Protein Expression and Purification 132 (2017) 138–142

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Purification and characterization of human dehydrodolychil diphosphate synthase (DHDDS) overexpressed in *E. coli*

Moshe Giladi [a, b, *], Ilan Edri [c], Michal Goldenberg [c], Hadas Newman [c, d], Roi Strulovich [a], Daniel Khananshvili [a, 1], Yoni Haitin [a, **, 1], Anat Loewenstein [c, d, 1]

[a] Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[b] Tel Aviv Sourasky Medical Center, Tel Aviv, Israel
[c] Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[d] Department of Ophthalmology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel

ARTICLE INFO

Article history:
Received 26 December 2016
Received in revised form
1 February 2017
Accepted 2 February 2017
Available online 3 February 2017

Keywords:
Heterologous expression
Prenyltransferase
Dolichol
Dehydrodolychil diphosphate synthase
DHDDS
Enzyme

ABSTRACT

Protein asparagine (N)-linked glycosylation is a post-translational modification that occurs in the endoplasmic reticulum; it plays an important role in protein folding, oligomerization, quality control, sorting, and transport. Accordingly, disorders of glycosylation may affect practically every organ system. Dehydrodolichyl diphosphate synthase (DHDDS) is an eukaryotic *cis* prenyltransferase (*cis*-PT) that catalyzes chain elongation of farnesyl diphosphate via multiple condensations with isopentenyl diphosphate to form dehydrodolichyl diphosphate, a precursor for the glycosyl carrier dolichylpyrophophate involved in N-linked glycosylation. Mutations in DHDDS were shown to result in retinitis pigmentosa, ultimately leading to blindness, but the exact molecular mechanism by which the mutations affect DHDDS function remains elusive. In addition, bacterial *cis*-PT homologs are involved in bacterial wall synthesis and are therefore potential targets for new antibacterial agents. However, as eukaryotic *cis*-PT were not thoroughly characterized structurally and functionally, rational design of prokaryotic *cis*-PT specific drugs is currently impossible. Here, we present a simple protocol for purification of functionally active human DHDDS under non-denaturating conditions using a codon-optimized construct. The purified protein forms a stable homodimer, similar to its bacterial homologs, and shows time- and substrate-dependent activity. Purification of this protein requires the presence of a detergent for protein solubility. The protocol described here may be utilized for the overexpression of other eukaryotic *cis*-PT. Future structural and functional studies of the recombinant DHDDS may shed light on the mechanisms underlying DHDDS-related retinitis pigmentosa and lead to novel therapeutic approaches.

1. Introduction

Protein asparagine (N)-linked glycosylation is a post-translational modification in which an oligosaccharide, composed of 14 sugar moieties, is covalently attached to a polypeptide [1–3]. This modification has an important role in protein folding, oligomerization, quality control, sorting, and transport [1,4]; accordingly, glycosylation disorders result in a wide range of clinical syndromes affecting practically every organ system [5]. In mammalian cells, synthesis of the initial universal 14-sugar core begins on the cytosolic surface of the ER membrane by the addition of sugars to membrane-embedded dolichylpyrophophate. After the addition of seven sugars, the oligosaccharide is flipped to the ER lumen and seven additional sugars are added [1]. The final 14-sugar dolichol-linked oligosaccharide is then transferred to the side chain of asparagine within a consensus Asn-X-Thr/Ser sequence [3].

Prenyltransferases are a group of enzymes that catalyze chain elongation of allylic diphosphate using isopentyl diphosphate (IPP) via multiple condensation reactions [6,7]. Z-type enzymes (*cis*-PT) catalyze the formation of *cis* double bonds from the condensation reaction, whereas E-type enzymes catalyze *trans* double bond

* Corresponding author. Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel, and Tel Aviv Sourasky Medical Center, 6 Weizmann st., Tel Aviv, Israel.
** Corresponding author. Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel.
E-mail addresses: moshegil@post.tau.ac.il (M. Giladi), yhaitin@post.tau.ac.il (Y. Haitin).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.pep.2017.02.001
1046-5928/

formation [8]. Dehydrodolichyl diphosphate synthase (DHDDS) is an eukaryotic *cis*-PT that catalyzes chain elongation of farnesyl diphosphate (FPP, an allylic diphosphate) via multiple condensations with isopentenyl diphosphate (IPP) [6,9,10]. This results in the formation of dehydrodolichyl diphosphate, a long chain (C_{55-100}) polyprenyl diphosphate serving as a precursor for dolichylpyrophosphate, the glycosyl carrier molecule involved in N-linked protein glycosylation [6]. In recent years, mutations in DHDDS were shown to result in retinitis pigmentosa, an inherited retinal eye disorder, which causes degeneration of rod and cone photorecptors, resulting in night vision and peripheral vision impairment, followed by impaired central vision [11–13]. Intriguingly, the mutations in DHDDS result in isolated ocular involvement (i.e., non-syndromic RP), despite the important role of dolichol synthesis in protein glycosylation in many other tissues. The isolated ocular phenotype seems to be related to the specific mutations involved, since a case of fatal congenital disorder of glycosylation was recently reported in a patient with splice site mutation and a non-sense mutation in DHDDS [14].

The exact molecular mechanisms involved in DHDDS-related retinitis pigmentosa may have major clinical implications, since patients with specific forms of retinitis pigmentosa may benefit from therapies directed towards the underlying molecular defect, whereas the same therapies are ineffective in patients with other genes implicated [12]. In addition, as bacterial *cis*-PTs are involved in bacterial wall synthesis, this group potentially forms targets for new antibacterial agents. However, as eukaryotic *cis*-PT were not thoroughly characterized structurally and functionally, rational design of prokaryotic *cis*-PT specific drugs is currently not possible.

