Eicosapentaenoic Acid Facilitates the Folding of an Outer Membrane Protein of the Psychrotrophic Bacterium, *Shewanella livingstonensis* Ac10

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Abstract

Polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA), are found in various cold-adapted microorganisms. We previously demonstrated that EPA-containing phospholipids (EPA-PLs) synthesized by the psychrotrophic bacterium *Shewanella livingstonensis* Ac10 support cell division, membrane biogenesis, and the production of membrane proteins at low temperatures. In this article, we demonstrate the effects of EPA-PLs on the folding and conformational transition of Omp74, a major outer membrane cold-inducible protein in this bacterium. Omp74 from an EPA-less mutant migrated differently from that of the parent strain on SDS-polyacrylamide gel, suggesting that EPA-PLs affect the conformation of Omp74 *in vivo*. To examine the effects of EPA-PLs on Omp74 protein folding, *in vitro* refolding of recombinant Omp74 was carried out with liposomes composed of 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoglycerol and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (1:1 molar ratio) with or without EPA-PLs as guest lipids. SDS-PAGE analysis of liposome-reconstituted Omp74 revealed more rapid folding in the presence of EPA-PLs. CD spectroscopy of Omp74 folding kinetics at 4°C showed that EPA-PLs accelerated β-sheet formation. These results suggest that EPA-PLs act as chemical chaperones, accelerating membrane insertion and secondary structure formation of Omp74 at low temperatures.

Introduction

Long-chain polyunsaturated fatty acids (LPUFAs) such as eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) are found in the cell membranes of various marine bacteria [1]. Many have been isolated from permanently cold environments, such as deep-sea and polar regions. Members of the *Shewanella* genus that produce varying amounts of EPA as an acyl chain of membrane phospholipids exhibit a good correlation between cold-adaptation and EPA production [2].

EPA functions as an antioxidative component at low temperatures and influences the hydrophobicity of the bacterial cell membrane, thus affecting the entry of hydrophilic and hydrophobic compounds [3]. In addition to these functions, EPA in bacteria growing at low temperatures is assumed to improve membrane fluidity and decrease phase transition temperature of phospholipid membranes due to the low packing order of its poly *cis* unsaturated acyl chain. Appropriate membrane physical properties are essential for many biological processes, such as membrane protein dispersion, protein-protein interaction, and protein-lipid interaction within the lipid bilayers [4]. LPUFAs modulate a variety of biological processes and alter the functions of several membrane proteins such as sarcolemmal ion channels and rhodopsin [5,6]. However, the molecular mechanisms by which PUFAs interact with these proteins and modulate their function in lipid bilayers remain unclear.

A psychrotrophic bacterium isolated from Antarctic seawater, *Shewanella livingstonensis* Ac10, produces EPA as the *sn*-2 acyl chain of phospholipids, constituting about 5% of the total fatty acids in cells grown at 4°C and less than 1% when grown at 18°C [7]. EPA plays an important role in cell growth, cell division, and membrane biogenesis of *S. livingstonensis* Ac10 at low temperatures [7]. EPA deficiency alters the composition of membrane proteins,

although the small amount of EPA produced in *S. livingstonensis* Ac10 did not cause a detectable increase in bulk membrane fluidity [7]. These results suggest that, rather than maintaining membrane fluidity, EPA more specifically influences the behaviors of membrane proteins in *S. livingstonensis* Ac10.

We found in the present study that in an EPA-less mutant, one of the major outer membrane cold-inducible proteins, Omp74 (GenBank ID: BAF64763), forms a conformation different from that in the parent strain at 4°C, suggesting that EPA-containing phospholipids (EPA-PLs) contribute to the folding of this protein at low temperatures. We constructed an *in vitro* Omp74 reconstitution system by using chemically synthesized phospholipids and found a novel function of EPA-PLs, facilitating folding of this membrane protein as chemical chaperones at low temperatures.

