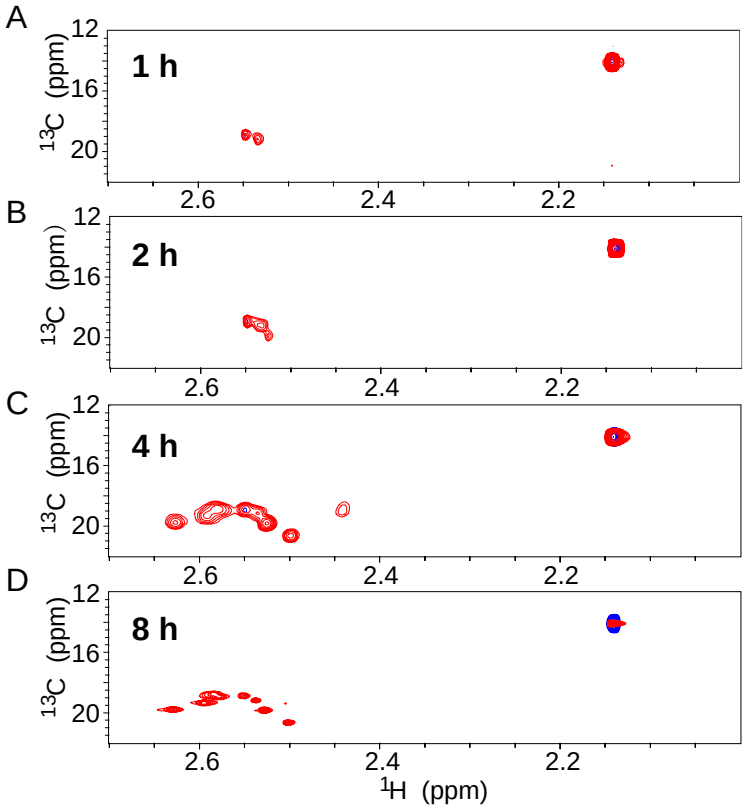

Figure S10. 2D ^{1}H-^{13}C HSQC NMR spectra of $^{13}CH_3$-Met incubated with 0.5 molar equivalent of cisplatin at 37 °C for different times: (A) 1 h, (B) 2 h, (C) 4 h and (D) 8 h. The 2D ^{1}H-^{13}C HSQC NMR spectrum of untreated $^{13}CH_3$-Met is shown, superimposed in blue, in Figure D. The reactions were conducted at 37 °C in 20 mM HEPES buffer (pH 7.4).

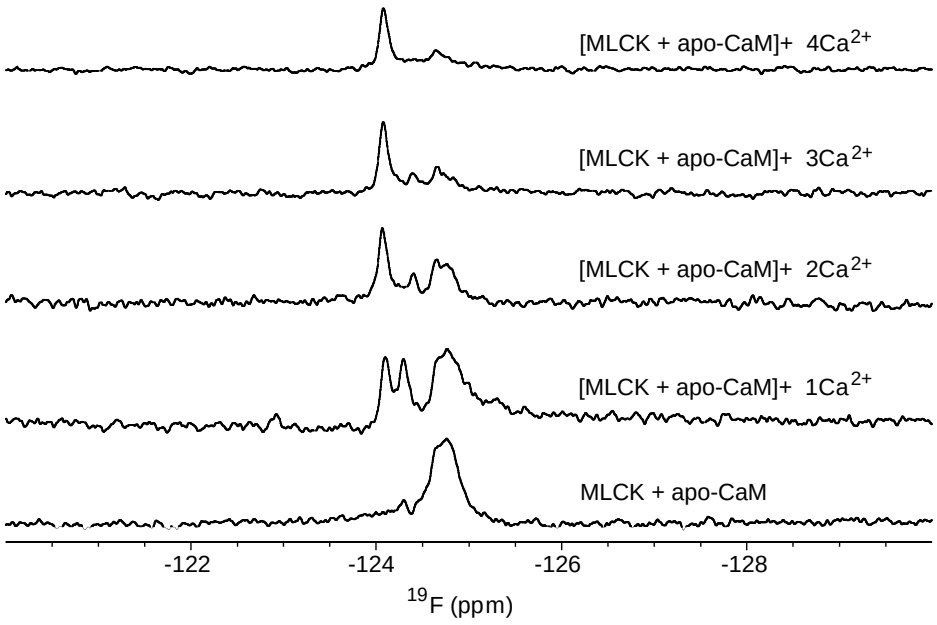

Figure S11. 1D ^{19}F NMR spectra of a 1:1 mixture of MLCK and apo-CaM treated with different amounts of Ca^{2+}. The reactions were conducted at 37°C and the spectra were recorded after 10 min post-incubation in 50 mM phosphate buffer (pH 7.4).

