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Review article

Analyzing protein-ligand and protein-interface interactions using high pressure

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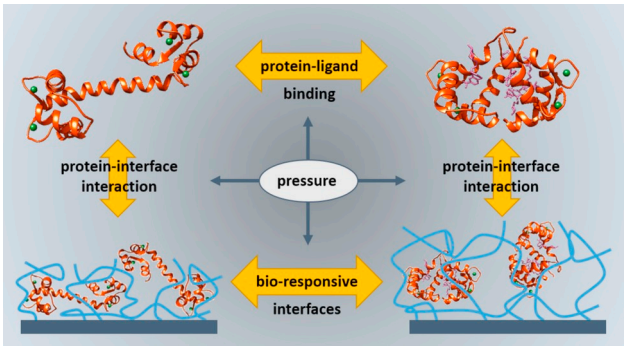
HIGHLIGHTS

- Protein-ligand and protein-interface interactions are studied using high pressure.
- Ligand binding affinity of calmodulin correlates with pressure response.
- Pressure experiments clarify protein adsorption mechanism at polyelectrolyte brushes.
- Designs of novel bio-responsive interfaces are presented.

GRAPHICAL ABSTRACT



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ABSTRACT

All protein function is based on interactions with the environment. Proteins can bind molecules for their transport, their catalytic conversion, or for signal transduction. They can bind to each other, and they adsorb at interfaces, such as lipid membranes or material surfaces. An experimental characterization is needed to understand the underlying mechanisms, but also to make use of proteins in biotechnology or biomedicine. When protein interactions are studied under high pressure, volume changes are revealed that directly describe spatial contributions to these interactions. Moreover, the strength of protein interactions with ligands or interfaces can be tuned in a smooth way by pressure modulation, which can be utilized in the design of drugs and bio-responsive interfaces. In this short review, selected studies of protein-ligand and protein-interface interactions are presented that were carried out under high pressure. Furthermore, a perspective on bio-responsive interfaces is given where protein-ligand binding is applied to create functional interfacial structures.

1. Introduction

The interaction of proteins with small molecules, polymers, other proteins, and interfaces is generally associated with a volume change. The volume of a system depends on the spatial fit of the interacting molecules, which can leave some void volume, and generally on the packing density of the system [1,2]. The latter is important to consider, when water molecules are making contact with hydrophobic moieties or electric charges [3–6]. When a salt bridge or ionic bond in aqueous solution is broken, water molecules form a hydration shell that has a higher packing density than bulk water (electrostriction). Thus, pressure destabilizes ionic bonds in aqueous solution [1,2].

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The overall effect of pressure on the thermodynamic stability of a system can be expressed by the pressure derivative of the free energy (Gibbs energy, G) of the system, which is given by the volume of the system, V:

$$\left(\frac{\partial G}{\partial p}\right)_T = V \tag{1}$$

where p and T are the applied pressure and temperature, respectively. Thus, any system has a higher Gibbs energy upon pressurization. However, when chemical reactions, molecular associations or phase transformations are considered, an underlying volume change, ΔV, can favor or disfavor this process depending on the sign of ΔV [1,2,7]:

$$\left(\frac{\partial \ln K}{\partial p}\right)_T = -\frac{\Delta V}{RT} \tag{2}$$

where K is the equilibrium constant and R is the gas constant. A similar equation results from the transition state theory, where the volume change upon formation of the transition state, the activation volume ΔV^*, affects the rate constant, k, of a chemical reaction [1,2,7]:

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^*}{RT}. \tag{3}$$

It is noticed that even a small activation volume of $+18\,\mathrm{mL\,mol^{-1}}$ (size of a water molecule) will decrease the rate constant to 48% at 1000 bar according to this equation. Thus, activation volumes of enzymatic reactions can be measured in the pressure range of about 1–5000 bar [8–14]. On the other hand, protein unfolding is often observed at higher pressures, i.e., in the range of 4000–10,000 bar [15–20]. In a similar way, the degree of binding of small molecules (ligands, substrates) to proteins can be affected in the pressure range of several kilobars depending on the magnitude of the volume change of binding [21,22].

In most of the studies that are presented in this short review, calmodulin (CaM) is used as model protein. This protein is involved in a signal transduction where a rise in the Ca^{2+} concentration is transformed into a change of protein activity [23,24]. Hence, there are three states of CaM: Apo-CaM without bound Ca^{2+} ions has an open conformation, holo-CaM with four bound Ca^{2+} ions has an open dumbbell shape, and the holo-CaM-ligand complex, which is formed when the two lobes of holo-CaM wrap around the target ligand, has a closed globular shape (Fig. 1) [25–30]. The ligand can be a small molecule, a short peptide or a protein and binds via electrostatic attraction and mostly hydrophobic interactions [23,26,31,32]. Electrostatic attraction stems from the dominant negative net charge of holo-CaM at neutral pH-values [31] and multiple positive charges of the ligands. Both electrostatic attraction and the likely existence of void volumes at the contact interface in a holo-CaM-ligand complex represent sources of pressure sensitivity, as outlined above. Thus, from an experimental point of view, CaM combines several advantages for pressure-dependent binding studies. It has a pronounced ability to bind Ca^{2+} ions, various small molecules and peptides. Furthermore, the huge conformational change upon ligand binding is a clear marker of the binding state, and the strong negative net charge at pH = 7 facilitates adsorption at surfaces via electrostatic interactions. Finally, CaM can be produced as the wild type and as various mutants by gene expression in an efficient way [33].

