IDENTIFICATION OF 3-METHOXY-4-HYDROXY-5-HEXAPRENYLBENZOIC ACID AS A NEW INTERMEDIATE IN UBIQUINONE BIOSYNTHESIS BY *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Recent evidence indicates that there are major differences in the biosynthetic pathway of ubiquinone in prokaryotes as compared with eukaryotes [1–3]. 3,4-Dihydroxy-5-hexaprenylbenzoic acid from a ubiquinone-deficient strain of *Saccharomyces cerevisiae* was isolated [4], demonstrating that eukaryotes, unlike prokaryotes, hydroxylate 4-hydroxy-5-polyprenylbenzoic acid prior to decarboxylation [4]. In addition, this protocatechuic acid derivative was found to accumulate in methionine auxotrophs of yeast when methionine is removed from the culture medium [5]. Using rat liver and heart mitochondrial preparations, both protocatechuic and vanillic acids can be prenylated in vitro [6]. Further, the prenylated protocatechuic acid was found to be converted to ubiquinone, suggesting this metabolite to be on a pathway to ubiquinone biosynthesis in mammals.

The purpose of this communication is to report the isolation and identification of 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid from a mutant strain of *S. cerevisiae* that is unable to synthesize ubiquinone-6. The accumulation of this prenylated vanillic acid in this mutant blocked in ubiquinone-6 biosynthesis suggests that this compound is an obligate intermediate on the major pathway to ubiquinone-6 biosynthesis in *S. cerevisiae*.

2. Materials and methods

2.1. *Chemicals*

[*methyl*-^{3}H]Methionine was purchased from Amersham-Searle at spec. act. 8.7 Ci/mmol. *p*-Hydroxy-[U-^{14}C]benzoate was prepared by sodium fusion of D,L-[U-^{14}C]tyrosine (435 μCi/mmol) purchased from New England Nuclear Co. *p*-Hydroxy-[7-^{14}C]benzoate was prepared by Grignard reaction of *p*-bromophenylether with $^{14}CO_2$ (Amersham-Searle) followed by catalytic reduction to the product with H_2/palladium.

3-Methoxy-4-hydroxy-5-nonaprenylbenzoic acid was synthesized by alkylation of vanillic acid with solanesol via BF_3 as a catalyst.

2.2. *Radiolabeling of yeast cultures*

Ubiquinone-deficient mutant of *S. cerevisiae* (strain 26H) was a gift from J. de Kok [7]. This strain was grown in the presence of radiolabeled *p*-hydroxybenzoate in liquid cultures of SD media (0.67% Difco Yeast nitrogen base without amino acids) with 2.0% dextrose at 31°C using a New Brunswick gyrorotary shaker. Cultures were grown several hours into stationary phase, harvested, washed with distilled H_2O, then lyophilized. The lyophilized pellets were extracted with acetone, followed by a second extraction with acetone/diethyl ether (1:1). The fractions were combined, the solvent removed,

and the remaining viscous material analyzed by thin-layer chromatography (TLC) using Brinkman silica gel G 0.25 mm plates. In a separate experiment, 20 mg of L-methionine were added to a 500 ml culture of SD media and strain 26H was allowed to grow to mid-log phase. The cells were harvested by centrifugation and resuspended in fresh media containing 5 mCi [*methyl*-^{3}H]methionine at the original concentration of total methionine and incubated for 3 h. The cells were prepared as before, lyophilized, extracted with diethyl ether, the solvent removed and the lipid extract analyzed by TLC.

2.3. *Analytical methods*

The presence of labeled radioactive intermediates was detected by scanning the developed TLC plates using a model 7201 Packard radiochromatogram scanner. Authentic metabolites in the biosynthetic pathway for ubiquinone were cochromatographed with both the initial crude lipid extract and in consequent purification steps. Radioactivity was measured in Bray's mixture [8] using a Packard Tri-Carb scintillation counter with [^{14}C]toluene as a standard. [*methyl*-^{3}H]Methionine was used as the tritium standard. Mass spectral analysis was performed by direct probe employing a LKB 9000 computer-interface mass spectrometer.

3. Results and discussion

When strain 26H was grown in the presence of the precursor *p*-hydroxy-[U-^{14}C]benzoic acid approx. 14% label was found to be incorporated into a lipid intermediate (peak 3, fig.1A) more polar than 2-nonaprenylphenol but less polar than either 3,4-dihydroxy-5-nonaprenylbenzoic acid or 4-hydroxy-5-nonaprenylbenzoic acid. The new compound was purified to constant specific activity (peak 3, fig.1B) by TLC employing several different solvent systems. Similar results were obtained using *p*-hydroxy-[7-^{14}C]benzoic acid. Since this intermediate possessed a unique R_F relative to other characterized ubiquinone intermediates, larger amounts of this strain were grown to isolate and characterize this compound.

