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Inhibitor and peptide binding to calmodulin characterized by high pressure Fourier transform infrared spectroscopy and Förster resonance energy transfer

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ABSTRACT

We compare the binding of an inhibitor with that of a natural peptide to Ca^{2+} saturated calmodulin (holo-CaM). As inhibitor we have chosen trifluoperazine (TFP) that is inducing a huge conformational change of holo-CaM from the open dumbbell-shaped to the closed globular conformation upon binding. On the other hand, melittin is used as model peptide, which is a well-known natural binding partner of holo-CaM. The experiments are carried out as a function of pressure to reveal the contribution of volume or packing effects to the stability of the calmodulin-ligand complexes. From high-pressure Fourier transform infrared (FTIR) spectroscopy, we find that the holo-CaM/TFP complex has a much higher pressure stability than the holo-CaM/melittin complex. Although the analysis of the secondary structure of holo-CaM (without and with ligand) indicates no major changes up to several kbar, pressure-induced exposure of α-helices to water is most pronounced for holo-CaM without ligand, followed by holo-CaM/melittin and then holo-CaM/TFP. Moreover, structural pressure resistance of the holo-CaM/TFP complex in comparison with the holo-CaM/melittin complex is also clearly visible by higher Ca^{2+} affinity. Förster resonance energy transfer (FRET) from the Tyr residues of holo-CaM to the Trp residue of melittin even suggests some partial dissociation of the complex under pressure which points to void volumes at the protein-ligand interface and to electrostatic binding. Thus, all results of this study show that the inhibitor TFP binds to holo-CaM with higher packing density than the peptide melittin enabling a favorable volume contribution to the inhibitor efficiency.

1. Introduction

Calmodulin has four Ca^{2+} binding sites that become saturated at Ca^{2+} concentrations of about 10^{-5} M [1]. Ca^{2+}-saturated calmodulin (holo-CaM, Fig. 1) has a net charge of -15 *e* at neutral pH-value and provides two hydrophobic surface patches [2–4]. It adopts an open dumbbell-shaped conformation that can bind numerous different target molecules, peptides and proteins, which interact via electrostatic and hydrophobic forces [5]. Upon binding, the two lobes of the holo-CaM structure usually wrap around the target to form a closed globular complex [6–8]. Target proteins can become activated in this way and can, for example, catalyze a reaction [9,10]. As a result, a Ca^{2+} signal is transformed into protein activity by calmodulin.

Melittin is a short peptide (26 residues, 2846 g mol^{-1}) with a net positive charge (Fig. 1) [11,12]. There are four basic amino acid residues close to the C-terminus and a further Lys residue. The remaining amino acid residues are mostly hydrophobic. It is a component of the honey bee venom and known to interact with lipid membranes, but also with holo-CaM [6,12]. In this way, it can be regarded as a competitive inhibitor of calmodulin function. However, we will use it as a model for target peptides of calmodulin in this study. The second ligand of calmodulin studied here is trifluoperazine (TFP, Fig. 1). It has a charge of +2 at neutral pH-value and hydrophobic aromatic groups that qualify this molecule as ideal binding partner of holo-CaM [8,13,14]. TFP is a well-known CaM antagonist [15]. For example, it suppresses binding and activity of CaMKII [16]. It has been reported that four TFP molecules bind to one holo-CaM [8]. In both cases, melittin and TFP, binding of the ligand to holo-CaM leads to a huge conformational change of holo-CaM from the open dumbbell to the closed globular shape [6,8,17,18]. Both complexes, holo-CaM/TFP and holo-CaM/melittin, are rather spherical with a maximum diameter of 48 Å and a radius of gyration of 18 Å, as determined from small-angle X-ray scattering [6,8].

