High pressure modulated transport and signalling functions of membrane proteins in models and in vivo

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Abstract. Cellular membranes serve in the separation of compartments, recognition of the environment, selective transport and signal transduction. Membrane lipids and membrane proteins play distinct roles in these processes, which are affected by environmental chemical (e.g. pH) or physical (e.g. pressure and temperature) changes. High hydrostatic pressure (HHP) affects fluidity and integrity of bacterial membranes instantly during the ramp, resulting in a loss of membrane potential and vital membrane protein functions. We have used the multiple drug transporter LmrA from Lactococcus lactis and ToxR, a membrane protein sensor from Photobacterium profundum, a deep-sea bacterium, and Vibrio cholerae to study membrane protein interaction and functionality in proteolioposomes and by the use of in vivo reporter systems, respectively. Both proteins require dimerization in the phospholipid bilayer for their functionality, which was favoured in the liquid crystalline lipid phase with ToxR and LmrA. Whereas LmrA, which resides in liposomes consisting of DMPC, DMPC/cholesterol or natural lipids, lost its ATPase activity above 20 or 40 MPa, it maintained its active dimeric structure in DOPC/DPPC/cholesterol liposomes up to 120 MPa. By using a specific indicator strain in which the dimerisation of ToxR initiates the transcription of lacZ it was demonstrated, that the amino acid sequence of the transmembrane domain influences HHP stability of ToxR dimerization in vivo. Thus, both the lipid structure and the nature of the protein affect membrane protein interaction. It is suggested that the protein structure determines basic functionality, e.g. principle ability or kinetics to dimerize to a functional complex, while the lipid environment modulates this property.

1. Introduction
Cellular membranes are complex associations of macromolecules consisting of lipids and (glyco)proteins. The lipids in these systems are mostly phospholipids with hydrophilic (ionic) head groups and lipophilic fatty acid chains. This amphiphilic nature forces them to spontaneously associate to form bilayers or vesicles. These phospholipid bilayers comprise compartments of biological systems, which allow cellular functions in a controlled cytoplasmic space. The biochemical nature of the phospholipid side chains determines the basic characteristics of biological membranes with respect to their phase behavior, which can adopt several physical states between liquid crystalline and gel. These are influenced by physical (temperature, pressure) and biochemical (lipid composition, pH, ions) parameters. While pure lipids and artificial mixtures of few different lipids show distinct parameter sets for phase transitions and areas of co-existence, complex natural lipid mixtures exhibit smooth phase transitions. In the phospholipid bilayers of bacterial membranes a large variety of...
proteins are inserted, serving vital cellular functions of recognition, signal transfer or transport of metabolites and ions.

The effect of high hydrostatic pressure (HHP) on cellular systems has been extensively studied with foodborne bacteria, directed at the inactivation of pathogens and food spoiling bacteria. The vast majority of publications in this area describe bacterial inactivation in model and food systems along sigmoid inactivation curves revealing a threshold pressure before any effect can be seen and a very small number of survivors within the population, which were somehow protected or intrinsically resistant to HHP. Below the threshold pressure sublethal damage of bacteria has been described, which involves a cease of metabolic functions [17], ribosomal biosynthesis [14], impaired membrane integrity and fluidity [17] or lateral tension [16]. This initially reversible loss of HHP induced membrane functionality has been described to be causative for the killing of cells as a function of the holding time upon which environmental factors exert detrimental effects. While low pH acts synergistically with pressure to induce bacterial inactivation, molar concentrations of ions or neutral solutes stabilize bacterial membranes to near complete pressure resistance [12].