A broad range of spectroscopic and scattering techniques can be applied to investigate protein interactions under high pressures. Protein solutions have long been studied under high pressures by Fourier transform infrared (FTIR) [15–18], NMR [19,34–37], and fluorescence [20,38–40] spectroscopy, among others, to reveal mostly the secondary and tertiary structure of the protein, whereas high-pressure X-ray and neutron scattering is applied to determine the radius of gyration and the pair distance distribution function of a protein in solution [41,42]. High pressure sample cells are often home-built, but in some cases also commercially available. More recently, high-pressure cells enabling the study of planar interfaces have also been developed [43–45]. For example, high-pressure X-ray and neutron reflectometry reveal the scattering length density of an interface. Similarly, the high-pressure total internal reflection fluorescence (TIRF) spectroscopy detects a fluorescence signal created in an evanescent wave at a planar water/quartz interface. In contrast to using colloidal particles or beads to provide an aqueous-solid interface, the analysis of a planar interface has the advantage of a clear separation of the interfacial signal from the bulk solution. Furthermore, the solution, which is in contact with the interface, can be exchanged by simple rinsing.

The studies discussed in this short review reveal that the application of high pressure yields novel and fundamental insights into protein interactions, because these are almost always associated with volume changes. For example, as shown in section 2, the binding of an inhibitor to CaM creates less void volume than that of a natural peptide in agreement with the observed CaM affinities of these binding partners. Moreover, as discussed in section 3, adsorption mechanisms of proteins

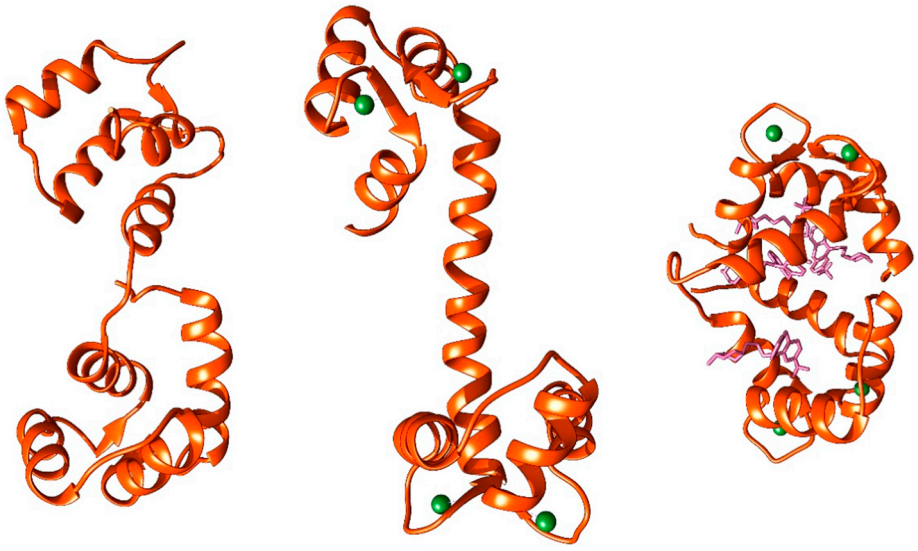

Fig. 1. Calmodulin (CaM) adopts three conformational states: apo-CaM (left), holo-CaM with four Ca^{2+} ions (middle), and holo-CaM with four Ca^{2+} ions and a ligand (right). Here, four TFP molecules are bound as ligands. The images were prepared with Chimera 1.10.2 using PDB IDs 1DMO, 3CLN, and 1LIN.

at polyelectrolyte interfaces that cannot be distinguished experimentally at ambient pressure can be identified at high pressures, because they are associated with different volume changes of adsorption. The review is concluded with a special outlook in section 4, where the design of bio-responsive interfaces is discussed. Two realized examples are highlighted here, which combine ligand binding, conformational changes, and interfacial adsorption of CaM in a favorable way.

2. High-pressure studies of protein-ligand interactions

Proteins bind various types of ligands. However, different ligands generally have different protein affinities, which is important for the natural protein function. In some cases, there are even artificial inhibitors that bind most strongly to a protein and prevent the binding of natural ligands. To understand the origin of different affinities between a protein and ligands, it is not sufficient to know the types of interaction, such as electrostatic or hydrophobic interactions, it is also important to analyze the spatial fit of a ligand into the binding pocket of the protein. An improper fit will create some void volume at the protein-ligand interface, which can be addressed in pressure dependent binding experiments. Hence, pressure modulation provides an additional tool for drug design.

