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Localization of ligands within human carbonic anhydrase II using ^{19}F pseudocontact shift analysis

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General remarks

Commercial reagents were used as received without further purification. The quality of the solvents for reactions corresponds at least to "puriss.", while for aqueous solutions ultrapure water was used. The numbering system for the residues of human carbonic anhydrase I (hCA I) was used for human carbonic anhydrase II (hCA II), where Thr-125 is followed directly by Lys-127. hCA I has an additional alanine residue at the position 126 which is missing for hCA II.[1]

Methods and Devices

Standard methods, e.g. primer design, deoxyribonucleic acid (DNA) extraction and purification, gel electrophoresis, protein affinity chromatography and spectroscopic quantification of cell growth were carried out according to published protocols. Commercial kits, e.g. PD10 ion exchange columns, Sep-Pak C18 columns, Amicon ultrafiltration tubes, were used according to instructions of the manufacturer.

NMR spectroscopy: All NMR spectra were recorded either on a Bruker Avance III NMR spectrometer operating at 600.13 MHz, equipped with a 5 mm BBFO probe head with z-axis pulsed field gradients or a 5 mm $^{1}H/^{13}C/^{15}N$ TXI probe head with z-axis pulsed field gradients, or on a Bruker Avance III HD spectrometer operating at 600.13 MHz, equipped with a 5 mm $^{1}H/^{19}F-^{13}C/^{15}N$-D QCI cryo probe head with z-axis pulsed field gradients.

HPLC: HPLC purification was performed on a Prep LC 4000 System in combination with a 2487 Dual λ Absorbance Detector both from Waters (Baden-Dättwil, Switzerland) equipped with a 150 × 20 mm column packed with ReproSil-Pur 120 ODS-3.5 µm from Dr. Maisch Gmbh (Ammerbach, Germany) using 0.1% TFA in degassed H_2O as aqueous phase and 90% acetonitrile with 10% H_2O and 0.085% TFA as organic phase. For each purification the following program was used: flow rate 10 ml/min 10% for 2 min, 10% to 55% in 20 min, 55% for 5 min, 55% to 100% in 2 min and 100% until complete elution of all compounds (values correspond to percentage of the organic phase).

PD MiniTrap G-25 columns: Single use PD MiniTrap G-25 Size exclusion columns from GE Healthcare (Uppsala, Sweden) for desalting of protein solutions were applied according to the instructions of the manufacturer. Columns were equilibrated with 8 ml of desired final buffer, the sample was loaded on the column in a volume of 0.5 ml and eluted with 1 ml of the desired final buffer.

Mass spectrometry: ESI-MS analysis of small molecules and lanthanide complexes was performed on a Bruker Daltonics Esquire 3000 plus spectrometer. Mass spectrometric analysis of protein samples was performed on a Bruker Daltonics microTOF ESI/time of flight (TOF)-MS spectrometer combined with an Agilent HPLC system.

Polymerase chain reaction (PCR): PCRs were carried out in an Mastercycler Gradient from Eppendorf (Schönenbuch, Switzerland).

UV spectroscopy: UV spectra for the determination of DNA concentrations were recorded on a Thermo Fisher Scientific NanoDrop 1000 spectrometer. Concentrations of proteins, M8-SSPy and LnM8-SSPy complexes were determined on an Agilent Technologies 8453 UV-Visible Diode Array spectrophotometer using cuvettes suitable for 50 µL sample volume from Hellma Analytics (Müllheim, Germany). Determination of cell growth was performed on a Varian Cary 50 Scan UV-Vis spectrometer.

Gel electrophoresis: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were performed on Bio-Rad Mini-PROTEAN and Sub-Cell systems, respectively. The gels were analysed using a Molecular Image Gel Doc XR (Reinach, Switzerland).

Protein expression: Expression of protein in shaking flasks were performed either in an Infors HT Ecotron (Bottmingen, Switzerland) or a New Brunswick Scientific INNOVA 44 (Edison NJ, USA). Expression of triple labelled protein was carried out in a 2 l Infors HT Labforce 5 Cell fermenter (Bottmingen, Switzerland) equipped with pH sensor, pO_2 sensor and four peristaltic pump channels.

Protein purification: Chromatographic purification of proteins was carried out on an ÄKTA prime plus from GE Healthcare (Glattbrugg, Switzerland).

Centrifugation: Centrifugation steps were carried out on the following centrifuges: A Heraeus Multifuge 4KR centrifuge equipped with a swinging bucket rotor (4440 rpm correspond to 5346 g), a Sorvall RC-6 plus centrifuge equipped with a fixed angel rotor

either for 6 x 250 ml samples (14000 rpm correspond to 30000 g) or for 6 x 1 l samples (9500 rpm correspond to 16880 g), a Heraeus Fresco 21 centrifuge for eppendorf samples (14800 rpm correspond to 21100 g) (all from Thermo Fisher Scientific, Waltham, MA USA)) or a Universal 320 from Hettich (Tuttlingen, Germany) equipped with a rotor for 12 x 15 ml falcon tubes (12000 rpm correspond to 13201 g).

Lyophilisation: Lyophilisation steps were carried out in a Labonco benchtop lyophilizer FreeZone 2.5 l (Kansas City, MO USA).

Ultrapure water: Ultrapure water was obtained either from a Merck Millipore Milli-Q Reference water purification system (Molsheim, France) or from a Labtec ELGA Pure lab ultra-water purification system (Villmergen, Switzerland).

Protein expression

The numbering system for the residues of hCA I was used for hCA II, where Thr-125 is followed directly by Lys-127. hCA I possesses an additional alanine residue at the position 126 which is missing for hCA II.[1] All solutions used were either heated in an autoclave at 121 °C for 15 min or filter-sterilized using a 0.22 µm sterile filter.

pACA plasmid

The pACA plasmid used for the production of hCA II was a generous gift from Prof. Carol A. Fierke (University of Michigan, USA).[2] Consisting of a hCA II gene[3] behind a T7 RNA polymerase promoter, a f1 origin of replication[4], and an amp^r gene as well as a cm^r gene in a pMa5-8 vector[5]. The gene for hCA II has an alanine instead of a serine at position 2 which does neither affect the expression of the protein nor its catalytic activity. This plasmid was used as a template for single point mutations by PCR.

Site-directed mutagenesis

Plasmid amplification: The plasmid containing the gene for hCA II was amplified by transformation into *E. coli* DH5α cells (genotype: F$^-$ Φ80*lac*ZΔM15Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r^-_K, m^+_K) *pho*A *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1) according to the Invitrogen protocol. 50 µL DH5α chemically competent cells were allowed to thaw on ice. 10 ng of the plasmid were mixed gently with the cells and incubated on ice during 30 min. Then the cells were heat-shocked by keeping the tube for exactly 20 s in a 42 °C water bath without shaking and then immediately place the tube on ice for 2 min. 950 µL of pre-warmed Super Optimal Broth with Catabolic repressor (SOC) medium were added and the mixture was incubated for 2 h in a shaking incubator (37 °C, 250 rpm). 20 µL to 200 µL of the medium from the transformation were spread on a pre-warmed lysogeny broth (LB) (LB-Miller) plate (containing 60 µg/ml ampicillin (amp) and 34 µg/ml chloramphenicol (cm)). The plates were incubated upside down at 37 °C overnight. Three colonies were selected for extraction of the plasmid using a Promega AG Wizard Plus SV Miniprep DNA purification system (Dübendorf, Switzerland) and were subsequently analysed by sequencing at Microsynth AG (Balgach, Switzerland).

Site-directed mutagenesis: hCA II double mutants were prepared by site-directed mutagenesis using the wt hCA II plasmid (pACA) as initial template. The site-directed mutagenesis steps were carried out according to the procedure described by Zheng et al.[6] and primer candidates were tested *in silico* to minimize hairpin formation.[7] Resulting primers were ordered from Microsynth (Balgach, Switzerland) and are listed in Table 1.

Table 1: List of primers (f: forward, r: reverse).

Mutant		Primers (5' → 3')	Tm (°C)	Length (bases)
C206S	f	CTT CTG GAG tct GTG ACC TGG ATT GTG CTC AAG	66.1	33
	r	CCA GGT CAC aga CTC CAG AAG AGG AGG GG	68.1	29
S50C	f	CTG TCT GTT tgc TAT GAT CAA GCG ACT TCC CTG	64.4	33
	r	TTG ATC ATA gca AAC AGA CAG GGG CTT CAG	62.5	30
S166C	f	GTG CTG GAT tgc ATT AAA ACA AAG GGG AAG AGT GC	64.5	35
	r	GT TTT AAT gca ATC CAG CAC ATC AAC AAC	58.2	29
S173C	f	AAG GGC AAA tgc GCT GAC TTC ACT AAC TTC G	63.5	31
	r	GAA GTC AGC gca TTT GCC CTT TGT TTT AAT G	61.0	31
S217C	f	AAG GAA CCC ATC tcg GTC AGC AGC GAG CAG GTG	69.8	33
	r	GCT GCT GAC gca GAT GGG TTC CTT GAG CAC AAT CC	69.0	35
S220C	f	AGC GTC AGC tgc GAG CAA GTG TTG AAA TTC CG	66.0	32
	r	CAC TTG CTC gca GCT GAC GCT GAT GGG TTC	67.8	30

Transformation of plasmids for protein expression

For protein expression, the desired plasmid was transformed into *E. coli* BL21(DE3)pLysS cells (genotype: F^- *ompT lon hsd*S_B (r^-_B, m^-_B) *dcm gal*λ(DE3) [pLysS (cm^r)] (made competent in house). 100 µL of ultra-competent E. coli BL21(DE3)pLysS cells were allowed to thaw on ice, 8 µl dithiothreitol (DTT) (200 mM) and 3 µL of the plasmid (0.2 µg to 0.5 µg of DNA) were added and mixed gently. The mixture was kept on ice for 15 min and then plated on pre-warmed LB plates (containing 60 µg/ml amp and 34 µg/ml cm and 2% w/v glucose). The plates were incubated upside down at 37 °C overnight.

Protein expression

Expression of uniform 15N labelled hCA II mutants in 1 l shaking flasks

Double mutants mentioned were expressed under the following conditions.

Inoculum: 15 ml of LB medium (containing 100 µg/ml amp and 34 µg/ml cm and 2% w/v glucose) in a 75 ml baffled shaking flask were inoculated with a tip dipped into a single colony from the transformation. The mixture was incubated at 37 °C for 6 h to 7 h at 250 rpm.

Preculture: The inoculum was centrifuged at 5346 g for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 2.5 ml preculture medium. Either 50 ml LB medium (containing 60 µg/ml amp and 34 µg/ml cm and 0.2% w/v glucose) or 50 ml of main culture medium in a 250 ml baffled shaking flask were inoculated with the 2.5 ml of resuspended inoculum. When using LB medium, the culture was incubated overnight at 37 °C, 250 rpm until the optical density at 600 nm (OD600) reached around 6.0. When using main culture medium, the culture was incubated overnight at a temperature between 25 °C to 33 °C in order to reach a final OD600 of 1.0 to 2.0. Then the preculture was centrifuged at 5346 g for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 50 ml main culture medium.

Main culture: 1 l of main culture medium in 3 l baffled shaking flasks was inoculated with the cells from the preculture. The medium was consisting of phosphate buffer (2 g/l Na_2HPO_4, 1 g/l KH_2PO_4, pH adjusted to 7.2), 0.5 g/l NaCl, 1 g/l $^{15}NH_4Cl$ (filter-sterilized), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% w/v glucose, 0.5 mM $ZnSO_4$, 100 µg/ml amp, 34 µg/ml cm, 1X BME vitamin solution (using a 100X stock solution from Sigma-Aldrich, Buchs, Switzerland) and 1X of a trace element solution (using a 200X stock solution consisting of: 6 g/l $CaCl_2 \cdot 2\ H_2O$, 6 g/l $FeSO_4 \cdot 7\ H_2O$, 1.15 g/l $MnCl_2 \cdot 4\ H_2O$, 0.8 g/l $CoCl_2 \cdot 6\ H_2O$, 0.7 g/l $ZnSO_4 \cdot 7\ H_2O$, 0.3 g/l $CuCl_2 \cdot 2\ H_2O$, 0.02 g/l H_3BO_4, 0.25 g/l $(NH_4)6Mo_7O_{24} \cdot 4\ H_2O$ and 5 g/l EDTA disodium salt). The culture was incubated at 37 °C in a shaking incubator at 250 rpm until an OD600 reached of 0.6. Then the temperature was decreased to 25 °C and the expression was induced when OD600 reached 1.0 to 1.3 by the addition of 250 µM Isopropyl-β-D-thiogalactopyranosid (IPTG) and 450 µM $ZnSO_4$. The cells were incubated for 4 h to 6 h after the induction and the OD600 was monitored continuously. 1 ml samples were taken before induction and then every hour until the end of expression. All samples were kept on ice and were treated the same way as the main culture after the end of the expression. In order to not compromise the labelling quality, the expression was not left for more

than 6 h after the induction, even if the OD600 was still increasing. Cells were harvested by centrifugation at 4 °C and 5346 g for 15 min. The supernatant was deactivated by the addition of NaClO and the cell pellet was stored at −20 ◦C.

Expression of selectively ^{15}N leucine labelled hCA II mutants

All double mutants listed in Table 3 were expressed ^{15}N leucine labelled.

Inoculum: 15 ml of LB medium (containing 100 µg/ml amp and 34 µg/ml cm and 2% w/v glucose) in a 75 ml baffled shaking flask were inoculated with a tip dipped into a single colony from the transformation. The mixture was incubated at 37 °C for 6 h to 7 h at 250 rpm.

Preculture: The inoculum was centrifuged at 5346 g for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 2.5 ml preculture medium. 50 ml LB medium (containing 60 µg/ml amp and 34 µg/ml cm and 0.2% w/v glucose) in a 250 ml baffled shaking flask were inoculated with 2.5 ml of resuspended inoculum. The culture was incubated overnight at 37 °C, 250 rpm until the OD600 reached 6.0. Then the preculture was centrifuged at 5346 g for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 20 ml main culture medium.