Materials and Methods

Strains, plasmid, and growth conditions

The bacterial strains and plasmid used in this study are summarized in Table S1. A rifampin-resistant mutant of *S. livingstonensis* Ac10 (parent strain) and the EPA-less mutant (ΔEPA) were grown in Luria–Bertani (LB) medium at 4°C. Seed cultures of the parent strain and ΔEPA were grown at 18°C. When required, antibiotics were added to the medium at the following concentrations: rifampin, 50 μg mL^{-1} and kanamycin, 30 μg mL^{-1}.

Preparation of purified recombinant Omp74

To obtain recombinant Omp74 as inclusion bodies in *Escherichia coli*, an *omp74*-overexpression vector was constructed and recombinant Omp74 was purified as described in Supporting Information.

Western-blot analysis of Omp74

The parent strain and EPA-less mutant of *S. livingstonensis* Ac10 were cultivated at 4°C and collected at late-log phase, OD_{600} = 0.5–1.0; early-stationary phase, OD_{600} = 1.0–2.5; and late-stationary phase, OD_{600} = 2.5–4.0. All samples were normalized by OD_{600}. Cells were disrupted by sonication. Crude membrane proteins were collected by centrifugation at 30,000 x *g* for 60 min and resuspended in 2% (w/v) *N*-lauroylsarcosine sodium salt (Sigma, St. Louis, MO, USA). Samples were not heated before loading onto SDS-PAGE if not noted. Omp74 was detected with 80,000-fold diluted anti-Omp74 serum (Supporting Information) and an ECL plus kit™ (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the standard

protocol.

EPA-PL synthesis

To mimic the cell membrane of *S. livingstonensis* Ac10, phospholipids containing palmitoleic acid and EPA were synthesized as described in Supporting Information [8]. All other phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

Liposome preparation

Liposomes were prepared as described previously, with slight modification [9]. Four micromoles of phospholipids dissolved in chloroform were dried to a thin film in glass tubes with nitrogen gas stream, and then placed under vacuum for more than 30 min to remove residual chloroform. The dried lipid films were hydrated in 400 μL of TED buffer (pH 8.0) containing 10 mM Tris-HCl, 1.5 mM EDTA, and 0.5 mM 1,4-dithio-D,L-threitol, agitated by vortexing for at least 30 min, and sonicated in a bath sonicator until the solutions became translucent. The liposome solutions were equilibrated at 4°C for 3 days before use. At all steps of the liposome preparation, nitrogen gas was added to the container to prevent oxidation of unsaturated acyl chains. The sizes of the liposomes used for folding kinetics studies were analyzed with a DynaPro 801 dynamic light scattering instrument (Protein Solutions, Chicago, IL, USA). The average diameters of liposomes made from 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphoglycerol (DPPG) (1:1 molar ratio) and those containing 1-palmitoleoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphoethanolamine (PEPE) and

1-palmitoleoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphoglycerol (PEPG) (2.5 mol% each) were 50–55 nm.

In vitro reconstitution of Omp74

Purified inclusion bodies of Omp74 were dissolved with 8 M urea in TED buffer (pH 8.0) and centrifuged for 30 min to remove insoluble materials. Protein concentration was measured with Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) and the final concentration was adjusted to 0.5 mM. *In vitro* reconstitution was performed by rapidly diluting recombinant Omp74 unfolded in 8 M urea by 50-fold in liposome solutions to a final lipid concentration of 5 mM and 1.25 mM for SDS-PAGE and circular dichroism (CD) spectroscopy, respectively, and incubated at the temperatures indicated in the Results. Folding was stopped by the addition of SDS sample buffer to a final concentration of 2% SDS, 2 mM β-mercaptoethanol, 4% glycerol, 0.2 M Tris-HCl, and 0.1% bromophenol blue (Takara Bio, Tokyo, Japan). Samples were stored at −30°C as necessary. Omp74 band intensity was quantified by densitometry with Image J software version 1.14. All values were normalized to the band intensities of the protein marker. Folding efficiency was calculated by dividing the intensity of the folded protein band by the total intensity of the unfolded and folded protein bands.

