Electronic Supplementary Information:

Quantitative Comparison of Protein Dynamics in Live Cells and *In Vitro* by In-Cell ^{19}F-NMR

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Table S1. Data collection and refinement statistics of **1**-hCA and **1**-hCA$_{AAZ}$.

	1-hCAI	**1**-hCAI$_{AAZ}$
-Data collection-		
X-ray Source	SPring-8 BL381	SPring-8 BL381
Detector	ADSC Q210	ADSC Q210
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions		
a, b, c (Å)	62.5, 69.6, 119.9	62.0, 65.3, 120.5
a, b, g (°)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å)	50.0-2.70 (2.80-2.70) *	50.0-3.00 (3.11-3.00) *
R_{merge} *1	11.7 (37.6)	15.7 (55.1)
I / sI	6.4	5.2
Completeness (%)	96.4 (90.0) *	99.9 (99.9) *
Unique reflections	14,504	10,494
Redundancy	3.6 (2.9)	4.7 (4.8)
-Refinement-		
Resolution (Å)	43.35-2.70	34.20-3.00
No. reflections	14,486	10,451
R_{work} / R_{free} *2	21.9 / 27.0	22.2 / 28.3
R.m.s. deviations		
Bond lengths (Å)	0.003	0.009
Bond angle (°)	0.688	1.235

*Values in parentheses are for highest-resolution shell. *1 $R_{merge} = \Sigma_h \Sigma_i | I(h)_i - \langle I(h) \rangle | / \Sigma_h \Sigma_i I(h)_i$, where $I(h)$ is the intensity of reflection h, Σ_h is the sum of all measured reflections and Σ_i is the sum of i measurements of reflection. *2 R_{work} and $R_{free} = (\Sigma_{hkl} ||Fo| - |Fc||) / \Sigma_{hkl} |Fo|$, where the free reflections (5% of 10 % of the total used) were used for R_{free} throughout refinement.

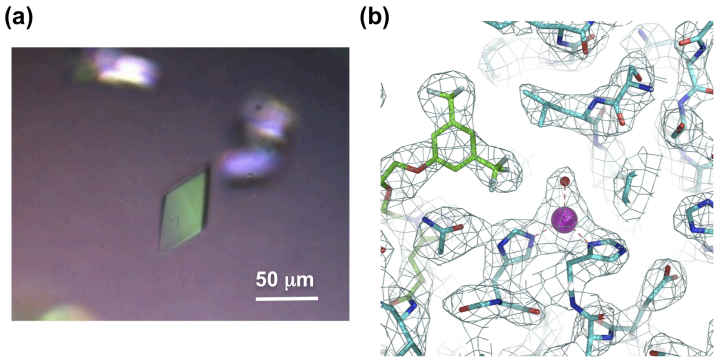

Figure S1. (a) Single crystal images of **1**-hCAI. (b) The magnified image of the active site of **1**-hCAI (ligand free) for showing the presence of coordinated water molecule (red) with zinc ion (purple) as sphere models. The 2|*F*o| - |*F*c| difference Fourier map (>1.0 σ) is shown in blue.

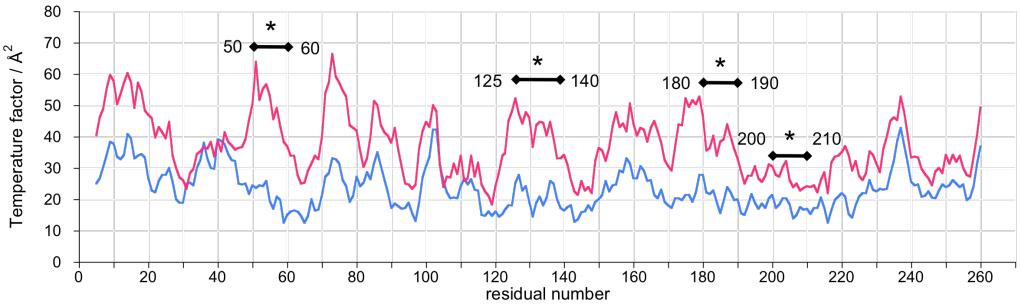

Figure S2. The average main-chain temperature factors of **1**-hCAI (ligand free, red line) and **1**-hCAI$_{AAZ}$ (blue line). Regions 50-60, 125-140, 180-190 and 200-205 (shown as *) showed slightly different patterns between two states.