Main culture: 1 l of main culture medium in 3 l baffled shaking flasks was inoculated with the cells from the preculture. The medium was consisting of phosphate buffer (4.5 g/l Na_2HPO_4, 3 g/l KH_2PO_4, pH adjusted to 7.2), 0.5 g/l NaCl, 1 g/l, amino acid mix (Table 2), 0.2% w/v glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 µM $ZnSO_4$, 100 µg/ml amp, 34 µg/ml cm, 1X BME vitamin solution (using a 100X stock solution from Sigma-Aldrich, Buchs, Switzerland) and 1X of a trace element solution (using a 200X stock solution consisting of: 6 g/l $CaCl_2 \cdot 2H_2O$, 6 g/l $FeSO_4 \cdot 7\ H_2O$, 1.15 g/l $MnCl_2 \cdot 4\ H_2O$, 0.8 g/l$CoCl_2 \cdot 6\ H_2O$, 0.7 g/l $ZnSO_4 \cdot 7\ H_2O$, 0.3 g/l $CuCl_2 \cdot 2\ H_2O$, 0.02 g/l H_3BO_3, 0.25 g/l $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ and 5 g/l EDTA disodium salt). The culture was incubated at 37 °C in a shaking incubator at 250 rpm until OD600 reached around 0.8. Then the temperature was decreased to 25 °C and the expression was induced when OD600 reached 1.0 to 1.3 by the addition of 250 µM IPTG, 450 µM $ZnSO_4$ and 60 mg ^{15}N leucine dissolved in 50 ml main culture medium (filter sterilized). The cells were incubated for 4 h to 6 h after the induction and the OD600 was monitored continuously. 1 ml samples were taken before induction and then every hour until the end of expression. All samples were kept on ice and were treated the same way as the main culture after the end of the expression. Cells were harvested by centrifugation at 4 °C and 5346 g for 15 min. The supernatant was deactivated by the addition of NaClO and the cell pellet was kept overnight at −20 ◦C.

Uniform ^{2}H ^{13}C ^{15}N labelled hCA II S50C C206S

Only hCA II S50C C206S was expressed in triple labelled medium. For expression of triple labelled protein all solutions were prepared in D_2O apart from the BME vitamin solution and the trace element solution where only small volumes were added to the medium. All solutions were filter sterilized and used directly after preparation.

Table 2: Recipe for amino acid mixture.

Order	Amino acid	mg/l
1	Alanine	500
2	Arginine	400
3	Aspartic acid	400
4	Glutamic acid	650
5	Glycine	550
6	Histidine	100
7	Isoleucine	230
8	Lysine · HCl	420
9	Methionine	250
10	Proline	100
11	Serine	2100
12	Threonine	230
13	Valine	230
14	Phenylalanine	130
15	Tryptophan	50
16	Asparagine	-
17	Cysteine · HCl	50
18	Tyrosine	170
19	Glutamine	400
20	Leucine (^{15}N labelled)	60
^{15}N labelled leucine was not added to the medium until the time of induction.		

Inoculum: 15 ml of LB medium (containing 100 µg/ml amp and 34 µg/ml cm and 2% w/v glucose) in a 75 ml baffled shaking flask were inoculated with a tip dipped into a single colony from the transformation. The mixture was incubated at 37 °C for 6 h to 7 h at 250 rpm.

Preculture: The inoculum was centrifuged at 5346 g for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 2.5 ml preculture medium. 50 ml of main culture medium (see below), with 0.375% w/v D_7-^{13}C-glucose instead of 0.08%, in a 250 ml baffled shaking flask were inoculated with the 2.5 ml of resuspended inoculum. The culture was incubated overnight at a temperature between 30 °C to 37 °C in order to reach a final OD600 of 1.0 to 2.0. Then the preculture was centrifuged at 5346 g for 10 min at 4 °C, the supernatant was discarded and the pellet was resuspended in 50 ml main culture medium.

Main culture: All parts of the fermenter with 2 l working volume were autoclaved and dried properly. The medium for the main culture was consisting of phosphate buffer (9 g/l Na_2HPO_4, 13 g/l KH_2PO_4, 10 g/l K_2HPO_4, 2.4 g/l K_2SO_4, pH adjusted to 7.2), 1 g/l $^{15}NH_4Cl$ (filter-sterilized), 0.08% w/v D_7-^{13}C-glucose, 5 mM $MgSO_4$, 500 µM ZnSO4, 100 µg/ml amp, 34 µg/ml cm, 1X BME vitamin solution (using a 100X stock solution from Sigma-Aldrich, Buchs, Switzerland) and 1X of a trace element solution (using a 200X stock solution consisting of: 6 g/l$CaCl_2 \cdot 2\ H_2O$, 6 g/l $FeSO_4 \cdot 7\ H_2O$, 1.15 g/l $MnCl_2 \cdot 4\ H_2O$, 0.8 g/l $CoCl_2 \cdot 6\ H_2O$, 0.7 g/l $ZnSO_4 \cdot 7\ H_2O$, 0.3 g/l $CuCl_2 \cdot 2\ H_2O$, 0.02 g/l H_3BO_3, 0.25 g/l $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ and 5 g/l EDTA disodium salt). 1 l of main culture medium was inoculated in the fermenter with the cells from the preculture. The fermentation was initiated at 37 °C with the stirring at 250 rpm and an oxygen level kept between 50% to 90%. Antifoam solution was applied manually if required. The glucose level was monitored using Merckoquant Glucose-Test strips (from Merck, Darmstadt, Germany). After around 3 h, when the initial glucose was consumed, a slow feed containing a total amount of 5.5 g D_7-^{13}C-glucose and 0.5 g $^{15}NH_4Cl$ was applied with such a feed rate that 60% of the glucose is delivered overnight to the culture. When the OD600 reached 3.0 to 3.5, the temperature was decreased to 20 °C and protein expression was induced by the addition of 250 µM IPTG and 450 µM $ZnSO_4$. 1 ml samples were taken before induction and then every hour until the end of expression. All samples were kept on ice and were treated the same way as the main culture after the end of the expression. The remaining 40% of the feed was delivered to the culture over a period of 5 h to 6 h. 3 h after induction, 0.5 mM phenylmethanesulphonylfluoride (PMSF) was added. When all glucose was consumed, the cells were harvested by centrifugation at 4 °C and 5346 g for 15 min. The supernatant was deactivated by the addition of NaClO and the cell pellet was kept overnight at −20 °C.

Protein purification

Cell pellets from the different expressions were treated the same way. In order to obtain the protein in the reduced state with regard to the free thiol group, all buffers used for purification were prepared with 1 mM DTT, dialysis buffer with a minimal amount of 100 µM DTT. If no DTT was used, the protein was obtained with glutathione bound to the thiol.

Cell lysis: Lysis of the cells was achieved by three freeze-thaw cycles of the cell pellet. The pellet was resuspended in 25 ml lysis buffer (50 mM Tris-SO_4, pH 8.0, 50 mM NaCl, 0.5 mM $ZnSO_4$, 1 mM DTT and 10 µg/ml PMSF). The resuspension was shaken vigorously (300 rpm) at rt for 30 min, then Deoxyribonuclease I (DNase I) (1 µg/l) was added and the mixture was shaken for 30 min. The lysate was centrifuged at 16880 g for 30 min at 4 °C. The supernatant was recovered and the pellet was resuspended two times in 25 ml lysis buffer (followed by 30 min shaking and centrifugation each time) to extract as much protein as possible. The total 75 ml solution from the extraction were filtered through a 0.45 µm filter and used directly for affinity chromatography.

Ligand affinity chromatography: Affinity chromatography was performed using 25 ml of p-amino-ethylbenzene sulphonamide agarose resin packed into a XK 16 column (GE Healthcare, Glattbrugg, Switzerland). The column was equilibrated with 5 column volumes (CV) activity buffer (50 mM Tris-SO_4, pH 8.0, 0.5 mM $ZnSO_4$, 1 mM DTT) and the protein in 75 ml lysis buffer was loaded onto the column at a flow rate of 1 ml/min. Then the column was washed with 5 CV of wash buffer (50 mM Na_2SO_4, 50 mM $NaClO_4$, 25 mM Tris-SO_4, pH 8.8, 1 mM DTT) and the protein was eluted with 10 column volumes of elution buffer (200 mM $NaClO_4$, 100 mM NaAc, pH 5.6, 1 mM DTT). 10 ml fractions were collected and those containing the protein were pooled and dialyzed at 4 °C against activity buffer for 12 h, followed by deionized H_2O for 24 h and finally against ultrapure water for another 24 h. Dialysis buffer contained 100 µM DTT and were exchanged at least three times a day. The resulting solution was frozen in liquid nitrogen and lyophilized. The resulting protein was stored at 4 °C.

Protein analysis by gel electrophoresis

Sample preparation: The samples taken during the expression were lysed by three freeze-thaw cycles of the cell pellet. The pellet was resuspended in ultrapure water. For each sample 40 µL water multiplied by the OD600 at the time of sampling was used to normalize the concentration over the number of cells. 1 µL of DNase I (1 mg/ml dissolved in 5 mM Tris-HCl, pH 7.5, 75 mM $MgCl_2$ and 50% v/v glycerol). The samples were vortexed and shaken vigorously (300 rpm) at RT for 30 to 60 min. The samples were centrifuged at 21100 g for 5 min at RT. The soluble fraction (supernatant) was separated and the insoluble fraction (pellet) was dissolved in 8 M urea (40 µL urea solution multiplied by the OD600). Each sample fraction (soluble and insoluble) as well as a positive control (around 5 to 20 µg pure hCA II dissolved in 20 µL water) were mixed with 4 µL 6X loading buffer (100 mM Tris-HCl pH 6.8, 2% v/v β-mercaptoethanol, 20% w/v sucrose, 0.012% w/v bromophenol blue). The samples were heated to 98 °C for 5 min and then loaded immediately on a 12% acrylamide gel.

SDS-PAGE: A 12% acrylamide running gel for gel electrophoresis was prepared as follows. 5 ml ultrapure water, 6 ml of 30%/0.8% w/v acrylamide/bis-acrylamide, 3.8 ml of 1.5 M Tris-HCl pH 8.8, 75 µL of 20% w/v sodium dodecyl sulphate (SDS), 100 µL of 15% w/v ammonium perchlorate (APS) and 6 µL of tetramethylethylenediamine (TMEDA) were mixed well and the solution was directly poured into a chamber between glass plates and left for polymerization for 30 min. A 5% acrylamide stacking gel was prepared by mixing 3.4 ml ultrapure water, 1 ml of 30%/0.8% w/v acrylamide/bis-acrylamide, 1.5 ml of 0.5M Tris-HCl pH 6.8, 30 µL of 20% w/v SDS, 40 µL of 15% w/v APS and 6 µL of TMEDA. The solution was poured directly after preparation on top of the running gel and a comb was inserted into the solution in order to prepare the spaces required for loading the different samples onto the gel. The stacking gel was again left for 30 min for polymerization. The gel was placed in a tank containing SDS buffer (25 mM Tris-HCl, 0.192 M glycine and 0.1% SDS). Then 20 µL of each sample were loaded onto the gel and 6 µL of the protein marker (Prestained Protein Marker, Broad Range, from New England BioLabs Inc., Bioconcept, Allschwil, Switzerland). The gel ran at 200 V until the blue front line reached the end of the gel. The gel was transferred into a container with 100 ml of staining solution (25% w/v Coomassie Brilliant Blue R-250, 50% v/v MeOH, 7.5% v/v glacial acetic acid) and was rocked gently for 1 h. Then the gel was placed in 100 ml of a destaining solution (20% v/v MeOH and 7.5% v/v glacial acetic acid) and was rocked gently until the protein pattern appeared. The gel was left in water overnight and a Kimwipe towel was placed in the solution to absorb the stain in the solution.

Refolding of hCA II S50C C206S

For denaturation, hCA II was dissolved in 5 M GdnHCl, 100 mM Tris-SO_4, 1 mM TCEP at pH 7.5 to obtain a final protein concentration of 14.25 µM. Protein concentration was controlled by UV absorption at 280 nm (ε_{280} =54700 $M^{-1}cm^{-1}$)[8] after every step of the protocol. Refolding of the protein was initialized by fast dilution of the whole sample into 100 mM Tris-SO_4 buffer at pH 7.5 containing 10 µM TCEP to obtain a final concentration of 0.3 M GdnHCl and 0.855 µM of protein. The resulting solution was stirred for 3 h to allow the protein to subsequently refold. To reduce the large sample volume, the protein solution was loaded on a sulphonamide affinity column according to the same conditions as described above with the exception that instead of buffer exchange to the described activity buffer the protein was loaded directly in the present buffer after the dilution onto the column. The resulting fractions containing the protein were pooled and dialyzed at 4 °C against activity buffer for 12 h, followed by deionized H_2O for 24 h and finally against ultrapure water for another 24 h. All dialysis buffer contained 10 µM TCEP. The final solution was concentrated using 15 ml Vivaspin ultrafiltration tubes (MW cut-off 10 kDa) to a volume of 2 ml by successive addition of the protein solution. The final buffer exchange to 10 mM PO_4^{3-} pH 6.8 was carried out using a 4 ml Amicon ultrafiltration tube to obtain a final volume of 280 µL.

Table 3: Yields [mg/l] of hCA II mutants for a given isotope labelling scheme and their calculated and determined masses (calculated mass corresponds to protein without N-terminal methionine).

Mutant	Yield (mg/L)	Calculated Mass (Da)	Determined Mass (Da)
unif. ^{15}N hCA II S50C C206S	95	29452	29447
unif. ^{15}N hCA II S166C C206S	95	29452	29449
unif. ^{15}N hCA II S173C C206S	85	29452	29450
unif. ^{15}N hCA II S217C C206S	60	29452	29450
unif. ^{15}N hCA II S220C C206S	80	29452	29450
sel. ^{15}N-Leu hCA II S50C C206S	230	29125	29116
sel. ^{15}N-Leu hCA II S166C C206S	140	29125	29116
sel. ^{15}N-Leu hCA II S173C C206S	180	19125	29116
sel. ^{15}N-Leu hCA II S217C C206S	180	29125	29117
sel. ^{15}N-Leu hCA II S220C C206S	165	29125	29117
unif. ^{2}D ^{13}C ^{15}N hCA II S50C C206S	84	32763	32157[a]
[a] Corresponds to a deuteration level of 85%.			

NMR experiments

NMR spectra were recorded on Bruker Avance III or Avance III HD spectrometers operating at 600.13 MHz proton frequency, equipped with 5 mm BBFO, $^{1}H/^{13}C/^{15}N$ TXI or $^{1}H/^{19}F$-$^{13}C/^{15}N$-D QCI (cryo) probe heads, all fitted with z-axis pulsed field gradients. A ^{1}H-^{15}N-HSQC experiment reported by Grzesiek et al. [9] with a pulse sequence including a water suppression scheme using selective water flip-back pulses and gradients in order to suppress radiation damping was used for the determination of the PCS, since it does not cause the small additional shift due to dipolar coupling (rdc) introduced in TROSY-$^{1}H^{15}N$-HSQC spectra.

Table 4: Parameters for 2D NMR experiments.