CD spectroscopy

CD spectra were measured using a JASCO J-820-L Circular Dichroism Spectropolarimeter (Jasco Inc., Tokyo, Japan). The far-UV spectrum was recorded in the spectral range 210–250 nm and averaged over 4 scans at 18°C, using a 1 mm path-length cell. Liposomes in TED buffer

(pH 8.0) were used to obtain a baseline, and the background was subtracted automatically during scanning. For folding kinetics at 4°C, the signal at 215 nm was monitored every 0.5 s for 15 min. To analyze the folding kinetics of Omp74, a moving average was calculated from the values of every 10 scans.

Results

EPA deficiency affected Omp74 folding *in vivo*

Crude membrane proteins were extracted from early stationary phase *S. livingstonens*is Ac10 and EPA-less mutant (ΔEPA) cells grown at 4°C and analyzed by western blotting with an anti-Omp74 serum. Figure 1 shows Omp74 in the parent and ΔEPA strains. When the crude membrane proteins were not boiled, 2 main bands of about 34 and 32 kDa were observed in the parent strain (F1 and F2, respectively). Bands of differing mobility presumably represent Omp74 in different tertiary structures, but not nonspecific cross-reacted or truncated Omp74, because boiled samples gave a single main band of 39 kDa corresponding to the theoretical molecular mass (Fig. 1). These results indicate that the defect of EPA affects the folding of Omp74 *in vivo*.

EPA-PLs facilitate folding of Omp74 *in vitro*

In order to examine the involvement of EPA-PLs in the folding of Omp74, we carried out *in vitro* reconstitution of Omp74 in the presence of *n*-octyl-β-D-glucopyranoside (OG) and chemically synthesized phospholipids. The phospholipids containing palmitoleic acid at *sn*-1 and EPA at *sn*-2 were used as the characteristic molecular species in *S. livingstonensis* Ac10 grown at 4°C. Omp74 in the F1 form was found when liposomes and OG were used (Fig. S1), and the F1 band disappeared after heat treatment. This demonstrates that Omp74 was folded into the F1 form, which was found in the living cells, by the presence of liposomes and micelles. The F2 form of Omp74 was, however, not induced under the present experimental conditions, probably due to the lack of a chaperone molecule involved in the *in vivo* formation of the F2

form.

To investigate the effects of EPA-PLs on Omp74 folding into the F1 form, the kinetics of *in vitro* folding was analyzed by SDS-PAGE. After incubation of unfolded Omp74 with the liposomes composed of DPPG/DPPE with or without EPA-PLs, Omp74 was applied to SDS-PAGE. In the presence of liposomes, the F1 form of Omp74 was detected at 20 min at 18°C and 4°C, and increased over time (Fig. 2 left panels). We determined the folding kinetics by calculating the band intensities of the F1 form and unfolded Omp74 (Fig. 2 right panels), which revealed that Omp74 refolding into the F1 form was facilitated by EPA-PLs. About 65% and 35% of Omp74 incubated with EPA-PLs was refolded within 360 min and 1,020 min at 18°C and 4°C, respectively. Without EPA-PLs, only about 40% and 20% of Omp74 was refolded at 18°C and 4°C, respectively.

β-Sheet formation of Omp74 in EPA-containing liposomes

Omp74 shares about 44% amino acid identity with *E. coli* OmpA (GenBank ID: AF234269) and is predicted to form transmembrane β-barrel structures. The CD spectrum of Omp74 folded in buffer lacking liposomes showed a negative Cotton effect at 215 nm [10], revealing that Omp74 adopted a β-sheet-rich structure in aqueous solution after urea dilution (Fig. 3A). To determine the effects of EPA-PLs on the main-chain conformation of Omp74, we analyzed CD spectra of Omp74 reconstituted in liposomes with or without EPA-PLs and found they were similar to each other after prolonged incubation (Fig. 3A).