In this study, we have characterized the structure of holo-CaM in the absence and the presence of a ligand (melittin or TFP) as a function of pressure. Using pressure as a thermodynamic variable, volume or packing density contributions to the structural stability of holo-CaM

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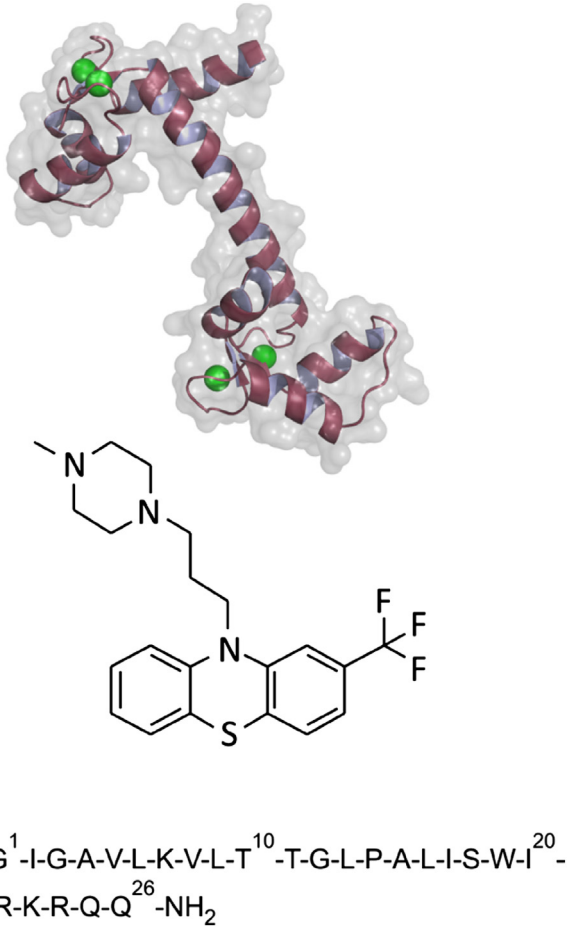

Fig. 1. Structures of holo-CaM (top, PDB ID: 3CLN) and the two ligands, TFP (middle) and melittin (bottom), as used in this study. The four Ca^{2+} ions of holo-CaM in the dumbbell-shaped conformation are shown as green spheres.

and the binding interaction of holo-CaM with ligands are revealed [19,20]. In particular, the spatial fit of a ligand to the binding pocket of holo-CaM determines the potential formation of void volumes, which represent an important source of pressure sensitivity of the formed holo-CaM-ligand complexes. Moreover, attractive Coulomb interactions (salt bridges) between holo-CaM and a ligand are weakened under pressures up to about 2000 bar, because the hydration of separated ions leads to a denser packing of water molecules [19]. The concomitant reduction in volume is favored upon pressurization. Contrary, negative volume changes have recently been observed for the binding of ligands to the human heat shock protein 90 leading to a stabilization of the protein upon compression [21,22]. In any case, pressure allows for the determination of volumetric properties of protein-ligand interactions and for novel mechanistic insights into ligand or inhibitor affinities.

To reveal the effect of pressure on holo-CaM-ligand interactions, we have used Fourier transform infrared (FTIR) and fluorescence spectroscopy. From FTIR spectroscopy, the penetration of water into the tertiary structure, the secondary structure distribution, and the Ca^{2+}-binding affinity of holo-CaM is directly accessible. Fluorescence spectroscopy is used to determine the Förster resonance energy transfer (FRET) from the Tyr residues of holo-CaM to the Trp residue of melittin. In this way, the pressure effect on the melittin association with holo-CaM has been monitored. Recently, we have studied the effect of pressure on holo-CaM-ligand complexes using small-angle X-ray and elastic incoherent neutron scattering [23]. From X-ray scattering, we have observed that the holo-CaM/TFP complex is pressure-stable up to 3 kbar, whereas the binding of a peptide to holo-CaM is pressure-sensitive. However, only the overall size and shape of the holo-CaM-ligand complexes could be resolved without insight into the internal structure. The neutron scattering data indicate a pressure-induced increase of the dynamics of a holo-CaM-ligand complex on the Å scale [23]. Thus, using FTIR spectroscopy and FRET, complementary techniques are applied in this study providing a view on the intramolecular structures. Moreover, the FTIR experiments are carried out with a diamond anvil sample cell giving access to extreme pressures of > 10 kbar, which is needed to detect small volume changes.