Parameter		^{15}N HSQC	^{15}N TROSY
NS		32-512	4
SW (Hz)	F1	2000	2270
	F2	10822	12019
Offset (ppm)	F1	116.5	116.6
	F2	4.7	4.74
TD (points)	F1	200	256
	F2	2048	2048
AQ (ms)	F1	50	56
	F2	95	85
Nucleus	F1	^{15}N	^{15}N
	F2	^{1}H	^{1}H

For backbone assignment of uniformly ^{2}H ^{13}C ^{15}N labelled hCA II S50C Lu-DOTA-M8 and all spectra recorded for protein characterization, TROSY[10] variants of HSQC, HNCO, HNCA, HN(CO)CA, HN(CA)CO and HNCACB experiments incorporating a WATERGATE water suppression were used.[11-13] The parameters to set up these experiments as well as details of the samples used are given in Table 5.

Table 5: Parameters for 3D NMR experiments used for backbone assignment of hCA II S50C C206S Lu-DOTA-M8.

Parameter		HNCO	HNCA	HN(CO)CA	HN(CA)CO	HNCACB
NS		8	16	32	32	36
SW (Hz)	F1	2113	1947	5634	2113	1947
	F2	2128	5585	1946	2270	10566
	F3	12019	11261	11261	11261	11261
Offset (ppm)	F1	175	116.5	175	175	116.5
	F2	116.5	56	116.5	116.5	45
	F3	4.74	4.74	4.74	4.74	4.74
TD (points)	F1	80	80	88	88	80
	F2	80	88	88	88	84
	F3	2048	2048	2048	2048	2048
AQ (ms)	F1	19	20	8	21	21
	F2	19	8	23	19	4
	F3	85	91	91	91	91
Nucleus	F1	^{13}C	^{15}N	^{13}C	^{13}C	^{15}N
	F2	^{15}N	^{13}C	^{15}N	^{15}N	^{13}C
	F3	^{1}H	^{1}H	^{1}H	^{1}H	^{1}H

Backbone assignment of hCA II S50C C206S-Lu-DOTA-M8

Table 6: Backbone chemical shifts of hCA II S50C C206S-Lu-DOTA-M8. All values are given in ppm. Deposition in BMRB (Accession number 27859).

Residue	HN	N	CO	Cα	Cβ	Residue	HN	N	CO	Cα	Cβ
P21[a]	-	-	175.7	62.2	28	A142	8.77	123.9	173.7	47.9	18.5
I22	7.92	121.1	171.7	58	35.4	V143	7.16	124.8	170.2	56.7	28.1
A23	8.55	123.3	176.8	50.4	15.6	L144	8.35	128.7	170.2	49.7	41.3
K24	7.07	115.3	173.6	52	27.8	G145	9.8	114.7	168	40.8	-
G25	8.17	109.3	171.5	41.9	-	I146	9.02	124.7	182	56	39.2
E26	9.24	118.2	173.6	53.7	27.3	F147	9.99	129.3	172	55.1	36.6
R27	8.71	122	172.6	50.2	27.9	L148	8.1	120.7	172.3	52.1	39.4
Q28	7.58	115.9	175.2	52.2	28.6	K149	8.85	119.8	171.8	49.8	32.7
S29	7.94	118.3	181.8	54.2	60.5	V150	8.32	121.8	175.2	59.4	27.9
P30	-	-	170.2	59	29.7	G151	8.97	118.5	169.9	43.7	-
V31	6.14	107.5	171	56	32.2	S152	8.22	125	170.1	55.5	60.5
D32	8.19	118.7	172	50.2	39.1	A153	8.24	121.5	174.8	49.3	15.6
I33	8.59	129	170.2	58	33.6	K154	8.59	124.4	174	48.5	29
D34	7.4	128.7	175.4	49.2	38	P155	-	-	-	-	-
T35	10.15	122.7	174.4	62.2	65.8	G156	-	-	-	-	-
H36	8.67	119.3	173.7	54.7	25.5	L157	7.42	117.3	175.3	50.6	40.5
T37	7.51	109.2	172.5	58.7	66.6	Q158	7.65	122.8	173.7	56.2	24.5
A38	7.34	128	173.7	49.3	14.4	K159	8.71	116.3	176.7	56.4	30
K39	7.98	123.1	174	51.8	30.8	V160	6.89	113.6	173.5	61.5	28.3
Y40	8.57	127.1	172.2	53.9	34.3	V161	7.15	114.8	175.6	62.5	27.8
D41	7.61	129.4	171.3	46	39.3	D162	8.26	117.9	174.7	53.6	37.4
P42	-	-	173.9	59.7	28.2	V163	7	114.5	174.7	58	29.8
S43	8.35	115.6	172.7	56.2	60.7	L164	6.92	122.1	175.9	54.3	36.2
L44	6.86	123.5	174.6	52.1	37.2	D165	8.08	116.6	175.3	53.7	36.7
K45	7.42	123.6	172	51.2	28	S166	7.85	114	171.9	57	60.9
P46	-	-	175.1	60	28	I167	7.27	117	171.7	55.8	34.5
L47	8.84	127	173.1	52	39.9	K168	7.25	117.9	173	57.4	30
S48	8.45	122	168.8	54.2	60.3	T169	6.44	99.6	171.4	55.3	68.3
V49	8.13	126.7	172.7	58.1	27.8	K170	7.34	121	175	54.8	28.1
S50	8.38	127.4	172	50.1	36.4	G171	8.74	117.3	172.2	41.4	-
Y51	8.73	123.9	174	49.8	35.2	K172	7.68	120.7	172.3	53	30
D52	8.61	122.2	173.9	55	37.2	S173	8.21	114.2	170.2	53.6	63.8
Q53	7.93	114.9	172.4	51.4	25.1	A174	8.9	123.9	173.5	47.9	18.3
A54	7.26	120.6	175.3	50.4	15.9	D175	8.48	122.4	173.1	52.8	37.8
T55	9.26	121.1	170.4	57.6	68.7	F176	7.88	125.8	170.7	54.7	36.1
S56	9.88	127	170.8	55.9	60.6	T177	7.97	115.4	171.8	57.3	68
L57	8.81	118	175.4	52.5	42	N178	9.58	115.9	171.5	51.4	33.9
R58	7.07	115.3	169	52	30.3	F179	8.14	119.4	171.9	55.3	37.6
I59	8.91	122.6	169.7	54.7	38.2	D180	7.89	128.6	173.2	46.6	37.9
L60	8.56	124.1	172.1	51.2	41.6	P181	-	-	174.2	60.9	27.7
N61	8.31	121.5	173.8	48.8	35.5	R182	8.19	118.7	176.4	56.2	25.7
N62	7.9	123.7	174.1	48.4	35.3	G183	7.16	103.3	171.2	42.8	-
G63	9.56	109.7	170.2	42.1	-	L184	7.1	117.8	170.5	50.6	38.1
H64	-	-	-	-	-	L185	6.49	113.6	174.1	49.7	37.7
A65	8.46	123.9	172.6	49	16.5	P186	-	-	171.9	59	28.7
F66	7.07	110.5	171.2	52	38.5	E187	8.03	116.9	174.2	55.4	26.3
N67	8.85	121.2	172.4	47.3	36.6	S188	7.5	111.9	173	53.5	61
V68	8.53	122.4	172.5	58.3	27.2	L189	8.51	126.2	173.7	50.3	37.1
E69	8.35	124.3	173.4	52.1	28.7	D190	7.3	122.4	174.2	52.7	36.9
F70	8.51	119.5	172.2	53.8	38.5	Y191	8.64	119.4	169.8	53.8	39.8
D71	8.32	117.4	173.5	51.4	38	W192	9.42	117.6	174.3	54.7	27.9
D72	8.82	132.1	173.8	49.8	37	T193	9.57	115.6	168.1	55.3	67.5

Residue	HN	N	CO	Cα	Cβ	Residue	HN	N	CO	Cα	Cβ
S73	8.59	116.1	171.4	57.7	60.9	Y194	7.9	126	168.3	53.2	36
Q74	7.53	118.1	172.7	50.4	30.1	P195	-	-	174.1	58.6	27.8
D75	8.76	123.4	172	51.8	35.6	G196	9.23	111.2	171.3	43	-
K76	7.85	124.6	172.5	54.7	31	S197	8.56	120.5	173.2	53.9	67.1
A77	7.93	122.9	172.2	48.1	15.6	L198	7.89	118.7	177.4	52.3	39.9
V78	8.23	117.3	169.8	55.2	33.1	T199	-	-	-	-	-
L79	8.84	123.2	171.8	49.7	42.4	T200	6.84	109.2	169.2	53.4	65.8
K80	8.28	120.4	172	51.4	33.2	P201	-	-	-	-	-
G81	8.92	107	171.4	41.2	-	P202	-	-	172.3	61	30.2
G82	6.97	109.5	171.4	41.4	-	L203	9.01	120.9	170.8	53.9	33.4
P83	-	-	172.6	59.5	28.4	L204	5.8	111.7	176.3	52.3	37.9
L84	7.66	120.9	174.3	51.4	37.5	E205	8.55	125.4	174.1	52.8	22.9
D85	8.95	125.6	173	50	38	C206	7.34	114.1	170.7	52.9	63.1
G86	7.79	110.1	170.3	41.2	-	V207	7.09	117.2	173.6	58.3	32.2
T87	8.35	116.9	170	59.5	67	T208	8.22	125.8	170.3	60.2	64.9
Y88	8.3	126.1	172.6	52.1	36	W209	8.1	129.7	173.9	55.5	27.7
R89	8.48	123.4	173.3	52.5	29.8	I210	8.65	127.8	170.7	58	35.9
L90	8.4	124.4	172	52.7	38	V211	9.6	128.8	172.7	58	30
I91	-	-	-	-	-	L212	8.8	126.9	173.9	52.2	36
Q92	7.15	115.6	172.3	51.8	26.4	K213	7.26	122.8	174.2	54.5	28.9
F93	8.47	113.9	169.7	52.8	41.3	E214	8.77	122.2	172.1	50.4	27.2
H94	8.06	113.5	168.3	52.6	28.6	P215	-	-	176.1	58.6	29.7
F95	9.01	117.6	169.8	52.7	40.1	I216	8.81	114	171.7	56.9	37.4
H96	8.62	115.6	171.6	51.2	28	S217	7.95	117	170.1	53.8	61.7
W97	9.39	119.6	172.7	55.5	28	V218	8.41	116.4	172	55.7	30.3
G98	7.94	108.4	172.1	41	-	S219	8.28	116.4	174.1	53.2	62.4
S99	8.26	112.4	170.7	55.4	59.4	S220	9.17	117.7	174	58.8	62.5
L100	7.21	119.5	174.8	50.4	41.7	E221	8.27	118.9	175.8	56.6	25.8
D101	8.76	120.9	173.6	54	36.8	Q222	7.4	118.4	173.6	56.3	24.8
G102	7.54	103.3	169.6	42.3	-	V223	7.23	115.2	174.8	61.4	27.9
Q103	7.71	115.5	168.6	50.1	27.2	L224	7.87	121.3	176.6	55.1	37.9
G104	8.11	106.6	168.2	43	-	K225	6.77	115.5	176.9	55.2	27.8
S105	7.21	108.2	170	55	60	F226	6.98	119.2	173.1	55.5	30.2
E106	8.16	119.5	175	55.4	27.1	R227	6.55	109.5	172.5	53.9	26.1
H107	10.56	118.2	170.9	56.9	25.5	K228	6.72	114.6	175.2	52	29.2
T108	7.22	109.8	170.7	56.2	68.3	L229	6.97	119.7	173.3	52.6	40.2
V109	7.5	119	174.4	57.9	30.1	N230	8.78	118.8	171.8	48.8	38.1
D110	9.57	132.8	173.6	53.3	35.8	F231	8.75	117.5	173.9	56.2	37.7
K111	9.72	110.4	173.1	55	26.5	N232	8.03	111	172.9	50.1	37.8
K112	8.1	125.1	172.1	53.5	29	G233	8.54	105.2	171.7	40.9	-
K113	7.84	122.4	173.9	51.9	31.7	E234	8.26	121.9	174.9	54.9	26.1
Y114	8.06	121.8	170.8	54.6	36	G235	8.94	114.6	171	42.1	-
A115	7.44	121.3	175.2	50.6	15.9	E236	7.23	120.5	170.6	50.6	26.5
A116	8.01	112.5	172.7	48.2	18.7	P237	-	-	173.8	60.1	-
E117	9.3	121.4	170.2	52.8	32.5	E238	8.12	123.7	174.1	54.8	27.4
L118	9.77	130.9	171.8	50	39.9	E239	8.89	130.3	172.5	52.2	29.5
H119	8.87	125.6	171	49	29.9	L240	8.62	126.3	173.1	53	38
L120	8.9	124.1	172	52.5	38.5	M241	8.5	123.5	168.3	50.9	28.3
V121	9.07	126.9	170.9	60.8	29.3	V242	6.59	115.4	171.7	56	34.1
H122	8.41	124.6	-	50.8	37.8	D243	7.01	115.2	172.2	53.3	34.8
W123	8.95	119.5	171.4	50.6	30.3	N244	8.26	119.8	170.4	47.5	32.4
N124	8.94	120.3	174.3	50.3	35.6	W245	6.35	114.7	170.2	47.9	29.3
T125	8.07	117.9	174	60.9	65.3	R246	10.29	127.3	171.1	49.5	29.1
K127	7.81	123.4	174.6	55.1	28.4	P247	-	-	173.3	58.7	29.4
Y128	7.56	116.1	174.3	54.7	35.1	A248	7.86	120.7	174.3	50.3	14.9

Residue	HN	N	CO	Cα	Cβ	Residue	HN	N	CO	Cα	Cβ
G129	7.73	107.9	170.5	44.7	-	Q249	8.58	122.5	170.1	48.5	24.9
D130	7.3	116.8	171.4	49.5	39.6	P250	-	-	174.6	59.5	28
F131	7.48	120.1	172.5	58.3	36.1	L251	8.55	126.3	176	54.1	38.6
G132	7.95	105	173.9	43.3	-	K252	8.74	115.1	174	54.4	25.9
K133	7.5	120.1	176.4	54.8	29.3	N253	8.57	120.2	172.2	50.6	34.7
A134	7.8	123.8	176.8	52.1	16.3	R254	7.17	119.1	171.7	52.9	30.8
V135	7.8	113.9	171	60.7	27.6	Q255	8.46	118.7	172	51.2	28
Q136	6.44	113.9	173.5	51.9	26.1	I256	8.79	124.8	173.5	58	34.1
Q137	7.54	119.6	172.7	49.2	25.2	K257	8.66	127	172.4	51.3	32.5
P138	-	-	173.5	61.9	28.3	A258	8.23	124	173.7	46.3	18.7
D139	7.77	115	175.6	48.8	35.7	S259	8.84	116.9	169.4	55.7	60.4
G140	8.15	108.7	171.7	45.3	-	F260	6.51	118.4	168.4	50.3	38.7
L141	8.63	116.1	174.5	49.3	43.3	K261	7.62	124.9	178.7	54	30
[a] Residues 1 to 20 were not assigned since no unambiguous assignment was possible.											

