Supporting Information

Probing conformational and functional substates of calmodulin by high pressure FTIR spectroscopy: influence of Ca^{2+} binding and the hypervariable region of K-Ras4B

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Experimental Details

Sample Preparation

Ca^{2+}-free calmodulin was obtained by exhaustively dialyzing 2 mg protein (c = 4 mg/mL) against 1000 volumes of 2 mM EGTA, 50 mM Tris HCl and 150 mM NaCl (pH = 7.4) at 4 °C in a 0.5 mL Pierce *Slide-A-Lyzer™ Dialysis* Cassette (3.5 K MWCO) for 2 days. As the carboxylic groups of EGTA exhibit strong IR bands in the region 1570-1630 cm^{-1} that overlap with the amide I' band of proteins (1600-1700 cm^{-1}), EGTA was removed by subsequent dialysis against 1000 volumes MilliQ water at 4 °C for 2 days. In both cases, the dialysis buffer and water, respectively, were exchanged every 9-12 h. Calmodulin concentrationwas determined by measuring UV/Vis absorption at 276 nm with ε_{276} = 3006 M^{-1} cm^{-1}. The appropriate amount of the protein was aliquoted and lyophilized overnight in order to remove H2O, which is necessary for FTIR spectroscopy. All measurements were performed in D_2O buffer containing 50 mM Tris HCl, 150 mM NaCl and 10 mM $CaCl_2$ (pD 7.4), except for the apo-calmodulin experiments, in which $CaCl_2$ was lacking in buffer. The *S-tert*-butylthio (StBu) protection group at the N-terminal cysteine of the synthetic peptide was removed by reduction with tris(2-carboxyethyl)phosphine (TCEP) before all experiments.

High Pressure FTIR Spectroscopy

For the pressure-dependent FTIR spectroscopy experiments of holo- and apo-CaM, the dried protein was dissolved to a concentration of 3 wt% in the above mentioned buffer in the presence or absence of $CaCl_2$, respectively. The FTIR measurements of the K-Ras4B peptide alone were carried out at the same concentration (3 wt%) in the presence of the Ca^{2+}-containing buffer. As the purchased peptide contains trifluoroacetic acid (TFA) as counter ion, which shows a strong absorption band at 1673 cm^{-1} and thus overlaps with the amide I' band of the protein-peptide complex or peptide, respectively, the peptide powder was dissolved in 0.1 M DCl and freeze-dried for at least 2 h. This was repeated for another time in order to exchange TFA^- by Cl^- as counter ion. For the protein-peptide interaction studies, buffer containing the C-terminal K-Ras4B peptide in addition to CaCl2 was added to the dried CaM yielding a molar ratio of protein to peptide of 1:10 in order to ensure saturation of CaM. The mixture was incubated for 30 min allowing protein-peptide complex formation before FTIR absorbance spectra collection started. Afterwards, the sample solution was filled into the central hole of a 50 µm thick stainless steel spacer placed onto a 730 µm-thick Type IIa diamond window (Almax easyLab) in a gas-membrane-driven diamond anvil cell (Diacell Vivo DAC, Almax. easyLab). For accurate pressure determination, BaSO4 powder was used as an internal pressure calibrant, since the shift of the band at 983 cm^{-1} assigned to BaSO4 stretching vibration is proportional to the applied pressure.[1] After assembling, the pressure cell was implemented into a Nicolet Magna 550 FTIR spectrometer equipped with a liquid-nitrogen-cooled MCT detector (Thermo Fisher Scientific). The spectrometer was continuously purged with dry air to minimize water vapor. High precision pressure application between 0.1-1200 MPa was achieved by the automated pneumatic pressure controller PACE 5000 from GE Sensing and the temperature was set to 25 °C and controlled by a circulating water flow. After each pressure change, the sample was allowed to equilibrate for 7 min before collection of the FTIR spectrum. Each spectrum was obtained by recording 256 interferometer scans with a spectral resolution of 2 cm^{-1} and the corresponding spectral evaluation was performed using GRAMS/AITM software (Thermo Electron). All spectra were buffer subtracted. For secondary structure analysis, the amide I' band between 1700 and 1600 cm^{-1} was baseline corrected and normalized to one. Initial peak wavenumbers for subband fitting to the amide I' band, whose peak wavenumbers are characteristic for secondary structure elements and whose areas are proportional to their fractions, were determined from second derivative spectra.

