FEBS Letters 588 (2014) 3375–3381

journal homepage: www.FEBSLetters.org

Directed evolution of squalene synthase for dehydrosqualene biosynthesis

Maiko Furubayashi [a,1], Ling Li [a,1], Akinori Katabami [a], Kyoichi Saito [a], Daisuke Umeno [a,b,*]

[a] Department of Applied Chemistry and Biotechnology, Chiba University, 1-33 Yayoi-cho, Inage, Chiba 263-8522, Japan
[b] Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

ARTICLE INFO

Article history:
Received 20 June 2014
Revised 20 July 2014
Accepted 22 July 2014
Available online 2 August 2014

Edited by Miguel De la Rosa

Keywords:
Squalene synthase
Carotenoid synthase
Phytoene desaturase
Directed evolution

ABSTRACT

Squalene synthase (SQS) catalyzes the first step of sterol/hopanoid biosynthesis in various organisms. It has been long recognized that SQSs share a common ancestor with carotenoid synthases, but it is not known how these enzymes selectively produce their own product. In this study, SQSs from yeast, human, and bacteria were independently subjected to directed evolution for the production of the C_{30} carotenoid backbone, dehydrosqualene. This was accomplished via high-throughput screening with *Pantoea ananatis* phytoene desaturase, which can selectively convert dehydrosqualene into yellow carotenoid pigments. Genetic analysis of the resultant mutants revealed various mutations that could effectively convert SQS into a "dehydrosqualene synthase." All of these mutations are clustered around the residues that have been proposed to be important for NADPH binding.

1. Introduction

Sterols and hopanoids, which are both triterpenes (C_{30} isoprenoids) derived from squalene (SQ), are widespread in nature. Sterols and hopanoids have various structures and functions, such as membrane fluidity controller, hormone precursor, or serving as the messenger for developmental signaling. In addition to its value as a universal triterpene precursor, SQ is gaining interest as a therapeutic/pharmacological chemical [1] and a biofuel candidate [2,3].

Squalene synthase (SQS) catalyzes the head-to-head condensation of two molecules of FPP to synthesize SQ (Fig. 1) [4]. SQSs have been extensively studied over the last 50 years due to their biological importance, their potential as targets for cholesterol lowering drugs, and their very unique and complex mechanism of action. SQ formation can be divided into two steps. The first step is the head-to-head condensation of two farnesyl diphosphate (FPP) molecules, yielding the intermediate presqualene pyrophosphate (PSPP). This intermediate subsequently undergoes a multi-step and reductive rearrangement into SQ. Very recently, a series of efforts [5,6] to elucidate the structure of SQS at various reaction stages provided a comprehensive and animated picture on how the entire process of SQ formation proceeds.

SQSs are known to be evolutionarily related to carotenoid synthases. In particular, *Staphylococcus aureus* dehydrosqualene (DSQ) synthase (CrtM), the first committed enzyme in staphyloxanthin (a C_{30} carotenoid pigment) synthesis [7,8], has recently been extensively studied structurally [5,9–11] as an important target for Methicillin-Resistant *S. aureus* (MRSA) treatment [9,12,13]. The first step of the reaction catalyzed by CrtM is identical to the first step catalyzed by SQS: CrtM condenses two molecules of FPP into a PSPP intermediate. CrtM differs from SQS in the second reaction: CrtM proceeds without NADPH and therefore results in the production of DSQ (Fig. 1).

It is not known how SQS and CrtM diverged during evolution. From the standpoint of reaction chemistry, it is expected to be much easier to generate CrtM from SQS than *vice versa*: if one can successfully impede hydride migration from NADPH to PSPP, the second reaction is completed in a non-reductive way, leading to DSQ formation. Indeed, it has long been known that SQS produces DSQ in the absence of NADPH [14–18]. Conversely, it has also been suggested that NADPH acts as the trigger factor for the first reaction [19]. To convert SQSs into DSQ synthases with meaningful cellular activity, one must disrupt hydride migration from NADPH to the reaction precursor PSPP *without* severely blocking NADPH binding.