Using holo-CaM as model protein, the binding of the inhibitor trifluoperazine (TFP) and natural peptides, such as melittin and the hypervariable region (HVR) of K-Ras4B, has been investigated as a function of pressure [46–48]. TFP is known as antipsychotic drug and calmodulin antagonist [49,50]. As an important result, the deconvolution of the amide I′ band in the FTIR spectrum of holo-CaM shows an intensity increase of the 1632 cm^{-1} sub-band with increasing pressure, which has been interpreted as an indication for an increasing hydration of α-helical secondary structure [46], in accordance with other studies of this infrared sub-band [51–53]. Moreover, when holo-CaM binds four TFP molecules and forms a globular complex (Fig. 1), this pressure-induced hydration is shifted to higher pressures by several kilobars [46]. Thus, the inhibitor complex appears more pressure stable, suggesting a denser packing with less void volume. When melittin is bound to holo-CaM, an intermediate behavior is observed in the FTIR spectrum, as compared to holo-CaM and holo-CaM/4TFP [46]. At much higher pressures, holo-CaM is losing its four Ca^{2+} ions [46,48], probably due to a distortion of the tertiary structure of the protein [16]. There are two marker bands at 1552 cm^{-1} and 1579 cm^{-1} in the FTIR spectrum of holo-CaM signaling Ca^{2+} binding [54,55]. In Fig. 2, the 2nd derivative of these bands is plotted as a function of pressure. The analysis of the sigmoidal data curves reveals midpoints of 5.5 kbar for holo-CaM, 7.5 kbar for holo-CaM/melittin, and 11 kbar for holo-CaM/4TFP, again showing the highest pressure stability for the inhibitor complex [46]. Interestingly, the associated van't Hoff volumes, as determined using eq. 2, are different for the three samples. Values of $-14\ mL\ mol^{-1}$ for holo-CaM, $-12\ mL\ mol^{-1}$ for holo-CaM/melittin, and $-6\ mL\ mol^{-1}$ for holo-CaM/4TFP have been determined, clearly showing that the volume changes are not only related to the Ca^{2+} dissociation, because the same value would be expected in this case for all samples [46]. Rather, the volume changes also characterize the pressure-induced unfolding of holo-CaM. Thus, the holo-CaM/4TFP complex is almost perfectly packed with a minimum of void volume, explaining in part the mechanism of holo-CaM inhibition by TFP [46].

Structural studies of holo-CaM with ligands have been performed in the pressure range up to 3000 bar [47]. Using small-angle X-ray scattering (SAXS) it has been found that the so-called pair distance distribution function, $p(r)$, of holo-CaM is slightly modified upon pressurization. $p(r)\,dr$ is proportional to the number of distances in a molecule between r and $r + dr$ [56]. The $p(r)$ function of holo-CaM has two peaks which are characteristic of a dumbbell shape. The second peak is enhanced under pressure suggesting a swelling of the two lobes due to water penetration into the protein [47]. This result is in favorable agreement with the FTIR experiments outlined above, from which a pressure-induced hydration of the α-helices was inferred [46]. Moreover, the SAXS measurements indicate a rather pressure-stable globular holo-CaM/4TFP complex up to 3000 bar, again in line with the corresponding FTIR experiments mentioned above [46,47]. Apparently, the four TFP molecules are small and flexible and can fill the binding cavity of holo-CaM in the globular conformation completely. In Fig. 3, three-dimensional structure models of holo-CaM without and with bound TFP are shown which were calculated from the corresponding $p(r)$ functions using the ATSAS software package [57]. The images show satisfying agreement between the SAXS and crystallographic protein structures at ambient conditions [47]. After pressurization, holo-CaM without ligand shows a swelling of the two lobes, whereas the shape of holo-CaM/4TFP remains rather similar. Indeed, the radius of gyration of this complex is slightly decreasing from 1.73 nm at 5 bar to 1.65 nm at 3000 bar [47].

From complementary neutron scattering experiments, the dynamics of holo-CaM without and with bound TFP or the HVR of K-Ras4B was studied up to 4000 bar [47]. Here, the elastic incoherent neutron scattering (EINS) of the samples was evaluated which arises from the H-atoms of the protein and the ligands in D_2O as the solvent. The EINS intensity depends on the quasi-elastic broadening of the elastic line in the neutron scattering spectrum due to internal fluctuations and diffusion on the sub-ns time scale. The data indicate a reduced mobility of the H-atoms of holo-CaM above about 2000 bar, which might be explained by water penetration into protein cavities and the concomitant hydration of the α-helices, as observed by SAXS [47] and FTIR spectroscopy [46]. In the case of the holo-CaM/4TFP complex, no such change in mobility is observed up to 4000 bar, again showing an enhanced pressure stability upon binding the inhibitor [47].

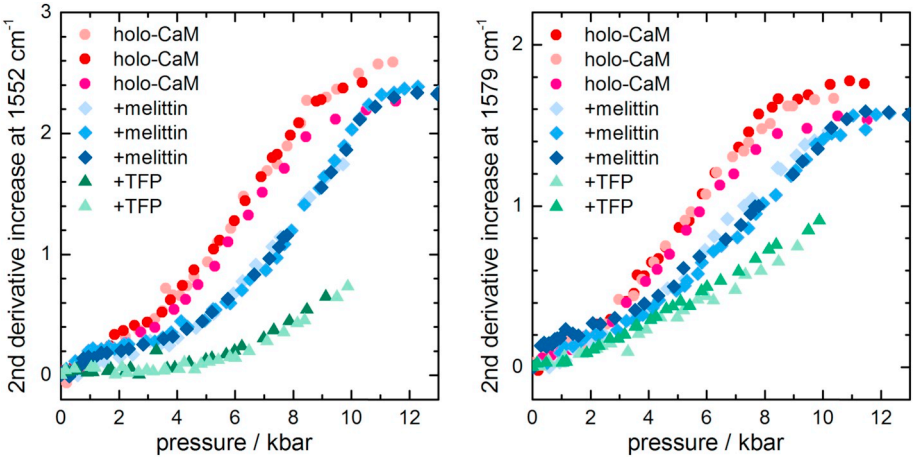

Fig. 2. Ca^{2+} binding of CaM as a function of pressure. The increase of the 2nd derivative of the 1552 cm^{-1} and 1579 cm^{-1} infrared bands indicates dissociation of Ca^{2+} ions from holo-CaM. Data are shown for holo-CaM without ligand, with bound melittin and with bound TFP. Image reproduced from [46] with permission from Elsevier.