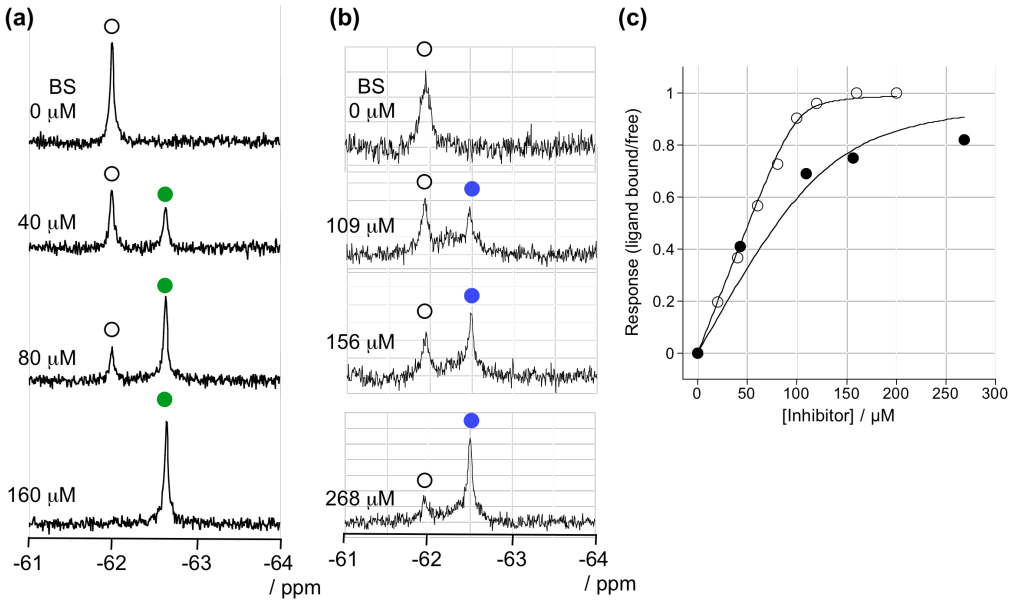

Figure S3. (a, b) ^{19}F-NMR spectra change of **1**-hCAI (100 μM) following addition of Benzenesulfonamide (BS, 0 – 160 μM) *in vitro* (a) or **1**-labeled RBCs following addition of BS (0 – 268 μM) in RBCs (b). The chemical shift of **1**-hCAI (○; -62.0 ppm) and **1**-hCAI$_{BS}$ (●; -62.7 ppm *in vitro*) or **1**-hCAI$_{BS}$ (●; -62.6 ppm in RBCs) were indicated. (c) Quantitative NMR detection of BS with **1**-hCAI. The response shown on the *y* axis is defined as the relative peak area of **1**-hCAI$_{BS}$ divided by the sum of the relative peak areas of **1**-hCAI and **1**-hCAI$_{BS}$, and was plotted against the concentration of BS *in vitro* (○) or in RBCs (●).

Table S2. The linewidth of 1D-^{19}F-NMR peaks *in vitro* and in RBCs.