Here, we conducted the directed evolution of SQSs from human, yeast, and bacteria in an attempt to mimic the activity of DSQ synthases. We successfully isolated various SQS mutants with significant DSQ synthase activity, and this clearly shows that it is

* Corresponding author at: Department of Applied Chemistry and Biotechnology, Chiba University, 1-33 Yayoi-cho, Inage, Chiba 263-8522, Japan.
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.febslet.2014.07.028
0014-5793/

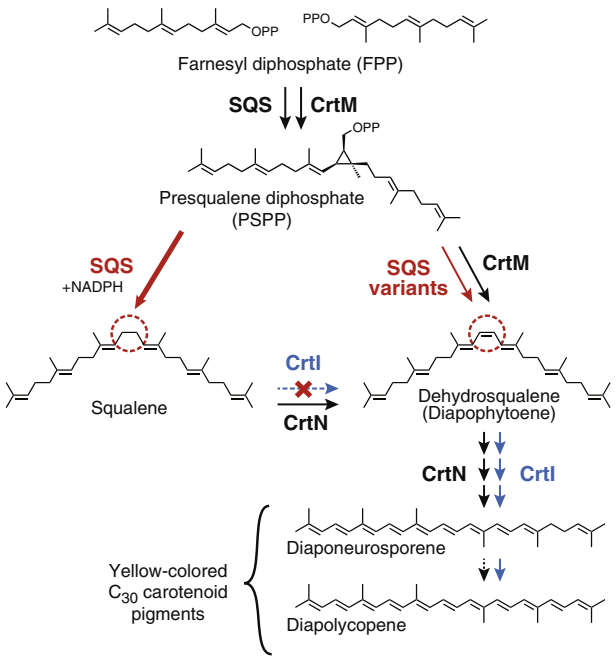

Fig. 1. Reaction mechanism of squalene (SQ) and dehydrosqualene (DSQ) formation and their conversion into carotenoid pigments. SQS mediates the condensation of two FPPs to form PSPP, and the subsequent rearrangement and reduction of PSPP using NADPH, to produce SQ. CrtM catalyzes the same first reaction, but the second rearrangement step produces DSQ instead of SQ. CrtN catalyzes the desaturation of DSQ, also using SQ as a substrate [20] to produce diaponeurosporene and/or diapolycopene, C_{30} carotenoid pigments. CrtI, conversely, uses DSQ but not SQ.

possible for SQS to acquire DSQ synthase activity by few mutations. We identified 10 DSQ-forming mutations that alone confer DSQ-forming activities to SQSs, all of which were found near the proposed NADPH binding domains for all three of the SQSs tested. In general, the DSQ-forming capacity comes with the severe cost of the original SQ-forming activity. Our results also provides unique experimental support for the latest reaction model of SQSs [6], where multiple regions dynamically interact with NADPH, the key molecule which controls both the product specificity and the reaction rate of SQSs.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli XL10-Gold Kan^R (Stratagene, La Jolla, CA, USA) was used for DNA cloning, while XL1-Blue (Stratagene) was used for carotenoid or SQ production analysis.

2.2. Plasmids

pAC-*hsqs* and pUCara-*crtI* were derived from Ref. [20]. Here, the *hsqs* gene was derived from pHSS16 from Thompson et al. [28], where 30 N-terminal residues and 47 C-terminal residues were truncated. There are 3 amino acid differences in the N-terminus of hSQS (Q33R, D34N, and S38N) because rat SQS primers were used initially to clone this gene [28]; however, these differences were not reported to affect the enzyme's behavior. *E. coli* codon-optimized *Thermosynechococcus elongatus* BP-1 SQS (*tsqs*) was purchased from DNA 2.0 Inc. (Menlo Park, CA). A ribosome binding sequence and a spacer (5′-AGGAGGATTACAAA-3′) were added before the open reading frame of *tsqs*, flanked by *Xba*I/*Xho*I. This construct was inserted into the *Xba*I/*Xho*I site of pAC-*hsqs*, thereby replacing the *hsqs* gene to yield pAC-*tsqs*. Yeast SQS gene (*ysqs*) was PCR-amplified from active dry yeast (Nisshin Super Kameria Dry Yeast, Nisshin Seifun Group Inc.). PCR primers were designed so that the C-terminal 24 amino acids of ySQS were removed. The resultant *ysqs*, compared with the *Saccharomyces cerevisiae* SQS gene (ERG9) sequence (NCBI Refseq NC_001140), contained three synonymous mutations (G1125A, T1128C, T1176C) and one non-synonymous mutation (G856A, which corresponds to Gly286Ser). pAC-*ysqs* was constructed by the replacement of *hsqs* in pAC-*hsqs* with the *ysqs* obtained as described above.