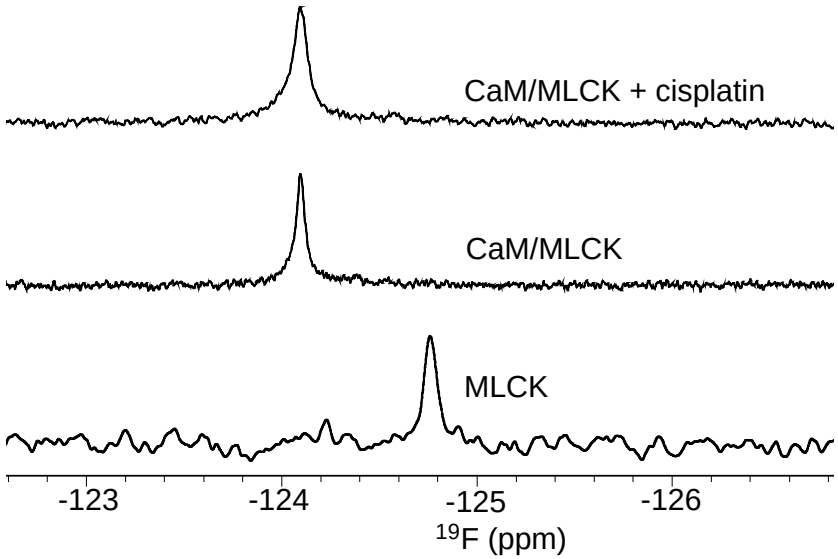

Figure S12. 1D ^{19}F NMR spectra of the CaM/MLCK complex upon reaction with 5 molar equivalents of cisplatin. The reaction was conducted at 37°C for 12 h in 50 mM phosphate buffer (pH 7.4).

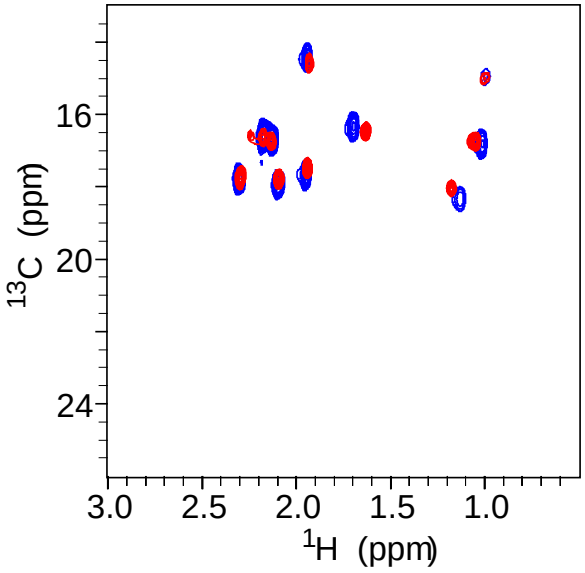

Figure S13. Superposition of 2D ^{1}H-^{13}C HSQC NMR spectra of $^{13}CH_3$-Met-labeled CaM/MLCK complex before (blue) and after (red) incubation with 2 molar equivalents of cisplatin. The reaction was conducted at 37 °C for 4 h in 20 mM HEPES buffer (pH 7.0). There are no peaks detectable in the region of 20 - 25 ppm (^{13}C) and 2.4 - 2.8 ppm (^{1}H) characteristic of Pt-coordinated methionines.