Strain 26H, 12 liters, was cultured in SD medium in the presence of 70 μCi *p*-hydroxy-[7-^{14}C]benzoic acid. An oily material, 0.45 g, was obtained from dry wt 18.7 g yeast and found to contain radioactivity,

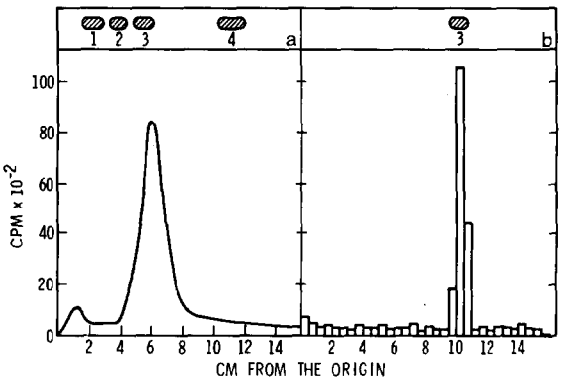

Fig.1a. Radiochromatography of TLC plate (silica gel 0.25 mm) of acetone/ether extract of yeast mutant 26H grown in the presence of *p*-hydroxy-[U-^{14}C]benzoate developed with cyclohexane/ether/acetic acid (50:50:0.5). A strip counter was used. Markers are (1) *p*-HBA; (2) 3,4-dihydroxy-5-nonaprenylbenzoate; (3) 3-methoxy-4-hydroxy-5-nonaprenylbenzoate; (4) 2-nonaprenylphenol. 1b. Radiochromatogram of TLC plate of purified 3-methoxy-4-hydroxy-5-hexaprenylbenzoate developed with benzene/acetone (1:1). Segments, 0.5 cm, were scraped and counted in a scintillation spectrometer.

2×10^7 dpm. The extracted material was dissolved in 25 ml hexane and the volume reduced to 5 ml with N_2 which produced precipitation of unmetabolized labeled *p*-hydroxybenzoic acid and extraneous lipids. 50% radioactivity was recovered in the hexane-soluble fraction with a 2.5-fold increase in specific activity. This hexane-soluble extract was subjected to preparative TLC using 1.0 mm silica gel G plates developed with benzene/acetone (1:1). 8% counts were recovered with 6-fold increase in specific activity as shown in table 1. Final purification was achieved using a 0.25 mm analytical TLC plate developed with ether/cyclohexane (8:2). Of intermediate, 32 μg was obtained, estimated from ultraviolet absorption using a molar extinction coefficient λ_{max}^{ether} 217 nm, $\epsilon = 22\,000$.

In a similar experiment in which [*methyl*-^{3}H]-methionine was employed, 0.11% of the label was incorporated into the intermediate, which was subsequently purified 90-fold by TLC to constant specific radioactivity. These results indicate that this new intermediate contains both a C-7 carboxyl group and at least 1 methyl constituent.

Synthetic 3-methoxy-4-hydroxy-5-nonaprenyl-

Table 1
Isolation of 3-methoxy-4-hydroxy-5-hexaprenylbenzoate from cells of *S. cerevisiae* 26H

Step	lipid (mg)	Radioact. (dpm)	Spec. act. (dpm/mg)
1. Acetone/Ether (1:1) crude extract	450	2.0×10^7	44 000
2. Hexane-soluble reside	90	1.1×10^7	116 666
3. TLC benzene/acetone (1:1)	1.3	9.0×10^5	692 307
4. TLC ether/cyclohexane (8:2)	0.032	2.6×10^4	854 838

benzoic acid was purified in a similar manner as the labeled intermediate and found to exhibit identical mobilities in three different solvent systems. Ultraviolet spectra for the natural and synthetic homologue were identical. Mass spectra were obtained for both the material and synthetic homologue. Both compounds produced the base peak *m/e* 69 corresponding to an ion produced by fragmentation of the isoprene side chain. A *m/e* 97 fragment, characteristic for vanillic acid [9] was the second most abundant ion in both spectra. The unstable tropylium ion, which formed with *m/e* 181, was readily decarboxylated to yield a prominent peak *m/e* 137. The chromenylium ion, a major peak at *m/e* 235 was also observed to produce the decarboxylated fragment at *m/e* 191. Further proof of the structure of the natural compound was obtained from the mass spectrum of its methyl ester, prepared by treatment with diazomethane. The molecular ion observed for this compound was *m/e* 590 corresponding to its calculated molecular weight. In addition, a major fragment *m/e* 195 corresponding to the methyl ester of the trophylium ion was also observed.

From the evidence presented, we conclude that the mutant yeast strain 26H accumulates 4-methoxy-3-hydroxy-5-hexaprenylbenzoic acid when given *p*-hydroxybenzoate. This suggests that the genetic block in this mutant is at the 'decarboxylase level'. This finding also constitutes the first clear evidence that *O*-methylation precedes decarboxylation of the benzoate derivatives in the biosynthesis of ubiquinone 6 in yeast.

It is now clear that the pathway of ubiquinone biosynthesis in prokaryotic organisms differs from that in eukaryotic organisms [10–12]. In prokaryotic organisms 4-hydroxy-5-polyprenylbenzoate is converted to the next common intermediate, i.e., 6-methoxy-2-polyprenylphenol in steps involving decarboxylation, hydroxylation and methylation. In eukaryotic organisms, the sequence is hydroxylation, methylation and finally 'decarboxylation'. The mechanism of this 'decarboxylation' is known and a subject of continuing study in this laboratory.

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