THE ISOLATION AND IDENTIFICATION OF A NOVEL INTERMEDIATE IN UBIQUINONE-6 BIOSYNTHESIS BY SACCHAROMYCES CEREVISIAE

Robert R. Goewert, C. Jeffrey Sippel and Robert E. Olson*

Edward A. Doisy Department of Biochemistry
St. Louis University School of Medicine
St. Louis, MO 63104

Received June 6,1977

SUMMARY

The mutant strain of Saccharomyces cerevisiae E3-24 is unable to synthesize ubiquinone-6. When this mutant is grown in the presence of p-hydroxybenzoate-U-^{14}C or p-hydroxybenzoate-7-^{14}C, radioactive 3,4-dihydroxy-hexaprenylbenzoate accumulates. This new metabolite has been isolated and found to have the same R_f as a synthetic homologue on thin layer chromatography with 4 solvent systems. Further, it shows an identical fragmentation pattern in the mass spectrometer. Its presence in S. cerevisiae establishes an alternate pathway for ubiquinone biosynthesis in eukaryotic organisms.

INTRODUCTION

The biosynthetic pathway of the electron transport chain component ubiquinone has been elucidated for prokaryotic organisms (1,2). For eukaryotic organisms, however, several steps in its biosynthesis remain obscure. Numerous observations made in the rat suggest that decarboxylation of prenylated benzoates does not occur directly, but follows ortho-hydroxylation of the ring which implicates 3,4-dihydroxy-5-polyprenylbenzoic acid as an intermediate (3,4). Furthermore, recent preliminary evidence has suggested that 3,4-dihydroxy benzoic acid can be both prenylated and further metabolized to ubiquinone in rat liver mitochondria (5).

The purpose of this communication is to report the isolation and identification of 3,4-dihydroxy-5-hexaprenylbenzoic acid from a mutant of S. cerevisiae that is unable to synthesize ubiquinone-6. It appears in this organism that a protocatechuic acid derivative is on the major pathway of ubiquinone-6 biosynthesis.

*To whom correspondence should be addressed.

ISSN 0006-291X

MATERIALS AND METHODS

p-Hydroxy (U-^{14}C) benzoate was prepared by sodium fusion of DL-(U-^{14}C) tyrosine (435 mCi per mmole) purchased from New England Nuclear Co. p-Hydroxybenzoate-7-^{14}C was prepared by Grignard reaction of p-bromophenyl ether with $^{14}CO_2$ followed by ethanolic alkaline hydrolysis of the benzyl group. p-Hydroxybenzoate G-^{3}H (800 mCi per mmole) was obtained from Amersham-Searle by custom synthesis. 3,4-dihydroxy-5-nonaprenyl benzoate was prepared by alkylation of magnesium protocatechuate with solanesol using BF_3 as a catalyst. The product was isolated by preparative thin layer chromatography and identified by ultraviolet and mass spectrometry.

A ubiquinone deficient mutant from Saccharomyces cerevisiae strain E3-24, was obtained from Dr. A. Tzagaloff (6). Yeast were grown in YPD media (Difco-Yeast Extract 1%, Bacto-peptone 2%, and dextrose 0.8%) in the presence of radiolabeled p-hydroxybenzoate at 31° in a rotary shaker. Cultures were grown several hours into stationary phase, harvested, washed with distilled H_2O and lyophilized. The lyophilized pellets were first extracted with acetone, decanted, and then extracted with an equal volume of ether. The two fractions were combined, the solvent removed and the remaining viscous material isolated and analyzed by thin layer chromatography using Brinkman Silica G 0.25 mm and Anatech 0.50 mm Silica Gel preparative plates. The presence of radioactive intermediates was shown by scanning the TLC plates with a Packard Radiochromatogram Scanner model 7201 using authentic metabolites in the ubiquinone pathway. Quantitation of radioactivity was determined in Bray's solution (7) using a Packard Tri-Carb Scintillation Counter.

Mass spectral analysis was performed by direct probe employing a LKB 9000 GLC-computer interfaced mass spectrometer.

RESULTS AND DISCUSSION

When strain E3-24 was grown in the presence of the ubiquinone precursor p-hydroxy (U-^{14}C) benzoate, approximately 11-20% of the label was found to be incorporated into a compound more polar than 4-hydroxy-3-hexaprenylbenzoic acid (Peak 2, Fig. 1A). In addition another peak, which has been shown to be the decarboxylation product of the new intermediate, was also seen (Peak 3, Fig. 1A). With repeated TLC the new intermediate was obtained in pure form (Fig. 1B) Similar results were obtained in experiments using p-hydroxybenzoate-7-^{14}C as a precursor with the exception that the decarboxylation product was not labeled. Since no other known ubiquinone metabolite or intermediate is more polar than 4-hydroxy-3-hexaprenyl benzoic acid it appeared that a new compound had been found and larger