For the construction of single-mutation SQS variants, using pAC-*hsqs*, *tsqs* or *ysqs* as a template, the whole plasmid sequences were amplified by using the primer containing the desired mutation and BsaI restriction site (primer sequence shown in Table S4). The PCR products were digested/ligated using Golden-Gate cloning method [29]. The ORF sequences were confirmed for each variant plasmids.

2.3. Carotenoid pigment analysis

Carotenoid pigment production in *E. coli* cells was measured using a previously described method [20]. Briefly, we cultured cells in 500 μL LB-Lennox medium in a 96-deep-well plate for 16 h at 37 °C. Aliquots (40 μL) of these pre-cultures were transferred to 2 mL Terrific Broth (TB) medium in 48-deep-well plates and shaken at 30 °C, 1000 rpm. Then, 0.2% (w/v) arabinose was added after 8 h, and cultures were shaken for an additional 40 h. Cells were harvested and washed with saline, and carotenoids were extracted from the cell pellet by adding 1 mL acetone, which was immediately followed by vortexing. The acetone layer was collected after centrifugation (15,000 rpm for 10 min), and carotenoids were analyzed by absorption spectroscopy (350–650 nm at 5-nm intervals). The pigmentation level of each culture was estimated from the lambda max (470 nm) of the resulting extract, using the molar absorption coefficients of diaponeurosporene (147,000 $M^{-1}cm^{-1}$).

2.4. Directed evolution of hSQS, tSQS, and ySQS

The *hsqs* gene was subjected to error-prone PCR by adding 10 or 50 μM Mn^{2+} to the PCR reaction (using 5U *Taq* polymerase from New England Biolabs, Ispwich, MA, and a PCR program of 94 °C for 5 min followed by 25 cycles of [94 °C for 30 s, 52 °C for 30 s, 72 °C for 1.5 min], and a final extension of 72 °C for 7 min), using the following primers: Fwd: 5′-AGCTGGGGATCCAGGTTTCCC-GACTGGAAAGCG-3′, and Rev: 5′-ACCATAGGATCCGTGAAATACCG-CACAGATGCG-3′. Similarly, *tsqs* and *ysqs* were PCR-amplified under the same mutagenic conditions using the same primers.

The resultant PCR products were digested using *Xba*I/*Xho*I and cloned into the *Xba*I/*Xho*I site of pAC-*hsqs*. Ligation products were transformed into XL10-Gold Kan^R (for hSQS and tSQS) or ElectroMax DH10B (for ySQS). For the hSQS and ySQS libraries, the transformants were inoculated into 40–50 mL LB (supplemented with 50 μg/mL carbenicillin) and cultured for 12 h, and plasmid libraries were obtained by miniprepping the respective cultures. A portion (approximately 1/1000) of each transformant was plated onto an agar plate to determine the library sizes (approximately 10^5). For the tSQS library, the transformants were plated onto several LB-agar plates to form colonies, and the plasmid library was obtained by miniprepping the collected colonies. The size of the tSQS library was approximately 7500.

Plasmid libraries were transformed into XL1-Blue harboring pUCara-*crtI*, plated onto an LB-agar plate topped with a nitrocellulose membrane, and incubated for 16 h at 37 °C to form colonies. The colonies on the membrane was transferred to an LB-agar plate containing 0.2% (w/v) arabinose and incubated for 24–48 h at room temperature (approximately 25 °C). The colonies were screened;