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Stereochemistry of reduction in digeranylgeranylglycerophospholipid reductase involved in the biosynthesis of archaeal membrane lipids from *Thermoplasma acidophilum*

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Abstract

The basic core structure of archaeal membrane lipids is 2,3-di-*O*-phytanylglyceryl phosphate, which is formed by reduction of 2,3-di-*O*-geranylgeranylglyceryl phosphate. This reaction is the final committed step in the biosynthesis of archaeal membrane lipids and is catalyzed by digeranylgeranylglycerophospholipid reductase (DGGGPL reductase). The putative DGGGPL reductase gene (Ta0516m) of *Thermoplasma acidophilum* was cloned and expressed. The purified recombinant enzyme appeared to catalyze the formation of 2,3-di-*O*-phytanylglyceryl phosphate from 2,3-di-*O*-geranylgeranylglyceryl phosphate, which confirmed that the Ta0516m gene of *T. acidophilum* encodes DGGGPL reductase. The stereospecificity in reduction of 2,3-di-*O*-phytylglyceryl phosphate by the recombinant reductase appeared to take place through addition of hydrogen in a *syn* manner by analyzing the enzyme reaction product by NMR spectroscopy.

Keywords: Archaea; Membrane lipid; Biosynthesis; Digeranylgeranylglycerophospholipid reductase; Stereochemistry

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1. Introduction

Archaea, which have been attracting considerable attention from both biochemical and evolutionary aspects, are distinct from bacteria and eukarya, and are now classified in the third independent domain [1]. Archaea thrive under extreme environments such as high temperature, high salt concentration and/or low pH. One of the most significant differences between archaea and other organisms is found in the chemical structure of core membrane lipids. Archaeal membrane lipids consist of ether linkages at *sn*-2 and 3-positions in glycerophosphate and isoprenoid chains, while other organisms consist of ester linkages at *sn*-1 and 2-positions between glycerophosphate and fatty acids [2]. The core lipid structure is one of the most crucial feature for the classification of archaea from other organisms. 2,3-Di-*O*-phytanylglyceryl phosphate is the most basic structure to archaeal membrane lipids [3–5]. It is known that 2,3-di-*O*-phytanylglyceryl phosphate (archaetidic acid) is biosynthesized by reduction of 2,3-di-*O*-geranylgeranylglyceryl phosphate as shown in Fig. 1. This reduction of isoprenyl chain is quite intriguing since some archaea are considered to adapt their membrane lipid properties to the environments by adjusting the number of double bonds in the isoprenoid chains. Actually, Nichols et al. reported that the number of the double bonds in the membrane lipids of *Methanococcoides burtonii* changes in response to environmental temperature [6].

Concerning this double bond reduction, in vivo incorporation experiments with mevalonolactone-d_9 have indicated that the reduction reaction of geranylgeranyl groups to phytanyl groups proceeds via *syn* addition of hydrogen for double bonds [7,8]. Further, we recently succeeded in isolating and purifying this reduction enzyme from thermoacidopophilic archaea *Thermoplasma acidophilum* [9]. The purified enzyme catalyzed

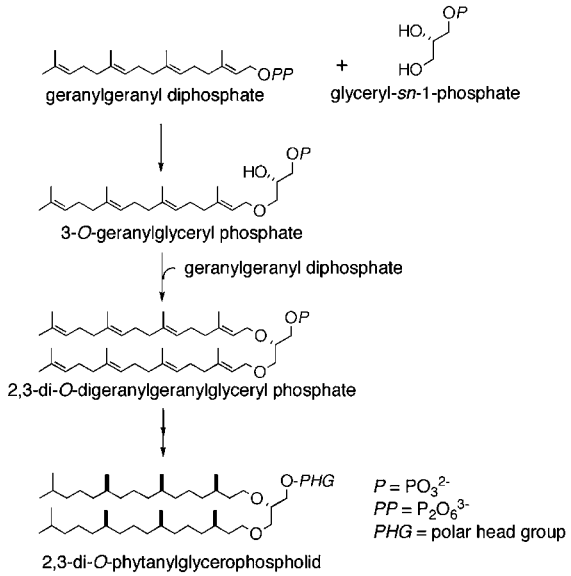

Fig. 1. Biosynthetic pathway of archaeal membrane lipids.