As we will show in this study, the TFP inhibitor is binding to holo-CaM with a higher packing density than the melittin peptide making the holo-CaM/TFP complex highly pressure stable, which will contribute to the inhibitor efficiency of TFP in a favorable way. Whereas the secondary structures of the holo-CaM-ligand complexes remain unaltered with increasing pressure, pressure-induced water penetration into the complexes is inferred from the FTIR spectra. Moreover, small volume changes of unfolding are determined that corroborate these findings.

2. Materials and methods

Recombinant CaM from rat was produced as wild type (WT) and as mutant T26W using the pET-14b vector from GenScript (Piscataway, NJ, USA) and *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells from Agilent Technologies (Santa Clara, CA, USA), as described in the literature [24]. TFP and melittin were obtained from Sigma-Aldrich (Darmstadt, Germany) and Biophoretics (Sparks, USA), respectively. $CaCl_2$, NaCl, and BisTris were also purchased from Sigma-Aldrich. Solutions used for FTIR spectroscopy were prepared by dissolving 30 mg mL^{-1} or 1.8 mM CaM, 10 mM $CaCl_2$, 150 mM NaCl, and 20 mM BisTris in D_2O. H_2O cannot be used as the solvent, because the amide I band of proteins, which is sensitive to the secondary structure, is overlapping with the bending mode of liquid H_2O. The BisTris buffer is characterized by only little pressure dependence of the pD-value (small reaction volume) [25]. The pD-value was adjusted to 6.8. The pD-value was determined by adding 0.4 to the pH-meter reading using a conventional pH-electrode [26]. Solutions were heated to 60 °C for 20 min to achieve H/D exchange of the protein, except in the case of the H/D exchange experiments. Then, TFP was added to the CaM solution in a molar ratio of 1 CaM to 5 TFP, or melittin was added in a molar ratio of 1 CaM to 1.5 melittin. Solutions used for fluorescence spectroscopy were prepared in a similar way except for using a lower CaM concentration of 0.6 mM and H_2O as the solvent. The pH-value was adjusted to 6.6. All measurements were performed at 25 °C as a function of increasing pressure.

FTIR spectra of CaM in D_2O solution were collected using a Nicolet 6700 FTIR spectrometer from Thermo Fisher Scientific, which was operated with a liquid nitrogen-cooled MCT detector and was purged with dry air. Each FTIR spectrum is the average over 256 interferometer scans with a spectral resolution of 2 cm^{-1}. Pressure-dependent measurements were performed using the P-series diamond anvil cell from High Pressure Diamond Optics (Tucson, AZ, USA). It has two type IIa diamonds with a surface diameter of 0.6 mm. They are separated by a 50 μm thick steel gasket with a central hole of 0.5 mm diameter containing the sample solution (about 10 nL). The temperature of the diamond anvil cell was set by a circulating water flow. Barium sulfate was added to the sample as an internal pressure sensor. The shift of the 983 cm^{-1} band to higher wavenumbers reflects the applied pressure according to p/kbar = 0.031 $(\Delta\tilde{\nu}/cm^{-1})^2$ + 2.097 $(\Delta\tilde{\nu}/cm^{-1})$, where p is the pressure and $\Delta\tilde{\nu}$ is the wavenumber shift [27]. Spectral analysis was carried out using the Grams/AI 8.1 software from Thermo Fisher Scientific.

Fluorescence experiments to determine the FRET from Tyr of CaM to Trp of melittin or to Trp of the CaM (T26W) mutant were carried out using the K2 fluorescence spectrometer from ISS (Champaign, IL, USA) with a high-pressure cell from ISS, which is temperature-controlled by a circulating water flow. Fluorescence of Tyr and Trp residues was excited with a Xe arc lamp and detected in photon counting mode.