	In vitro		In RBCs	
	1-hCAI (-62.0ppm)	**1**-hCAI$_{BS}$ (-62.7 ppm)	**1**-hCAI (-62.0ppm)	**1**-hCAI$_{BS}$ (-62.6 ppm)
Half width / ppm	0.054	0.054	0.087	0.070
Half width / Hz	19	19	31	26

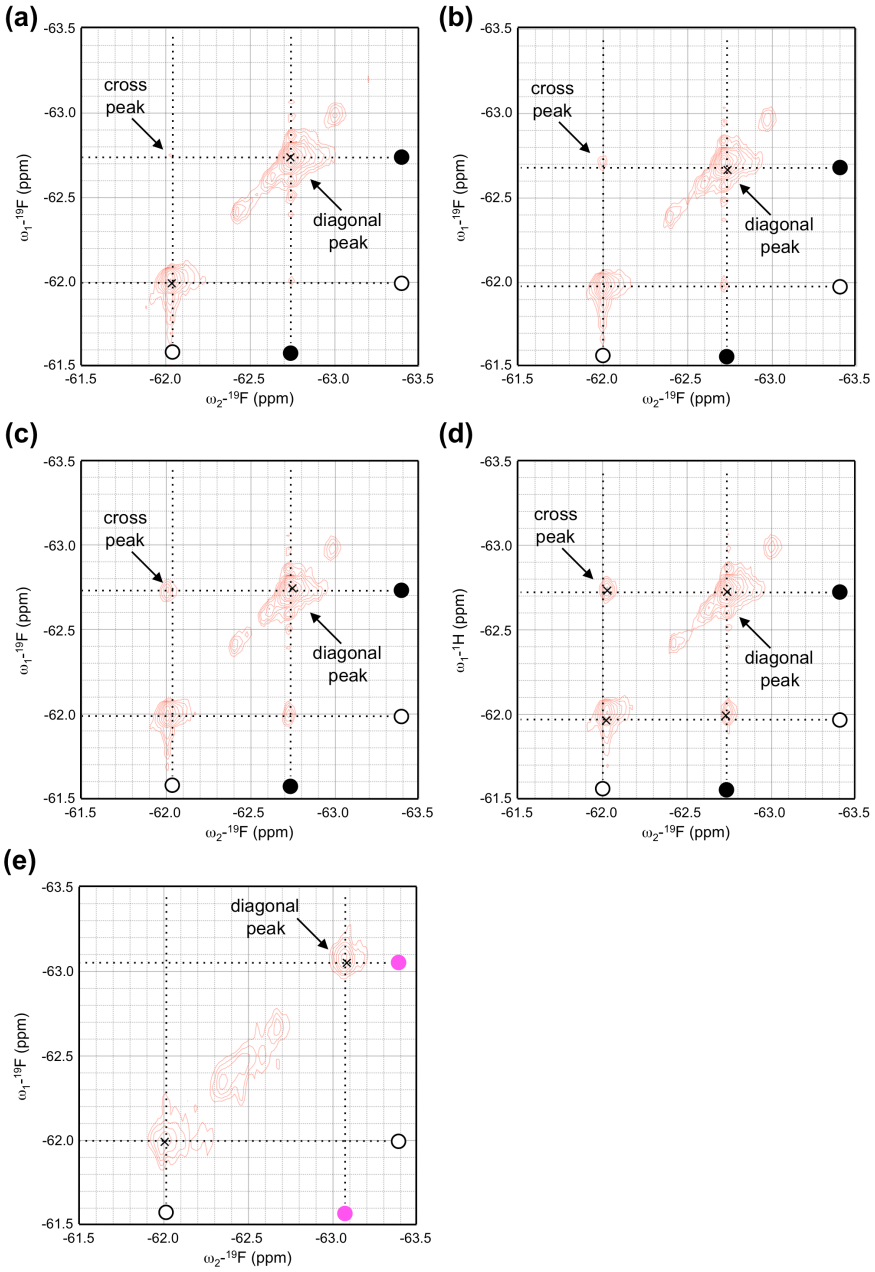

Figure S4. (a-d) Selected regions of the two-dimensional **1**-hCAI (150 μM) NOESY spectra of the complex with benzenesulfonamide (BS, 100 μM) with various mixing times (a; 25 msec, b; 50 msec, c; 125 msec, d; 200 msec). The chemical shift of **1**-hCAI (○; -62.0 ppm) and **1**-hCAI$_{BS}$ (●; -62.7 ppm) were indicated. The spectra were measured in 50 mM HEPES buffer (200 μM TFA, 10% D_2O) at 35 °C. (e) Selected region of the two-dimensional **1**-hCAI (150 μM) NOESY spectrum of the complex with acetazolamide (AAZ, 100 μM). The NOESY mixing times were 500 msec. The chemical shift of **1**-hCAI$_{AAZ}$ (●; -63.0 ppm) was indicated. The spectra were measured in 50 mM HEPES buffer (200 μM TFA, 10% D_2O) at 35 °C.

