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Eicosapentaenoic acid plays a role in stabilizing dynamic membrane structure in the deep-sea piezophile *Shewanella violacea*: A study employing high-pressure time-resolved fluorescence anisotropy measurement

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ABSTRACT

Shewanella violacea DSS12 is a psychrophilic piezophile that optimally grows at 30 MPa. It contains a substantial amount of eicosapentaenoic acid (EPA) in the membrane. Despite evidence linking increased fatty acid unsaturation and bacterial growth under high pressure, little is known of how the physicochemical properties of the membrane are modulated by unsaturated fatty acids *in vivo*. By means of the newly developed system performing time-resolved fluorescence anisotropy measurement under high pressure (HP-TRFAM), we demonstrate that the membrane of *S. violacea* is highly ordered at 0.1 MPa and 10 °C with the order parameter S of 0.9, and the rotational diffusion coefficient D_w of 5.4 μs^{-1} for 1-[4-(trimethylamino)pheny]-6-phenyl-1,3,5-hexatriene in the membrane. Deletion of *pfaA* encoding the omega-3 polyunsaturated fatty acid synthase caused disorder of the membrane and enhanced the rotational motion of acyl chains, in concert with a 2-fold increase in the palmitoleic acid level. While the wild-type membrane was unperturbed over a wide range of pressures with respect to relatively small effects of pressure on S and D_w, the Δ*pfaA* membrane was disturbed judging from the degree of increased S and decreased D_w. These results suggest that EPA prevents the membrane from becoming hyperfluid and maintains membrane stability against significant changes in pressure. Our results counter the generally accepted concept that greater fluidity is a membrane characteristic of microorganisms that inhabit cold, high-pressure environments. We suggest that retaining a certain level of membrane physical properties under high pressure is more important than conferring membrane fluidity alone.

1. Introduction

High hydrostatic pressure and low temperature characterize the majority of oceanic environments in terms of the volume occupied. Deep-sea organisms have adapted to survive under such extreme conditions [1–3]. High pressure and low temperature exert profound physiological impacts on biological membranes, primarily resulting in tighter packing and restricting the rotational motion of acyl chains [4,5]. It is assumed that the functions of membrane proteins such as electric transport, nutrient uptake, ion influx, and receptor activation are diminished by high pressure because these functions largely depend on an appropriate membrane structure [4,6–8]. Therefore, the maintenance of appropriate membrane fluidity is crucial for life under low-temperature and high-pressure conditions. The packing effects of membranes are circumvented by modifying the lipid compositions in a broad range of organisms. Generally, cold adaptation is associated with the incorporation of greater proportions of unsaturated fatty acids (UFAs) [9–11]. Fatty acids containing one or more double bonds take a more expanded conformation than their counterparts with saturated bonds. The large free volume created within intermolecular spaces allows greater conformational freedom of acyl chains and less packing

Abbreviations: EPA, eicosapentaenoic acid; POPC, palmitoyl-oleylphosphatidylcholine; UFA, unsaturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylamino) pheny]-6-phenyl-1,3,5-hexatriene; TCSPC, time-correlated single-photon counting; r_s, steady-state anisotropy; r_0, maximum anisotropy; r_∞, limiting anisotropy; θ, rotational correlation time; S, order parameter; D_w, rotational diffusion coefficient; τ, fluorescence lifetime; HP-TRFAM, high-pressure time-resolved fluorescence anisotropy measurement

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of the lipids. Consequently, the membrane becomes more fluid. This homeostatic adaptation has been termed "homeoviscous adaptation" [7,12].

Elevated hydrostatic pressure orders lipid membranes in a manner analogous to lowering temperature. For example, a pressure increase by 100 MPa increases the main transition (L_β/L_α) temperature of the stearoyl-oleylphosphatidylcholine (SOPC) and dioleylphosphatidylcholine (DOPC) membrane by 18.1 °C and 23.3 °C, respectively [13]. Macdonald and colleagues investigated the fluidity of the membranes of a number of fish that were confined to the shallow-water zone or the deep ocean floor by means of fluorescence anisotropy measurement. Anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the brain myelin was compared between different species of the genus *Coryphenoides* which lived over different depth ranges and was distinctly lower in deep-sea species, indicating a lower order of the membrane [14]. The fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in liver mitochondria was also compared among different species [15]. The ratio of UFAs to saturated fatty acids showed a statistically significant increase with depth of capture, implying the acclimation of fish membranes to ambient pressure. Similarly, many high-pressure-adapted bacteria (termed piezophiles) in the deep sea contain high proportions of UFAs in their membrane lipids, which in some cases increase with increasing growth pressure [16–19]. Particularly, the occurrence of omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) within phospholipids is a characteristic of piezophiles and psychrophiles that are adapted to cold environments [20,21]. Because of their extremely low melting temperatures, the incorporation of PUFAs is thought to exert a significant promoting effect on membrane fluidity. While the main transition temperature of the distearoylphosphatidylcholine (DSPC) membrane is 55.6 °C, that of stearoyl-oleylphosphatidylcholine (SOPC) and stearoyl-arachidonoylphosphatidylcholine (SAPC) is 6.7 °C and −13.0 °C, respectively [22]. However, the criterion of incorporating double bonds in membrane fatty acids is not always relevant to biological significance in homeoviscous adaptation [12]. According to the thermodynamic properties of phase transitions in artificial lipid bilayers, the volume change (ΔV) associated with the gel-to-liquid crystalline phase is 31.6 ml/mol, 18.9 ml/mol, and 10.1 ml/mol in the DSPC, SOPC, and SAPC membrane, respectively [22]. The clear difference in the ΔV can be explained on the basis of the large free volumes of UFAs in the gel phase without any significant difference in their volumes in the liquid crystalline phase. In the stearoyl-docosahexanoylphosphatidylcholine (SDPC) membrane, the polyunsaturated fatty acyl chains take a helical configuration, which reduces the effective chain length and stabilizes the gel phase [23]. Because biological membranes are complex mixtures of lipids in terms of the chain length, degree of unsaturation, polar head species, and proportion of individual phospholipids in a membrane, the role of monounsaturated fatty acids (MUFAs) and PUFAs on membrane properties cannot be understood straightforwardly. EPA and DHA also have clinical importance in human health and development. These PUFAs serve as precursors for hormones such as inflammatory mediators [24]. DHA, which is synthesized from EPA as a precursor, constitutes the major PUFA of brain lipids [25]. It has beneficial effects on cognition and learning ability and inhibits amyloid levels in the cerebral cortex of Alzheimer's disease model rats [26]. Nevertheless, the effects of PUFAs on the structural properties of the plasma membrane of nerve cells are still unclear.