DHDDS from the yeast *S. cerevisiae* was previously overexpressed in *E. coli* and was mostly unsoluble despite the presence of a thioredoxin (TRX) fusion protein. The protein was thus recovered from the pellet, solubilized and purified under denaturating conditions, followed by refolding using different protocols; the oligomeric state of the purified protein was not reported [15]. In order to resolve the biochemical and structural features of human DHDDS and the mechanisms underlying protein dysfunction leading to retinitis pigmentosa, we developed a method for its heterologous overexpression and purification in *E. coli* using a codon-optimized TRX-fusion construct. Our method does not require refolding and results in quantity and purity of protein amendable for functional and structural studies. We also show that DHDDS forms a homodimer, similarly to its bacterial homolog, and is catalytically active. Thus, our approach reported here will serve future studies of human DHDDS structure and function in health and disease.

2. Materials and methods

2.1. Strains and reagents

Codon-optimized human DHDDS (UniProt Q86SQ9) was synthesized and cloned into pET-32a plasmid between the *Nde*I and *Bam*HI restriction sites (GenScript, USA) as a TRX fusion protein. Proteins were overexpressed in T7 express *E. coli* cells (New England Biolabs, USA). TALON-superflow resin, HiPrep 26/10 desalting column, superdex-200 increase 5/150 GL column and HiLoad 16/60 superdex-200 column were purchased from GE healthcare. *Trans*, *trans*-farnesyl diphosphate (FPP) was purchased from Sigma and ^{14}C-isopentenyl pyrophosphate (^{14}C-IPP) was purchased from Perkin Elmer. All other reagents (buffers, salts, etc.) were of analytical grade.

2.2. Overexpression of human DHDDS in E. coli

E. coli T7 express competent cells were transformed with the TRX-fusion DHDDS construct and plated on LB-agar under ampicillin selective conditions (200 mg/L). Selected colonies were inoculated into 100 mL of LB medium in 250 mL flask and incubated for 2 h with constant shaking at 200 rpm. 80 mL of the culture were used to inoculate two 5 L flasks (40 mL per flask), each containing 1.5 L of 2xYT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) with 100 mg/L ampicillin at 37 °C with constant shaking at 180 rpm. Cells were grown at 37 °C until reaching $OD_{600nm} = 0.5$ and induced at 16 °C by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Proteins were expressed at 16 °C for 16–20 h and harvested by centrifugation (10,000xg for 10 min).

2.3. Purification of human DHDDS

Cells were suspended in buffer A (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10 mM β-mercaptoethanol), 1 μg/mL DNase I, and protease inhibitor mixture. The cells were homogenized and disrupted in a microfluidizer. The soluble protein was then recovered by centrifugation at ~40,000xg for 45 min at 4 °C. The supernatant was loaded onto a TALON-superflow column with 10 mM imidazole, followed by thorough washing with buffer A and 10 mM imidazole to reduce nonspecific protein binding. Next, overexpressed proteins were eluted with buffer A and 250 mM imidazole.

Imidazole was removed using a HiPrep 26/10 desalting column equilibrated with buffer A, followed by the addition of 6xHis-tagged TEV protease (1 mg TEV protease per 50 mg protein) to the eluted proteins to remove their 6xHis-tagged TRX fusion protein at 4 °C overnight. The cleaved proteins were then loaded again onto a TALON-superflow column with 5 mM imidazole to remove the cleaved 6xHis-tagged TRX fusion protein and TEV protease.

The flow-through was collected, concentrated to 3–4 mL, and loaded onto a HiLoad 16/60 superdex-200 column (GE healthcare, UK) equilibrated with buffer A for final purification. Protein concentration was determined by Lowry assay. Purified proteins were flash-frozen in liquid nitrogen and stored at −80 °C.

2.4. Analytical size-exclusion chromatography (SEC)

Experiments were performed using an analytical SEC column. The proteins were thawed, centrifuged at 21,000xg for 10 min, and loaded onto a superdex-200 increase 5/150 GL column pre-equilibrated with 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.02% Triton X-100, 10 mM β-mercaptoethanol, on an ultra-performance liquid chromatograph (Shimadzu Corporation) [16,17].

2.5. Enzyme kinetics

The activity of purified DHDDS was assayed as previously described [15,18,19]. To demonstrate the time-dependent IPP consumption, 5 μM of purified DHDDS were mixed with 10 μM FPP and 50 μM ^{14}C-IPP to initiate the reaction in buffer A with 0.5 mM $MgCl_2$. The reaction was performed at 22 °C. 15 μL were withdrawn from the reaction at 2, 4, 6 and 24 h and the reaction was quenched by the addition of 15 μL buffer A with 20 mM EDTA (to a final concentration of 10 mM). To demonstrate the substrate dependent activity, 1 μM of purified DHDDS were mixed with 10 μM FPP and 50 μM ^{14}C-IPP to initiate the reaction in buffer A with 0.5 mM $MgCl_2$. The reaction was performed at 30 °C. ^{14}C-IPP concentration was varied in the presence of 10 μM FPP or the concentration of FPP was varied in the presence of 20 μM ^{14}C-IPP. 50 μL were withdrawn from the reaction at 24 h and the reaction was quenched by the