The kinetics of β-sheet formation of Omp74 at 4°C was analyzed by monitoring changes in the CD signal at 215 nm. After dilution into the liposome solutions, there was an initial decrease

(upward in the graph) in CD signal derived from Omp74 diluted with liposomes with (1.5 min) and without (3 min) EPA-PLs, as indicated by arrowheads in Figure 3B. Then the signal increased (downward in the graph) rapidly. This result indicates that Omp74 rearranged its secondary structures as it folded into the lipid bilayer. In the presence of EPA-PLs, the CD signal at 215 nm rapidly decreased within 1.5 min and then increased until 9 min. In the absence of EPA-PLs, the increase in CD signal after 3 min was slower. These results demonstrate that EPA-PLs facilitated the rearrangement and formation of Omp74 β-sheet structures in lipid bilayers. When Omp74 was diluted in buffer lacking liposomes, the CD signal increased over time, indicating that, in aqueous solution, Omp74 was folded into β-sheet-rich structures without rearrangement.

Discussion

In cold environments, the efficiency of molecular dynamics decreases, influencing enzyme activity and membrane fluidity [11]. To survive such unfavorable conditions, a psychrotrophic bacterium, *S. livingstonensis* Ac10, developed unique cold-adaptation mechanisms. To characterize the cold-adaptation mechanism of this bacterium, we focused on the physiological function of its cell membrane. *S. livingstonensis* Ac10 produces EPA as an acyl chain of its membrane phospholipids, PG and PE [7]. It is known that phospholipids containing LPUFAs affect the physicochemical properties of lipid bilayers, such as membrane thickness, permeability, elasticity, and curvature [4]. We found that an EPA-less mutant grown at 4°C has defects in cell division, membrane biogenesis, and biosynthesis of several membrane proteins including outer membrane porins, strongly suggesting that EPA-PLs are required for function of various membrane proteins at low temperatures [7,12].

In this study, we focused on an outer membrane protein, Omp74, a cold-inducible protein, to analyze its interaction with EPA-PLs. Omp74 is a predicted outer membrane porin that serves as a membrane passage for hydrophilic compounds and stabilizes the outer membrane structure, as an *omp74*-disrupted strain exhibited osmotic sensitivity (unpublished data). Western blotting of Omp74 in the EPA-less mutant grown at 4°C indicates that Omp74 migrates differently than that of the parent strain (Fig. 1). Since this mobility shift is caused by altered protein folding, EPA might interact with Omp74 and modulate its folding [14,15].

We created an *in vitro* Omp74 reconstitution system by using chemically synthesized PGs and PEs containing palmitoleic acid, which are major phospholipids in the cell membrane of *S. livingstonensis* Ac10, and EPA-PLs to mimic the physiological membrane. Omp74

reconstituted with various liposomes and micelles showed a band on SDS-PAGE corresponding to the F1 form observed *in vivo* (Figs. 1 and S1). However, under this *in vitro* condition, the F2 form of Omp74 produced in the presence of EPA *in vivo* was not detected. The formation of the F2 form probably requires a chaperone molecule, in addition to EPA, that was not included in the *in vitro* reaction mixture. Although the F1 form of Omp74 was produced even in the absence of EPA *in vivo* (Fig. 1), we found that EPA-PLs significantly affect the production of this form *in vitro* (Fig. 2). Folding speed to produce the F1 form increased in the presence of EPA-PLs. Thus, EPA-PLs support Omp74 folding in lipid bilayers.

The CD signal (215 nm) derived from Omp74 incubated with liposomes with and without EPA-PLs decreased (upward in the graph) until 1.5 and 3 min, respectively, then increased (downward in the graph) (Fig. 3B). During this period, the secondary structures of denatured Omp74 was supposed to be rearranged on the lipid bilayers surface. The increase in CD signal was likely due to reconstruction of the β-sheet structures in hydrophobic environments [16]. During refolding of Omp74, EPA-PLs probably facilitate interaction with the membrane surface, insertion into the hydrophobic environment, and reconstruction of the β-sheet structures in the lipid bilayer. DHA, an analogue of EPA, forms diverse conformations compared to monounsaturated fatty acids, which allows DHA to solvate the rough surface of membrane proteins with lower energetic costs [17]. Solvation by LPUFAs would facilitate folding of membrane proteins at low temperatures.