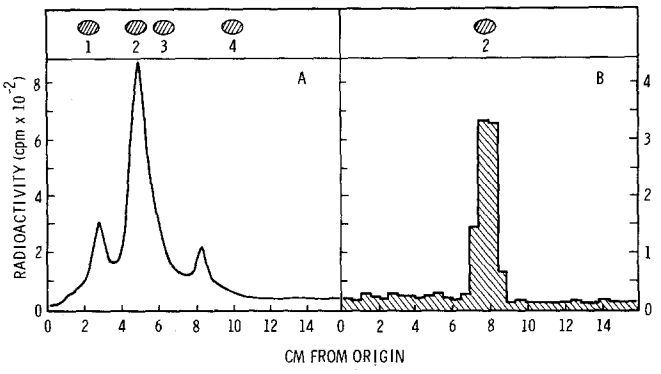


Figure 1. A. Radiochromatography of TLC plate (Silica G. 0.25 mm) of acetone:ether extract of yeast mutant E3-24 grown in the presence of p-OH-benzoate-U-^{14}C developed with cyclohexane:ether:acetic acid, 50:50:0.5. A strip counter was used. Markers are 1) p-HBA; 2) 3,4-dihydroxy-5-nonaprenylbenzoate; 3) 4-hydroxy-3-nonaprenyl-benzoate; 4) 2-nonaprenylphenol. B. Radiochromatogram of TLC plate of purified 3,4-dihydroxy-5-nonaprenyl developed with benzene:acetone, 1:1. 0.5 cm segments were scraped and counted in a scintillation spectrometer.

amounts of this yeast strain were grown in an attempt to isolate this compound.

Strain E3-24 was cultured in 43 liters of SD medium (0.67% Difco Nitrogen Base without amino acids and 0.8% dextrose). An additional one liter culture of strain E3-24 was grown in the presence of p-hydroxy (G-^{3}H) benzoate. This labeled culture was later combined with the other 43 liters for extraction to label the entire pool of putative intermediates. An oily material in the amount of 0.9 grams containing 264,000 c.p.m. was extracted from the total 32.9 grams of dried yeast harvested from the labeled culture. The extracted material was dissolved in a small volume of petroleum ether and applied to a 10 x 1 cm Alumina (Woelm) column and eluted with increasing concentrations of anhydrous ether and petroleum ether in steps. The new intermediate was eluted with 2.5% ether and 97.5% petroleum ether. This fraction contained 42% of the total counts or 112,000 c.p.m. as shown in Table I. The

Table I

ISOLATION OF SUSPECTED 3,4-DIHYDROXY-5-HEXAPRENYL BENZOATE FROM S. CEREVISIAE E3-24

Step	Weight Lipid mg	Radioactivity c.p.m.	Specific Activity c.p.m./mg
1. Acetone:Ether Extract	900	264,000	293
2. Alumina Fraction 2.5% ether	93	112,000	1,204
3. TLC Benzene:Acetone 1:1	2.2	88,500	40,227
4. TLC $CHCl_3$:MeOH:Acetone 10:3:20	0.20*	50,220	251,100
5. TLC Benzene:Acetone 1:1	0.06*	32,500	541,666

*Weight estimated from U.V. spectrum of synthetic 3,4-dihydroxy-5-polyprenylbenzoate, $\lambda^{max}_{\text{diethyl ether}}$ 253 nm, ε = 2,500.

radioactive fraction was then subjected to preparative TLC purification on silica gel G plates using benzene:acetone 1:1. Thirty-three percent of the counts were recovered with a 33 fold increase in specific activity. From this point forward three additional TLC systems were employed in the purification of the metabolite: cyclohexane:ether 8:2, benzene:acetone:ammonia 6:2:2, chloroform:methanol acetone 10:3:20 with a rising specific activity. The new compound was finally rechromatographed on Silica Gel G with benzene:acetone 1:1. The final yield was 60 µg, estimated from the molar extinction coefficient of the synthetic homologue at $\lambda^{max}_{\text{diethyl ether}}$ 253 nm, ε = 2500.

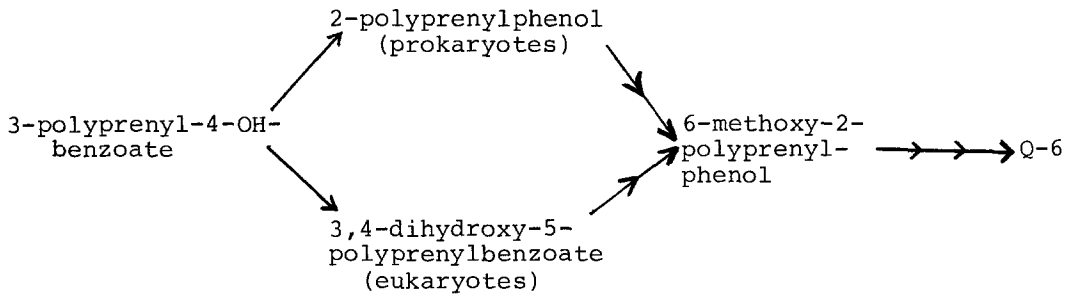
It was noted that during the purification scheme a considerable portion of the labeled intermediate broke down spontaneously to form a less polar compound. The breakdown product, as noted earlier, was not labeled with p-hydroxybenzoate-7-^{14}C which suggested that decarboxylation had occurred to yield 1,2-dihydroxy-3-hexaprenyl-benzene.

