Vol. 85, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1978 Pages 867-873

SYNTHESIS OF DEHYDROSQUALENE IN MICROSOMAL FRACTION OF *SACCHAROMYCES CEREVISIAE*

Tokuzo Nishino, Hiroshi Takatsuji, Shingo Hata, and Hirohiko Katsuki

Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606, Japan

Received October 2,1978

SUMMARY When the microsomal fraction of *Saccharomyces cerevisiae* was incubated with farnesyl pyrophosphate or presqualene pyrophosphate in the presence of Mn^{2+}, dehydrosqualene was formed. Incubation of the reaction mixture in the presence of NADPH gave squalene, not dehydrosqualene, as the product. Little dehydrosqualene was formed when Mn^{2+} was replaced with Mg^{2+}. These observations suggest that dehydrosqualene formation is closely associated with squalene synthesis in yeast, which synthesizes neither carotenes nor related pigments.

In the early stages of studies on sterol biosynthesis, dehydrosqualene was for a time believed to be an intermediate by some group of workers. However, the idea was soon abandoned. The isolation of dehydrosqualene was first achieved from *Staphylococcus aureus* (1, 2). After that the compound was found also from *Halobacterium cutirubrum* (3) and *Streptococcus faecium* (4). These organisms all belong to the procaryotes which are not able to synthesize sterols. With *Staph. aureus*, the possibility was pointed out that the yellow pigment of the bacterium which had been believed to be a C_{40}-carotene was a C_{30}-apocarotene and that dehydrosqualene serves as a precursor for the pigment (5). Recently, in fact, the pigment was demonstrated to be a C_{30}-apocarotene (6).

Dehydrosqualene is presumed to be formed by condensation of two molecules of farnesyl pyrophosphate. Rilling, in his studies on squalene synthesis from farnesyl pyrophosphate with microsomal fraction of yeast,

Abbreviations used: TLC, thin-layer chromatography; GC-MS, gas chromatography-mass spectrometry; GLC, gas chromatography.

0006-291X/78/0853-0867$01.00/0

found that presqualene pyrophosphate, an intermediate of squalene synthesis, accumulated in the reaction mixture when NADPH was omitted (7). The accumulation of presqualene pyrophosphate under similar conditions was also found in the reaction with microsomes of animal liver by Popják *et al.* (8).

Recently we found that when microsomal fraction of *Saccharomyces cerevisiae* was incubated with [^{14}C]farnesyl pyrophosphate under similar conditions, radioactivity was incorporated into dehydrosqualene as well as into presqualene alcohol and its pyrophosphate. Addition of NADPH to the reaction mixture gave rise to squalene instead of dehydrosqualene and presqualene alcohol. *S. cerevisiae* belongs to the eucaryotes which differ from the above described bacteria in their ability to synthesize sterols but not carotenes and related pigments. The observation which the present paper will describe suggests a close relation of dehydrosqualene formation to squalene synthesis. Investigation of the problem with yeast should be useful for elucidation of the mechanism for squalene synthesis.

MATERIALS AND METHODS

[^{14}C]Farnesyl pyrophosphate (specific radioactivity, 66 μCi/μmol) was prepared according to the method of Holloway and Popják as follows: the ultracentrifugal supernatant (105,000 x g) of pig liver homogenate was incubated with [2-^{14}C]mevalonate (obtained from Radiochemical Center, Amersham) and the radioactive polar product was extracted with *n*-butanol (9). Inspection by TLC revealed it to be mostly composed of radioactive farnesyl pyrophosphate (9). Non-radioactive farnesyl pyrophosphate was synthesized chemically from *trans, trans*-farnesol (10). [^{14}C]Presqualene pyrophosphate (specific radioactivity, 340 μCi/μmol) was prepared according to the method by Rilling (7) and identified by TLC (11). Dehydrosqualene used as the authentic sample was prepared as follows (2): *Staph. aureus* cells were grown in the presence of 6 x 10^{-5} M diphenylamine and saponified with alkali. Non-saponifiable lipids were extracted with petroleum ether and subjected to a column chromatography on alumina (2). The obtained dehydrosqualene was identified by GC-MS (column, 1.5 m 3% Dexsil 300; temperature, 265°C) (2).