The standard volume change accompanying the conformational change of a protein from state 1 to state 2 at a given temperature T can be determined from

$$\Delta V^o = V_2^o - V_1^o = \left(\frac{\partial \Delta G^o(p)}{\partial p}\right)_T \quad ()$$

The standard free Gibbs energy change $\partial \Delta G^o(p)$ at pressure p is derived from the equilibrium constant $K_{eq}(p)$ calculated from the fraction of the secondary structure elements of the conformers in these two states:

$$\Delta G^o = -RT\ln K_{eq}(p) = -RT\ln\left(\frac{x_1 - x(p)}{x(p) - x_2}\right) \quad (2)$$

where $x(p)$ is the secondary structure fraction at pressure p, and $x_1$and x_2 are asymptotic secondary structure fractions of state 1 and state 2, respectively. The pressure-dependent curve progression of the secondary structure elements was fitted by the Boltzmann equation[2]:

$$x(p) = \frac{x_1 - x_2}{1 + e^{-(p - p_m)\cdot\left(\frac{\Delta V^o}{RT}\right)}} + x_2 \quad (3)$$

Fluorescence correlation spectroscopy (FCS)

Binding of the K-Ras4B peptide to CaM was ascertained by fluorescence correlation spectroscopy (FCS). For this purpose, the dye fluorescein-5-maleimide was covalently bound to the deprotected thiol moiety of N-terminal cysteine in the peptide. The coupling step was performed in 20 mM Tris HCl and 3 mM TCEP at pH = 7.0 with a 1.5-fold molar excess of fluorescein-5-maleimide for 1 h. Unbound fluorophores were removed by using 5 mL HiTrap desalting column (GE Healthcare, Freiburg, Germany). FCS measurements were performed in a confocal fluorescence microscope (MicroTime 200, PicoQuant) consisting of an inverted microscope from Olympus IX 71 as microscope body, which is equipped with a water immersion objective (UplansApo, 60X, NA 1.2). For fluorescein excitation, a pulsed diode laser of wavelength 485 nm (FWHM = 600 ps, repetition rate 20 MHz) was used. The output of the pulsed diode laser is coupled to the main optical unit by using polarization maintaining single mode optical fiber, in which the excitation light is guided through a dichroic mirror reflecting it to the entrance port of the microscope. The objective lens focuses the laser beam in the sample drop placed on a coverslip. Fluorescence is collected in the same path, passing through the dichroic mirror and 510 nm long pass emission filter (HQ, Chroma, 510 LP), before passing a 50 µm pinhole and impinging onto SPCM-AQR series SPAD single photon avalanche diode (SPAD). Autocorrelation traces were generated from the SPAD by using fluorescence lifetime correlation spectroscopy (FLCS) method. Here, the fluorescence signals based on the lifetimes can be separated and the autocorrelation trace for each lifetime component can be individually recorded. The detector afterpulsing effect that usually distorts the autocorrelation curve at the shorter correlation times in a conventional FCS measurement can be suppressed for the most part by applying this method. TimeHarp 200 TCSPC module in a time-tagged time-resolved (TTTR) mode was used for data acquisition and the subsequent analysis of the autocorrelation curves was performed by SymPhoTime software provided by PicoQuant. The autocorrelation curves $G(\tau)$ for C-terminal K-Ras4B peptide in the absence of CaM is fitted to a simple 1-component diffusion model according to equation 1 ($i = 1$), and in the presence of CaM, a further exponential term is required to provide an adequate fit to the data:

$$G(\tau) = 1 - T + T\,exp\left(-\frac{\tau}{\tau_f}\right)\sum_{i=1}^{k}\rho_i\left(1 + \frac{\tau}{\tau_i}\right)^{-1}\left(1 + \frac{\tau}{\tau_i\kappa^2}\right) \quad (4)$$

