Colloids and Surfaces B: Biointerfaces 135 (2015) 735–741

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Activity of squalene-hopene cyclases in bicontinuous microemulsions

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ARTICLE INFO

Article history:
Received 4 July 2015
Received in revised form 19 August 2015
Accepted 20 August 2015
Available online 22 August 2015

Keywords:
Bicontinuous microemulsion
Activity of squalene-hopene cyclase (*Aac*SHC)
Circular dichroism
Conformation of *Aac*SHC in microemulsions
Biocatalysis in non-conventional media

ABSTRACT

The paper at hand deals with biocatalysis in bicontinuous microemulsions. The latter consist of a dynamic network of oil and water domains separated by a monolayer of surfactant molecules, i.e. the interfacial layer. A microemulsion with the composition buffer – *n*-octane – nonionic surfactant was tested as reaction medium for an enzyme-catalysed reaction with a focus on the conversion of hydrophobic substrates, which are difficult to convert in aqueous buffer solutions. For the study at hand, we chose to investigate the activity of the squalene-hopene cyclase from *Alicyclobacillus acidocaldarius* (*Aac*SHC) towards its natural substrate squalene in bicontinuous microemulsions. Firstly, the study revealed that the activity of *Aac*SHC depends linearly on the enzyme concentration. Secondly, a hyperbolic curve was found for the dependence of the activity on the substrate concentration and a saturation of the *Aac*SHC at substrate concentrations above 20 mM was observed. Thirdly, the composition of the interfacial layer was found to have no significant influence on the activity or on the conformation of *Aac*SHC. Surprisingly and unexpectedly, a distinctly enhanced selectivity towards hopene was discovered in the microemulsion. To conclude, bicontinuous microemulsions were found to be a suitable reaction medium for biocatalytic reactions with the enzyme *Aac*SHC.

1. Introduction

Squalene-hopene cyclases (SHC) are able to synthesise cyclic triterpenoic compounds like hopanoids and sterols in just one step from linear precursors such as squalene. Hopanoids have a condensing effect on biological membranes due to their rigid ring structures, which help to stabilise the membranes once the hopanoids are integrated in the membrane [1]. The aim of this study is to clarify whether cyclases can be used as biocatalysts in bicontinuous microemulsions, i.e. to investigate the stability and activity of cyclases in this reaction medium.

In general, squalene-hopene cyclases catalyse some of the most complex reactions, namely the formation of cyclic terpenoids from linear terpenes. The products obtained are well defined with regard to both structure and stereochemistry [2]. To achieve these highly complex products from quite simple and flexible polyisoprenoid substrates, the active site is assumed to be a template that steers the reaction towards the desired product. Interestingly, triterpene cyclases were recently found to act as general Brønsted acid catalysts. By way of example, the squalene-hopene cyclase from *Alicyclobacillus acidocaldarius* (*Aac*SHC), a triterpene cyclase whose crystal structure is well known [3,4], converts homofarnesol to the fragrance compound ambroxan. Further examinations of SHCs revealed their ability to convert a wide range of substrates, i.e. to catalyse a broad variety of cyclisation reactions with excellent selectivities [5,6]. Moreover, SHCs can catalyse a Friedel–Crafts alkylation [7]. In summary, truncated squalene analogues (C_{15}–C_{25}) can be converted by triterpene cyclases, however, many alternative nucleophiles are also accepted; hence this class of enzymes is highly promising for the use in organic synthesis.

In vivo, squalene-hopene cyclases catalyse the formation of hopanol and hopene from squalene, where five ring structures, 13 carbon-carbon bonds and nine stereocenters are formed [3]. It is assumed that squalene is in a fixed conformation that facilitates the conversion to hopene and hopanol. This reaction is one of the most complex enzymatic one- step reactions and not yet chemically feasible [1]. Hopanoids can adjust the properties of bacterial membranes as they are able to stabilise the lipid layers and to make them more rigid [8]. Consequently, they have the same tasks as sterols in eukaryotic membranes, namely to inhibit gel phase formation and to order the lipids [9]. Generally speaking, in nature terpenoids are important for signalling, defence and communication, whereas in the chemical industry they can be used as chiral building blocks, as anti-cancer or anti-malaria drugs [6].

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http://dx.doi.org/10.1016/j.colsurfb.2015.08.034
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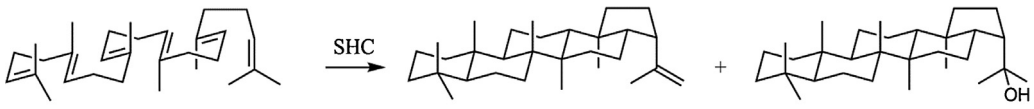

Squalene Hopene Hopanol

Scheme 1. Conversion of squalene to hopene and hopanol. The reaction is catalysed by squalene-hopene cyclases (SHCs).

In the paper at hand, we studied whether cyclases are still active in the presence of organic solvents and surfactants, and whether or not their conformation is disturbed. As a model reaction the natural substrate squalene was converted by the squalene-hopene cyclase from *A. acidocaldarius* (*Aac*SHC) to hopene and hopanol. The reaction is shown in Scheme 1.