the reduction of double bonds of several digeranylgeranylglycerophospholipids in the presence of FAD and NADH (or dithionite). In this present paper, we report cloning and expression of the putative digeranylgeranylglycerylphospholipid reductase gene (Ta0516m) from *T. acidophilum*, in which membrane lipids are known to be composed of archaeol and caldarchaeol containing 0–8 cyclopentane ring [10]. As a result, the recombinant enzyme catalyzed the reduction of geranylgeranyl groups of 2,3-di-*O*-geranylgeranylglyceryl phosphate to phytanyl groups. In addition, when the enzyme reaction was carried out in deuterium oxide buffer, the stereospecific incorporation of deuterium to double bonds in 2,3-di-*O*-phytylglyceryl phosphate was observed by NMR spectroscopy.

2. Materials and methods

2.1. Generals

^{1}H and ^{2}H NMR spectra were recorded on a LA-400 spectrometer (JEOL). A fast atom bombardment mass spectrometry (FAB MS) was performed with an MS station JMS-700 mass spectrometry system (JEOL). Chloroform was used as a solvent for ^{2}H NMR spectrum. ^{2}H chemical shift was reported in δ value based on natural abundance signal of $C^{2}HCl_3$ ($\delta^{2}_{H} = 7.26$) as the reference. Column chromatography was carried out with Kiesegel 60 (70–230 mesh, Merck). Proteins were detected at 280 nm and all purification steps ran at 4 °C. Ultrafiltration was performed by using *Mr* 10,000 cut-off tubing (VIVASIENCE). Protein concentrations were determined by the method of Bicinchoninate (BCA) using bovine serum albumin as a standard [11]. Protein samples were analyzed by 12.5% SDS–PAGE and visualized by staining with Coomassie brilliant blue. 2,3-Di-*O*-geranylgeranylglyceryl phosphate and 2,3-di-*O*-phytylglyceryl phosphate were synthesized in our laboratory [9]. Other chemicals were of the highest grade commercially available.

2.2. Cloning of DGGGPL reductase gene from T. acidophilum

Thermoplasma acidophilum JCM 9062 was cultured in the medium described previously [9] for 48–72 h at 60 °C and harvested to prepare genomic DNA. The primers were designed on the basis of the Ta0516m genomic sequence of *T. acidophilum*. Two synthetic oligonucleotides, 5′-CATATGGAAACCTATGATGTTCTTGTGG-3′ and 5′-ATGAATGGATCCAAATCAGATGAGG-3′, including *Nde*I or *Bam*HI restriction site (respective underline) were used as primers for amplification of the putative DGGGPL reductase gene. PCR was carried out with *T. acidophilum* genomic DNA as a template by using LA *Taq* DNA polymerase (TaKaRa). The initial denaturation step was 2 min at 94 °C, followed by 30 cycles of 15 s at 94 °C, 30 s at 55 °C, and 70 s at 72 °C. The PCR product was sub-cloned into a pT7Blue T vector (Novagen).

Eschericha coli DH5R competent cells were transformed with the ligation mixture and plated onto LB/Amp/IPTG/X-gal plates. White colonies were screened by restriction analysis using *Bam*HI and *Nde*I, and positive clones were sequenced. The sub-cloned fragment, extracted from a 1% agarose gel after electrophoresis, was digested with *Nde*I and *Bam*HI, and then ligated into the *Nde*I-*Bam*HI site of the pET-30b vector (Novagen). This plasmid was named pTa0516m.