These results suggest that EPA acts as a chemical chaperone in the hydrophobic lipid core. Although our findings have not revealed the molecular mechanism of EPA-modulated folding, the results showed that EPA-PLs have a chaperone-like function, which explains the

physiological advantage of LPUFA synthesis in cold environments. Considering that the lack of EPA causes a defect in *S. livingstonensis* Ac10 cell division, proper function of cell division proteins may require EPA-PLs [7,18]. Further studies should address whether the chaperone-like function of LPUFAs generally contributes to efficient membrane insertion, folding, and physiological modulation of membrane proteins in various organisms.

Because the folding efficiency and conformational change of Omp74 increased depending on the presence of EPA, this protein is a useful model to understand the physiological function of acyl chains in membrane phospholipids. Synthetic phospholipids with diverse acyl-group chain length and unsaturation might help to elucidate the structure-function relationship of LPUFAs during folding of membrane proteins.

Acknowledgments

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phospholipids with polyunsaturated hydrocarbon chain, J Biol Chem (2012).

Figure legends

Figure 1. EPA deficiency in *S. livingstonensis* Ac10 affects the *in vivo* folding of a cold-inducible protein, Omp74

The parent strain (Ac10) and the EPA-less mutant (ΔEPA) were grown at 4°C to the late-log phase, and the membrane proteins were extracted. The crude membrane proteins were heated with SDS sample buffer (Takara Bio, Japan) at 100°C for 5 min (boiled) to obtain the unfolded Omp74. Boiled and unboiled samples were applied to SDS-PAGE. Omp74 was detected with anti-Omp74 serum. "F1" and "F2" indicate Omp74 in 2 different folded forms; "U" indicates unfolded protein. A degraded Omp74 product is marked with an asterisk.

Figure 2. Folding kinetics of Omp74

Omp74 dissolved in 8 M urea was folded in liposomes of DPPE/DPPG with or without 2.5 mol% PEPE and 2.5 mol% PEPG at 18°C (A) and 4°C (B). Aliquots were removed at specific time intervals as indicated and subjected to SDS-PAGE analysis without boiling. The folded fraction of Omp74 was quantified by densitometry with Image J software and plotted as a function of time (right panels). Samples with and without EPA-PLs are represented by closed or open symbols, respectively. The lipid:protein molar ratio was 500:1. The error bars indicate the standard deviation from 3 independent experiments.

Figure 3. EPA-PLs facilitate Omp74 secondary structure formation

(A) CD spectra of Omp74 reconstituted with liposomes with and without EPA-PLs. Unfolded Omp74 was incubated with liposomes composed of DPPE/DPPG with (black) or

without (gray) 2.5 mol% PEPE and 2.5 mol% PEPG for 7 days at 18°C to ensure reconstitution, and the samples were analyzed by CD spectroscopy. The spectrum of Omp74 folded in buffer lacking liposomes is represented by a dashed line. **(B)** The kinetics of secondary structure formation of Omp74 at 4°C. The changes in CD signal at 215 nm characteristic of β-sheet structure were monitored. Omp74 reconstituted with or without EPA is represented by a black or gray line, respectively. The dotted line indicates the change in β-sheet CD signal of Omp74 folded in buffer lacking liposomes. The lipid:protein molar ratio was 125:1. The arrowhead indicates the start point of β-sheet structural rearrangement. Reproducibility of CD measurements was confirmed by 3 independent experiments.