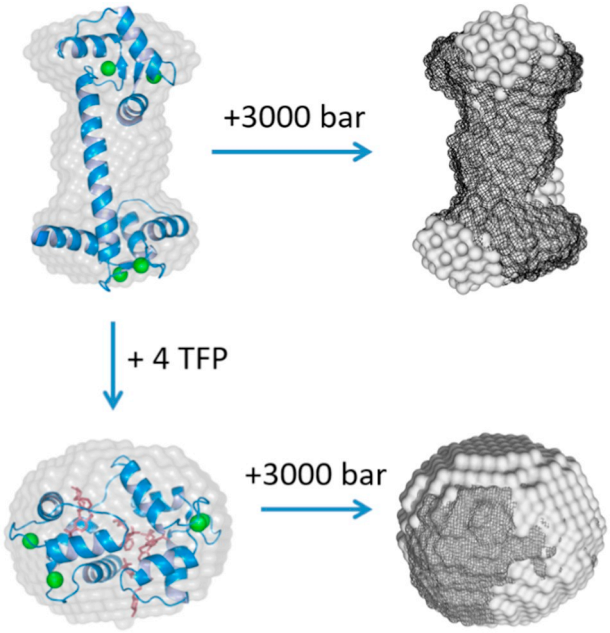

Fig. 3. Ab initio calculations of the three-dimensional shape of holo-CaM without and with bound TFP at 5 and 3000 bar, which are based on experimental SAXS data. At ambient pressure (left images), SAXS and crystallographic structures are overlaid. At 3000 bar (right images), small deviations are observed. The gray spheres and the black nets represent the 3000 bar and 5 bar structures, respectively. Image adapted from [47] with permission from the PCCP Owner Societies.

3. High-pressure studies of protein-interface interactions

3.1. Polyelectrolyte brushes

The immobilization of proteins, such as enzymes or antibodies, on solid supports is of great interest for biotechnological and biomedical applications, the industrial production of chemicals, and the academic research [58–63]. In particular, immobilized proteins are used for the synthesis of organic molecules, the treatment and transformation of food, the production of pharmaceuticals, drug delivery, and the sensing of other molecules. The main advantage of immobilized proteins in comparison with proteins that are free in solution is the easy recovery and separation of the proteins from the reaction solution. A planar solid support with attached proteins can be removed and rinsed with other solutions, whereas colloidal carrier particles can be separated by centrifugation, sedimentation, or magnetic forces, if the particles have a magnetic core. It has also been pointed out that a careful immobilization technique can even lead to a conformational stabilization of the protein, thereby enhancing the biological activity [60], although this seems not to be the rule. Rather, immobilized proteins can suffer from a reduced biological activity due to adsorption-induced partial unfolding, blocking of the active site, and limited dynamics [64–66]. Thus, the surface of the solid support (planar material or nanoparticles) needs to be modified to provide a benign environment for the immobilized protein molecules.

There are many studies in the literature that characterize polymer and polyelectrolyte brushes as an appropriate surface modification for the immobilization of proteins [67–75]. A polyelectrolyte brush consists of long polyelectrolyte chains that are densely grafted to the solid surface [76,77]. In particular, a polyelectrolyte brush consisting of poly (acrylic acid) chains (PAA brush) largely maintains the conformation and biological activity of adsorbed proteins [69,70,72,73]. Interestingly, the degree of protein adsorption at a PAA brush depends on the ionic strength of the protein solution that is in contact with the brush. At low ionic strength, both positively and negatively charged proteins adsorb at a PAA brush. In contrast, protein resistance of a PAA brush is observed, when the ionic strength of the protein solution is increased to a few 100 mM [74,75,78]. Adsorption of positively charged proteins at a PAA brush is typically governed by electrostatic attraction. However, there are documented examples of adsorption above the isoelectric point, where the protein should experience net electrostatic repulsion. Several models have been proposed to account for this counter-intuitive behavior. When the protein molecules carry a weak negative net charge, it has been argued that a lower pH-value inside the PAA brush will reverse the net charge of the protein resulting in electrostatic attraction [79,80]. However, protein adsorption is also observed at 2–3 pH units higher than the isoelectric point. Then, the release of counterions has been identified as the dominant entropic driving force for adsorption, when positively charged patches on the protein surface interact with the negatively charged PAA chains [81,82]. Apparently, all proposed mechanisms for protein adsorption at a PAA brush are based on charge interactions in water. Thus, pressure can be expected to affect the degree of protein adsorption, because volume changes are associated with the breaking or formation of ionic bonds [1,2].