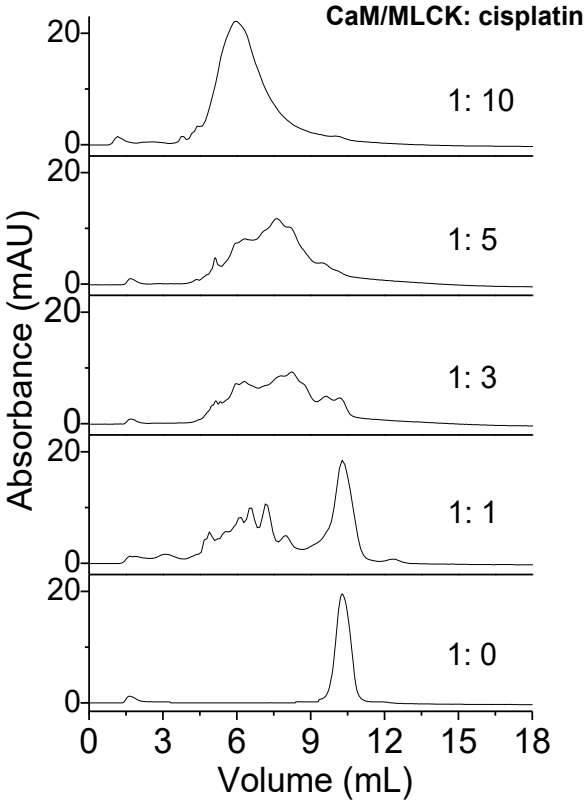

Figure S14. Anion exchange profiles of the CaM/MLCK complex after reaction with cisplatin. The reactions were conducted by incubation of the CaM/MLCK complex (0.1 mM) with different molar equivalents of cisplatin in 20 mM HEPES buffer (pH 7.0) at 37°C for 24 h. Ca^{2+} ions (1 mM) were added in both mobile phases A and B to maintain the stability of the CaM/MLCK complex during the chromatographic process.