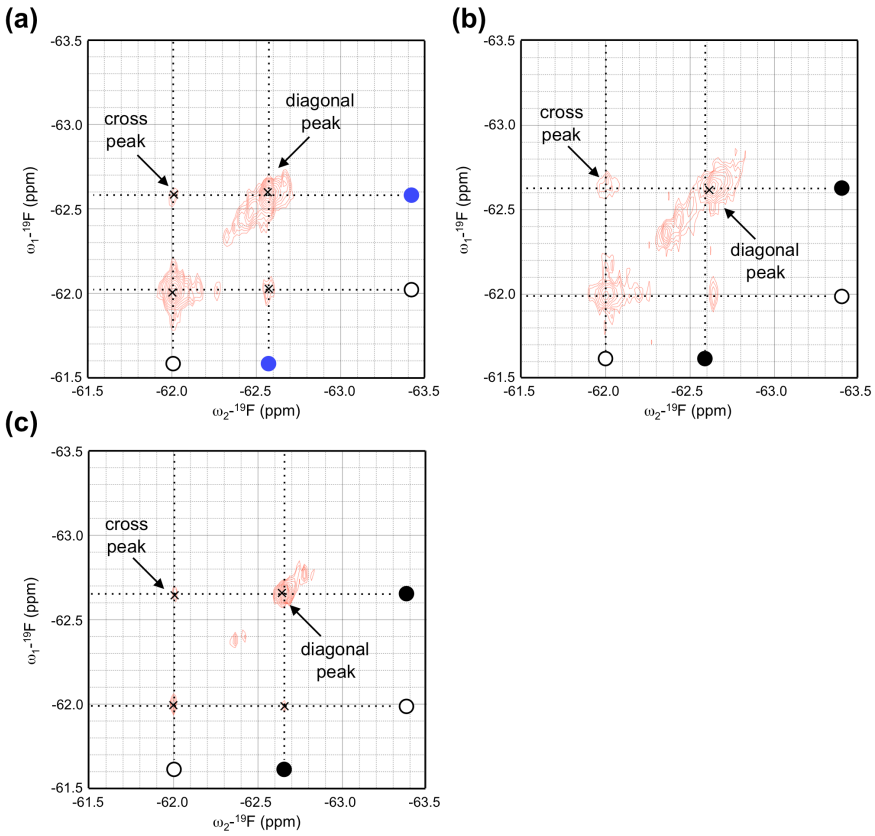

Figure S5. (a) Selected regions of the two-dimensional NOESY spectrum of **1**-labeled RBCs with benzenesulfonamide (BS) (300 μL **1**-labeled RBCs and 300 μM BS). The chemical shifts of **1**-hCAI (○; -62.0 ppm) and **1**-hCAI$_{BS}$ (●; -62.6 ppm) are indicated. The spectrum was measured in a sample containing HBS buffer (20 μM TFA, 10% D_2O) at 35 °C. The NOESY mixing time was 500 msec. (b, c) Selected regions of the two-dimensional NOESY spectra of **1**-labeled RBC lysate (b, undiluted solution) or 2.5-fold diluted lysate (c) with BS (100 μM). The NOESY mixing times were 500 ms. The chemical shift of **1**-hCAI (○; -62.0 ppm) and **1**-hCAI$_{BS}$ (●; -62.6 ppm) were indicated.