In this equation, the diffusion time τ_i describes the average time that a fluorophore stays in the detection volume, T and τ_f signify the fraction of the molecules undergoing faster fluctuations and the time duration of the faster fluctuations, respectively. τ denotes the lag time and ρ_i is given by

$$\rho_i = \frac{\alpha_i}{\langle N\rangle\,(1 - T)} \quad (5)$$

considering the fraction of the molecule α_i with the diffusion time τ_i and the mean number $\langle N\rangle$ of molecules in the detection volume. κ represents the structure parameter of the observation volume and is given by $\kappa = \frac{\omega_z}{\omega_{xy}}$ with ω_z and ω_{xy} as longitudinal and transverse radii of the observation volume, respectively. This structure parameter of the detection volume was calibrated using the known diffusion coefficient of Atto488 in water (400 µm²/s)[3] and amounts to 6 fL. The translational diffusion coefficient D_t employing the τ_i value was obtained from the following equation:

$$\tau_i = \frac{\omega_{xy}^2}{4D_t} \quad (6)$$

The concentration of labelled K-Ras4B peptide was maintained around 16 nM throughout all FCS measurements, whereas the concentration of CaM was varied from 0 to 1000 μM. All measurements were carried out at room temperature in the above mentioned buffer including 10 mM $CaCl_2$ for 5-10 times. Before data collection, the samples containing both the peptide and protein were incubated for at least 15 min. Errors shown in the figure represents standard deviations. Since the binding of the C-terminal K-Ras4B peptide to CaM is relatively weak, the exchange of the fluorescein-labelled peptide between the free and CaM-bound state might be faster than the diffusion time through the detection volume. For this reason, the determined diffusion coefficient is the average one with contributions from both the free and CaM-bound K-Ras4B peptide. Al-Soufi *et al.* have deduced the relation between the mean diffusion time τ_i and the binding constant K_b considering the fast exchange dynamics of the molecule in detail.[4] Briefly, the association of the fluorescein-labelled K-Ras4B peptide with the non-fluorescent CaM results in fluorescent K-Ras4B peptide · CaM complex and can be represented as

$$K - Ras4B\ peptide + CaM \underset{k_-}{\overset{k_+}{\rightleftharpoons}} K - Ras4B\ peptide \cdot CaM \quad (7)$$

where k_+ and k_- denote the association and dissociation rate constant, respectively, and describe the binding constant $K_b = \frac{k_+}{k_-}$. The mean diffusion coefficient $\bar{D}_t$ is described as the

sum of the individual diffusion coefficients, D_{Pep} and $D_{Pep \cdot CaM}$, as well as the respective mole fractions x_{Pep} and $x_{Pep \cdot CaM}$:

$$\bar{D}_t = x_{Pep} \cdot D_{Pep} + x_{Pep \cdot CaM} \cdot D_{Pep \cdot CaM} \quad (8)$$

With equation (3) and (5), the average diffusion time $\bar{\tau}_i$ can be expressed directly as a function of the binding constant and the CaM concentration $[CaM]$:

$$\bar{\tau}_i = \frac{\tau_{Pep}\,(1 + K_b\,[CaM])}{1 + \frac{\tau_{Pep}}{\tau_{Pep \cdot CaM}} K_b [CaM]} \quad (9)$$

τ_{Pep} and $\tau_{Pep \cdot CaM}$ denote the diffusion time of the peptide in the absence of CaM and at the CaM concentration when all peptides available in solution are bound, respectively. With increasing $[CaM]$, the observed diffusion time shifts from τ_{Pep} to $\tau_{Pep \cdot CaM}$.