We first investigated the influence of *Aac*SHC and of the substrate squalene, respectively, on the phase behaviour of the microemulsion. Second, we studied the activity of *Aac*SHC for different enzyme and substrate concentrations. Third, the composition of the interfacial layer was modified, and its influence on the activity and conformation of *Aac*SHC was determined. Fourth, we studied whether the activity of *Aac*SHC is influenced by the composition of the oil phase.

2. Material and methods

2.1. Enzyme and material

The nonionic surfactant $C_{10}E_5$ (pentaethylene glycol monodecyl ether, >97%), DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 95%), β-$C_{10}G_1$ (*n*-decyl β-D-glucopyranoside, 98%), *n*-octane (99%), squalene (98%), and sodium chloride (99.5%) were purchased from Sigma–Aldrich. The squalene-hopene cyclase from *A. acidocaldarius* was cultivated and purified according to the protocol given in the Supporting information. The buffer is a 60 mM citrate buffer with pH 6.0 containing 2 mM $MgCl_2$. For the preparation of the microemulsions and of the buffer solutions bi-distilled water was used.

2.2. Preparation of the microemulsions

The parameters describing the composition of the microemulsion are described in the following equations. The total surfactant ($C_{10}E_5$ + cosurfactant) mass fraction in the mixture is

$$\gamma = \frac{m_{\text{Surfactant}}}{m_{\text{Surfactant}} + m_{\text{Oil}} + m_{\text{Buffer}}}, \quad (1)$$

the cosurfactant (β-$C_{10}G_1$ and DOPC, respectively) mass fraction in the surfactant mixture is

$$\delta = \frac{m_{\text{Cosurfactant}}}{m_{\text{Cosurfactant}} + m_{\text{Surfactant}}}, \quad (2)$$

the oil volume fraction in the mixture of oil and aqueous phase is

$$\phi = \frac{V_{\text{Oil}}}{V_{\text{Oil}} + V_{\text{Buffer}}} \quad (3)$$

and the NaCl mass fraction in the aqueous phase is

$$\epsilon = \frac{m_{\text{NaCl}}}{m_{\text{NaCl}} + m_{\text{Buffer}}}. \quad (4)$$

It holds for the *Aac*SHC concentration in the aqueous phase

$$C_{\text{AacSHC}} = \frac{m_{\text{AacSHC}}}{V_{\text{Buffer}}} \quad (5)$$

For the preparation of microemulsions, equal volumes ($\varphi = 0.5$) of the buffer solution and *n*-octane were mixed. Where possible, stock solutions of squalene in *n*-octane and of *Aac*SHC in buffer were prepared. For the phase diagrams 0.3 mL of each phase, while for the reactions only 0.075 mL of each phase were mixed. The corresponding surfactant mass was calculated and the three components were weighed in with an accuracy of 0.5 mg. The sample was stirred before the reaction was carried out.

2.3. Optical detection of phase behaviour

The microemulsions were prepared in a glass vial, which was closed with a plastic stopper and sealed with laboratory film. The vial was placed in a thermostated water bath equipped with a magnetic stirrer and a digital thermometer (accuracy 0.01 °C) next to the vial. The phase boundaries enclosing the one phase region at surfactant mass fractions $\gamma > \tilde{\gamma}$ were optically detected in an accuracy of ±0.5 °C and extrapolated to the *X*-point.

2.4. Circular dichroism spectroscopy

The circular dichroism spectra were obtained using a JASCO-185 spectrometer, which is equipped with a N_2 purge and a Peltier system (PTC-4235) to control the temperature. The spectra were recorded from 200 to 300 nm. In order to improve the signal-to-noise level, 8 spectra were acquired and averaged for each sample. The measurements were carried out with the following parameters: the scan speed was set to 100 nm min^{-1}, the response time was 1 s and the bandwidth 1 nm. Cells made of Quartz Suprasil (Hellma) with a path length of 0.1 mm up to 10 mm were used to record the spectra. The intensity of the CD spectra were normalised as follows

$$\theta_{\text{M}} = \frac{100 \times \theta_{\text{measured}}}{C_{\text{enzyme}} \times d} \quad (6)$$

with θ_{measured} being the measured ellipticity in mdeg, d the cuvette path length in cm and c_{enzyme} the enzyme concentration in mol L^{-1}.

2.5. Biotransformations

0.65–2.6 mg mL^{-1} of partially purified enzyme (molecular mass = 71.57 kDa) was used for the biotransformation of 2–80 mM substrate. The total sample volume was 1 mL and the biotransformations were carried out in glass vials identical to those used to measure the phase diagrams. The oil phase contained squalene and the aqueous phase the *Aac*SHC. The sample was shaken thoroughly and placed in the pre-heated water basin. The biotransformations were carried out for 4–35 days at the phase inversion temperature of the respective microemulsion.

For analysis, the internal standard (1-decanol) was added to the reaction mixture, which was extracted twice with 750 μL of *n*-octane by mixing with a vortex mixer and subsequent centrifugation at 12,000 rpm for 60 minutes each. The combined organic phases were then analysed by gas chromatography. The GC-FID analysis was carried out with a Shimadzu GC2010 equipped with an AOC-20i auto injector using H_2 as carrier gas (linear velocity 30 cm s^{-1}). A 5% phenyl-polysil-phenylene-siloxane phase column (CS-Chromatographie, Langerwehe, Germany, 30 m, 0.25 mm, 0.25 μm) was used. The cyclic products were characterised by