Synthesis of Ln-DOTA-M8-(8S)-SSPy

Ln-DOTA-M8-(8S)-SSPy and Ln-M7PyThiazole-DOTA used in this study were synthesized according to the published procedures by Häussinger et al. and Müntener et al.[14,15]

Protein tagging

General procedure for Ln-DOTA-M8-(8S)-SSPy tagging: 2 to 7.5 mg of protein were dissolved in 200 µL to 500 µL of 10 mM PO_4^{3-} pH 6.8 to yield a 300 to 350 µM solution. The concentration was confirmed by UV absorption at 280 nm (ε_{280} =54700 $M^{-1}cm^{-1}$)[8]. 100 mM TCEP solution at neutral pH was added to obtain a final concentration of 1 mM TCEP. The sample was kept at 4 ◦C overnight. The full reduction of the protein was confirmed by ESI-MS (in the case of not complete reduction, concentrations of up to 10 mM TCEP in the protein sample were successfully applied). The buffer was exchanged to 10 mM PO_4^{3-}, 100 µM TCEP pH 6.8 by ultrafiltration using 4 ml Amicon Ultrafiltration tubes (MW cut-off 10 kDa) in 4 centrifugation steps. In each step the spinning time was adjusted that the sample volume did not decrease below 500 µL to avoid aggregation of the protein. The TCEP concentration was further decreased using a PD MiniTrap G-25 desalting column and the protein was eluted directly into 3.0 eq. of the desired Ln-DOTA-M8-(8S)-SSPy in aqueous solution and kept under agitation overnight.

Modification of the procedure for Ln-M7PyThiazole-DOTA tagging: The reduced monomeric protein was tagged directly after the buffer exchange to 100 µM TCEP pH 6.8 by adding 6.0 eq. of Ln-M7PyThiazole-DOTA in acetonitrile and keeping the solution under agitation overnight.

Table 7: Overview on the Ln-DOTA-M8-(8S)-SSPy protein mutants and the determined m/z by ESI-MS.

Product	Concentration[a] (µM)	ESI-MS (m/z)
unif. ^{15}N hCA II S50C C206S Tm-DOTA-M8	90	30187
unif. ^{15}N hCA II S50C C206S Lu-DOTA-M8	180	30193
sel. ^{15}N-Leu hCA II S50C C206S Tm-DOTA-M8	560	29856
sel. ^{15}N-Leu hCA II S50C C206S Lu-DOTA-M8	550	29862
unif. ^{15}N hCA II S166C C206S Tm-DOTA-M8	320	30189
unif. ^{15}N hCA II S166C C206S Lu-DOTA-M8	210	30195
sel. ^{15}N-Leu hCA II S166C C206S Tm-DOTA-M8	230	29857
sel. ^{15}N-Leu hCA II S166C C206S Lu-DOTA-M8	240	29864
unif. ^{15}N hCA II S173C C206S Tm-DOTA-M8	340	29859
sel. ^{15}N-Leu hCA II S173C C206S Tm-DOTA-M8	440	29864
sel. ^{15}N-Leu hCA II S173C C206S Lu-DOTA-M8	520	30180
unif. ^{15}N hCA II S217C C206S Tm-DOTA-M8	280	30192
unif. ^{15}N hCA II S217C C206S Lu-DOTA-M8	200	30199
sel. ^{15}N-Leu hCA II S217C C206S Tm-DOTA-M8	240	29859
sel. ^{15}N-Leu hCA II S217C C206S Lu-DOTA-M8	400	29864
unif. ^{15}N hCA II S220C C206S Tm-DOTA-M8	240	30190
unif. ^{15}N hCA II S220C C206S Lu-DOTA-M8	200	30195
sel. ^{15}N-Leu hCA II S220C C206S Tm-DOTA-M8	270	29857
sel. ^{15}N-Leu hCA II S220C C206S Lu-DOTA-M8	500	29863
unif. ^{2}H ^{13}C ^{15}N hCA II S50C C206S Tm-DOTA-M8	330	32952
unif. ^{2}H ^{13}C ^{15}N hCA II S50C C206S Lu-DOTA-M8	460	32902
[a] Final concentration of the NMR sample (Volume = 280 µL)		

Upon detection of completion by ESI-MS (> 95%), the excess of tag was removed by ultrafiltration with 10 mM PO_4^{3-} pH 6.8 in 4 centrifugation steps to ensure that more than 99% of the tag has been removed. Apart from the last step, again the sample volume was not allowed to decrease below 500 µL. In the final step, the sample volume was reduced to around 250 µL. The sample was removed and the centrifugation tube was washed to obtain a final volume of 280 µL. 5% D_2O were added and the pH was adjusted to 6.80. The concentration was determined again by UV absorption and the sample was transferred into a Shigemi NMR tube.

Refinement of the magnetic susceptibility tensors

The anisotropy tensors were obtained by fitting the obtained pseudocontact shifts to the X-ray structure of ligand-bound human carbonic anhydrase II (3KS3) using the program Numbat. Protons were added to the crystal structure using the HAAD algorithm.[16]

When the calculated PCS were compared with the experimental shifts apart from the S166C mutant, few PCS showed deviations of more than 0.2 ppm. After close examination of the NMR spectra and the X-ray structure possible reasons explaining these deviations were found:

• The approach in this study is based on the assumption that the protein structure in buffered solution is comparable to the X-ray structure used for the determination of the magnetic susceptibility tensor. Based on the large number of PCS that could be assigned, the protein in solution has to have high structural similarity to the available X-ray structure. This is not necessarily true for every part of the protein. Crystal artefacts arising from the crystal packing, as well as dynamic behaviour of flexible loop regions in solution are both well-known phenomena explaining structural differences in certain parts of the protein.

• The X-ray structure available was recorded from wild type protein. But the PCS were determined from double mutants which were tagged with Ln-DOTA-M8-SSPy. Both, the tagging as well as the mutations can cause small structural differences. Especially for residues close to the tag only a very small displacement in their position compared to the X-ray structure can lead to large differences in the PCS due to their r^{-3} distance dependence.

• In the ^{1}H-^{15}N HSQC spectra of uniformly labelled protein, peak overlap can limit the precision of the determined PCS. This is more pronounced for the ^{15}N dimension because of lower resolution and a larger spectral window.

Based on these considerations we defined three criteria upon which we excluded some PCS from the final tensor calculation:

i) If the weighted chemical shift distance of Lu-DOTA-M8-SSPy tagged compared to untagged protein is larger than 5σ, where σ is the standard deviation of unaffected residues, ^{1}H and ^{15}N PCS were removed regardless of the according PCS deviation.

ii) If the PCS deviation is larger than a defined cut-off and the residue is either in a flexible loop region of the protein or at the edges of a secondary structure element close to the tag, ^{1}H and ^{15}N PCS were removed.
iii) If the PCS deviation is larger than a defined cut-off and peak overlap in the ^{1}H-^{15}N HSQC spectra could explain the observed PCS deviation. In this case only the PCS of the concerned nucleus was removed.

The aim of the first criterion is to detect residues whose position could be affected by the tagging with Ln-DOTA-M8-SSPy. We decided to follow the approach of chemical shift mapping, where chemical shift differences are used to determine and characterize protein ligand or protein-protein interaction sites.[17] In this case, the site where the tag is bound to the protein is already known, but information could be obtained about which residues are strongly affected by the tagging. Chemical shift changes show changes in the chemical environment of the observed nuclei. This has not necessarily to be caused by a structural change in the protein, but nevertheless it is a possibility. The number of PCS compared to the full set, that were excluded based on this criteria, consisted of only 5% or even less PCS. Therefore, priority was laid on exclusion of candidates whose structure may differ instead of maximizing the number of PCS per subset. Chemical shift changes between untagged and Lu-DOTA-M8-SSPy tagged protein were determined as weighted chemical shift distance with a weighting factor of 0.25 for the nitrogen shift.
Well separated peaks in the ^{1}H-^{15}N HSQC spectra which were not shifted after tagging were used to determine the standard deviation of the chemical shift differences (σ) of unaffected peaks. Only residues showing larger weighted shift differences than 5σ were excluded from the PCS set. The value of 5σ was obtained experimentally by comparing different sizes of this cut-off, in order to keep the number of excluded residues as small as possible, but still observe a significant improvement of the determined tensor values.
The second criterion takes the possibility into account that loop regions of a protein in solution can adopt a different conformation than in the X-ray structure. As well as to cover for possible structural changes due to the serine to cysteine mutation, which could not be approached by chemical shift changes, because no wild type protein was available for such a comparison.
The third criterion considers residues where the corresponding peak in the ^{1}H-^{15}N HSQC overlaps with another signal either the diamagnetic or in the paramagnetic spectra. It was found that in several cases one of the two PCS (in most cases the ^{1}H PCS) fitted well to the tensor whereas the deviation of the other PCS could be explained by the lowered accuracy of the shift in the respective dimension due to the overlap. Therefore, only the deviating PCS was removed.
To see whether the exclusion of different number of PCS has a large influence on the tensor, we decided to define three different subsets of PCS each with a different cut-off for the PCS deviation. The values chosen were 0.2 ppm, 0.15 ppm and 0.1 ppm respectively. The three corresponding subsets will be called further on set 1 to 3 where for set 1 all PCS with deviations exceeding 0.2 ppm were excluded leaving set 3 to be the one with the smallest PCS deviations. The defined cut-off was chosen as 0.2, 0.15 and 0.1 ppm to have even with the strictest criteria ~90% of the residues left in order to guarantee a sufficient number of PCS for the determination of the anisotropy tensor.
In the following section, it will be discussed in detail for each mutant individually, which PCS have been removed and what impact on the tensor the three different subsets have.

S50C tensor

For this mutant a total of 410 PCS (^{1}H and ^{15}N) for 205 residues could be assigned unambiguously. This corresponds to 85% of all residues or to 95% of those residues where a reasonable assignment was available.

Set 1: In total 28 PCS were removed for set 1 to obtain a set where the largest PCS deviation was below 0.2 ppm. This set consists of 93% of all available PCS.
• Residues 22, 23, 36, 47, 54, 72, 74, 82, 87, 88, 95 and 198 showed a shift of more than 5σ when the protein was tagged with Lu-DOTA-M8-SSPy. Of these residues 47 to 88 are all found in the β-sheet where the tag is attached, in the parallel β-sheet or in the adjacent loop regions to the two β-sheet. Except residue 88 is the first residue of the second next β-sheet. For all these residues it is very likely that they are influenced by the attachment of the tag. Residues 22, 23, 36, 95 and 198 were found further away from the tag. Nevertheless, they were removed from the tensor set to exclude all residues with possible structural changes. Of these residues both PCS (^{1}H and ^{15}N) were removed.
• After excluding the residues mentioned above, residues 71 and 89 still showed a PCS deviation of more than 0.2 ppm. Both residues were found in a loop region close to the tag and next to a residue that showed a large chemical shift change. Therefore, both PCS of these residues were excluded from set 1.

Set 2: In total 32 PCS were removed for set 2 (all PCS that were excluded for set 1 and 4 additional), to obtain a set where the largest PCS deviation was below 0.15 ppm. This set consists of 92% of all available PCS.
• Residue 189 was found in a loop region close to the tag. Both PCS of this residue were excluded.
• The deviation of ^{15}N PCS of residue 197 could be explained by peak overlap in the ^{1}H-^{15}N HSQC spectra.

• After exclusion of all PCS above, the ^{15}N PCS of residue 119 still showed a PCS deviation of more than 0.15 ppm. For this PCS no explanation of the deviation could be found. Nevertheless, it could be shown that the exclusion of this PCS did not have an influence on the tensor parameters. Therefore, it was decided to remove this PCS in order to exclude any possible source of errors.

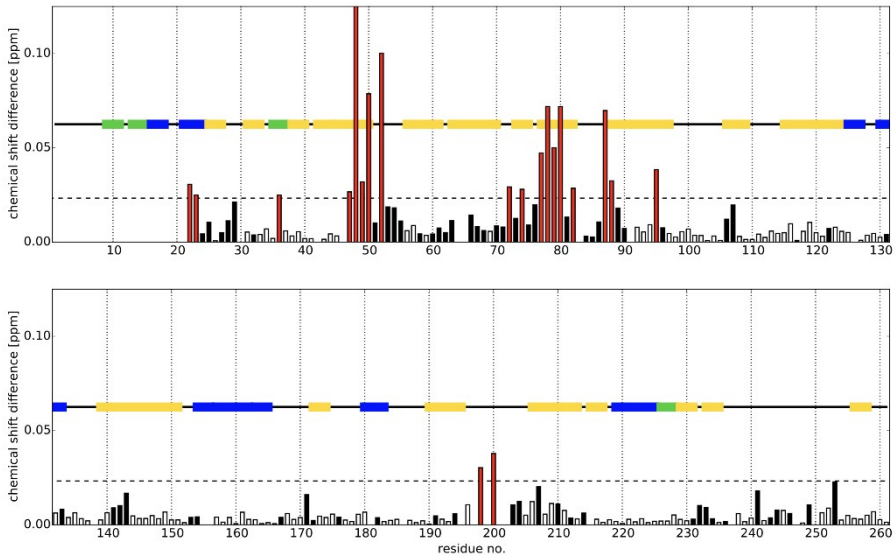

Figure 1: Plot of weighted chemical shift differences of hCA II S50C C206S-Lu-DOTA-M8 compared to untagged protein, calculated using $0.5[(\Delta\delta_{1H})^2+(0.25(\Delta\delta_{15N}))^2]^{0.5}$. Residues with well separated signals in the ^{1}H-^{15}N HSQC spectra used for calculation of the standard uncertainty (σ) of the peak position are shown in white. The dashed line indicates the 5σ cut-off. Residues exceeding this value were excluded from tensor calculation (shown in red). Secondary structure elements of the protein are indicated (β-sheets: yellow, helices: blue and hydrogen bonded turns: green).

Set 3: In total 44 PCS were removed for set 3 (all PCS that were excluded for set 2 and 12 additional), to obtain a set where the largest PCS deviation was below 0.10 ppm. This set consists of 89% of all available PCS.
• Residue 84 and 190 were found in a loop region close to the tag. Both PCS were removed for these residues.
• The deviation of ^{15}N PCS of residues 32, 60, 96, 106, 128 and 203 could be explained by peak overlap in the ^{1}H-^{15}N HSQC spectra.

• After exclusion of all PCS above, the ^{15}N PCS of residues 29 and 135 still showed a PCS deviation of more than 0.1 ppm. For these PCS again no explanation of the deviation could be found. Nevertheless, it could be shown, that the exclusion of these PCS did not have an influence on the tensor parameters. Therefore, it was decided to remove these PCS too in order to exclude any possible source of errors.