Synthetic 3,4-dihydroxy-5-nonaprenyl benzoic acid, a homologue of the new compound, was purified by the same procedure as the labeled intermediate and behaved precisely the same on the four thin layer systems. In addition, mass spectra obtained for both compounds showed identical findings with regard to certain critical fragmentation parts. For example, the typical isoprene fragment m/e 69 was the base peak. The unstable tropylium ion was formed with m/e 167 which yielded a very prominent peak m/e 123 upon decarboxylation. The catecholic tropylium compound characteristically gave up CO to yield m/e 95 which was also a prominent ion. The spontaneous decarboxylation product of synthetic 3,4-dihydroxy-5-nonaprenyl benzoic acid gave a strong signal at 123 and at 95 and in other respects was identical with that of the natural product.

In view of the evidence presented we conclude that the labeled intermediate accumulating in yeast strain E3-24 is 3,4-dihydroxy-5-hexaprenyl benzoic acid. This finding constitutes the first clear evidence that the eukaryotic organism has an alternate pathway for generating 6-methoxy-2-nonaprenyl phenol which appears to be a common intermediate in both pro- and eukaryotic organisms (7,8). The initial alkylation product of p-hydroxybenzoate (3-polyprenyl-4-hydroxybenzoate) has also been identified in both pro- and eukaryotic organisms (8,9). We (10) and others (11) have been unable to detect 2-nonaprenylphenol in animal tissues and were unable to demonstrate the conversion of radioactive synthetic 2-nonaprenyl phenol to ubiquinone-9 in rat liver slices.

Attempts in our laboratory to show the enzymatic decarboxylation of 3-nonaprenyl-4-hydroxybenzoate-7-^{14}C have also been unsuccessful (12). It is also of interest that the E. coli mutant (ubi D) devoid of 3-octaprenyl-4-hydroxybenzoate decarboxylase (8) retained an alternate pathway accounting for 20% of ubiquinone synthesis. It is possible that this alternate pathway in E. coli is identical with the one which becomes dominant in eukaryotic organisms.

These data suggest that eukaryotic organisms differ from prokaryotic ones in the metabolism of 3-polyprenyl-4-hydroxybenzoate as shown below:



In prokaryotes, 2-polyprenylphenol is converted to ubiquinone by alternating hydroxylations and methylations (2). The precise order of events by which 3,4-dihydroxy-5-polyprenylbenzoate is converted to 6-methoxy-2-polyprenylphenol is unknown. Since methionine auxotrophs of S. cerevisiae also accumulate this new intermediate (13), it is possible that methylation precedes decarboxylation, or is simultaneous with it. Further studies to elucidate this alternate pathway in ubiquinone synthesis are in progress.

ACKNOWLEDGEMENT

This research was supported in part by grant AM 10004 from the National Institutes of Health, U.S.P.H.S., Bethesda, Maryland.

REFERENCES

1. Young, I.G., Stroobant, P., MacDonald, C.G., and Gibson, F., (1973) J. Bact. 114, 42-52.
2. Friis, P., Daves, G.D., and Folkers, K. (1966) J. Amer. Chem. Soc. 88, 4754-4756.
3. Nilsson, J.L., Farley, T.M., and Folkers, K. (1968) Anal. Biochem. 23, 422-428.
4. Parsons, W. and Rudney, H. (1965) Proc. Natl. Acad. Sci. 53, 599-606.
5. Nambudiri, A.M.S., Brockman, D., Alam, S.S., and Rudney, H., (1976) Fed. Proc. 35, 1644.
6. Tzagaloff, A., Akai, A., and Needleman, R.B. (1975) J. Biol. Chem. 250, 8228-8235.
7. Bray, G.A. (1960) Anal. Biochem. 1, 279-285.
8. Cox, G.B., Young, I.G., McCann, C.M., and Gibson, F. (1969) J. Bact. 99, 450-458.
9. Raman, T.S., Rudney, H., and Buzzelli, N.K. (1969) Arch. Biochem. Biophys. 130, 164-174.
10. Trumpower, B.L., Houser, R.M., and Olson, R.E. (1974) J. Biol. Chem. 249, 3041-3048.
11. Nilsson, J.L., Farley, T., Scholler, J., and Folkers, K. (1968) Arch. Biochem. Biophys. 123, 422-426.
12. Gardner, E.J. and Olson, R.E., unpublished results.
13. Goewert, R.R. and Olson, R.E., unpublished results.