HHP has also widely been applied to investigate protein structure. The pressures used are very high (up to 1 GPa and beyond) and models used mostly employ high protein concentrations in buffer solutions. Therefore, the information obtained cannot easily be transferred to the conditions of a biological system. Furthermore, most proteins used were soluble and investigated in liquid systems, while information on membrane proteins in their natural lipid environment is scarce. With whole cells it has been shown that membrane fluidity affects the activity of membrane transport proteins [18]. It was therefore the aim of our study to elucidate the role of the phospholipid membrane’s ability to influence membrane protein function in response to environmental changes, which affect membrane biophysical properties. Two systems were developed and employed. Proteoliposomes with the multiple drug transporter LmrA from *Lactococcus lactis* were used to investigate the influence of membrane lipid composition and fluidity on membrane protein functionality. On the other hand, a reporter system was developed which allowed investigation of membrane protein interaction as a function of protein structure *in vivo*. In both systems, HHP was used to adjust and vary membrane physical parameters, namely transitions from liquid crystalline to gel phases and induction of raft formation.

2. Materials and Methods

2.1. HHP dependence of LmrA activity in proteoliposomes

2.1.1. Production of LmrA. The plasmid pNHlmrA containing the His-tagged *lmrA* gene under the control of a nisin-inducible promoter was transformed into *Lactococcus lactis* [9]. Expression of LmrA was induced by nisin addition, and LmrA protein was purified by affinity chromatography using Ni²⁺ sepharose beads following a modified protocol from van den Berg van Saporea *et al.* [20].

2.1.2. Production of membrane vesicles. Vesicles were constructed from natural glycolipids of *Lactobacillus plantarum*, dimyristoylphosphatidylcholine (DMPC), DMPC/10% (mol/mol) cholesterol and dioleoylphosphocholine (DOPC)/dipalmitoylphosphocholine (DPPC)/cholesterol (1:2:1). Lipid mixtures were swelled in 50 mM HEPES-KOH buffer pH=7.0 and agitated to form multi lamellar vesicles (MLV) and extruded through a carbon membrane filter with 200nm pores to obtain large unilamellar vesicles (LUV). Liposomes were destabilized with DDM as detergent, LmrA was added to a lipid/protein ratio of 20:1 and DDM was adsorbed in two successive steps.

2.1.3. Activity of LmrA. Vesicles were subjected to pressures of 20 to 120 MPa in 50 mM HEPES-KOH buffer pH=7.0 immediately after addition of ethidium bromide as transport substrate and ATP as energizing substrate. After 100 min of pressurization in the presence of the substrates functionality and activity of LmrA were determined along the release of Pi using a colorimetric method based on the
protocol from Lanzetta et al. [8] and compared with a phosphorous standard. All experiments were compared with parallel experiments in the same vessel in the presence of ortho-vanadate. Readings were subtracted to exclude artifacts and selectively get the vanadate sensitive ethidium assisted ATPase activity of LmrA. Ortho-vanadate inhibits the activity of LmrA [19] [21].

2.2. HHP dependence of ToxR dimerization in vivo

2.2.1. Construction of an Escherichia coli ToxR reporter strain. The ompL promoter of Photobacterium profundum SS9 was fused to an E. coli lacZ encoding sequence. This fragment was inserted in vector pLDR10, which allowed, in combination with helper vector pLDR8, the integration into the λ attachment site atB of E. coli Top10. This strain was designated POLA. The ToxR gene was cloned into the arabinose-inducible plasmid vector pBADK- myc-hisB. The resulting vector was termed pBADK-toxR-his. The reporter strain POLA was then transformed with pBADK-toxR-his.

2.2.2. Determination of ToxR activity. Cells were grown at 37°C for 22 h at pressures from 0.1 to 35 MPa. Upon binding of the P. profundum ToxR dimer to the ompL promoter, lacZ is expressed, whose activity can be determined in an enzymatic assay using the chromogenic substrate ONPG. Pressure induced dissociation of the ToxR dimer results in decrease of the lacZ transcription and finally lower β-galactosidase activity.

