Influence of High Pressure on the Dimerization of ToxR, a Protein Involved in Bacterial Signal Transduction[▽]

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High hydrostatic pressure (HHP) is suggested to influence the structure and function of membranes and/or integrated proteins. We demonstrate for the first time HHP-induced dimer dissociation of membrane proteins in vivo with *Vibrio cholerae* ToxR variants in *Escherichia coli* reporter strains carrying *ctx::lacZ* fusions. Dimerization ceased at 20 to 50 MPa depending on the nature of the transmembrane segments rather than on changes in the ToxR lipid bilayer environment.

Bacteria are exposed to high hydrostatic pressure (HHP) in deep-sea environments and when HHP is used as a nonthermal food preservation method. Multiple effects on microbial physiology have been described for sublethal HHP treatments (20 to 150 MPa), which induce stress response, influence ribosomal protein synthesis, and directly affect membrane properties (2, 20). The physical state of the membrane affects the structure and function of membrane proteins, while in turn, integrated proteins can influence the phase state and lateral organization of the membrane. Until now, experiments on the influence of pressure on proteins and lipids have essentially been performed using monomeric and oligomeric proteins in aqueous solution and pure lipid bilayer systems, respectively (1, 4, 19). Still, little is known about the behavior upon pressurization of membrane proteins integrated in their natural lipid bilayer environment, and their putative function in pressure sensing and any in vivo data are missing. Therefore, we studied HHP-induced dimer dissociation of membrane proteins in vivo by using *Vibrio cholerae* ToxR variants in *Escherichia coli* reporter strains.

In *Vibrio cholerae*, ToxR is the central regulator of virulence gene expression which initiates transcription from the cholera toxin gene promoter *ctx* (12, 13), while it regulates adaptation to high pressure in the deep-sea bacterium *Photobacterium profundum* (18). This renders ToxR a relevant model to study HHP-modulated membrane protein interaction. Dimerization of ToxR is essential to initiate the required signaling function (3, 5, 9, 10, 15). We used a system in which the cytoplasmic domain of *V. cholerae* ToxR was linked to a periplasmic maltose binding protein (MalE) moiety via different transmembrane segments (TMS). An *E. coli* strain in which the *ctx* promoter was fused to a *lacZ* gene allowed the determination of ToxR dimerization according to β -galactosidase activity.

Four different TMS were tested: AZ2, a simplified version of a membrane-spanning leucine zipper interaction domain (LLAALLAALLALL); EG4, a mutant consisting of a simplified

version of the leucine zipper (LLAALAAALAALLAAL); GpA13, a wild-type glycoporphin A TMS (LIIFGVMAGVIGT); and GpAG83A, a mutant of the wild-type glycoporphin A TMS (LIIFGVMAAVIGT). A nondimerizing TMS consisting of the ToxR cytoplasmic domain and MalE was used for comparison. The reporter strain *E. coli* FHK12 was transformed with plasmids pToxRIV+AZ2, pToxRIV+EG4, pToxRIV+GpA13, pToxRIV+GpAG83A, and pToxRIV+ Δ TM carrying the ToxR variants (6, 11). Aliquots of 3-ml cultures were incubated for 22 h at 37°C and 30°C under different pressure conditions. The measurement of β -galactosidase activity (30- μ l samples) was performed as described by Kolmar et al. (10). The fatty acid composition was determined from late-exponential-phase cells grown at 30°C and 37°C. The fatty acids were extracted, transesterified, and analyzed by gas chromatography. Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) fluorescence spectroscopy was used to study the polarity of the lipid interface and to detect potential phase changes of the lipid membrane (16). Protoplasts of stationary phase cells were prepared as described by Weiss (17). One-milliliter samples (Laurdan embedded in *E. coli* protoplasts) were pressurized at 37°C by using a pressure cell with optical windows (7).