Figure 1

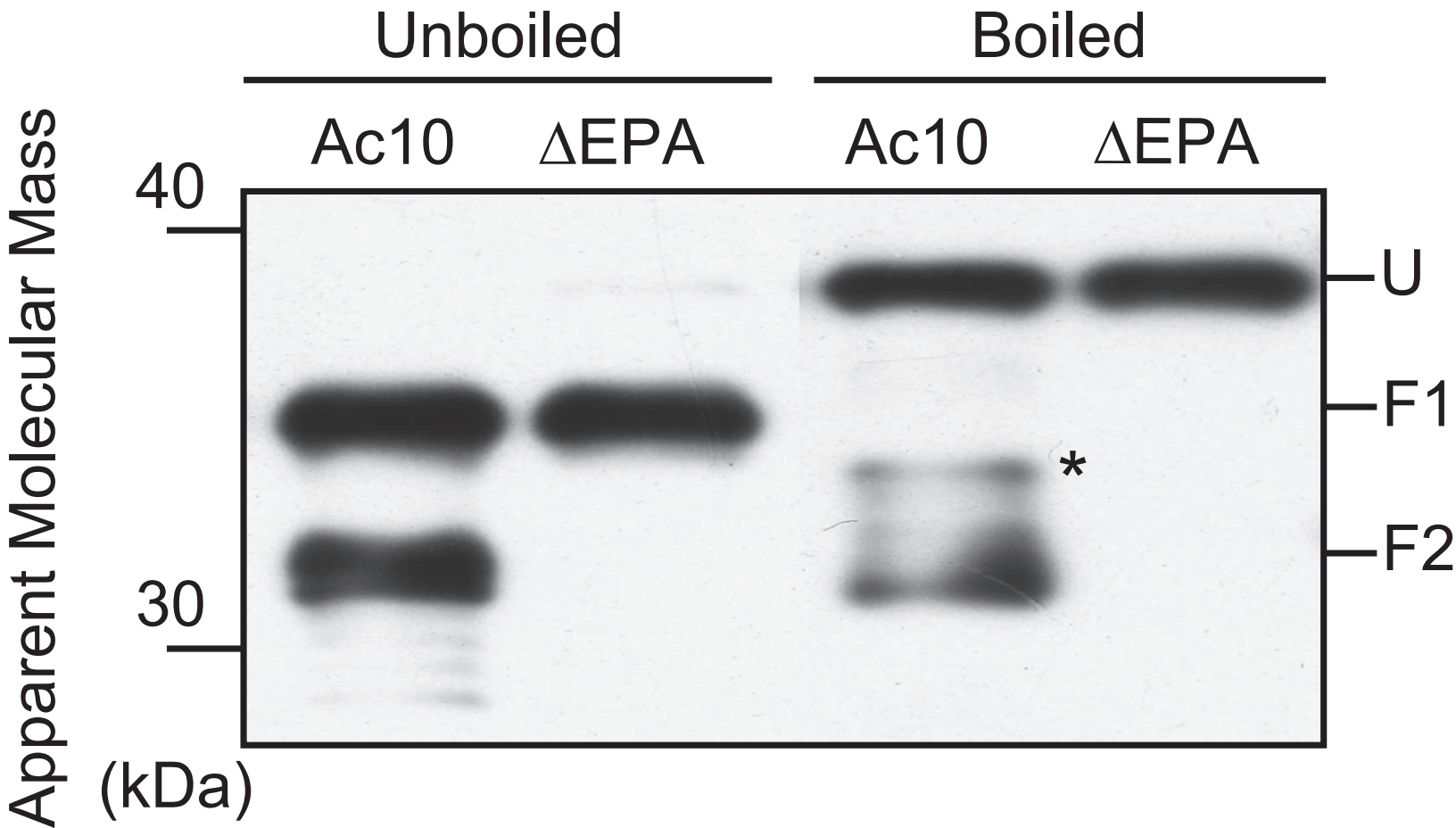
Unboiled
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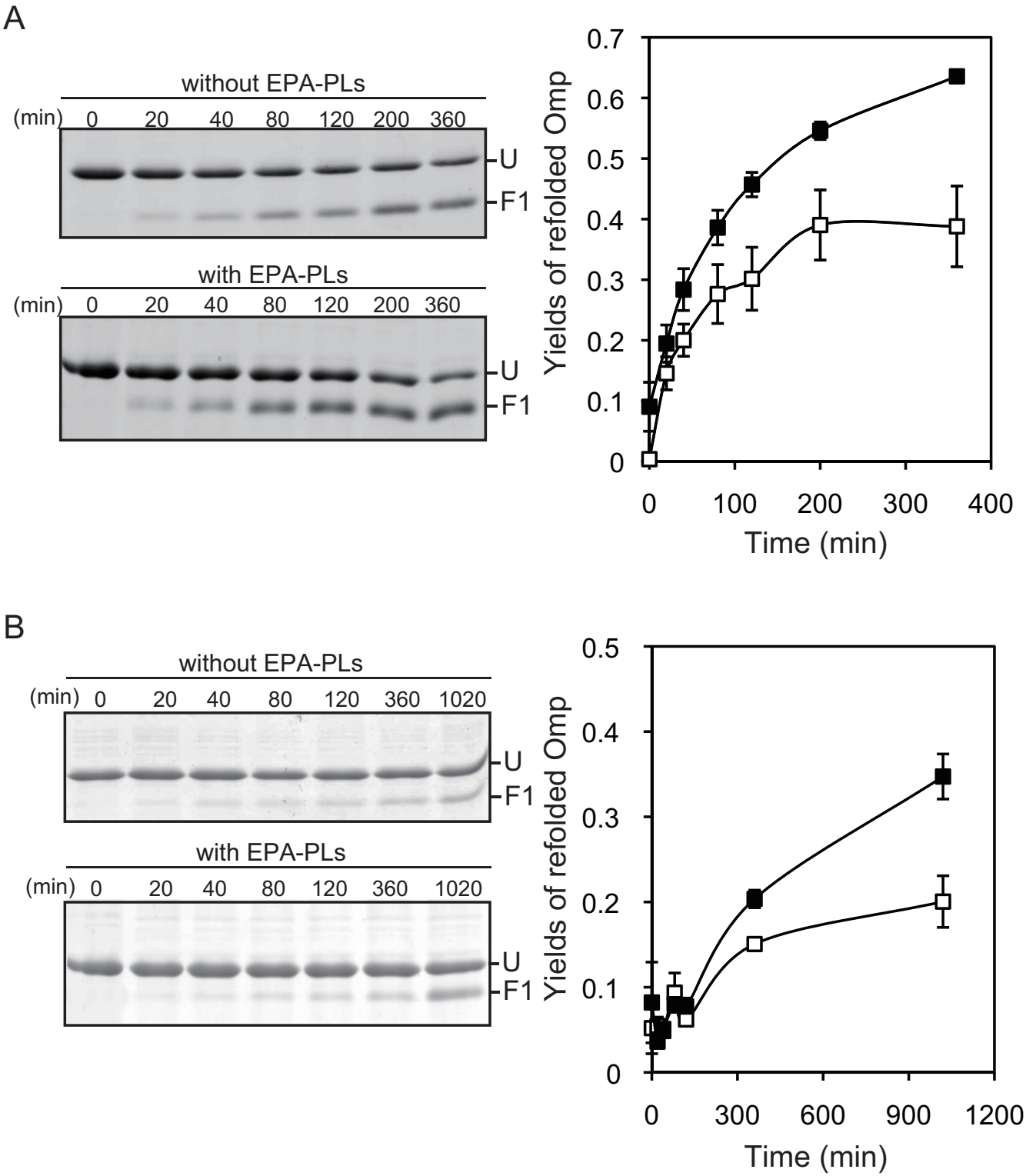