2.3. Expression and purification of the recombinant DGGGPL reductase

Competent cells of *E. coli* Rosetta DE3 (Novagen) were transformed with the plasmid pTa0516m and were grown in Luria-Bertani medium containing 30 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37 °C. When the cell density reached $OD_{600} = 0.6$, IPTG was added to a final concentration of 0.1 mM to induce expression and the culture was allowed to grow for an additional 14 h at 18 °C before the cells were harvested by centrifugation. The activity guided purification of the recombinant enzyme was performed as follows. Cells were suspended in buffer A (50 mM potassium phosphate, 5% glycerol, pH 6.5) by sonication on ice. Cell debris was removed by centrifugation at 10,000*g* for 30 min. The supernatant was ultracentrifugation at 150,000*g* for 6 h at 4 °C. The precipitate was resuspended in buffer A, and then homogenized at 4 °C. The homogenate was added to 2 ml of 10% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and then the mixture was incubated overnight at 4 °C. The mixture was ultracentrifugated at 70,000*g* for 30 min at 4 °C. The supernatant was loaded onto a 1 × 21-cm DEAE F.F. column (Amersham) equilibrated with buffer B (10 mM potassium phosphate, 10% glycerol, and 0.3% CHAPS, pH 6.5), and the column was washed with buffer B. The column was eluted with a 200 ml linear gradient of 0–200 mM KCl at a flow rate of 0.5 ml/min. Fractions containing DGGGPL reductase, as determined by SDS–PAGE, were pooled and concentrated. The buffer of the mixture was exchanged with buffer C (10 mM potassium phosphate, 10% glycerol, and 0.3% CHAPS, pH 6.8) by ultrafiltration. The concentrated sample was loaded onto a 1 × 6-cm CHT ceramic hydroxyapatite column (Bio-Rad) equilibrated with buffer C. The column was washed with 20 ml of buffer C and eluted with 120 ml of a 10–180 mM linear gradient of potassium phosphate at 0.2 ml/min. Fractions containing DGGGPL reductase were pooled and concentrated.

2.4. Assay for DGGGPL reductase

An assay mixture (500 μl) containing the purified recombinant enzyme (60 μg), 100 mM potassium phosphate (pH 6.5), 0.2 mM FAD, 0.7 mM 2,3-di-*O*-geranylgeranylglyceryl phosphate, 0.3% CHAPS, 10% glycerol, and 20 mM dithionite was incubated at 37 °C for 48 h. The reaction was terminated by addition of 2 M hydrochloric acid. The reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ extracts were washed with brine and concentrated. The residue was suspended in 0.5 ml of ether and a solution of diazomethane in ether (1 ml, 0.4 M) was added at 0 °C. After 1 h, the mixture was concentrated to dryness. The residue was chromatographed over silica gel with hexane–ethyl acetate (2:1) to give methyl esters of lipids, which were analyzed by FAB MS.

2.5. Stereospecificity of the enzyme reaction

An assay mixture (2 ml) containing the purified recombinant enzyme (2 mg), 100 mM potassium phosphate (pD 6.5), 0.2 mM FAD, 0.7 mM 2,3-di-*O*-phytylglyceryl phosphate, 0.3% CHAPS, 10% glycerol, and 20 mM dithionite in deuterium oxide was incubated at 37 °C for 5 day. The reaction was terminated by addition of 2 M hydrochloric acid. The enzyme reaction products were treated as described above, converted into phytanyl acetate according to the previous method [7], and then analyzed by NMR spectroscopy.

3. Results and discussion

3.1. Cloning and expression of the putative DGGGPL reductase

Recently, we purified DGGGPL reductase from a cell free extract of *T. acidophilum*. The Ta0516m gene of *T. acidophilum* genome was tentatively assigned to encode DGGGPL reductase based on the *N*-terminal amino acid sequence of the purified enzyme [9]. To confirm the function of this putative DGGGPL reductase gene, cloning and expression of this gene were performed. Primers were designed for the Ta0516m gene and PCR was carried out using these primers and the *T. acidophilum* genome DNA as a template. A DNA fragment with the expected length (about 1.2 kbp) was amplified, and sub-cloned into a pT7Blue vector. After confirmation of the sequence, the plasmid was digested with *Nde*I and *Bam*HI, and ligated into the identical sites in pET-30b for expression. The recombinant DGGGPL reductase was expressed in *E. coli* Rosetta (DE3) cells harboring the pTa0516m plasmid. The *E. coli* cells harboring pTa0516m were grown until mid-log phase and the gene expression was induced by IPTG. The activity guided purification of the recombinant enzyme was performed as described previously [9]. SDS–PAGE of the purified enzyme revealed a single band at 43 kDa (Fig. 2), which is corresponding to the calculated molecular mass of the putative DGGGPL reductase. Since DGGGPL reductase was presumed to be a flavin dependent enzyme from the amino acid sequence