Saccharomyces cerevisiae ATCC 12341 was grown semi-anaerobically for 48 h and then adapted aerobically for 2 h according to the method by Katsuki and Bloch (12) and harvested. The cells, suspended in 0.05 M potassium phosphate buffer (pH 7.4) containing 0.1 mM dithiothreitol (5 g wet cells/10 ml) and disrupted in a Vibrogen Cell Mill for 3 min at 4°C. The suspension was centrifuged at 15,000 x g for 10 min and the resulting supernatant was centrifuged at 105,000 x g for 1 h. The precipitated microsomal fraction, after washed once with the same buffer and then passed through a Sephadex G-50 column, was used as the enzyme.

The mixture for the enzyme reaction contained, in a final volume of 1.0 ml, 2 μmol of $MnCl_2$ (or 5 μmol of $MgCl_2$), 20 μmol of KF, 30 μmol of potassium

phosphate buffer (pH 7.4), microsomal fraction (1 mg of protein), [^{14}C]-farnesyl pyrophosphate (50,000 cpm) (or [^{14}C]presqualene pyrophosphate, 48,000 cpm). After the reaction for 2 h at 30°C, nonpolar and polar products were extracted from the mixture as follows: When [^{14}C]farnesyl pyrophosphate was used as substrate, nonpolar products were extracted with petroleum ether and then polar products with *n*-butanol, and when presqualene pyrophosphate was used as substrate, nonpolar products were extracted with petroleum ether after the mixture was hydrolyzed with alkali for 1 h at 75°C.

For separation of lipids, the following two kinds of TLC were used: normal-phase TLC on Silica Gel G plate using ethyl acetate/benzene (1:4) and reversed-phase TLC on Kieselguhr G plate impregnated with liquid paraffin using acetone/water (19:1) saturated with liquid paraffin as solvent (1). Radio-GLC was carried out with a Shimadzu gas chromatograph GC-5A equipped with a Shimadzu RID-2E radioisotope detector using the column (1 m long) of 1.5% SE-30 adsorbed on Chromosorb WAW at 205°C or using the column (1 m long) of 2% Dexsil 300GC adsorbed on Chromosorb WAW at 205°C.

The radioactivities were measured in a Beckman LS-230 liquid scintillation spectrometer using toluene scintillator.

Separation of lipids by column chromatography was carried out through the column (1 cm x 10 cm) of Woelm neutral alumina (activity, grade II). Elution was made with 1.1, 1.2, 1.3 and 5% diethyl ether in *n*-hexane, successively, and absorbance at 285 nm, λ_{max} of dehydrosqualene (3), and radioactivity were measured on every fraction.

Identification of dehydrosqualene by conversion to perhydro-derivative was carried out as follows: the radioactive reaction product to be identified was hydrogenated using about 1 mg of PtO_2 under hydrogen at room temperature for 2 h. The radioactive hydrogenated product was mixed with the authentic sample of squalane and the mixture was subjected to a radio-GLC.

UV spectra were measured with a Hitachi 124 recording spectrophotometer.

RESULTS

The microsomal fraction of yeast was incubated with [^{14}C]farnesyl pyrophosphate in the presence of NADPH and Mn^{2+}. Analysis of nonpolar products extracted with petroleum ether by normal-phase TLC showed an presence of radioactivity in the spot corresponding to squalene (Fig. 1-A). Incubation in the absence of NADPH showed an presence of radioactivity in the spots corresponding to presqualene alcohol (R_f, 0.6) and squalene (R_f, 0.72) (Fig. 1-B). Inspection by reversed-phase TLC of the radioactive compounds which were eluted from the spots corresponding to squalene in the above two experiments showed that the former compound migrated with squalene (R_f, 0.25) whereas the latter compound was located at different spot (R_f, 0.39)(Fig. 1-C). For identification of the latter compound, the enzyme reaction was carried out in the absence of NADPH in a large scale using 0.1 μmol of farnesyl pyrophosphate which was prepared by dilution of [^{14}C]farnesyl pyrophosphate 170-fold with nonlabeled farnesyl pyrophosphate. Measurement of the UV-spectrum