Applying high-pressure neutron reflectometry, the mass of adsorbed α-chymotrypsin at a PAA brush has been measured in the pressure range of 1–2000 bar [83]. At pH = 7, α-chymotrypsin has a positive net charge and can interact with a PAA brush under electrostatic attraction. The data show a pressure-dependent degree of adsorption with 2.3 mg m^{-2} of protein at ambient pressure and 1.8 mg m^{-2} at 2000 bar. Thus, the pressure experiments are in nice agreement with the expected underlying electrostatic interactions, because pressure favors the dissociation of ionic bonds and the concomitant hydration of free charges.

In a more systematic study, the effects of pH-value and pressure on the degree of CaM adsorption at a PAA brush has been investigated applying high-pressure TIRF spectroscopy [84]. By varying the pH-value, the net charge of the protein could be changed from negative (high pH) to positive (low pH). Surprisingly, when CaM carries an excess of negative charges (two pH units above the isoelectric point), a pressure-induced enhancement of the adsorbed protein mass has been observed, whereas the opposite pressure effect has been found with a positive net charge of the protein [84]. The latter observation goes in line with the pressure effect on α-chymotrypsin adsorption described above [83]. However, a pressure-induced protein adsorption at a PAA brush under conditions of a net electrostatic repulsion calls for the formation and hydration of free charges, which would be favored under high pressure. Considering the proposed mechanisms of adsorption, a simple interaction of the negatively charged PAA chains with positively charged protein surface patches can be ruled out because of a positive volume change (Fig. 4a). In the same way, a charge reversal of the protein inside the PAA brush leads to fewer charges and a concomitant volume increase (Fig. 4b). On the other hand, a release of protein or PAA counterions that are already fully hydrated is not expected to cause any significant volume change. However, the release of “condensed” and poorly hydrated counterions that become fully hydrated are in agreement with a volume decrease and a pressure-induced protein adsorption at a PAA brush (Fig. 4c). Indeed, about two third of all PAA counterions are “condensed”, if the Bjerrum length is calculated and compared with the monomer distance of the polyelectrolyte chains [84]. Thus, based on the pressure-dependent studies of the degree of protein adsorption at a PAA brush, adsorption of negatively charged CaM is likely driven by a release of “condensed” counterions. Overall, the use of pressure provides a way to distinguish interaction mechanisms between a protein and its environment experimentally.

3.2. Lipid membranes

Besides functioning as a barrier for polar molecules, lipid membranes also serve as an anchoring plane for proteins. In this way, proteins can interact with other proteins, form ion channels through the membrane, or induce fusion with another membrane. The interaction of

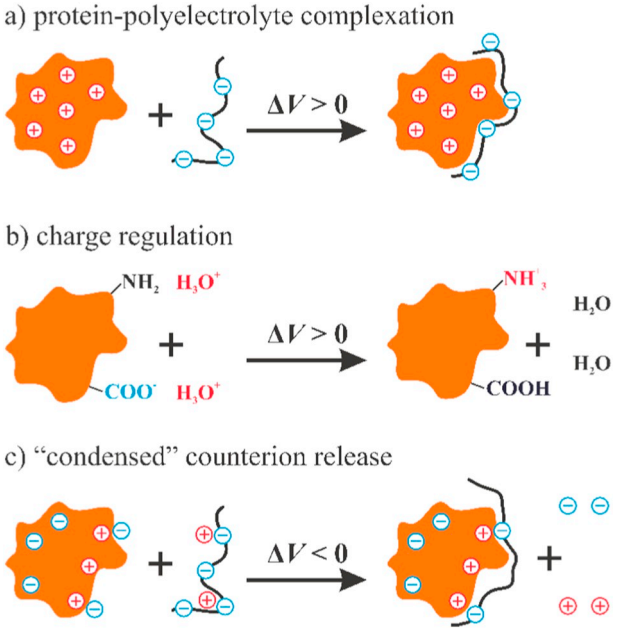

Fig. 4. Proposed volume changes upon interaction of a protein with a negatively charged polyelectrolyte chain. It is assumed that the formation and hydration of free charges (ions) is associated with a volume decrease. Image reproduced from [84] with permission from Elsevier.

a protein with a lipid membrane can result from attractive electrostatic forces, which provide a rather weak and reversible protein-membrane attachment. However, membrane proteins are generally characterized by hydrophobic surface areas that facilitate penetration into the lipid membrane and strong interaction with the hydrophobic membrane core. When proteins or protein segments are inserted into a lipid membrane, the packing density of the lipid chains is expected to be changed which can be probed by high pressure experiments.

There is a series of studies in the literature where the effect of so-called fusion peptides on the phase behavior of lipids is analyzed [85–87]. Fusion peptides are hydrophobic segments of viral fusion proteins that steer the fusion of a viral membrane with a cell membrane during formation of a pore [88,89]. A fusion pore and a hemifusion stalk, which is an intermediate in the fusion process, are characterized by lipid membranes that have a saddle-like curvature (Fig. 5). Lipid mesophases consisting of lipid membranes with a saddle-like curvature are inverse bicontinuous cubic phases, Q_{II}. Indeed, it has been reported in some studies that fusion peptides can induce inverse cubic phases [86,90,91]. However, to reveal the contribution of packing effects to the lipid phase control of the fusion peptides, pressure experiments are needed.