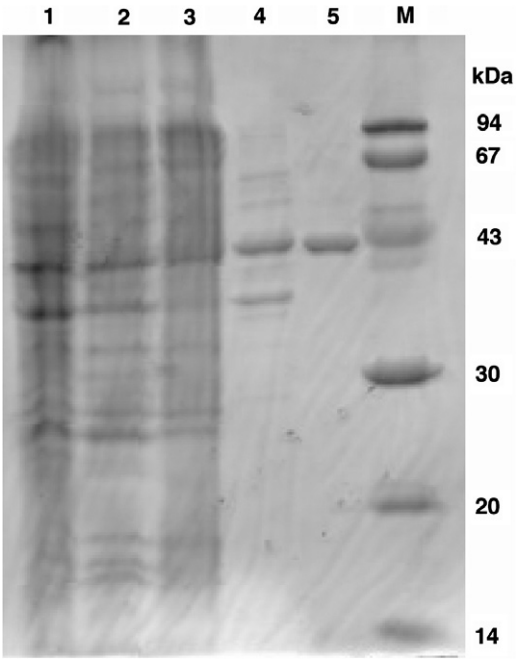

Fig. 2. SDS–PAGE analysis of the recombinant DGGGPL reductase. 12.5% SDS–PAGE of the proteins at each step in the purification. Lane M, protein mass markers; lane 1, supernatant from a crude extract of *E. coli* Rosetta-pET-30b; lane 2, supernatant from a crude extract of *E. coli* Rosetta-pTa0516m; lane 3, after ultracentrifugation; lane 4, after DEAE chromatography; lane 5, after hydroxyapatite chromatography.

analysis, the recombinant enzyme actually showed yellow color and the UV–visible spectrum indicated the absorbance maxima characteristic to flavoproteins. The absorption at 447 nm indicated that FAD bound to the enzyme at ca. 1:1 ratio (data not shown).

3.2. Enzyme activity of the recombinant DGGGPL reductase

The purified recombinant enzyme was incubated with 2,3-di-*O*-geranylgeranylglyceryl phosphate and dithionite as a reductant in the presence of CHAPS as a detergent. After extraction of the enzymatic mixture with chloroform and derivatization into phosphoric acid methyl ester with diazomethane, FAB MS analysis resulted in detection of 16 mass units higher molecular ion peak than the substrate as reported previously [9]. Thus, the present study clearly indicates that the function of the Ta0516m gene of *T. acidophilum* is assigned to encode DGGGPL reductase involved in the biosynthesis of archeal membrane lipids.

3.3. Stereospecificity of the recombinant DGGGPL reductase

As already mentioned above, in vivo incorporation experiments in halophilic and methanogenic archaea with mevalonolactone-d_9 have indicated that the reduction reaction of geranylgeranyl groups to phytanyl groups proceeded via *syn* addition of hydrogen for double bonds [7,8]. In the present study, in vitro analysis of the stereospecificity of the recombinant DGGGPL reductase from acidothermophilic archaea was pursued. Since this enzyme utilizes FAD as a cofactor, when an enzyme reaction is performed in deuterium oxide buffer, stereospecific incorporation of deuterium atoms into the double bonds of substrate can be expected. Further, we have already assigned the prochiral methylene proton signals at the C-2 position of phytanyl group in ^{1}H NMR spectroscopy [7]. Thus, the enzyme reaction using the recombinant reductase with 2,3-di-*O*-phytylglyceryl phosphate was carried out in deuterium oxide buffer. The reaction products were extracted with chloroform, followed by hydrolysis of the phosphate group. The obtained lipids were subjected to transformation into phytanyl acetate according to the reported method [7] as shown in Fig. 3.