2.2.3. Chimeric ToxR proteins. To determine the effect of the transmembrane domain structure on ToxR dimerization, ToxR chimeric proteins obtained from Vibrio cholerae were used. The proteins were encoded on the plasmid ToxRIV+TMS [5] in which the cytoplasmic domain of ToxR is linked to the periplasmic maltose binding protein (MalE) moiety via different trans membrane segments (TMS). The following TMS were used: AZ2: Simplified version of a membrane-spanning leucine zipper interaction domain (LLAALLALLALLALL), EG4: Mutation of the simplified version of the leucin zipper (LLAALAAALAALAAAL), GpA13: Wild type glycoporphin A transmembrane segment (LIIFGVMAGVIGT), G83A: Mutant of wild type glycoporphin A transmembrane segment (LIIFGVMAAVIGT) [6][7]. As these ToxR constructs originated from V. cholerae, the V. cholerae ctx promoter was used to initiate lacZ transcription in the indicator cells.

3. Results and Discussion

3.1. HHP dependence of LmrA activity in proteoliposomes

LmrA is an ATP-dependent multiple drug transporter from Lactococcus lactis. It requires dimerization in the phospholipid bilayer for its functionality [4]. Its substrate spectrum reaches from hydrophobic drugs to ionic molecules, e.g. the ethidium cation, which are transported outwards from the interior of the membrane [2]. The hydrophilic part of this transporter comprising an ATPase activity reaches into the cytoplasm and its transport energy results from the cleavage of ATP to ADP and inorganic phosphate (Pi). In the proteoliposomes used in this work, LmrA was inserted after the formation of the vesicles pushing in an unidirectional mode, with the ATPase function located outside of the vesicle, which renders it experimentally accessible. This enables easy substrate addition and observation of the released phosphate. For the activity of LmrA, dimerization of two LmrA molecules is required. Recent studies established that the minimal unit in the functional LmrA transporter is the homodimer [21]. To exclude any side effects, we determined the vanadate-sensitive ATPase activity of LmrA. As it has been demonstrated for an ATP dependent calcium-transporter that HHP did not affect the inhibitory capability of vanadate [15], vanadate sensitive ATPase activity of LmrA under HHP likely reflects changes in enzyme activity. Several types of lipids were used to investigate their influence on LmrA dimerization/activity. In vesicles from natural phospholipids from Lactobacillus plantarum LmrA activity was slightly reduced at 20 MPa and ceased above 40 MPa. In vesicles consisting solely of
DMPC employing saturated fatty acid chains LmrA activity ceased above 20 MPa. In DMPC/cholesterol vesicles, ATPase activity was strongly reduced above 20 MPa but still present at a low level up to 40 MPa. As an example, figure 1 shows the LmrA activity in DOPC/DPPC/cholesterol vesicles. LmrA activity was slightly reduced at 20 and 40 MPa, as observed for natural phospholipid mixtures. At 80 MPa the activity significantly increased again, and it was reduced above 120 MPa. Under these conditions the coexistence of lipid phases of such raft mixtures has been described [22]. LmrA activity may therefore be maintained at elevated pressures in transporter dimers residing in the DOPC islands, which are still in the liquid crystalline phase at these pressures because of the double bonds in the fatty acid chains.

Figure 1: Activity of LmrA in raft mixture (DOPC/DPPC/Cholesterol 1:2:1). Large unilamellar vesicles (LUV) with LmrA 20:1 (w/w) were used to measure the vanadate sensitive ethidium assisted ATPase activity under HHP.

3.2. HHP dependence of ToxR dimerization in vivo

It is essential for bacteria to recognize changes in environmental conditions to adapt their metabolism, stress tolerance or virulence. The ToxRS system is one of many systems residing in the bacterial membrane to receive environmental signals and initiate an intracellular signal cascade. The principle of sensing is the formation of a heterotrimer consisting of two ToxR and one ToxS molecules. This complex specifically binds to promoter regions and thus initiates transcription of the respective genes [11][13]. In P. profundum, the ToxRS system is involved in HHP adaptation as it regulates the formation of outer membrane pore proteins (Omp) by switching between induction of OmpH and OmpL formations. Under HHP conditions of the deep sea, the ToxRS complex is dissociated and OmpH is formed, while OmpL is induced at ambient pressure when the ToxRS complex is formed and binds to the OmpL promoter [1] [23] [24]. In Vibrio cholerae the ToxRS system is involved in the formation of the cholera toxin by binding to the ctx promoter [10].