Applying high-pressure SAXS in combination with high-pressure FTIR spectroscopy, the pressure-temperature phase diagram of monoolein at limited hydration has been determined in the absence and the presence of two different fusion peptides, and the secondary structures of the two peptides in the monoolein membrane have been analyzed [92]. It has been found that the peptides affect the monoolein phase behavior to very different extents, but both are favoring the formation of the Q_{II} phase and destabilize the fluid lamellar phase. Moreover, the induction of negative curvature of the lipid membrane by interaction with the fusion peptides can partially be reversed upon pressurization. Thus, the pressure-dependent SAXS experiments clearly prove a less dense packing of the lipid chains in the presence of the fusion peptides [92]. Pressure jumps across the cubic-lamellar phase boundary have also been carried out. Based on a simple model, the ratio of the rate constants in forward and backward directions, k_1 / k_2, has been related to the activation volume, $\Delta V^*_{1,2}$, proposing the following Eq. [92]:

$$\frac{k_1(p_2)}{k_2(p_1)} = \exp\left(-\frac{\Delta V^*_{1,2}\cdot(p_2 - p_1)}{RT}\right) \quad (4)$$

where p_1 is the lower and p_2 is the higher pressure in the jump experiments. Applying this equation, the authors have found an activation volume of $\Delta V^*_{1,2} = +65\,\mathrm{mL\,mol^{-1}}$, which represents a substantial volume barrier between the cubic and lamellar phase. Recalling that the formation of the cubic phase from the lamellar phase resembles the formation of fusion pores, this activation volume might be assigned to the transient void volume of a hemifusion stalk where the opposing cis monolayers of the two membranes are already fused but the distal trans monolayers are still separated (Fig. 5). From the pressure-dependent FTIR measurements, the secondary structures of the fusion peptides are found unaffected by the cubic-lamellar phase transition [92]. Apparently, the structure of the peptides is more stable than the structure of the lipid phases. Overall, the pressure-dependent studies of the interaction of fusion peptides with lipid membranes have revealed fundamental structural details that helped to clarify the mechanism of fusion pore formation by viral fusion proteins.

4. Design of bio-responsive interfaces

Our understanding of protein-ligand and protein-interface interactions has led to the design of bio-responsive interfacial structures composed of polymer films with embedded proteins [93,94]. Modification of an aqueous-solid interface with a judiciously selected polymer can yield a surface (hydrophilic, hydrophobic, or electrically charged, as desired) capable of adsorbing selected small molecules, proteins, or cells. The degree of adsorption can be influenced by manipulation of solution properties, if a stimuli-responsive polymer used. For example, brushes consisting of poly(N-isopropylacrylamide) or poly (2-(dimethylamino)ethyl methacrylate) chains will transition between the hydrated and collapsed polymer conformations in response to heating [68,95–97], often with an accompanying increase in protein-adsorption capacity. Alternatively, polyelectrolyte brushes are sensitive to pH. A reduction in pH will neutralize anionic groups or create cationic charges, in turn affecting the degree of protein adsorption or cell adhesion [71,98]. In cases where the modification of pH or temperature may result in protein denaturation, manipulation of the ionic strength can provide a more gentle strategy for increasing protein-binding capacity [74,75,78]. For example, the adsorptive capacity of a poly

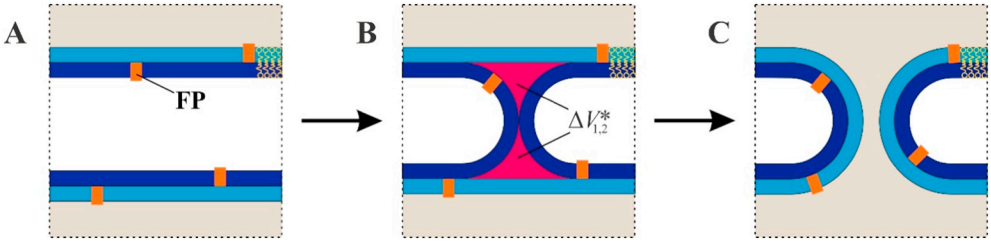

Fig. 5. Schematic illustration of the formation of a pore by the fusion of two lipid membranes containing fusion peptides (FP). A hemifusion stalk develops as intermediate (middle). It is characterized by areas of low packing density or void volumes, $\Delta V^*_{1,2}$.

(acrylic acid) brush is sensitive to ionic-strength variations in the range of a few 100 mM.

An interface that is strongly responsive to physico-chemical solution parameters (temperature, pH, ionic strength) may not be compatible with many biomedical or biophysical applications. Therefore, the design of bio-responsive interfaces that change their structure and properties as a function of biochemical stimuli is needed. The basic approach for the design is taken from bio-responsive polymer gels that contain proteins which undergo a huge conformational change upon ligand binding [99–101]. If this conformational change is coupled to a polymer network, a bio-responsive system is obtained. Here, holo-CaM is a highly favorable candidate, because it can be switched between an open dumbbell shaped and a closed globular conformation in the absence and presence of a ligand, respectively (Fig. 1). The inhibitor TFP is the ligand of choice for such purpose, because holo-CaM forms a stable globular complex upon binding four TFP molecules [25,47]. A further example where the hinge motion of a protein is translated to a polymer matrix is a poly(acrylamide) hydrogel that includes the glucose binding protein (GBP). When GBP is binding glucose, a volume change of the biomaterial is observed [100]. Similarly, addition of ATP to an N-(2-hydroxypropyl)methacrylamide polymer cross-linked with adenylate kinase produces a volume reduction on the order of 15% [100]. As shown below, the application of high pressure might be a further key parameter to control the bio-responsiveness of interfaces.