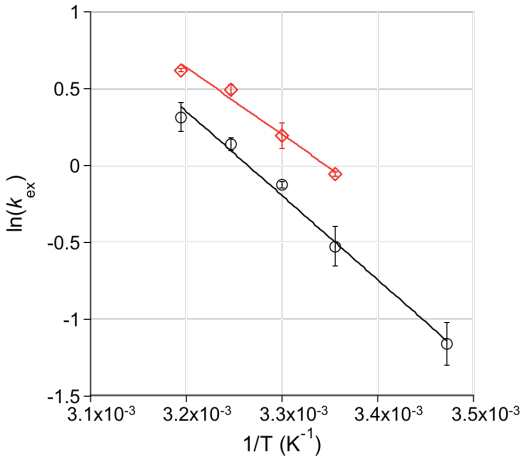

Figure S6. Arrhenius plots of ln(k_{ex}) vs $1/T$ for the **1**-hCAI – **1**-hCAI$_{BS}$ cross peak *in vitro* (○) and in RBCs (◇). Error bars represent the standard deviations of three experiments.

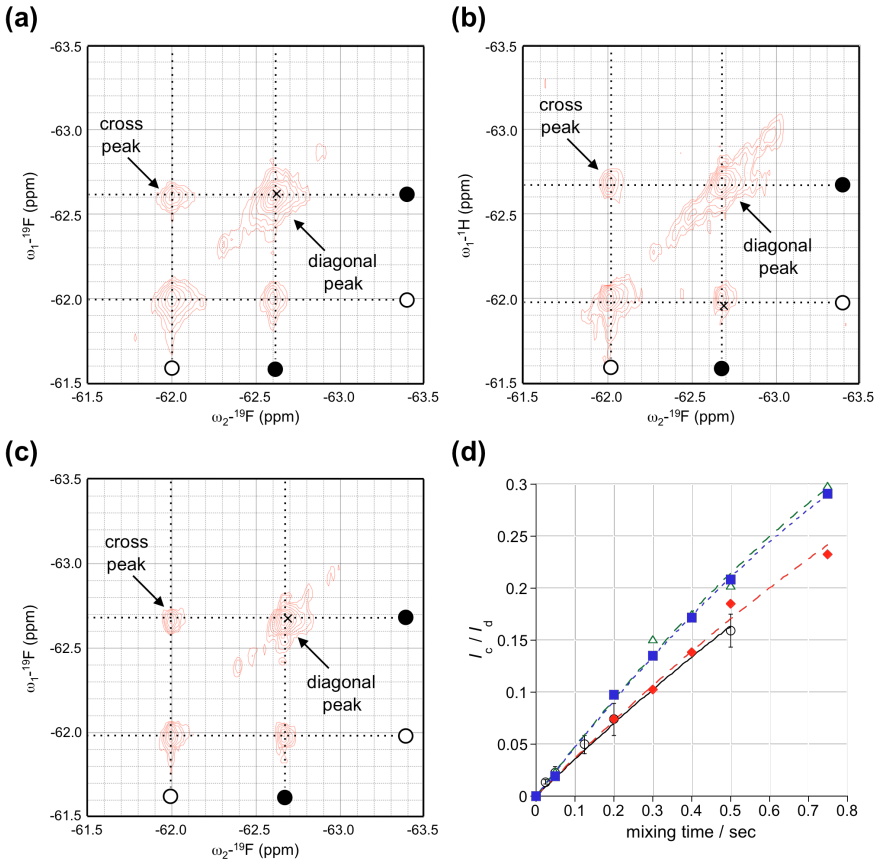

Figure S7. (a-c) Selected regions of the two-dimensional **1**-hCAI NOESY spectra of the complex with benzenesulfonamide (BS) in test tube with several crowders (a; 15% PEG6K, b; 15% PEG 20K, c; 10% Hemoglobin). The chemical shifts of **1**-hCAI (○; -62.0 ppm) and **1**-hCAI$_{BS}$ (●; -62.7 ppm) are indicated. The spectra were measured in a sample containing 50 mM HEPES buffer (a, 200 μM TFA, 10% D_2O) at 35 °C. The NOESY mixing times were 500 msec. (d) The ratios of cross-peak intensities [I_c/I_d] are plotted for **1**-hCAI and **1**-hCAI$_{BS}$ as a function of NOESY mixing times without crowder (○), with 15wt% PEG6K (◆), with 15wt% PEG20K (△), and with 10wt% Hemoglobin (■).

Supporting Methods.

General materials and methods. All proteins and chemicals were obtained from commercial suppliers (SIGMA, TCI, Dojindo, Wako or Acros Organics) and used without further purification. UV-visible spectra were recorded on a Shimadzu UV-visible 2550 spectrometer. MALDI-TOF MS spectra were recorded on a Bruker Autoflex III using sinapinic acid (SA) as the matrix. 1D ^{19}F NMR spectra were recorded on a JEOL ECX-400P (376.5 MHz) spectrometer and calibrated with TFA (-75.6 ppm). The number of accumulations was 1024.

Preparations and crystallization of 1-labeled hCAI with or without AAZ. Purified hCAI (5 mg, SIGMA, C4396) was dissolved in 50 mM HEPES buffer (pH 7.2). The concentration of hCAI was determined by absorbance at 280 nm using the molar extinction coefficient of 49,000 $M^{-1}cm^{-1}$.