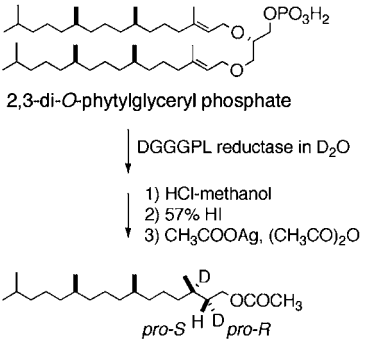

Fig. 3. Enzyme reaction and derivatization of the reaction products for stereochemical analysis of DGGGPL reductase reaction.

The phytanyl acetate derived from the enzymatic reaction was first analyzed by ^{2}H NMR spectroscopy. The ^{2}H NMR spectrum clearly showed two peaks at 1.53 and 1.64 ppm, which are assigned to the H-3 and the *pro-R* position of H-2, respectively [8], as shown in Fig. 4b. No signal was observed at the *pro-S* position of H-2 (1.38 ppm). Further, 1D-TOCSY ^{1}H NMR spectrum of the same sample irradiated at 4.05 ppm of H-1 indicated that the *pro-S* position (1.38 ppm) was protonated. No proton signal was observed at the *pro-R* position of H-2. Therefore, it appeared that the *pro-R* position of H-2 was specifically deuterated during the enzyme reaction. Since the stereochemistry at C-3 is *R*-configuration in the natural phytanyl structure, the reduction upon C-2 and C-3 of the phytyl groups occurred in *syn*-fashion. Because the reduction reaction is catalyzed by the single enzyme [9], the stereochemical course of the reduction for the remaining double bonds proceeds in the same manner. This observation agrees with previous reports that reduction reaction of a geranylgeranyl group to a phytanyl group proceed through *syn* addition of hydrogen for double bonds in halophilic and methanogenic archaea.

Saturated isoprenyl chains like archaeal membrane lipids are frequently found in nature, for examples, chlorophyll, menaquinone, and cholesterol and so on. DGGGPL reductase is homologous to enzymes involved in the biosynthesis of chlorophyll and menaquinone in some extents (25–30%) in amino acid level [12–14]. Although the stereochemistry of these enzyme reactions was not clarified, the reduction at C_{24}–C_{25} of desmosterol in cholesterol biosynthesis was reported to take place by *anti*-addition [15].

In summary, the putative DGGGPL reductase gene (Ta0516m) of *T. acidophilum* was cloned and expressed. The purified recombinant enzyme appeared to catalyze the formation of 2,3-di-*O*-phytanylglyceryl phosphate from 2,3-di-*O*-geranylgeranylglyceryl phosphate. Further, the stereochemistry in reduction of 2,3-di-*O*-phytylglyceryl phosphate by the recombinant reductase was found to take place through addition of hydrogen in a *syn* manner.

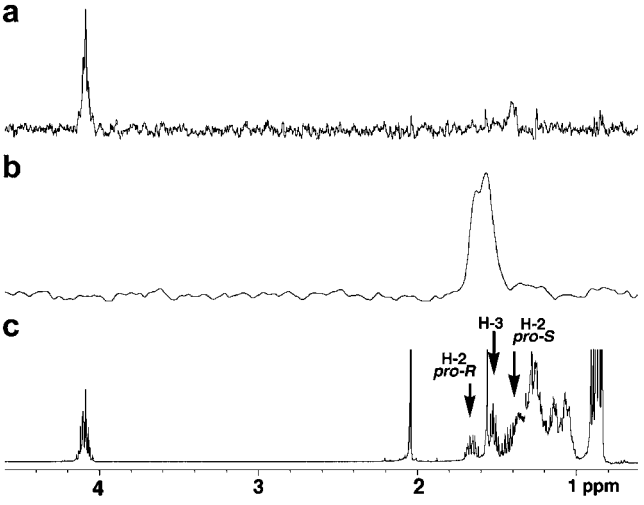

Fig. 4. NMR spectra of phytanyl acetate derived from the enzyme reaction of DGGGPL reductase in D_2O. (a) 1D-TOCSY ^{1}H NMR spectrum irradiated at 4.05 ppm, (b) ^{2}H NMR spectrum, (c) authentic phytanyl acetate. Assignments of the signals were performed according to Ref. [7].

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