4.1. Bio-responsive interfaces composed of poly(ethylene glycol)

The first bio-responsive interface that is based on the ligand binding of holo-CaM has been realized recently [93]. Here, poly(ethylene glycol) (PEG) chains are covalently bound to a silicon wafer and are cross-linked by a double-Cys mutant of CaM (Fig. 6 top). The two Cys mutations are located on the two lobes of CaM and are used to bind the PEG chains. It is noted that the two Cys mutations have a maximum distance in holo-CaM without ligand, but a very close distance when holo-CaM has bound four TFP molecules. In this way, the PEG chains are pulled together upon ligand binding of holo-CaM. Applying X-ray reflectometry, the thickness of this film has been determined after rinsing with various solutions and full hydration in a humidity sample chamber [93]. Interestingly, a reversible variation of the film thickness can be observed, when solutions of TFP/Ca^{2+} and EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) are used. A TFP/Ca^{2+} solution will generate holo-CaM/4TFP in the compact globular state, whereas rinsing with the EGTA solution will remove the Ca^{2+} ions from holo-CaM, thereby generating apo-CaM that does not bind TFP. There is always an increase of the film thickness after rinsing with TFP/Ca^{2+} and a decrease after rinsing with EGTA [93]. Thus, a bio-responsive interface that is changing its structure upon a biochemical trigger has been established. As illustrated in Fig. 6, apo-CaM without ligand allows for a relaxed PEG network and an optimum flat adsorption of the protein at the silicon surface. In contrast, holo-CaM/4TFP induces tension in the PEG network. Moreover, the minimum diameter of the globular protein-ligand complex is much larger than that of apo-CaM (44 Å vs. 15 Å, respectively). Thus, the observed structural changes can be explained in a reasonable way.

4.2. Bio-responsive polyelectrolyte multilayers

It has also been investigated, if bio-responsiveness can be induced into a polyelectrolyte multilayer (PEM) by incorporation of holo-CaM [94]. A PEM consists of alternating layers of a polycation and a polyanion [102–104]. During the build-up of a PEM, a protein can replace the polycation or the polyanion, depending on the protein net charge. In this way, a bio-functionalization of an interface is achieved [105,106]. There are many examples in the literature that show preservation of the native structure and the enzymatic activity of proteins when they are embedded in or adsorbed on a PEM [107–109]. Some degree of conformational flexibility of a protein inside a PEM can be inferred from enzymatic studies, but there is a lack of knowledge about the extent of such conformational freedom. Thus, it is very interesting to study the huge conformational changes of CaM upon binding Ca^{2+} ions and TFP, when CaM is embedded in a PEM.

To this end, PEMs with the sequence Si-PEI-$(PSS\text{-}PAH)_x$-CaM-PAH-CaM-PAH (x = 1,2) have been analyzed applying X-ray and neutron reflectometry (PEI, poly(ethylenimine); PSS, poly(styrenesulfonate); PAH, poly(allylamine hydrochloride)) [94]. The data suggest that no binding of TFP by holo-CaM is possible, when holo-CaM is forming a layer inside the PEM, because holo-CaM-PAH interactions appear much stronger than holo-CaM-TFP interactions. Thus, the conformational freedom of holo-CaM is strongly suppressed by the neighboring PAH layers. However, interestingly, reversible shrinking and swelling of the PEMs are observed upon rinsing with Ca^{2+} and EGTA solutions, respectively [94]. This behavior can easily be explained by a change of osmotic pressure (Fig. 6 bottom). When CaM binds four Ca^{2+} ions, intrinsic charge compensation dominates inside the PEM, resulting in a reduced osmotic pressure and a low water content. In contrast, after rinsing with EGTA, the bound Ca^{2+} ions of holo-CaM are removed and replaced by eight free monovalent ions (Na^+ or H^+). Then, extrinsic charge compensation leads to an increased osmotic pressure inside the PEM succeeded by water uptake and swelling of the PEM. Thus, although no ligand-induced conformational change of holo-CaM could be observed when the protein is embedded in a PEM, bio-responsiveness is still achieved via Ca^{2+} binding of CaM.