[S1] The solution of hCAI (100 μM) was incubated with **1** (200 μM) in 50 mM HEPES buffer (pH 7.2, 1.5 mL) at 37 °C. After 24 h, the completion of the labeling was confirmed by MALDI-TOF MS measurement using SA as the matrix. The labeled hCAI was purified twice by size-exclusion chromatography using a TOYOPEARL HW-40F (Tosoh Corporation, 10ϕ × 120 mm), and dialyzed twice against 50 mM Tris-sulfate buffer (pH 8.7) with a Spectra/Por dialysis membrane (MWCO: 10 kDa) and concentrated to 11 mg/mL using an Amicon Ultra (10 kDa, Millipore). The purified forms of **1**-labeled hCAI was crystallized by the hanging drop vapor diffusion method as previously described with a slight modification.[S2] Crystals of **1**-hCAI were grown in a reservoir solution containing 23 or 25% polyethylene glycol (PEG) 3,350, 200 mM NaCl and 100 mM MES (pH 6.3) at 20 ^{o}C. Crystals formed within 3 days. Crystals of the **1**-$hCAI_{AAZ}$ were obtained by soaking **1**-hCAI crystals in solution containing 10 mM AAZ in the reservoir solution for 30 min. Obtained crystals were transferred to a drop containing 30% glycerol and 70% reservoir solution.

X-ray crystallographic analyses. X-ray diffraction data were collected at a wavelength at 1.0 Å in the beam line BL38 of the SPring8 (Harima, Japan). The diffraction data of **1**-hCAI and **1**-hCA_{AAZ} were processed and scaled with *HKL2000*[S3] at 2.70 Å and 3.00 Å resolution, respectively. The crystal structures of **1**-hCAI and **1**-$hCAI_{AAZ}$ were solved by a molecular replacement method with the program *MOLREP*,[S4] using the coordinate

of native hCAI complexed with AAZ (PDB code 1AZM) as a search model. The initial model was built with the program *Coot*,[S5] and was refined with the program *PHENIX*.[S6] Several rounds of refinement give final R_{work} and R_{free} values of 21.9% and 27.0% for **1**-hCAI, respectively. The final model of the complex of **1**-hCAI$_{AAZ}$ was converged with R_{work} and R_{free} values of 22.2% and 28.3%, respectively. The data collection and refinement statics are summarized in Table S1. Figure 2 was generated with the program PyMOL.[S7]