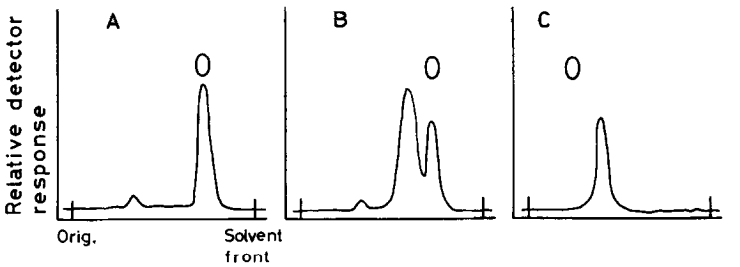


Fig. 1. Normal-phase and reversed-phase thin-layer chromatograms of nonpolar products. The reaction mixture containing 20 μmol of $MnCl_2$, 20 μmol of KF, 30 μmol of phosphate buffer (pH 7.4), [^{14}C]farnesyl pyrophosphate (50,000 cpm) and yeast microsomes (1 mg of protein) was incubated in the presence and absence of 2.5 μmol of NADPH for 2 h at 30°C (final volume of 1 ml). Nonpolar products extracted with petroleum ether were separated by normal-phase TLC and then by reversed-phase TLC. Reference compound was squalene which is shown with circle in the figure.
A. Normal-phase TLC of the product obtained by the incubation with NADPH.
B. Normal-phase TLC of the product obtained by the incubation without NADPH.
C. Reversed-phase TLC of the lipid corresponding to squalene in B.

of the purified lipid by normal-phase TLC and subsequent reversed-phase TLC showed an absorption spectrum, very similar to that of phytoene (13) or dehydrosqualene (1, 3), having a maximum at 285 nm and shoulders at 275 and 298 nm. For identification of carbon skeleton of the radioactive product, it was subjected to a catalytic hydrogenation. Radio-GLC (2% Dexsil 300GC 205°C) of the resulting hydrogenated product showed an identity of the compound with squalane. No radioactive peak corresponding to perhydrophytoene appeared at programmed temperatures up to 265°C. By a separate experiment, it was confirmed that authentic sample of perhydrophytoene was eluted under these conditions. These results suggested that the radioactive reaction product was dehydrosqualene. For further identification, the product was mixed with the authentic sample of dehydrosqualene isolated from *Staph. aureus* and the mixture was subjected to a column chromatography on alumina. As shown in Fig. 2, the elution pattern of radioactivity coincided with that of absorbance at 285 nm. Coincidence of the radioactive and authentic dehydrosqualene was obtained also by Radio-GLC. They appeared at relative retention times of 1.40 on Dexsil 300GC column and of 1.24 on

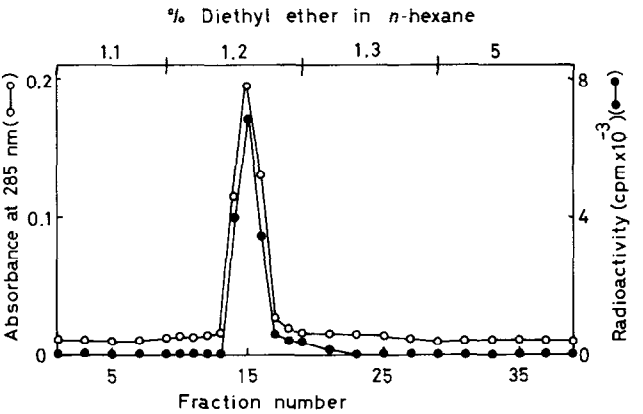


Fig. 2. Coincidence of elution pattern of radioactivity with that of absorbance due to dehydrosqualene in alumina chromatography. The mixture of radioactive reaction products and authentic dehydrosqualene was applied to the alumina column. Elution was done stepwise with diethyl ether in *n*-hexane as indicated at the top of the figure, and 4-ml fractions were collected. The radioactivity and absorbance at 285 nm were measured on each fraction.

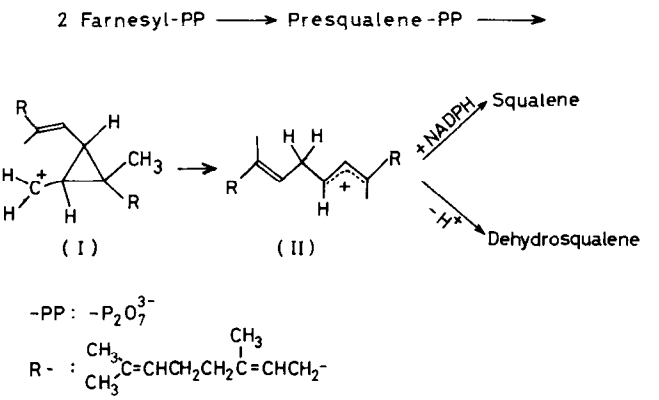
SE-30 column, using squalene as an internal standard. From these results, the radioactive nonpolar product formed in the absence of NADPH was identified as dehydrosqualene.