High pressure in the range of 2000 bar is known to favor dissociation of salt bridges (ionic bonds) in water, because the hydration of the separated charges leads to a higher packing density of water and hence a smaller volume [1,2]. Thus, the application of high pressure should also weaken the electrostatic interactions between the polycation and polyanion layers inside a PEM. Furthermore, it can be expected that the

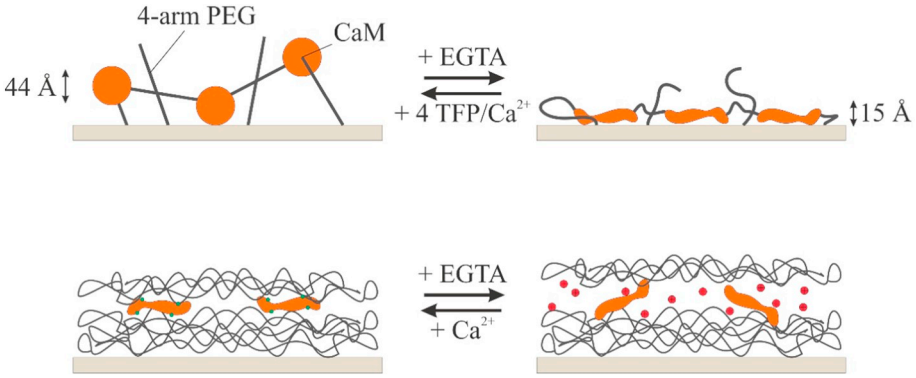

Fig. 6. Bio-responsive interfaces that are based on the TFP and Ca^{2+} binding of calmodulin [93,94]. Rinsing with an EGTA solution removes the bound Ca^{2+} ions.

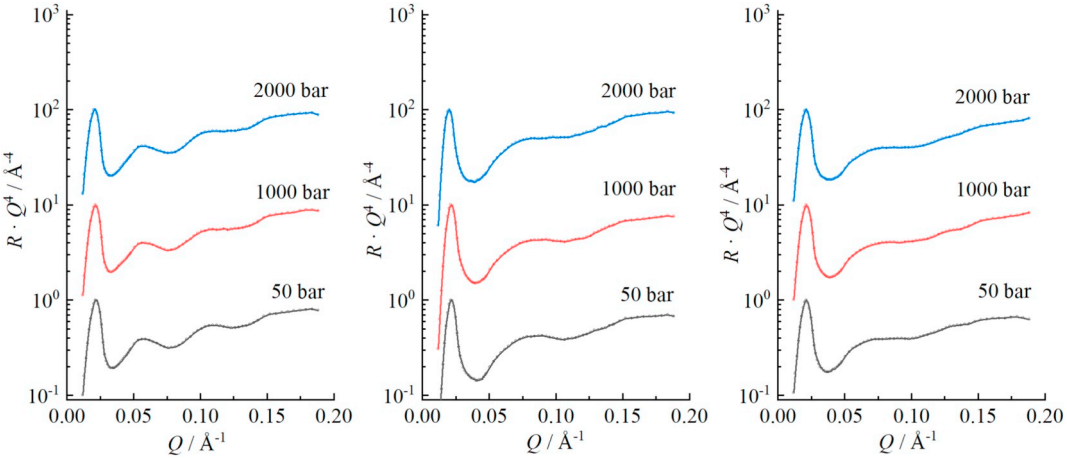

Fig. 7. High-pressure X-ray reflectivity data of a PEM without CaM (left), with embedded CaM (middle), and with embedded CaM in the presence of TFP (right) [110]. The data are shifted by factors for clarity.

conformational freedom of CaM embedded between two layers of PAH is enhanced due to weaker electrostatic attraction under high pressure. Therefore, high-pressure X-ray reflectivity has been applied to investigate PEMs with incorporated holo-CaM [110]. The data show Kiessig fringes that are consistent with approximate PEM thicknesses of 124 Å in the absence of holo-CaM, 97 Å when holo-CaM is embedded, and 98 Å when holo-CaM is embedded and the ligand TFP is present in the solution (Fig. 7). It is interesting to note that there are only little pressure effects on the structures of the investigated samples. A slight flattening of the minima at about $Q = 0.12\,\text{Å}^{-1}$ can be observed as the pressure is increased suggesting an increased roughness of the multilayers. It might also point to a pressure-induced conformational change of the embedded protein molecules. Apparently, the PEMs are densely packed films with little void volume. This would indicate fully hydrated charges of the polycation and the polyanion which do not form direct ionic bonds. However, these systems need to be explored in more detail to verify any pressure effect on the structure of CaM inside a PEM.

5. Conclusions

The few experimental studies highlighted in this short review illustrate that high-pressure experiments are very useful to study volume changes that are associated with the binding of ligands to proteins or with the interaction of proteins with various interfaces. In the case of ligand binding, pressure-dependent studies suggest that the binding strength is correlated with the pressure stability of the protein-ligand complex. Comparing an inhibitor with a natural peptide, smaller void volumes are generated upon binding the inhibitor, enabling a better spatial fit of the ligand and stronger binding to the protein. Thus, pressure studies may also be helpful to identify potential drug molecules that interact with proteins. In the case of protein-interface interactions, pressure studies can reveal electrostatic interactions and clarify interaction mechanisms. A postulated protein adsorption mechanism is always associated with a volume change if ionic bond formation or breaking is involved. Then, depending on the magnitude and sign of this volume change, a particular pressure effect on the degree of protein adsorption is expected and can be compared with experimental data. Furthermore, a change of the molecular packing density, such as that related to peptide binding to lipid membranes, can easily be detected by applying high pressure to these systems. Here, even volume barriers upon formation of lipid cubic phases are accessible by pressure-jump experiments, which might be related to the hemifusion stalk formation during membrane fusion. Finally, high pressure will also be of great use to design new bio-responsive interfaces where protein-ligand and protein-interface interactions are combined, because pressure modulation allows for a smooth adjustment of electrostatic interactions of the embedded protein with its environment, which is essential for its bio-responsive function at the interface.

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