^{19}F-labeling procedure in RBCs. The labeling processes in RBCs were performed as shown in our previous report.[S8] Blood was taken from one of the authors. After anti-coagulation treatment with heparin, RBCs were separated from plasma by centrifugation. The separated RBC suspensions were washed three times with HEPES-buffered saline (20 mM HEPES, 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM glucose, pH 7.4, HBS). A 1 mL solution of reagent **1** (133 μM) in HBS was mixed with a 1 mL suspension of RBCs, incubated at room temperature for a few minutes, and centrifuged (1500 rpm, 10 min). After removing the supernatant, the same procedure was repeated two more times to treat the cells with a total of 400 nmol of **1**. After 48 h of incubation at 25 °C, the labeled RBCs were collected by centrifugation, and resuspended in fresh HBS containing 20% D_2O (v/v) and 200 μM TFA (HBS^{DT}, 1 mL). The *in-cell* titration experiments and EXSY experiments were carried out using a portion of the RBC suspension (150 μL bed volume and 150 μL HBS^{DT}) in a NMR tube (ϕ 5 mm, shigemi tube). The concentration of hCAI in RBCs has already been determined in our previous study, that is, 260 μM for the total hCAI, and about 100 μM for the labeled hCAI.[S8]

Determination of BS concentration in RBCs. To determine the concentration of BS uptaken into the cells, the supernatants obtained during the titration experiments of BS in RBCs were mixed with 4-nitrobenzaldehyde (100 μM, as an internal standard), which were subjected to RP-HPLC on a YMC-triart C18 column (5 μm, 250 × 4.6 mm). The cocentration of BS that was not uptaken into the cells was estimated by determining the ratio between the peak area of BS and the area of internal standard. By subtracting the obtained value from initial mole number in the supernatant, BS concentration inside the cells was determined. The incorporated concentrations of BS were determined as 43,

109, 156 and 268 μM when the initial concentrations were 100, 200, 300 and 500 μM, respectively.

^{19}F-EXSY experiments. ^{19}F EXSY spectra were recorded at 15-40 °C on a Bruker Avance 600 spectrometer (565 MHz) equipped with a TCI cryophore head and calibrated with TFA (-75.6 ppm). Standard parameters were used with a 36 kHz spectral width, 8 ms pulse length, 0.46 s acquisition time, and 0.50 s relaxation delay. A 0.1 Hz line broadening was applied. The number of accumulations was 16 (*in vitro*) or 32 (in RBC). The intensity ratio of the diagonal and cross-peaks is given by

$$I_c / (I_d + I_N/3) = a\,[1 - \exp(-2k_{ex}t)] / \{(1-a) + a\,[1 + \exp(-2k_{ex}t)]\} \qquad (1)$$

where k_{ex} is the rate of the chemical exchange, I_c and I_N are the intensities of the exchange and NOE cross-peaks, respectively (in this case, I_N is zero), I_d is the intensity of the diagonal peaks, and a is the ratio of **1**-hCAI complexed with the inhibitor BS (a = **1**-hCAI/(**1**-hCAI + **1**-hCAI$_{BS}$), calculated from the ^{19}F-signal intensities).[S9] Least-squares fitting of the time dependency of $I_c/(I_d + I_N/3)$ in eq (1) yielded k_{ex} values (as shown in Figure 3d). Then, the activation energy, E_a, for the exchange process was determined by fitting, the k_{ex} values against the temperature between 15 and 40 °C according to the Arrhenius equation (2):

$$k_{ex} = A \exp(-E_a/RT) \qquad (2)$$

where A is a constant and T is the temperature in kelvin. The k_{ex} values at various temperatures are described well by the equation.

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