Magnetic susceptibility tensors of the different subsets (S50C tensor)

When the tensor parameters obtained from the different subsets were compared with each other, only very small effects on the tensor parameters were found (Table 8). Obviously the determined uncertainties decreased when a more consistent set of PCS was used for the tensor calculation. The differences in the tensor parameters for the two Monte-Carlo methods were much smaller for the three subsets than for the full set of PCS for this mutant.

Table 8: Tensors of the S50C mutant for the different subsets of PCS.

Parameter	Unit	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
$\Delta\chi_{ax}$	[10^{-32} m^3]	20.8 ± 1.2	21.1 ± 1.2	21.6 ± 1.2	20.7 ± 1.1	20.9 ± 1.1	21.1 ± 0.9
$\Delta\chi_{rh}$	[10^{-32} m^3]	8.4 ± 0.7	8.4 ± 0.7	8.5 ± 0.7	8.5 ± 0.7	8.5 ± 0.7	8.5 ± 0.6
x	[Å]	-27.5 ± 0.3	-27.6 ± 0.3	-27.8 ± 0.3	-27.3 ± 0.4	-27.4 ± 0.4	-27.5 ± 0.3
y	[Å]	13.7 ± 0.3	13.7 ± 0.3	13.7 ± 0.3	13.7 ± 0.3	13.7 ± 0.3	13.6 ± 0.2
z	[Å]	18.3 ± 0.3	18.3 ± 0.3	18.1 ± 0.3	18.3 ± 0.3	18.3 ± 0.3	18.2 ± 0.2
α	[°]	104.8 ± 1.6	104.8 ± 1.6	104.1 ± 1.6	103.9 ± 2.3	103.8 ± 2.2	104.0 ± 1.8
β	[°]	141.8 ± 1.1	141.8 ± 1.1	142.3 ± 1.1	141.8 ± 1.0	141.7 ± 1.0	141.8 ± 0.8
γ	[°]	116.4 ± 1.7	116.4 ± 1.7	116.2 ± 1.7	116.2 ± 2.1	115.9 ± 1.9	115.9 ± 1.7
Error analysis:		Monte-Carlo structure variation with a σ = 0.5 Å			Monte-Carlo protocol for random subset selection of 20% of PCS		

S166C tensor

For this mutant in total 416 PCS corresponding to 208 residues could be assigned. These are 86% of all residues that show a resonance in the ^{1}H-^{15}N HSQC spectra, or 97% of those residues, where a reasonable assignment was available.

Set 1: Only 2 residues (4 PCS) were removed for this set. It consists of 99% of all available PCS. Residue 167 and 168 both showed a PCS deviation smaller than 0.2 ppm but their PCS could only be assigned tentatively, because unambiguous assignment of their corresponding peaks in the diamagnetic spectra was not possible due to the large change in the chemical shift upon tagging. Anyway these residues would have been excluded due to the criteria that was defined based on the change in the chemical shift. Every possible candidate in the diamagnetic spectra for these two residues would have shown a change in the chemical shift of more than 5σ compared to the untagged protein.

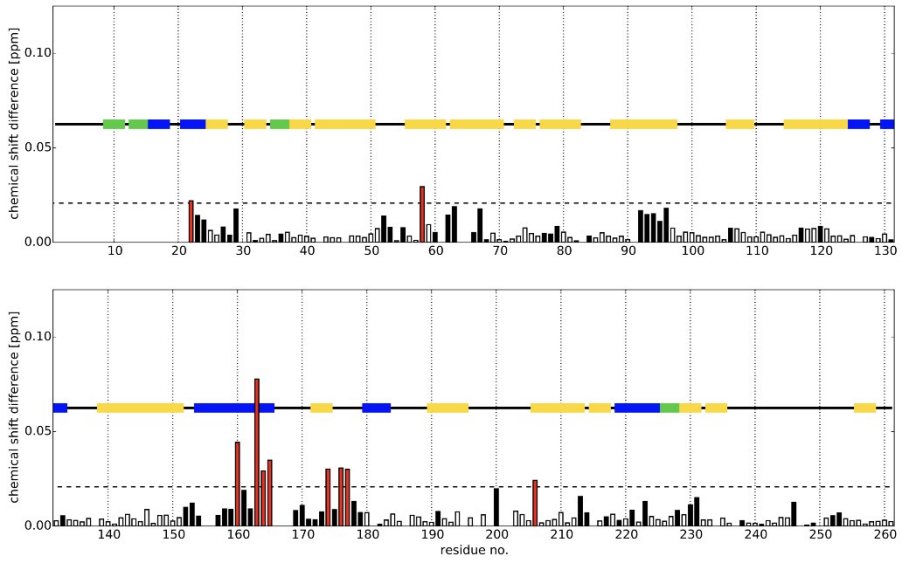

Figure 2: Plot of weighted chemical shift differences of hCA II S166C C206S-Lu-DOTA-M8 compared to untagged protein, calculated using $0.5[(\Delta\delta_{1H})^2+(0.25(\Delta\delta_{15N}))^2]^{0.5}$. Residues with well separated signals in the ^{1}H-^{15}N HSQC spectra used for calculation of the standard uncertainty (σ) of the peak position are shown in white. The dashed line indicates the 5σ cut-off. Residues exceeding this value were excluded from tensor calculation (shown in red). Secondary structure elements of the protein are indicated (β-sheets: yellow, helices: blue and hydrogen bonded turns: green).

Set 2: Set 2 consists of 402 PCS which corresponds to 96% of all assigned PCS. In total 14 PCS of 7 different residues were excluded.
- Residues 22, 58, 160 and 206 were removed due to the chemical shift change.
- Residue 172 was excluded according criteria (ii) as it was found at the edge of a β-sheet close to the tag.

Set 3: In total 19 PCS were removed for this set, all from set 2 and 5 additional PCS which showed a PCS deviation of more than 0.1 ppm. This set consists of 95% of all available PCS.

• The ^{15}N PCS of residues 75, 79, 197 and 235, as well as the ^{1}H PCS of residue 171 were excluded due to peak overlap.

Magnetic susceptibility tensors of the different subsets (S166C tensor)

The tensor parameters show almost no differences, depending on the different PCS subsets and even compared to the full set (Table 9). This shows the excellent agreement of PCS and protein structure for this mutant. When compared to the S50C mutant, larger differences are found for the two Monte-Carlo methods. Where the uncertainties for the subset selection were in the same range as for S50C, the uncertainties for the structure variation turned out to be around twice as large. This supports the good agreement between X-ray structure and experimental PCS, since the distortion of the protein structure leads to a decreased agreement to the recorded PCS. This effect could be amplified by the larger average PCS determined for this mutant, because the larger a PCS the higher the sensitivity to structural changes.

Table 9: Tensors of the S166C mutant for the different subsets of PCS.

Parameter	Unit	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
$\Delta\chi_{ax}$	[10^{-32} m^3]	38.7 ± 2.0	38.7 ± 1.9	38.5 ± 2.0	37.3 ± 1.1	37.6 ± 1.0	37.4 ± 0.9
$\Delta\chi_{rh}$	[10^{-32} m^3]	7.5 ± 1.2	8.4 ± 1.0	8.4 ± 1.0	7.8 ± 0.9	8.3 ± 0.8	7.8 ± 0.6
x	[Å]	-16.1 ± 0.5	-16.4 ± 0.4	-16.3 ± 0.4	-16.1 ± 0.3	-16.3 ± 0.3	-16.2 ± 0.2
y	[Å]	-3.5 ± 0.3	-3.6 ± 0.4	-3.6 ± 0.4	-3.5 ± 0.2	-3.6 ± 0.2	-3.6 ± 0.2
z	[Å]	-11.2 ± 0.4	-11.2 ± 0.4	-11.2 ± 0.4	-10.9 ± 0.3	-11.0 ± 0.2	-11.0 ± 0.2
α	[°]	51.5 ± 2.0	52.8 ± 1.8	52.2 ± 1.8	52.7 ± 1.5	53.3 ± 1.4	52.7 ± 1.2
β	[°]	123.1 ± 1.3	123.6 ± 1.4	123.6 ± 1.4	122.7 ± 0.9	123.0 ± 0.8	123.1 ± 0.7
γ	[°]	143.1 ± 5.0	140.5 ± 5.6	140.3 ± 5.6	142.7 ± 3.6	141.5 ± 3.0	141.1 ± 2.7
Error analysis:		Monte-Carlo structure variation with a σ = 0.5 Å			Monte-Carlo protocol for random subset selection of 20% of PCS		

S217C tensor

In total 398 PCS could be assigned for this mutant, these cover 82% of all backbone amide NH groups or 91% of the residues where a reasonable assignment was possible.

Set 1: Set 1 consists of 94% of all assigned PCS. 22 PCS were excluded.
• Residues 62, 93, 113, 116, 148, 190, 220, 221, 223 and 246 were removed due to their chemical shift change
• Residue 112 was found in a loop region close to the tag.
As already discussed, residue 148 showed a strong indication that the determined PCS does not fit to the position in the X-ray structure. Here this residue was excluded because of the chemical shift change. This shows that this method is suitable to sort out residues which are affected by structural changes due to the tagging of the protein.

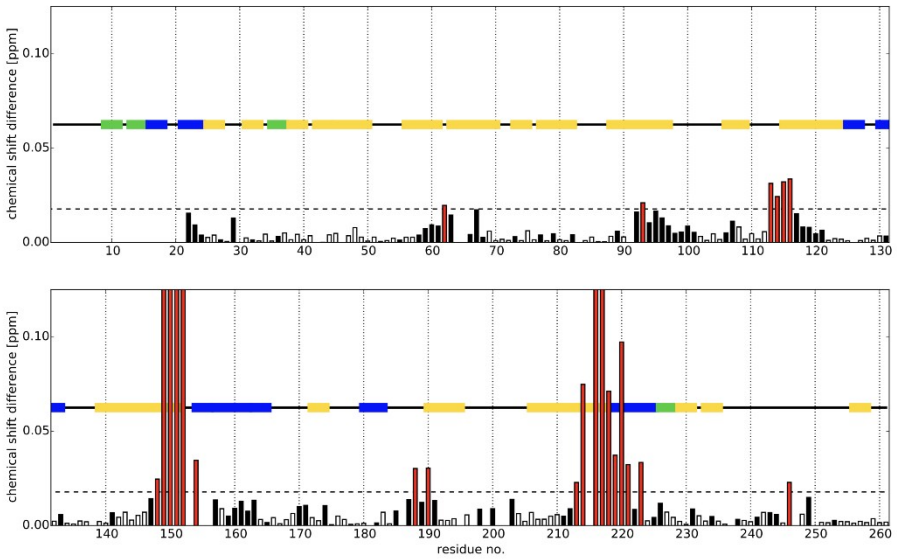

Figure 3: Plot of weighted chemical shift differences of hCA II S217C C206S-Lu-DOTA-M8 compared to untagged protein, calculated using $0.5[(\Delta\delta_{1H})^2+(0.25(\Delta\delta_{15N}))^2]^{0.5}$. Residues with well separated signals in the ^{1}H-^{15}N HSQC spectra used for calculation of the standard uncertainty (σ) of the peak position are shown in white. The dashed line indicates the 5σ cut-off. Residues exceeding this value were excluded from tensor calculation (shown in red). Secondary structure elements of the protein are indicated (β-sheets: yellow, helices: blue and hydrogen bonded turns: green).

Set 2: For set 2 all PCS of set 1 and 4 additional (in total 26 PCS) were removed. Set 2 consists of 93% of all available PCS.
• Residue 147 was found at the edge of a β-sheet close to the tag. This residue is next to L148 which already showed several indications for a very likely structural change.
• Residue 191 was found in a loop region close to the tag.

Set 3: This set consists of 91% of all assigned PCS. In total 34 PCS were excluded (all from set 2 and 8 additional).
• Residues 101, 109, 110 and 111 were removed according to criteria (ii). Their PCS deviation exceeded 0.1 ppm and in addition they were found in a loop region close to the tag.
For this subset, 34 PCS were removed to obtain a set where the largest PCS deviation is below 0.1 ppm. In contrast to 86 PCS that showed a PCS deviation larger than 0.1 ppm when the full set of PCS was used. This clearly shows that it was possible to increase the overall agreement of PCS and X-ray structure by a proper selection of the right subset.

Magnetic susceptibility tensors of the different subsets (S217C tensor)

The tensor parameters of the different subsets for this mutant do not show differences worth mentioning (Table 10). Whereas the differences to the tensor parameters determined for the full set of PCS clearly show the effect of the refinement.

Table 10: Tensors of the S217C mutant for the different subsets of PCS.

Parameter	Unit	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
$\Delta\chi_{ax}$	[10^{-32} m^3]	26.0 ± 1.0	25.8 ± 1.0	25.7 ± 1.0	25.5 ± 1.4	25.4 ± 1.3	25.5 ± 1.1
$\Delta\chi_{rh}$	[10^{-32} m^3]	13.4 ± 0.6	13.4 ± 0.5	13.2 ± 0.6	13.2 ± 0.7	13.2 ± 0.7	13.1 ± 0.6
x	[Å]	-24.9 ± 0.2	-24.9 ± 0.2	-24.9 ± 0.2	-24.7 ± 0.3	-24.7 ± 0.3	-24.8 ± 0.2
y	[Å]	-17.9 ± 0.3	-17.8 ± 0.3	-17.7 ± 0.3	-17.6 ± 0.4	-17.6 ± 0.3	-17.5 ± 0.3
z	[Å]	19.6 ± 0.2	19.7 ± 0.3	19.6 ± 0.2	19.7 ± 0.3	19.7 ± 0.3	19.6 ± 0.2
α	[°]	143.7 ± 0.8	143.7 ± 0.8	143.7 ± 0.8	143.4 ± 1.0	143.3 ± 0.9	143.3 ± 0.8
β	[°]	70.4 ± 0.6	70.3 ± 0.5	70.9 ± 0.5	70.6 ± 0.9	70.7 ± 0.8	71.2 ± 0.7
γ	[°]	126.1 ± 1.0	126.2 ± 1.1	125.5 ± 1.0	125.7 ± 1.3	125.7 ± 1.3	125.1 ± 1.1
Error analysis:		Monte-Carlo structure variation with a σ = 0.5 Å			Monte-Carlo protocol for random subset selection of 20% of PCS		

The tensor parameters showed clear differences depending on the Monte-Carlo method, when the full set of PCS was used. This was not the case any more for the three subsets, showing that PCS and protein structure become more consistent and less dependent on the mode of error estimation.

S220C tensor

For this mutant 396 PCS were assigned, covering 81% of all possible backbone amide PCS, or 90% of those residues where an assignment was available.