Figure 1 shows the β -galactosidase activities resulting from HHP-dependent ToxR dimerization at 37°C. A temperature change to 30°C had no significant influence on the β -galactosidase activities. The β -galactosidase activities of all constructs were not significantly affected by pressures up to 20 MPa. The leucine zipper (EG4) and glycoporphin A (GpAG83A) segments showed lower and decreasing β -galactosidase activities with increasing pressure above 20 MPa, leading to an almost-complete loss of activity at 50 MPa (ca. 20% of the activity at 0.1 MPa). The β -galactosidase activity of the leucine zipper TMS AZ2 drastically decreased at pressures above 20 MPa, resulting in 25% β -galactosidase activity at 50 MPa compared to the activity at 0.1 MPa. The wild-type glycoporphin A segment (GpA13) had the highest β -galactosidase activity up to 20 MPa, and, most interestingly, its β -galactosidase activity was affected to a much lesser extent between 20 MPa and 40 MPa than the activity of the other constructs. Its activity at 40 MPa was still around 66% of that at ambient pressure. However, at 50 MPa, GPA showed a reduced activity similar to that of AZ2. For the construct without a TMS (Δ TM), only a very weak

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FIG. 1. Maximal values of the β -galactosidase activity in Miller units from the strains *E. coli* FHK12(pToxRIV+AZ2) (●), *E. coli* FHK12(pToxRIV+EG4) (○), *E. coli* FHK12(pToxRIV+GpA13) (▼), *E. coli* FHK12(pToxRIV+GpAG83A) (△), and *E. coli* FHK12(pToxRIV+TM) (■) after high-pressure treatment in the range of 0.1 MPa to 50 MPa at 37°C. The error bars represent standard deviations ($n = 10$). p , pressure.

β -galactosidase activity was measured, with almost no change in activity up to 50 MPa.

The fatty acid composition at 30°C showed only marginally larger amounts of unsaturated fatty acids than that at 37°C (data not shown). The effect of pressure on the generalized polarization (GP) of Laurdan-labeled *E. coli* protoplasts is shown in Fig. 2, which exhibits GP data at 37°C as a function of pressure. The GP values increase steadily with increasing pressure, starting from a relatively high value at ambient pressure ($GP = 0.34$) which is already characteristic of a membrane with a rather rigid conformational order of the lipid chains. Typically GP values range from about -0.2 to 0.2 for pure-fluid-like, disordered phases of lipid bilayers up to ≈ 0.55 to 0.60 in all-solid-ordered, gel-like lipid phases (14). Upon pressurization up to 50 MPa, GP values of 0.43 are reached, which is close to the tight packing of membranes reached for all-ordered conformational states of lipid bilayers.

In this study, the effects of HHP on the dimerization ability of an integral membrane protein were examined *in vivo*. The four transmembrane constructs showed different degrees of *lacZ* expression and, accordingly, protein dimerization at low pressure, which declined above 20 MPa. The (cytoplasmic) construct lacking a TMS supported little *lacZ* expression (i.e., little dimerization) and did not change with pressure. *LacZ* reporter gene expression patterns as a function of pressure were similar at 30°C and 37°C, probably because the membrane fluidities were similar at 30°C and 37°C as the result of a slightly higher unsaturated fatty acid content at the lower temperature. The GP value of the amphiphilic Laurdan fluorophore increased linearly with pressure from 0.34 to 0.43 over the range of pressures tested, indicating the absence of any phase transition in the membrane to which the dimer dissociation described could be attributed. This result corresponds with the results of the related work of Kato et al. (8), who also found a (reversible) conformational change in a protein (ATPase) below the lipid phase transition.

FIG. 2. GP values of *E. coli* protoplasts stained with Laurdan at 37°C under pressure conditions ranging from 0.1 to 50 MPa. p , pressure.

Our results show that pressures between 20 and 50 MPa are sufficient to promote the dissociation of this regulatory membrane protein. Evidence is also provided that integral membrane proteins may be essential in pressure sensing and thus influence bacterial signal transduction. Moreover, dimerization ability under low pressure conditions *in vivo* is strongly controlled by the nature of the TMS. Pressure-dependent changes in the TMS environment probably are restricted to changes in the rigidity of the membrane and modulate the dimerization, only. Specifically, the glycophorin TMS dimerization is markedly less pressure sensitive than the leucine zipper, which is probably due to the closer packing at the dimer interface and, hence, a larger activation volume for dissociation of the dimer.

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