Figure 2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with EPA-PLs
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Yields of refolded Omp
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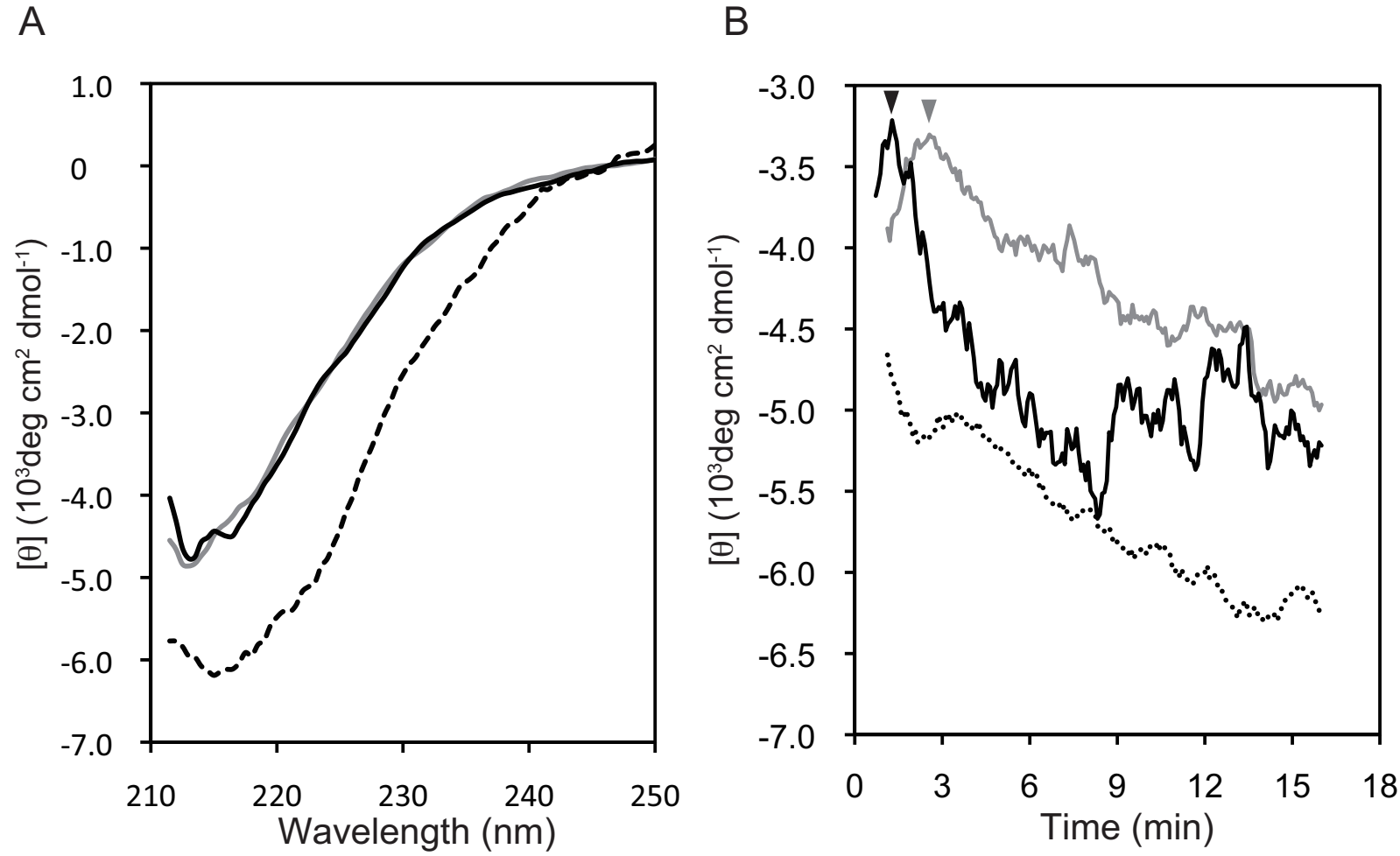
Time (min)

Figure 3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