Effects of eicosapentaenoic acid-containing phospholipids on the formation of membrane proteins from *Shewanella livingstonensis* Ac10

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Eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid with a 20-carbon chain and five *cis* double bonds, has been shown to play beneficial roles in various organisms. In this study, to specify the role of EPA in production and functionalization of membrane proteins, I characterized the effects of EPA-containing phospholipids (EPA-PLs) on the major β-barrel outer membrane protein Omp74 of a cold-adapted bacterium *Shewanella livingstonensis* Ac10 at low temperatures. In Chapter 1, I characterized the effects of EPA-PLs on the functions and conformation of Omp74. In Chapter 2, I investigated the chaperone-like role of EPA on the folding of Omp74 *in vitro*. In Chapter 3, I evaluated the roles of genes for EPA synthesis on the expression of another major outer membrane protein, Omp417. The results suggested that not EPA itself but the insertion of a knockout plasmid for EPA-biosynthesis genes down-regulated the expression of this protein. These studies suggested that EPA-PLs facilitated the late folding step(s) of Omp74 with a transient initiation structure containing α-helix. The results also implied that organization of the genes for the synthesis of this fatty acid can affect the expression of other proteins such as Omp417.

CHAPTER 1

Effects of EPA-containing phospholipids on the conformation of an outer membrane protein, Omp74, at low temperature in a cold-adapted bacterium, *Shewanella livingstonensis* Ac10

To evaluate the effects of EPA on the conformation of Omp74 in the lipid bilayer, I performed *in vitro* reconstitution assay of Omp74 by employing limited proteolysis of Omp74 refolded in the presence of liposomes containing EPA-PLs. The digestion patterns of Omp74 folded in the presence of EPA-PLs were distinct from those partially folded in the absence of EPA-PLs. Using single-tryptophan (Trp) mutants, I analyzed the fluorescence dynamics of Omp74 and specified the segments responding to EPA-PLs. These results suggested that the multiple segments in N-terminal and C-terminal regions interacted with EPA-PLs. Omp74 is predicted to consist of the N-terminal pore-forming β-barrel domain and the C-terminal peptidoglycan (PGN)-binding domain. I assessed the effects of EPA-PLs on them by liposome swelling assay and PGN-binding assay. Swelling assay of Omp74-containng liposomes showed that, once the folding had proceeded, the pore-forming activity of Omp74 was not significantly affected by EPA-PLs. PGN-binding assay using two membrane-permeable cross-linkers showed that the distance between Omp74 and PGN was approximately 6 Å~12 Å and EPA-PLs was also unlikely to affect the interaction between Omp74 and PGN after folding. These results implied that EPA-PLs facilitated the formation of a certain folding intermediate(s) of Omp74, whereas this lipid did not affect the pore size of the established conformation and its distance from PGN.

CHAPTER 2

EPA-containing phospholipids facilitate a late step in the folding of an outer membrane protein, Omp74, of the psychrotrophic bacterium, *Shewanella livingstonensis* Ac10

Previously it was shown that EPA-PLs facilitate the folding of the urea-denatured Omp74 in the presence of liposomes at low temperatures. In this study, I investigated the role of the initial structure of the polypeptide of Omp74 in the interaction with EPA-PLs by *in vitro* reconstitution assay. I found that the folding of Omp74 that had been transferred to a SDS-containing solution was faster than that of the urea-denatured one. CD spectrum analysis showed that the structure of the protein in urea was random, whereas SDS-denatured one contained α-helix-like secondary structure. These results suggested that an α-helical intermediate of the N-terminal region of Omp74 interacts with EPA-PLs to facilitate the folding.

CHAPTER 3

Regulatory mechanism of membrane protein production in an EPA-producing bacterium, *Shewanella livingstonensis* Ac10

I found that the level of the major outer membrane protein, Omp417, was markedly decreased in the EPA-less mutant (ΔEPA) cells of *S. livingstonensis* Ac10. To examine the effects of EPA on the folding of Omp417, I performed *in vitro* reconstitution assay of recombinant Omp417 with liposomes in the presence or absence of EPA-PLs. Trp fluorescence dynamics of the refolded Omp417 indicated that

EPA-PLs did not affect the local environments of Omp417 Trp residues and suggested that EPA-PLs are not involved in the folding of this protein at low temperatures. On the other hand, I analyzed real-time RT-PCR to analyze the transcription of *omp417* in *S. livingstonensis* Ac10 cells and ΔEPA cells. The results demonstrated that the amount of *omp417* transcript in the ΔEPA mutant was less than 2% of that in the wild-type strain. To analyze the effects of EPA-PLs on *omp417* expression, exogenous supplementation of EPA to ΔEPA cells and rescue of Δ*orf2* cells, a gene-disrupted mutant of a phosphopantetheinyl transferase required for the *de novo* synthesis of EPA, by using an *orf2*-expression vector were performed. Although these treatments restored EPA-PLs in the ΔEPA mutants, the transcriptional defect was not suppressed. These results suggested that the suppression of the transcription of *omp417* was not due to the lack of EPA, but due to the insertion of a knockout plasmid for EPA-biosynthesis genes into the genomic DNA.