The distribution of EPA-producing bacteria in the environment has been considered as evidence of a requirement for EPA for growth under cold, high-pressure conditions. Particularly, numerous deep-sea bacteria contain substantial amounts of PUFAs, leading to the speculation that PUFAs have a role in membrane-mediated functions [17,27–29]. The deep-sea piezophile *Photobacterium profundum* strain SS9 contains EPA at a proportion of 11% when cultured under the optimal growth conditions (28 MPa, 9 °C). It was reported that a mutant lacking EPA production exhibits normal growth under high pressure and low temperature, whereas another mutant defective in the production of *cis*-vaccenic acid fails to grow under the same conditions [17]. Accordingly, MUFAs but not PUFAs are required for growth in this bacterium under high pressure and low temperature. In the cold-adapted bacterium *Shewanella livingstonensis* Ac10 isolated from Antarctic seawater, EPA plays a role in organizing the cytoplasmic membrane at low temperature [30]. However, it may not be required for the maintenance of membrane fluidity, based on the finding that the diffusion rate of a small lipophilic molecule, pyrene, in the membrane was almost identical between the wild-type strain and the EPA-deficient mutant. Instead, EPA is specifically required for normal cell division at low temperature [30]. *Shewanella piezotolerans* WP3 isolated from a sediment sample of the western Pacific Ocean at the depth of 1914 m is a psychrotolerant and piezotolerant bacterium, growing optimally at 15–20 °C under pressures of 0.1–20 MPa [31]. *S. piezotolerans* cells contain EPA in the membrane. The loss of EPA results in growth defects at low temperature (4 °C, 0.1 MPa) and high pressure (20 °C, 20 MPa), indicating the requirement of EPA under the extreme conditions [19]. *Shewanella violacea* strain DSS12 is a deep-sea bacterium isolated from the Ryukyu Trench at the depth of 5110 m. It exhibits moderate piezophily with optimal growth at 30 MPa and 8 °C but it can also grow at 0.1 MPa [32]. The exploration of the piezophily of this bacterium has become an active area of research in deep-sea microbiology with respect to taxonomy, gene expression, protein function, and the respiratory system [32–35]. Its complete genome sequence is available to the public [36]. *S. violacea* cells contain a substantial amount of EPA in the membrane. EPA also plays a role in cell division under high pressure because an EPA-deficient *S. violacea* mutant displayed filamentation at 30 MPa [37]. Although the functional and physiological significance of microbial EPA is evident, its role in dynamic membrane structure under low temperature and high pressure remains to be resolved.

Fatty acid analysis provides valuable information on membrane physiology, although the variability in lipid composition and complexity of lipid behavior that occur in a heterogeneous natural cell membrane make it difficult to characterize how the physicochemical properties of the membrane respond to varied environmental conditions. To achieve an understanding of the role of UFAs, it is necessary to quantify membrane fluidity *in vivo* in terms of membrane order, rotational motion of acyl chains, or lateral diffusion of lipid molecules. Of the spectroscopic techniques available to study membrane properties, fluorescence anisotropy measurement is a common useful method providing information on dynamic membrane properties [38–40]. Although this technique has been widely employed for the study of model membranes, few results have been reported in whole, living cell systems. DPH and its cationic derivative 1-[4-(trimethylamino)pheny]-6-phenyl-1,3,5-hexatriene (TMA-DPH) are commonly used for such analyses. DPH primarily distributes perpendicular to the bilayer plane near the center of the membrane but partially distributes parallel to it within the acyl chain tails [39]. TMA-DPH is anchored at the lipid–water interface due to its charged moiety and thereby reflects only the interfacial region of the membrane. Trevors and colleagues have been extensively investigating structural and chemical changes that occur in bacterial membranes exposed to varied environmental factors including temperature, ions, pH, and chemicals [41,42].

In this study, we developed a new system to enable fluorescence anisotropy measurement under high pressure. Using this system, we elucidated the dynamic properties of the membrane in *S. violacea* cells under high pressure to elucidate the structural role of EPA in this bacterium. Specifically, we employed time-resolved fluorescence anisotropy measurement based on time-correlated single-photon counting (TCSPC), which provided quantitative information on membrane order, rotational motion of acyl chains, and the degree of water penetration within the membrane in a single measurement. Our results revealed an unexpected action of EPA to maintain cell membrane rigidity and to affect membrane hydration under varied pressure conditions.