Analysis by TLC of radioactive polar products which were extracted with *n*-butanol from water layer after extraction of nonpolar products, using *iso*-propanol/conc. ammonia/water (6:3:1) as solvent, revealed the existence of presqualene pyrophosphate (R_f, 0.41) and unchanged farnesyl pyrophosphate (R_f, 0.27)(11).

A similar enzyme reaction was carried out using presqualene pyrophosphate as substrate in the absence of NADPH. Extraction and analysis of the nonpolar products showed a formation of presqualene alcohol and dehydrosqualene in yields of 23 and 7%, respectively.

Replacement of Mn^{2+} by Mg^{2+} in the reaction resulted in only a small yield of dehydrosqualene.

These results indicate that dehydrosqualene is formed in the system for squalene synthesis and that Mn^{2+} plays an important role in the reaction.



Scheme I. Proposed mechanism for dehydrosqualene synthesis.

DISCUSSION

Dehydrosqualene has thus far been reported only from procaryotes. The possibility was suggested (5) that it serves as a precursor for C_{30}-apocarotene. The present study demonstrates the formation of dehydrosqualene with the cell-free extracts of yeast which can synthesize neither carotenes nor apocarotenes. The observation that presqualene pyrophosphate as well as dehydrosqualene were formed in the reaction and that squalene, instead of dehydrosqualene, was formed when the reaction was carried out in the presence of NADPH suggest a close relation between dehydrosqualene formation and squalene synthesis. The mechanism for squalene synthesis has been studied extensively by Rilling, Poulter and their coworkers (14, 15). We propose a mechanism for dehydrosqualene synthesis (Scheme I) based on the mechanism for squalene synthesis proposed by them: cyclopropylcarbinyl cation (I) is first formed by cleavage of C-O bond of presqualene pyrophosphate which is formed by condensation of two molecules of farnesyl pyrophosphate. Then, (I) undergoes a rearrangement of C-C bond in it accompanied by the ring opening to yield cation (II). When NADPH is present, hydride is captured by (II) to form squalene, whereas when NADPH is absent, proton is eliminated to yield triene, that is dehydrosqualene. It may be concluded that the compound is formed secondarily in the presence of Mn^{2+} in squalene synthetase reaction.

The authors wish to thank Prof. S. Seto and Dr. K. Ogura of Tohoku University for valuable discussions and for their kind supply of terpene alcohols. Thanks are also due to Prof. M. Kito and Dr. T. Suzuki of Kyoto University for GC-MS measurement. This work was supported in part by research grants from the Ministry of Education of Japan.

REFERENCES

1. Suzue, G., Tsukada, K., and Tanaka, S. (1967) Biochim. Biophys. Acta 144, 186-188.
2. Suzue, G., Tsukada, K., and Tanaka, S. (1968) Biochim. Biophys. Acta 164, 88-93.
3. Kushwaha, S. C., Pugh, E. L., Kramer, J. K. G., and Kates, M. (1972) Biochim. Biophys. Acta 260, 492-506.
4. Taylor, R. F., and Davies, B. H. (1974) Biochem. J. 139, 751-760.
5. Suzue, G. (1961) Biochim. Biophys. Acta 50, 593-594.
6. Shinoi, K., Katayama, K., Hayami, M., and Kanamasa, Y. (1977) Proc. Jap. Conf. Biochem. Lipids (Japan) 19, 11-13.
7. Rilling, H. C. (1966) J. Biol. Chem. 241, 3233-3236.
8. Popják, G., Edmond, J., Clifford, K., and Williams, V. (1969) J. Biol. Chem. 244, 1897-1918.
9. Holloway, P. W., and Popják, G. (1967) Biochem. J. 104, 57-70.
10. Kandutsch, A. A., Poulus, H., Levin, E., and Bloch, K. (1964) J. Biol. Chem. 239, 2507-2515.
11. Epstein, W. W., and Rilling, H. C. (1970) J. Biol. Chem. 245, 4597-4605.
12. Katsuki, H., and Bloch, K. (1967) J. Biol. Chem. 242, 222-227.
13. Davies, B. H. in "Chemistry and Biochemistry of Plant Pigments," ed. Goodwin, T. W., Academic Press, New York (1965) p. 489.
14. Rilling, H. C., Poulter, C. D., Epstein, W. W., and Larsen, B. (1971) J. Amer. Chem. Soc. 93, 1783-1785.
15. Poulter, C. D., Muscio, O. J., and Goodfellow, R. J. (1974) Biochem. 13, 1530-1538.