SAXS

Small-angle X-ray scattering (SAXS) measurements were carried out on a SAXSess mc² instrument (Anton Paar, Graz, Austria) using a monochromatic X-ray beam (λ = 1.54 Å) with line focus and an imaging plate detector (Cyclone, Perkin Elmer, USA). CaM was dissolved in the above mentioned buffer including $CaCl_2$ to a concentration of 1 wt% in the presence and absence of 10-fold molar excess of the C-terminal K-Ras4B peptide. Before data acquisition, the samples were equilibrated for 15 min. The temperature during all experiments was set to 25 °C and was controlled by an external water bath. 2D scattering patterns were integrated to obtain 1D scattered intensities, $I(q)$, as a function of scattering vector, $q = \frac{4\pi}{\lambda} sin(\frac{\theta}{2})$, where θ is the scattering angle, by using SAXSquant 3.1 supplied from Anton Paar. All data were background subtracted using the scattering profiles of pure buffer. Scattering patterns were collected over 30 min per image. Desmearing of the data and calculation of the pair-distance distribution function, $P(r)$, were performed using the software GNOM.[5]

Additional Figures

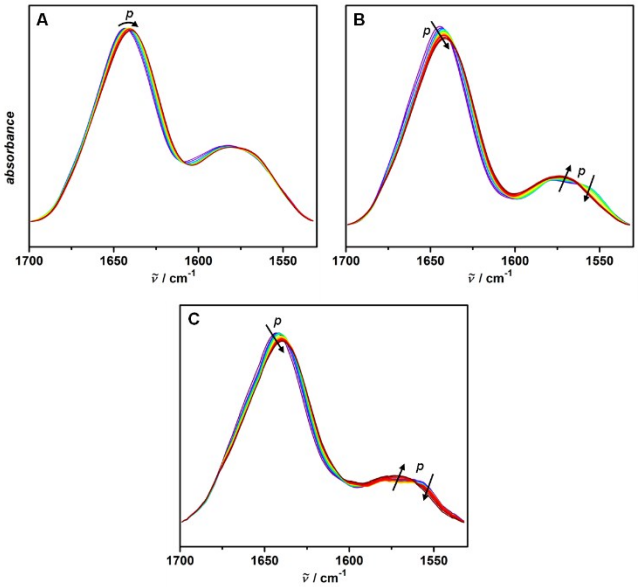

Fig. S1 Pressure-dependent absorbance IR spectra (buffer subtracted, baseline corrected, normalized) of apo- (A), holo-CaM (B), and the CaM-K-Ras4B peptide complex (C) in D_2O buffer at 25 °C.

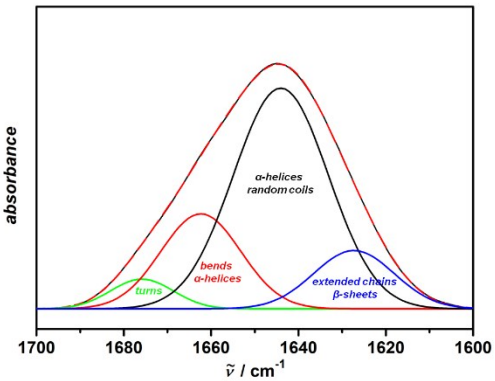

Fig. S2 Decomposition of the amide I' band (buffer subtracted, baseline corrected and normalized) of holo-CaM at 25 °C. The measured spectrum is shown in black. The fitted spectrum in red results from the underlying band components assigned to secondary structure elements.

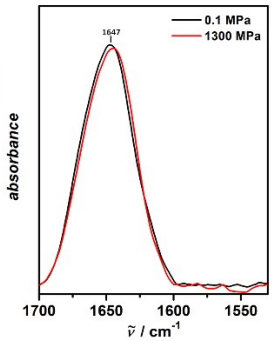

Fig. S3 FTIR spectra (buffer subtracted, baseline corrected, normalized) of the C-terminal K-Ras4B peptide at 0.1 MPa and 1300 MPa in the region of 1700-1530 cm^{-1} in D_2O buffer at 25 °C.

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