Set 1: For set 1 in total 7 residues (14 PCS) were excluded. The set consists of 96% of all assigned PCS.
• Residues 99, 102, 104, 148, 150, 153 and 161 showed a large chemical shift change, when the protein was tagged. All of these residues are found close to the tag.

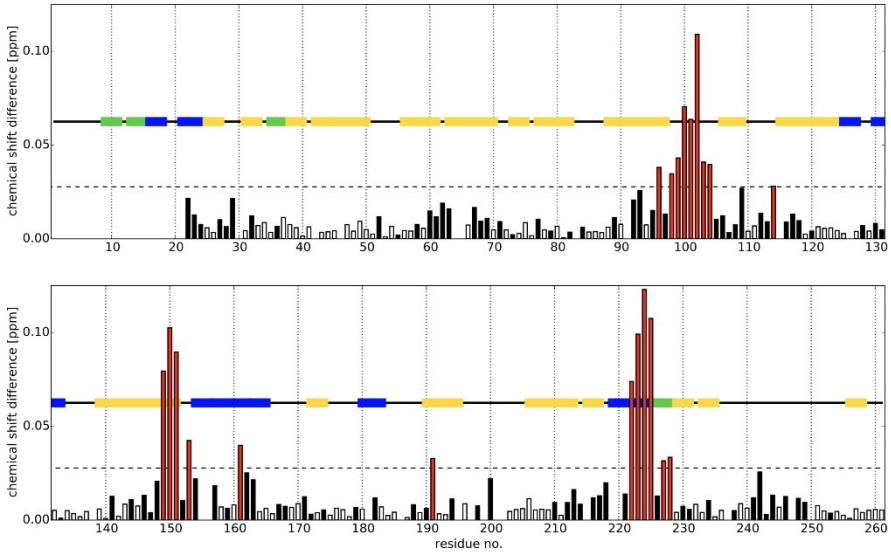

Figure 4: Plot of weighted chemical shift differences of hCA II S220C C206S-Lu-DOTA-M8 compared to untagged protein, calculated using $0.5[(\Delta\delta_{1H})^2+(0.25(\Delta\delta_{15N}))^2]^{0.5}$. Residues with well separated signals in the ^{1}H-^{15}N HSQC spectra used for calculation of the standard uncertainty (σ) of the peak position are shown in white. The dashed line indicates the 5σ cut-off. Residues exceeding this value were excluded from tensor calculation (shown in red). Secondary structure elements of the protein are indicated (β-sheets: yellow, helices: blue and hydrogen bonded turns: green).

Set 2: All residues of set 1 and 4 additional residues (in total 22 PCS) were removed for set 2. It consists of 94% of all available PCS.

• Residues 96, 98 and 114 were removed also because of their chemical shift differences.
• Residue 116 was excluded due to the PCS deviation and because it was found in a loop region close to the tag (according to criteria (ii)).

Set 3: For set 3 in total 28 PCS were removed. It consists of 93% of all assigned PCS.
• Residue 157 was found at the edge of a α-helix close to the tag.
• Residue 229 was found in a loop region close to the tag.
• The ^{15}N PCS of residues 58 and 197 were excluded due to peak overlap in the HSQC spectrum.

Magnetic susceptibility tensors of the different subsets (S220C tensor)

Compared to the tensor parameters determined with the full set of PCS the values clearly show smaller uncertainties (Table 11). When the different subsets are compared with each other the differences in the values of the tensor parameters are only marginal. Except for the Euler angles, where the differences and larger uncertainties (especially for γ) are attributed to the smaller $\Delta\chi_{rh}$, hence a tensor closer to axial symmetry. But what attracts attention, is the fact that the uncertainties for the parameters determined by structure variation are more than twice as small as the uncertainties determined by random subset selection. This is the other way round as found for the S166C mutant before. Possibly the uncertainties determined by structure variation are dependent on the size of the corresponding PCS. For S166C the largest average absolute PCS were determined, where S220C these were the smallest of all four mutants (for S50C and S217C these were in the same order in between the other two mutants). An explanation for the smallest uncertainties obtained for this mutant from the structure variation could be that structure variation with the same magnitude has a smaller effect on the resulting tensor parameters if the average absolute PCS are getting smaller.

Table 11: Tensors of the S220C mutant for the different subsets of PCS.

Parameter	Unit	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
$\Delta\chi_{ax}$	[10^{-32} m^3]	23.2 ± 1.0	23.6 ± 0.9	23.6 ± 0.9	22.5 ± 2.4	22.9 ± 2.3	23.0 ± 2.1
$\Delta\chi_{rh}$	[10^{-32} m^3]	4.0 ± 0.3	4.3 ± 0.3	4.3 ± 0.3	4.1 ± 0.9	4.4 ± 0.9	4.4 ± 0.8
x	[Å]	-13.0 ± 0.2	-13.4 ± 0.3	-13.1 ± 0.3	-13.0 ± 0.7	-13.3 ± 0.7	-13.0 ± 0.6
y	[Å]	-26.2 ± 0.3	-26.6 ± 0.3	-26.7 ± 0.3	-26.0 ± 0.7	-26.3 ± 0.6	-26.4 ± 0.6
z	[Å]	3.1 ± 0.2	3.3 ± 0.2	3.2 ± 0.2	3.1 ± 0.6	3.2 ± 0.6	3.2 ± 0.5
α	[°]	16.7 ± 1.6	14.9 ± 1.3	14.9 ± 1.4	17.8 ± 4.7	16.7 ± 4.0	16.2 ± 3.5
β	[°]	154.2 ± 0.6	154.3 ± 0.6	153.6 ± 0.6	154.3 ± 1.8	154.4 ± 1.7	153.7 ± 1.5
γ	[°]	1.2 ± 3.0	1.8 ± 2.6	1.0 ± 2.6	4.7 ± 8.1	5.4 ± 7.1	3.9 ± 6.6
Error analysis:		Monte-Carlo structure variation with a σ = 0.5 Å			Monte-Carlo protocol for random subset selection of 20% of PCS		

Comparison of tensor parameters for the different protein mutants

As it was shown above, there was no significant difference depending on which subset was used for determination of the tensor parameters. For convenience in this section, the comparison between the four mutants is based only on the tensor parameters determined with the PCS set 3.

After the refinement, the magnitude of the tensor parameters remained the same as for the full set of PCS. $\Delta\chi_{ax}$ for S166C is still almost twice as large as for the other three mutants, where as for $\Delta\chi_{rh}$, the biggest value was found for S217C and the smallest for S220C (Table 12). The most likely reason for the differences is that for the S166C mutant, the paramagnetic metal has the least motional freedom with respect to the protein. For the other mutants, residual dynamic behaviour of the tag could allow different orientations of the lanthanide metal that result in averaged and therefore reduced tensor parameters. Interestingly, this motional freedom has to be similar for all three mutants to result in such comparable tensor parameters.

For the different mutants, an excellent agreement between experimental and back-calculated PCS was found (Figure 5). The plot is shown for both tensor parameters obtained with the different Monte-Carlo methods to show again that there is basically no difference any more between these two approaches of the error analysis.

Table 12: Refined magnetic susceptibility tensor parameters The values and their uncertainties are calculated by a Monte Carlo protocol applying two different random inputs given below.

Parameter	Unit	S50C	S166C	S217C	S220C
$\Delta\chi_{ax}$	[10^{-32} m^3]	21.6 ± 1.2	38.5 ± 2.0	25.7 ± 1.0	23.6 ± 0.9
$\Delta\chi_{rh}$	[10^{-32} m^3]	8.5 ± 0.7	8.0 ± 1.0	13.2 ± 0.6	4.3 ± 0.3
x	[Å]	-27.8 ± 0.3	-16.3 ± 0.4	-24.9 ± 0.2	-13.1 ± 0.3
y	[Å]	13.7 ± 0.3	-3.6 ± 0.4	-17.7 ± 0.3	-26.7 ± 0.3
z	[Å]	18.1 ± 0.3	-11.2 ± 0.4	19.6 ± 0.2	3.2 ± 0.2
α	[°]	104.1 ± 1.6	52.2 ± 1.8	143.7 ± 0.8	14.9 ± 1.4
β	[°]	142.3 ± 1.1	123.6 ± 1.4	70.9 ± 0.5	153.6 ± 0.6
γ	[°]	116.2 ± 1.7	140.3 ± 5.6	125.5 ± 1.0	1.0 ± 2.6
Monte-Carlo structure variation with σ = 0.5 Å.					
$\Delta\chi_{ax}$	[10^{-32} m^3]	21.1 ± 0.9	37.4 ± 0.9	25.5 ± 1.1	23.0 ± 2.1
$\Delta\chi_{rh}$	[10^{-32} m^3]	8.5 ± 0.6	7.8 ± 0.6	13.1 ± 0.6	4.4 ± 0.8
x	[Å]	-27.5 ± 0.3	-16.2 ± 0.2	-24.8 ± 0.2	-13.0 ± 0.6
y	[Å]	13.6 ± 0.2	-3.6 ± 0.2	-17.5 ± 0.3	-26.4 ± 0.6
z	[Å]	18.2 ± 0.2	-11.0 ± 0.2	19.6 ± 0.2	3.2 ± 0.5
α	[°]	104.0 ± 1.8	52.7 ± 1.2	143.3 ± 0.8	16.2 ± 3.5
β	[°]	141.8 ± 0.8	123.1 ± 0.7	71.2 ± 0.7	153.7 ± 1.5
γ	[°]	115.9 ± 1.7	141.1 ± 2.7	125.1 ± 1.1	3.9 ± 6.6
Monte-Carlo protocol where random subsets consisting of 20% of the available PCS were used.					

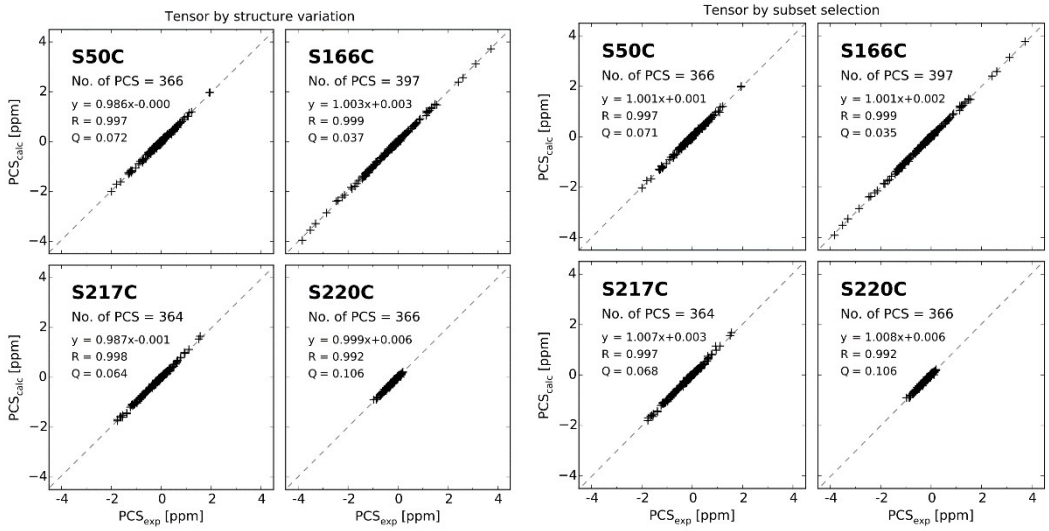

Figure 5: Correlation of experimental and back calculated PCS obtained from tensors determined with the given type of Monte-Carlo protocol using the PCS of set 3.

hCA II ligand complexes

The following ligands were used to form hCA II-ligand complexes: N-(2,3-Difluorobenzyl)-4-sulphamoylbenzamide (9) abbreviated as F2-Inh, N-(4-fluorobenzyl)-4-sulphamoylbenzamide abbreviated as FM-519 and 4-sulphamoyl-N-(4-(trifluoromethyl)benzyl) benzamide abbreviated as FM-520. F2-Inh, FM519 and FM 520 were synthesized by Dr. F. Monnard.[18]

F2-Inh / FM-519 / FM-520 ⊂ hCA II: To a protein sample 1.1 eq. of ligand dissolved in deuterated dimethyl sulphoxide (DMSO-d_6) were added and the sample was stirred well. The excess of ligand was removed by ultrafiltration using 4 ml Amicon ultrafiltration tubes (MW cut-off 10 kDa). Trifluoroacetate as internal standard for fluorine chemical shift calibration, 5% v/v D_2O were added and the pH was adjusted to 6.80.

FM-519 and FM-520 bound to different hCA II constructs

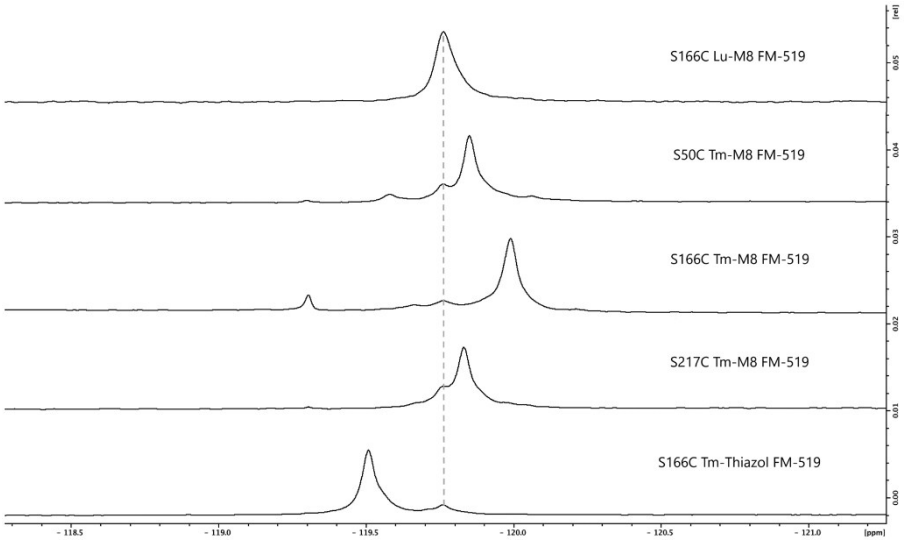

Figure 6: One-dimensional ^{19}F NMR spectra (565 MHz, 298 K) of FM-519 in the presence of different hCA II constructs. ^{19}F chemical shift was calibrated to an internal signal of trifluoroacetate at −79.0 ppm.