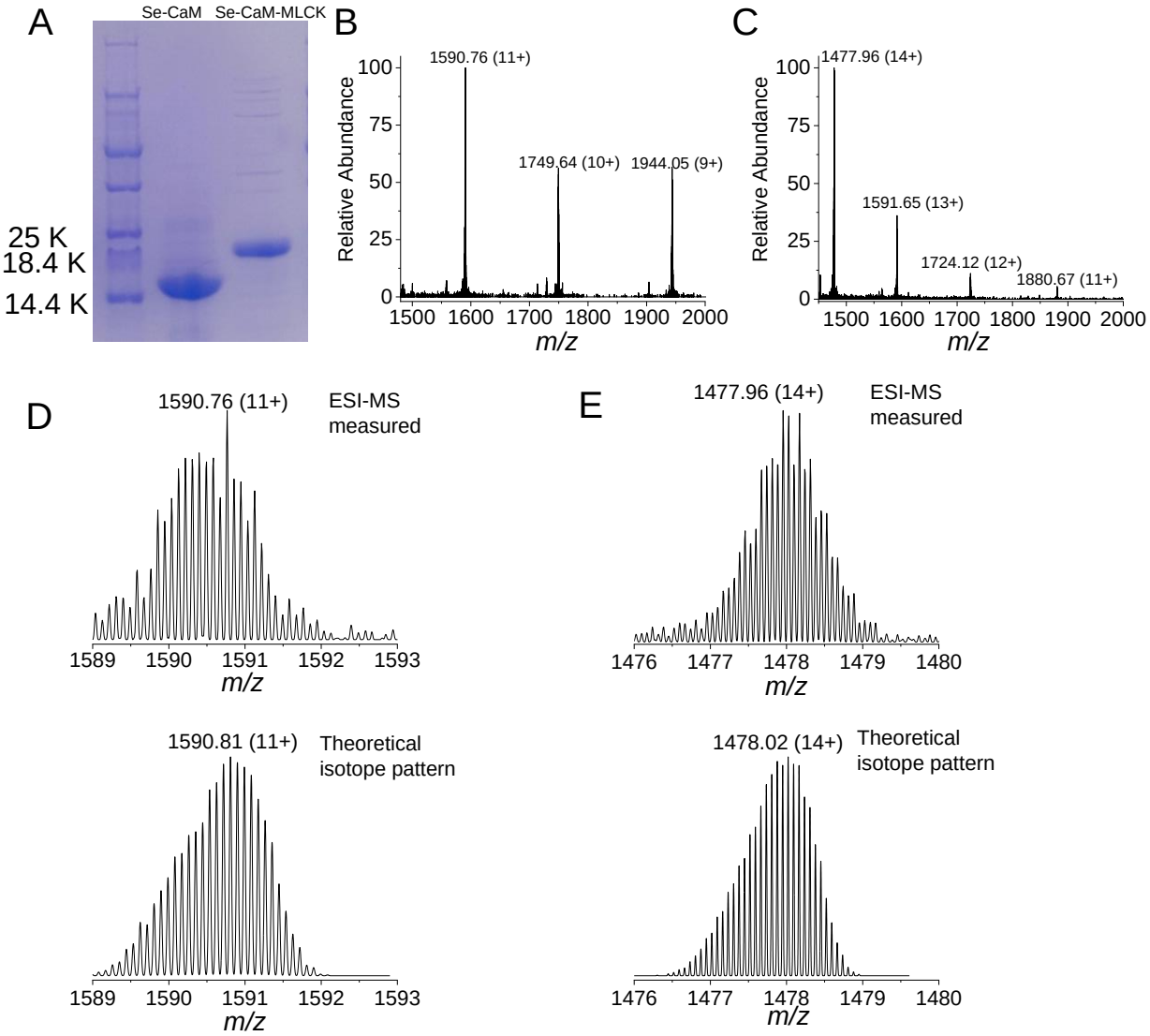

Figure S15. Characterization of seleno-L-methionine-labeled CaM (Se-CaM) and CaM-MLCK (Se-CaM-MLCK) proteins. (A) Electrophoretic analysis of Se-CaM and Se-CaM-MLCK with tricine-SDS-PAGE. (B) ESI-MS characterization of apo-Se-CaM. The observed peaks correspond to the Se-CaM protein containing 10 SeMet residues [m/z 1590.76 (11+, calculated 1590.81), 1749.64 (10+, calculated 1749.79) and 1944.05 (9+, calculated 1944.10)]. (C) ESI-MS characterization of apo-Se-CaM-MLCK. The observed peaks correspond to the Se-CaM-MLCK fusion protein containing 10 SeMet residues [m/z 1477.96 (14+, calculated 1478.02), 1591.65 (13+, calculated 1591.64), 1724.12 (12+, calculated 1724.19) and 1880.67 (11+, calculated 1880.84)]. (D) Comparison between observed ESI-MS peaks and theoretical isotopic patterns for Se-CaM. (E) Comparison between observed ESI-MS peaks and theoretical isotopic patterns for Se-CaM-MLCK.

