Synthesis and application of ω-ethynyl fatty acids to analyze the physiological functions of eicosapentaenoic acid

Tomohisa Tokunaga

Eicosapentaenoic acid (EPA) is an ω-3 polyunsaturated fatty acid with a 20-carbon chain and five *cis* double bonds and is known to have various beneficial effects on human health, such as anti-inflammatory and anti-tumor effects. However, detailed mechanisms of its function are unclear. In this study, I developed an ω-ethynyl EPA analog (eEPA), which has an ethynyl group at the ω-end of EPA, and applied it to physiological analysis of EPA. eEPA reacts with azides by "click chemistry" and is useful for investigation of physiological roles of EPA. In Chapter I, I established an efficient synthetic method for eEPA and assessed the function of eEPA by using an EPA-producing bacterium, *Shewanella livingstonensis* Ac10. In Chapter II, I performed click chemistry-based imaging of eEPA in this bacterium to get information on *in vivo* localization of EPA. In Chapter III, I identified eEPA-modified proteins by click chemistry-based affinity purification. These studies suggested ununiform subcellular distribution of EPA and recruitment of EPA for posttranslational protein modification in *S. livingstonensis* Ac10. eEPA will contribute to the analysis of the mechanisms underlying the physiological functions of EPA not only in *S. livingstonensis* Ac10 but also in other organisms.

CHAPTER I

Synthesis and functional assessment of a novel fatty acid probe, ω-ethynyl eicosapentaenoic acid analog, to analyze the *in vivo* behavior of eicosapentaenoic acid

I designed and synthesized an ω-ethynyl EPA analog (eEPA) as a tool for analyzing the *in vivo* behavior and function of EPA based on the following strategies. In order to introduce four C-C double bonds, a coupling reaction between terminal acetylene and propargylic halide or tosylate was employed, and then, by simultaneous and stereoselective partial hydrogenation with P-2 nickel, the triple bonds were converted to *cis* double bonds. One double bond and an ω-terminal C-C triple bond were introduced by Wittig reaction with a phosphonium salt harboring an ethynyl group. By using this scheme, eEPA was synthesized in 12 steps (yield: 5.2%). Then, I evaluated the *in vivo* function of eEPA by using an EPA-producing bacterium, *S. livingstonensis* Ac10. When eEPA was exogenously supplemented to the EPA-deficient mutant of this strain (ΔEPA), eEPA was incorporated into the membrane phospholipids as an acyl chain, and the amount of eEPA was about 5% of the total fatty acids in the membrane, which is comparable to the amount of EPA in the membrane of the parent strain. Notably, by supplementation with eEPA, the growth retardation and abnormal morphology of ΔEPA were almost completely suppressed. Thus, eEPA mimics native EPA well and is expected to provide information on the behavior of native EPA *in vivo*.

CHAPTER II

Elucidation of subcellular localization of eicosapentaenoic acid in *Shewanella livingstonensis* Ac10 by using its ω-ethynyl analog

I reported visualization of an ω-ethynyl EPA analog (eEPA) incorporated into *S. livingstonensis* Ac10. As a control, I synthesized an ω-ethynyl palmitoleic acid analog (ePAL), confirmed its incorporation into phospholipids when added to *S. livingstonensis* Ac10, and visualized it. After these ω-ethynyl fatty acids incorporated into the cells were conjugated with Alexa Fluor 488 azide by click chemistry, I analyzed their subcellular localization by super-resolution fluorescence microscopy (SIM and STED). As the result, I found that eEPA was more ununiformly distributed in the cells than ePAL. The results suggested the occurrence of EPA-enriched microdomains in this bacterium, which may contribute to proper function of membrane proteins. The imaging methods employed in this study may be applicable to other organisms, and eEPA would be useful to gain an insight into the subcellular distribution of EPA.

CHAPTER III

Identification of lipidated proteins in *Shewanella livingstonensis* Ac10 by using ω-ethynyl eicosapentaenoic acid analog

I searched for proteins posttranslationally modified with EPA in *S. livingstonensis* Ac10. The cells were grown with eEPA, and eEPA-modified proteins were visualized and affinity-purified. I successfully detected eEPA-modified proteins by coumarin-labeling by click chemistry. In the competition experiment between eEPA and native EPA by using coumarin-labeling, an EPA-dose-dependent reduction in in-gel fluorescence intensity was observed. These results suggested the occurrence of

EPA-modified proteins in this bacterium. eEPA-modified proteins were conjugated with biotin-azide by click chemistry and purified by using streptavidin beads. Five of them were identified as Dcp, PepO, AcrA, Omp74, and FkpA. Since Dcp, PepO, and AcrA have a conserved N-terminal lipobox sequence conserved in lipoproteins of Gram-negative bacteria, EPA is probably recruited for *N*-acyl or *S*-diacylglyceryl modification at the N-terminal cysteine residue of these proteins. Protein lipidation is a major posttranslational protein modification process in bacteria. Through a click chemistry-based analysis, I successfully discovered the recruitment of EPA for the posttranslational protein modification. This approach will provide a platform for further analyses of EPA-modified proteins in various organisms and a novel insight into physiological roles of EPA.