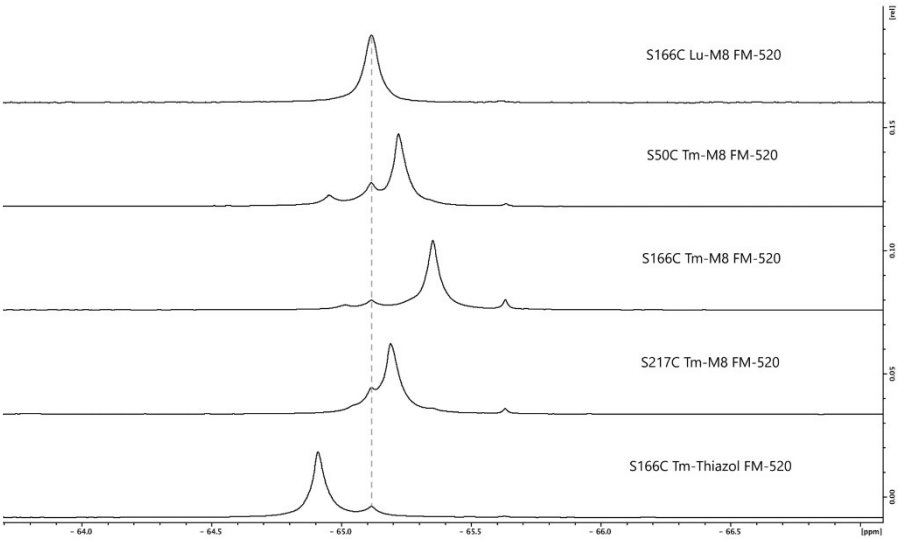

Figure 7: One-dimensional ^{19}F NMR spectra (565 MHz, 298 K) of FM-520 in the presence of different hCA II constructs. ^{19}F chemical shift was calibrated to an internal signal of trifluoroacetate at −79.0 ppm.

Table 13: Observed pseudocontact shifts for FM-519 and FM-520 bound to different hCA II constructs.

Inhibitor	hCA II construct	Observed PCS [ppm]
FM-519	sel. ^{15}N-Leu hCA II S50C C206S Tm-DOTA-M8	-0.093
	sel. ^{15}N-Leu hCA II S166C C206S Tm-DOTA-M8	-0.242
	sel. ^{15}N-Leu hCA II S217C C206S Tm-DOTA-M8	-0.100
	sel. ^{15}N-Leu hCA II S166C C206S Tm-Thiazolo	0.250
FM-520	sel. ^{15}N-Leu hCA II S50C C206S Tm-DOTA-M8	-0.116
	sel. ^{15}N-Leu hCA II S166C C206S Tm-DOTA-M8	-0.238
	sel. ^{15}N-Leu hCA II S217C C206S Tm-DOTA-M8	-0.078
	sel. ^{15}N-Leu hCA II S166C C206S Tm-Thiazolo	0.208

Modification of PDB file 1G54 for FM-519 and FM-520

The PDB file 1G54[19] (*N*-(2,3,4,5,6-pentafluorobenzyl)-4-sulphamoylbenzamide ⊂ hCA- II) was modified using the molecular editor Avogadro[20] applying the following procedures.

FM-519: All fluorine atoms except for the fluorine atom in para-position were deleted from the original ligand structure.

FM-520: All fluorine atoms in the original ligand structure were deleted and a CF_3 group was attached in para-position. The following force field optimization of the newly added atoms yielded the following bond lengths: C-CF_3 = 1.494 Å and C-F = 1.364 Å.

Coordinates: The used coordinates of all fluorine atoms are given in the reference frame of PDB entry 3KS3 in table 14.

Table 14: Used coordinates of the fluorine atoms of F2-Inh, FM-519 and FM-520.

Inhibitor	Fluorine atom	x	y	z
F2-Inh	meta	-3.049	10.475	15.432
	ortho	-3.909	8.122	14.186
FM-519	para	-1.027	12.945	14.759
FM-520	CF_3	-1.201	12.335	14.479

Shift list comparison

Shift lists for Tm- and Lu- M8-DOTA attached to hCA II S50C, S166C, S217C, and S220C are available in Suturina et al.[21]

Table 15: Shift list comparison of ^{1}H-^{15}N HSQC spectra of Tm- and Lu- M7PyThiazole-DOTA attached to selectively ^{15}N-Leu labelled hCA II S166C.

Residue	Reson. 1	Shift Tm	Shift Lu	Reson. 2	Shift Tm	Shift Lu
44Leu	H	7.098	7.001	N	123.231	123.081
47Leu	H	9.106	8.975	N	126.191	126.080
57Leu	H	9.268	8.935	N	117.751	117.581
60Leu	H	12.706	8.727	N	128.234	123.742
79Leu	H	9.191	9.016	N	123.670	123.536
84Leu	H	7.927	7.782	N	120.438	120.356
90Leu	H	8.767	8.549	N	124.047	123.832
100Leu	H	8.062	7.360	N	119.689	119.015
118Leu	H	10.766	9.899	N	131.435	130.441
120Leu	H	9.712	9.037	N	124.490	123.676
141Leu	H	8.986	8.754	N	115.899	115.616
144Leu	H	8.949	8.466	N	128.729	128.232
148Leu	H	8.962	8.242	N	121.085	120.396
157Leu	H	8.307	7.547	N	117.632	116.763
184Leu	H	7.683	7.252	N	117.783	117.312
185Leu	H	6.996	6.650	N	113.631	113.159
189Leu	H	8.809	8.653	N	125.834	125.699
198Leu	H	8.364	8.013	N	118.725	118.381
203Leu	H	9.520	9.156	N	120.804	120.390
204Leu	H	6.251	5.952	N	111.714	111.328
212Leu	H	9.365	8.978	N	127.070	126.717
224Leu	H	9.401	8.017	N	122.388	120.863
229Leu	H	10.381	7.104	N	122.775	119.255
240Leu	H	9.512	8.699	N	126.887	125.841
251Leu	H	8.896	8.749	N	126.024	125.954

Comparison of hCA II S50C C206S and hCA II S50C C206S-Lu-DOTA-M8

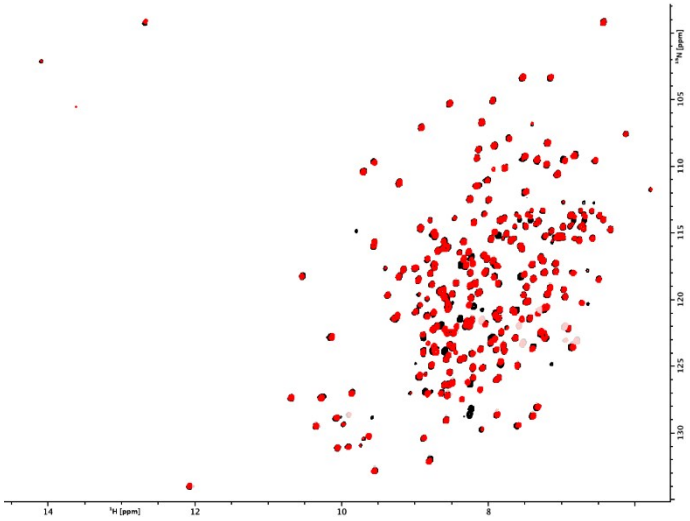

Figure 8: Overlay of ^{1}H-^{15}N HSQC spectra of hCA II S50C C206S (black) and hCA II S50C C206S-Lu-DOTA-M8 (red) in order to confirm that the global folding of the protein remains unchanged upon ligation to the lanthanide chelating tag.