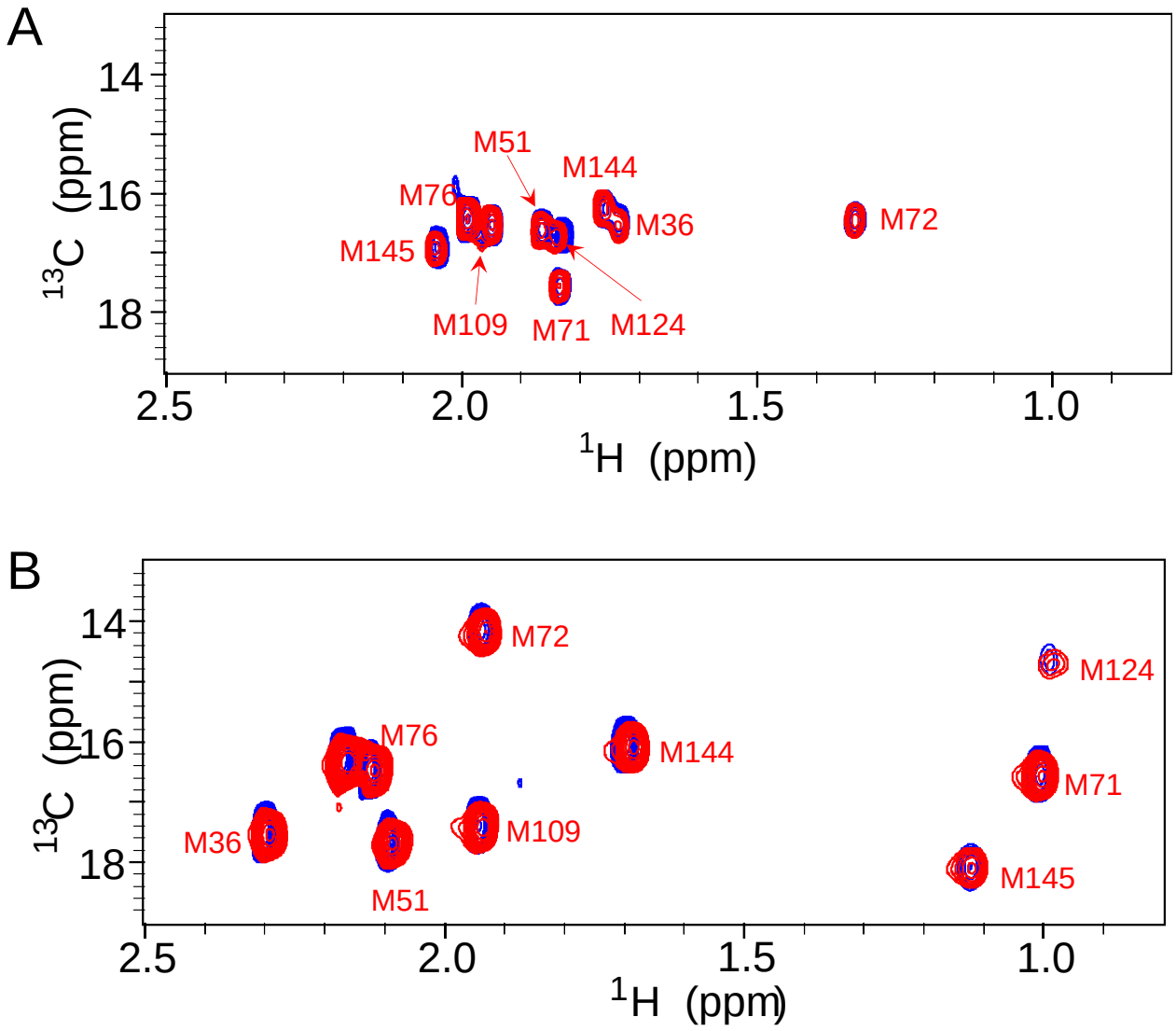

Figure S16. Superposition of 2D ^{1}H-^{13}C HSQC NMR spectra of 1:1 apo-CaM + MLCK proteins (red) and of CaM-MLCK fusion protein (blue) (both labeled with $^{13}CH_3$-Met) registered *in vitro* in the absence of Ca^{2+} (A) and in the presence of 4 molar equivalents of Ca^{2+} (B). Recognition between the two domains occurs only in the presence of Ca^{2+}.

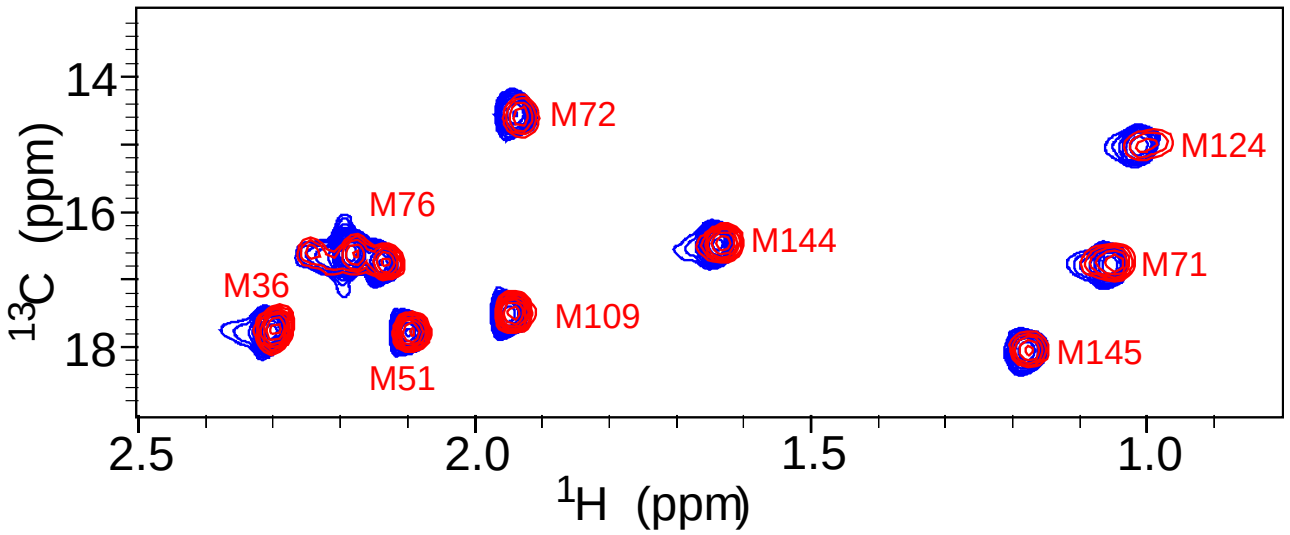

Figure S17. Superposition of 2D ^{1}H-^{13}C HSQC NMR spectra of 1:1 CaM + MLCK proteins (red) and of CaM-MLCK fusion protein (blue) (both labeled with $^{13}CH_3$-Met) treated *in vitro* (20 mM HEPES buffer, pH 7.0) with $CaCl_2$ (4 molar equivalents) and then incubated with 2 molar equivalents of cisplatin at 37°C for 4 h.