3.2.1. Photobacterium profundum ToxR. The reporter system used in this work only employs ToxR, which can dimerize in the reporter strain under ambient pressure without the aid of ToxS, because it is overexpressed as compared to the natural situation [13]. In this reporter strain, LacZ (β-galactosidase) activity indicating ToxR dimerization is increased by 20% at 10 MPa and remains unchanged at 20 MPa and 0.1 MPa. At pressures above 20 MPa it is successively reduced with increasing pressure. Above 40 MPa LacZ activity is no longer observed. Above this pressure, ribosomal synthesis starts to be affected, and therefore any measurements loose their significance.
3.2.2. *Chimeric Vibrio cholerae ToxR.* The interaction of proteins, namely their binding capability to each other depends on the biochemical nature of the contacting peptide chains. In membrane proteins the determinative domains for protein/protein interaction reside in the hydrophobic part of the membrane. The chimeric proteins used in this work consisted of the intracellular promoter-binding domain of ToxR and the periplasmic MalE protein, which helped correct orientation in the membrane. These domains were linked by 4 different TMS, which have previously been characterized with respect to their ability to dimerize in *E. coli* reporter strains [6][7]. Figure 2 shows the expression of lacZ as a result of HHP dependent ToxR hybrid dimerization. The basic levels of dimerization at 0.1 MPa, as determined by lacZ expression, varied for the different constructs and confirmed previously published data at ambient pressure. The behaviour of the construct with the leucin zipper (AZ2) mostly resembled that one obtained with ToxR from *P. profundum* characterized by a slight increase and subsequent decrease of dimerization with increasing pressure. The modified leucin zipper (EG4) and glycoporin segment (G83A) showed constantly decreasing dimerization with increasing pressure. Most interestingly, the dimerization of the construct containing the unmodified glycoporin TMS (A13) was little affected by the pressures applied. These results demonstrate, that the nature of the protein domain involved in dimerization of ToxR influences its dimerization independently of the lipid phase in which the protein resides. The different behaviour of the ToxR hybrids, namely the relative pressure stability of the A13 construct, also indicates that HHP induced inhibition of other cellular functions, including ribosomal synthesis, does not interfere with the *in vivo* reporter system at the pressures used here.

Figure 2: β-Gal-activity indicating ToxR dimerization in *E. coli-FHK12+pToxRIV+AZ2*, *E. coli-FHK12+pToxRIV+EG4*, *E. coli-FHK12+pToxRIV+GpA13* and *E. coli-FHK12+pToxRIV+GpAG83A* after 24 h of growth at HHP.

4. Conclusion

In this work, HHP and *in vivo* reporter systems were combined for the first time to investigate the structure function relationship of integral membrane proteins involved in transport and cellular signaling. We could show that the interaction of membrane proteins is pressure dependent *in vivo* and is maximal in the systems used at pressures of 10 – 20 MPa. *In vitro* experiments with raft mixtures of selected lipids suggest lipid sorting to coexisting islands allowing the proteins to find an area with the optimal fluidity required for their specific function. Also, changes in the protein structure affect
dimerization of membrane proteins. Not only are basal levels of functionality altered, but pressure dependent activity is observed again indicating the influence of membrane physical structure on the protein functionality. Certainly, the hen/egg question is obsolete when mirrored in the protein/lipid field in search of causative responsibility for membrane protein activity changes. Both the lipid structure and the nature of the protein affect membrane protein interaction. It is suggested that the protein structure determines basic functionality, e.g. principle ability or kinetics to dimerize to a functional complex, and may even affect principle structure of its lipid environment. The lipid environment modulates this property. Therefore, the lipid structure state needs to be considered as a major effector on protein functionality in living systems. Based on these findings it is tempting to speculate that environmentally caused changes in the membrane’s physical structure selectively modulates membrane protein activity and, possibly, the sorting of proteins to membrane areas which enable their maximal activity, thus helping cells to adapt to the environmental change.

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