Script used for the Monte-Carlo calculations and the angle score for evaluation of the suitability of different tensors and their combinations

```
tensorSet = 1

if tensorSet == 1:
    # refinement with 0.5Angstrom
    tensorS50C = Tensor(-27.8, 13.7, 18.1, 21.6, 8.5, 104.1, 142.3, 116.2)
    errorS50C = [0.3, 0.3, 0.3, 1.2, 0.7, 1.6, 1.1, 1.7]
    tensorS166C = Tensor(-16.3, -3.6, -11.2, 38.5, 8.0, 52.2, 123.6, 140.3)
    errorS166C = [0.4,0.4,0.4,2.0,1.0,1.8,1.4,5.6]
    tensorS217C = Tensor(-24.9, -17.7, 19.6, 25.7, 13.2, 143.7, 70.9, 125.5)
    errorS217C = [0.2,0.3,0.2,1.0,0.6,0.8,0.5,1.0]
    tensorS220C = Tensor(-13.1, -26.7, 3.2, 23.6, 4.3, 14.9, 153.6, 1.0)
    errorS220C = [0.3,0.3,0.2,0.9,0.3,1.4,0.6,2.6]

elif tensorSet == 2:
    # refinement with 20% set
    tensorS50C = Tensor(-27.5, 13.6, 18.2, 21.1, 8.5, 104.0, 141.8, 115.9)
    tensorS166C = Tensor(-16.2, -3.6, -11.0, 37.4, 7.8, 52.7, 123.1, 141.1)
    tensorS217C = Tensor(-24.8, -17.5, 19.6, 25.5, 13.1, 143.3, 71.2, 125.1)
    tensorS220C = Tensor(-13.0, -26.4, 3.2, 23.0, 4.4, 16.2, 153.7, 3.9)
else:
    raise Exception


tensorSet = 2

if tensorSet == 1:
    # Fit all NH
    tensorS166C_T = Tensor(-11.9, -0.7, -11.7, 38.6, 11.3, 118.3, 157.6, 40.8)
    errorS166C_T = [0.7,0.4,0.4,1.1,2.2,6.8,1.1,3.1]

elif tensorSet == 2:
    # Fit all NH with max dev 0.1 ppm (exclude 251 240 229)
    tensorS166C_T = Tensor(-11.8, -1.7, -11.0, 34.7, 13.3, 119.4, 162.1, 44.8)
    errorS166C_T = [0.5, 0.1, 0.2, 0.6, 1.1, 4.4, 0.5, 3.7]
else:
    raise Exception

tensors = [tensorS50C, tensorS166C, tensorS217C, tensorS220C, tensorS166C_T]
tensor_names = ['S50C','S166C','S217C','S220C','S166C_T']
tensor_errors = [errorS50C, errorS166C,errorS217C, errorS220C, errorS166C_T]
for t in tensors:
    t.active2passive_transformation()

do_create_pymol_potential_file = False
if do_create_pymol_potential_file:
    #create_pymol_macro_with_tensor_only(tensorS50C,0.5,1,'tensorS50C','../data/hCA/')
    #create_pymol_macro_with_tensor_only(tensorS166C,0.5,1,'tensorS166C','../data/hCA/')
    #create_pymol_macro_with_tensor_only(tensorS217C,0.5,1,'tensorS217C','../data/hCA/')
    #create_pymol_macro_with_tensor_only(tensorS220C,0.5,1,'tensorS220C','../data/hCA/')
    create_pymol_macro_with_tensor_only(tensorS166C_T, 0.5, 1, 'tensorS166C_T', '../data/hCA/')


# Select Inhibitor for calculation here:
# 1a = meta F 1b= ortho F; 2 = CF3; 3 = para F


inhibitors = ['1a']
```

```
for inhibitor in inhibitors:
    shiftS50C =  None
    shiftS166C = None
    shiftS217C = None
    shiftS220C = None
    shiftS166C_T= None



    if inhibitor == '1a':
        #meta F
        # HETATM 2082  F21 F2B A 555      -1.393  11.672  12.388  1.00  0.96         Fmeta
        # -3.049 10.475 15.432
        shiftS50C =  -0.142
        shiftS166C = -0.280
        shiftS217C = -0.126
        shiftS220C = -0.126
        shiftS166C_T= 0.2694

        # X-ray
        x_xray, y_xray, z_xray = -3.049, 10.475, 15.432

        #start guess
        x0, y0, z0 = -7.1, 8.4, 16.7

    elif inhibitor== '1b':
        #ortho F
        # HETATM 2083  F22 F2B A 555      -2.122   9.396  10.934  1.00 -2.48         Fortho
        # rotate pdb -3.909 8.122 14.186
        shiftS50C =  -0.135
        shiftS166C = -0.347
        shiftS217C = -0.150
        shiftS220C = -0.144
        shiftS166C_T= 0.4087

        #X-ray
        x_xray, y_xray, z_xray = -3.909, 8.122, 14.186

        #start guess
        x0, y0, z0 = -7.4, 6.1, 15.5

    elif inhibitor=='2':
        #CF3
        #HETATM   25  DX  FFB A 555      -1.115  12.651  14.619  1.00  0.00         F
        shiftS50C =  -0.116
        shiftS166C = -0.238
        shiftS217C = -0.078
        shiftS220C = None
        shiftS166C_T= 0.208

        #X-ray
        x_xray, y_xray, z_xray =   -1.027 , 12.945,  14.759

        #start guess
        x0, y0, z0 = -1.201, 12.335, 14.479


    elif inhibitor=='3':
        #para F
        #HETATM 2084  F23 FFB A 555      -1.201  12.335  14.479  1.00  3.64         F
        shiftS50C =  -0.093
        shiftS166C = -0.242
```

```
    shiftS217C = -0.100
    shiftS220C = None
    shiftS166C_T= 0.250

    # X-ray
    x_xray, y_xray, z_xray = -1.201, 12.335, 14.479

    # start guess
    x0, y0, z0 = -1.201, 12.335, 14.479
else:
    raise Exception


shifts = [shiftS50C, shiftS166C, shiftS217C, shiftS220C, shiftS166C_T]


use_this_shifts = []
use_this_tensors = []
use_this_tensor_names = []
use_this_tensor_errors = []
for i, shift in enumerate(shifts):
    if shift == None:
        pass
    else:

        use_this_shifts.append(shift)
        use_this_tensors.append(tensors[i])
        use_this_tensor_names.append(tensor_names[i])
        use_this_tensor_errors.append(tensor_errors[i])



do_monte_carlo = True



if do_monte_carlo:

    print('------------------------------------------------------------')
    print('GPS-Location')
    print('')
    X = []
    Y = []
    Z = []
    atoms_pdb=''
    for j in range(10000):
        use_this_tensors_with_errors = []
        for i, tensor in enumerate(use_this_tensors):
            x, y, z, ax, rh, a, b, g = tensor.get_tensor_parameters()

            x = x + random.randint(-100,100)*use_this_tensor_errors[i][0]/100
            y = y + random.randint(-100,100)*use_this_tensor_errors[i][1]/100
            z = z + random.randint(-100,100)*use_this_tensor_errors[i][2]/100
            ax = ax + random.randint(-100,100)*use_this_tensor_errors[i][3]/100
            rh = rh + random.randint(-100,100)*use_this_tensor_errors[i][4]/100
            a = a + random.randint(-100,100)*use_this_tensor_errors[i][5]/100
            b = b + random.randint(-100,100)*use_this_tensor_errors[i][6]/100
            g = g + random.randint(-100,100)*use_this_tensor_errors[i][7]/100
            t = Tensor(x,y,z,ax,rh,a,b,g)
            use_this_tensors_with_errors.append(t)
```

```
        x,y,z = find_location(use_this_tensors_with_errors, use_this_shifts,
                        x0=x0, y0=y0, z0=z0)

        X.append(x)
        Y.append(y)
        Z.append(z)

        print("%8.3f, %8.3f, %8.3f" % (x,y,z)+ "  Distance to X-ray: %8.3f" % (distance_between_two_points_nd((x,y,z), (x_xray,  y_xray,
z_xray))))
        atoms_pdb=atoms_pdb+str('ATOM  90000  OM  TSR  9000' + '{:12.3f}{:8.3f}{:8.3f}'.format(x, y, z) + '  1.00  0.00\n')
    print('')
    print('Statistics')
    print('----------------------------------------------------------------')
    with open('../data/hCA/monte_final_' +str(inhibitor)+'.pdb', "w") as text_file:
        text_file.write(atoms_pdb)

    print('Average position')
    print('')
    print("%8.3f +/- %5.3f, %8.3f +/- %5.3f , %8.3f +/- %5.3f" % (sum(X)/len(X),np.std(X), sum(Y)/len(Y),np.std(Y), sum(Z)/len(Z),
np.std(Z)))
    print('')
    print('Distance to X-ray position')
    print('')
    print("%8.3f" % (distance_between_two_points_nd((sum(X)/len(X),sum(Y)/len(Y),sum(Z)/len(Z)), (x_xray,  y_xray,  z_xray))))
    print('----------------------------------------------------------------')


  else:



    print('----------------------------------------------------------------')
    print('GPS-Location')
    x,y,z =  x_all, y_all, z_all = find_location(use_this_tensors, use_this_shifts,
                    x0=x0, y0=y0, z0=z0)
    print('')
    print('Coordinates')
    print('')
    print("%8.3f, %8.3f, %8.3f" % (x,y,z))
    print('')

    print('Distance to X-ray position')
    print('')
    print("%8.3f" % (distance_between_two_points_nd((x,y,z), (x_xray,  y_xray,  z_xray))))
    print('')


    print('Back-calculated PCS')
    print('')
    div = 0
    for i, shift in enumerate(use_this_shifts):
        c_shift = calculate_pseudocontact_shift(x,y,z,use_this_tensors[i])
        d = float(c_shift - shift)
        div = div + abs(d)
        print("%8s" % use_this_tensor_names[i] + "%8.3f, %8.3f, %8.5f" % (c_shift, shift, float(c_shift - shift)))

    print('')
    print('Total deviation : ' + "%8.3f" % div)
    print('----------------------------------------------------------------')
```

```
location_all_text = 'ATOM  99999  OZ  TSR  9999' + '{:12.3f}{:8.3f}{:8.3f}'.format(x, y, z) + '  1.00  0.00\n'
print('ATOM  99999  OZ  TSR  9999' + '{:12.3f}{:8.3f}{:8.3f}'.format(x, y, z) + '  1.00  0.00\n')


skip_combo = False

if skip_combo == True:
    pass
else:


    if len(use_this_shifts)-1 >= 3:
        combs = combinations(np.arange(len(use_this_shifts)), len(use_this_shifts) - 1)
        combs = combinations(np.arange(len(use_this_shifts)), 3)
    else:
        raise Exception

    solutions = []

    for j, combination in enumerate(combs):

        comb_shifts = []
        comb_tensors = []
        comb_tensor_names = []

        for i in combination:

            comb_shifts.append(use_this_shifts[i])
            comb_tensors.append(use_this_tensors[i])
            comb_tensor_names.append(use_this_tensor_names[i])

        print('-----------------------------------------------------------------')
        print('GPS-Location Combination ' + str(j+1))
        x, y, z = find_location(comb_tensors, comb_shifts,
                                x0=x0, y0=y0, z0=z0)

        print('')
        print('Coordinates')
        print('')
        print("%8.3f, %8.3f, %8.3f" % (x, y, z))
        print('')

        print('Distance to X-ray position')
        print('')
        print("%8.3f" % (distance_between_two_points_nd((x, y, z), (x_xray, y_xray, z_xray))))
        print('')

        print('Back-calculated PCS')
        print('')
        div = 0
        for i, shift in enumerate(comb_shifts):
            c_shift = calculate_pseudocontact_shift(x, y, z, comb_tensors[i])
            d = float(c_shift - shift)
            div = div + abs(d)
            print("%8s" % comb_tensor_names[i] + "%8.3f, %8.3f, %8.5f" % (c_shift, shift, float(c_shift - shift)))
        print('')
        print('Total deviation : ' + "%8.3f" % div)
        print('')
        print('Angles between isosurfaces:')

        comb1 = combinations(np.arange(len(comb_tensors)), 2)
```

```
    angles1 = []

    for k, comb in enumerate(comb1):
        tensors5 = []
        tensors_name = []
        for i in comb:
            tensors5.append(comb_tensors[i])
            tensors_name.append(comb_tensor_names[i])

        p1, p2 = calculate_gradient_at_xyz(tensors5[0], x,y,z)
        v1 = find_vector(p1, p2)



        p1, p2 = calculate_gradient_at_xyz(tensors5[1], x,y,z)
        v2 = find_vector(p1, p2)


        a = angle_between_two_vectors(v1,v2)


        if a > 90:
            a = 180-a

        angles1.append(a)

        print("%8s" % tensors_name[0], "%8s" % tensors_name[1], "%8.3f"%  a)


    div = 0
    for a in angles1:
        div = div + (90-a)

    div = div/len(angles1)

    print('')
    print('absolute angle score: ' + "%8.3f" % div)
    print('')

    solutions.append((x,y,z,div))

    print('-----------------------------------------------------------------')


x_av, y_av, z_av = 0,0,0


print('-----------------------------------------------------------------')
print('GPS-Location Combination Position Analysis')
print('')
w = 0
for i in solutions:
    if i[3]>30:
        pass
    else:
        w=w+1
        x_av = x_av + i[0]
        y_av = y_av + i[1]
        z_av = z_av + i[2]

x_av=x_av/(w)
y_av=y_av/(w)
```

```
        z_av=z_av/(w)

        atoms_pdb = ''
        angle_score_color_good = ''

        atoms_pdb=atoms_pdb + 'ATOM  88888  OX  TSR  8888' + '{:12.3f}{:8.3f}{:8.3f}'.format(x_av, y_av, z_av) + '  1.00  0.00\n'

        print("%40s"% "Average Position Angle Score below 30"+ "%8.3f, %8.3f, %8.3f" % (x_av, y_av, z_av))
        print("%40s"%"Average Position all Tensors"+ "%8.3f, %8.3f, %8.3f" % (x_all, y_all, z_all))
        print('')
        print('            x       y       z        div to av    div to all   Angle Score')
        atom_number = 90000
        ligand_number = 9000
        for j,i in enumerate(solutions):
            atom_number=atom_number+1
            ligand_number=ligand_number+1
            print("Combination " + str(j+1) + "%8.3f, %8.3f, %8.3f, %12.3f, %12.3f %12.3f" %
                (i[0], i[1], i[2],distance_between_two_points_nd((i[0], i[1], i[2]),(x_av, y_av, z_av)),distance_between_two_points_nd((i[0],
i[1], i[2]),(x_all, y_all, z_all)),i[3]))

            atoms_pdb = atoms_pdb + 'ATOM  '+str(atom_number)+'  OY  TSR  '+str(ligand_number) + '{:12.3f}{:8.3f}{:8.3f}'.format(i[0], i[1],
i[2]) + '  1.00  0.00\n'

            if i[3]>90:
                angle_score_color_good = angle_score_color_good + 'sel resid ' + str(ligand_number) + '\n'
                angle_score_color_good = angle_score_color_good + 'color red, sele' + '\n'
            elif i[3]<90 and i[3] > 45:
                angle_score_color_good = angle_score_color_good + 'sel resid ' + str(ligand_number) + '\n'
                angle_score_color_good = angle_score_color_good + 'color orange, sele' + '\n'

            elif i[3]<45 and i[3] > 0:
                angle_score_color_good = angle_score_color_good + 'sel resid ' + str(ligand_number) + '\n'
                angle_score_color_good = angle_score_color_good + 'color green, sele' + '\n'
        print('-----------------------------------------------------------------')

        print(atoms_pdb)

        path_atom = '../data/hCA/combo' + str(inhibitor) + '.pml.pdb'
        create_empty_file(path_atom)

        path_pml = '../data/hCA/combo' + str(inhibitor) + '.pml'
        create_empty_file(path_pml)


        with open(path_atom, 'w') as file:
            file.write(location_all_text)
            file.write(atoms_pdb)


        with open(path_pml, 'w') as file:


            if inhibitor == '1a':
                file.write('load 1g52.pdb\n')
                file.write('load ./inh/orthoF_metaF_inh.pdb\n')
                file.write('load ' + './combo' + str(inhibitor) + '.pml.pdb\n')

            elif inhibitor== '1b':
                file.write('load 1g52.pdb\n')
                file.write('load ./inh/orthoF_metaF_inh.pdb\n')
                file.write('load ' + './combo' + str(inhibitor) + '.pml.pdb\n')
```

```
elif inhibitor=='2':
    file.write('load 1g54.pdb\n')
    file.write('load ./inh/paraCF3_inh.pdb\n')
    file.write('load ' + './combo' + str(inhibitor) + '.pml.pdb\n')


elif inhibitor=='3':
    file.write('load 1g54.pdb\n')
    file.write('load ./inh/paraF_inh.pdb\n')
    file.write('load ' + './combo' + str(inhibitor) + '.pml.pdb\n')
else:
    raise Exception



file.write('bg_color white\n')
file.write('set sphere_scale, 0.3,all\n')
file.write('sel resid 9999\n')
file.write('set_name sele, allTensor \n')
file.write('sel resid 8888\n')
file.write('set_name sele, avTensor \n')
file.write('color gold, allTensor\n')
file.write('color silver, avTensor\n')


file.write('load ./tensorS50C.pml.xplor, isomap1, 1, xplor\n')
file.write('isosurface iso50, isomap1,' + str(shiftS50C)+'\n')
file.write('set transparency, 0.5, iso50\n')
file.write('set surface_color, red, iso50\n')
file.write('color red, iso50\n')

file.write('load ./tensorS166C.pml.xplor, isomap2, 1, xplor\n')
file.write('isosurface iso166, isomap2,' + str(shiftS166C)+'\n')
file.write('set transparency, 0.5, iso166\n')
file.write('set surface_color, blue, iso166\n')
file.write('color blue, iso166\n')

file.write('load ./tensorS217C.pml.xplor, isomap3, 1, xplor\n')
file.write('isosurface iso217, isomap3,' + str(shiftS217C)+'\n')
file.write('set transparency, 0.5, iso217\n')
file.write('set surface_color, green, iso217\n')
file.write('color green, iso217\n')

if not shiftS220C == None:
    file.write('load ./tensorS220C.pml.xplor, isomap4, 1, xplor\n')
    file.write('isosurface iso220, isomap4,' + str(shiftS220C)+'\n')
    file.write('set transparency, 0.5, iso220\n')
    file.write('set surface_color, yellow, iso220\n')
    file.write('color yellow, iso220\n')

file.write('load ./tensorS166C_T.pml.xplor, isomap5, 1, xplor\n')
file.write('isosurface iso166_T, isomap5,' + str(shiftS166C_T)+'\n')
file.write('set transparency, 0.5, iso166_T\n')
file.write('set surface_color, black, iso166_T\n')
file.write('color black, iso166_T\n')

file.write(angle_score_color_good)
```

^{1}H-^{15}N HSQC spectrum of hCA II S173C C206S-Tm-DOTA-M8 and the positioning of residue 173 in the protein

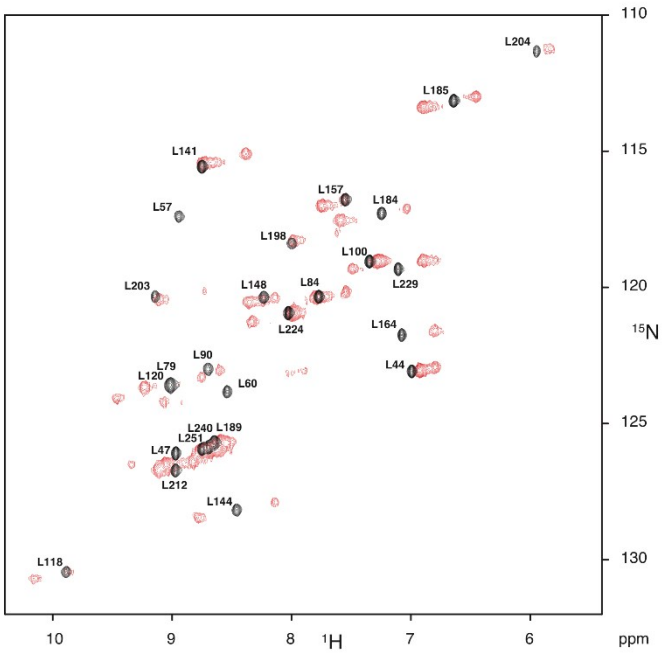

Figure 9: Overlay of ^{1}H-^{15}N HSQC spectra of hCA II S173C C206S-Tm-DOTA-M8 (red) and hCA II S50C C206S-Lu-DOTA-M8 (black) exhibiting two equally populated species for the Tm construct. Peak broadening indicates that dynamic factors are involved.

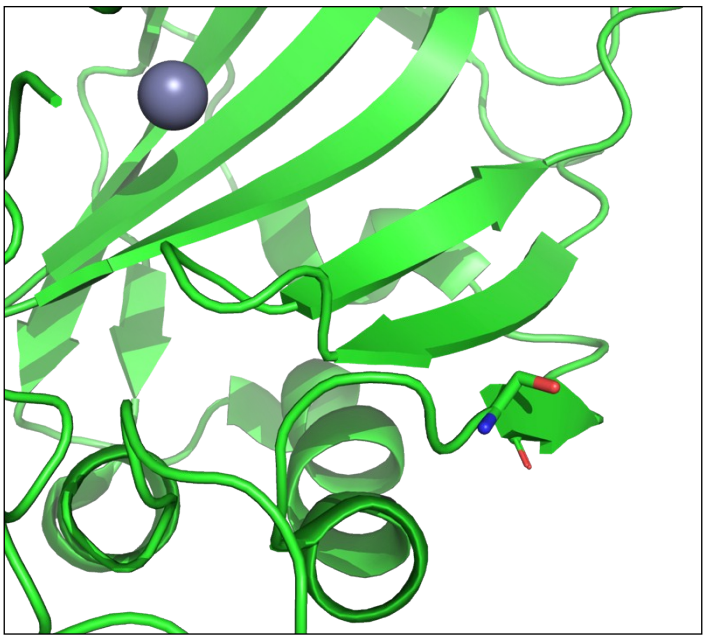

Figure 10: Positioning of residue 173 (right) within hCA II on the edge of a beta-sheet; blue sphere: Zn^{2+} ion.

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