MEVALONIC ACID AS A PRECURSOR OF THE ALKYL SIDECHAIN OF HEME *a* OF CYTOCHROME *c* OXIDASE IN YEAST *SACCHAROMYCES CEREVISIAE*

Jacqueline KEYHANI and Ezzatollah KEYHANI

Laboratory for Cell Biology and Biochemistry, Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 314-1700, Tehran, Iran

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1. Introduction

Heme *a* of cytochrome *c* oxidase exhibits a strikingly different structure and properties from those of protoheme. The distinction between these two hemes resides in structure of the side chains. In heme *a*, a methyl group at position 8 is replaced by a formyl group, while the vinyl group at position 2 is replaced by an α-hydroxyfarnesylethyl group [1–4]. The mechanism of the transformation of protoporphyrin IX or protoheme into heme *a* is not yet understood. On the basis of chemical degradation of the alkyl side chain, the hypothesis was proposed [5] that the vinyl group in position 2 of the protoporphyrin ring is alkylated by farnesyl pyrophosphate [5] and that pyrophosphate elimination occurs simultaneously with the introduction of a hydroxyl group on the α-carbon atom, in a concerted reaction. However, attempts to demonstrate the incorporation of farnesyl chain into heme *a* of yeast cell have not been successful [6].

We show here that mevalonic acid, a precursor of farnesyl pyrophosphate [7], can be incorporated into heme *a* of cytochrome *c* oxidase in *Saccharomyces cerevisiae* yeast cells. This is the first reported evidence of this incorporation, which supports the hypothesis [5] concerning this aspect of heme *a* biosynthesis.

2. Materials and methods

Yeast cells *Saccharomyces cerevisiae*, strain 777-3c (α OP1 *ade2 mit*$^+$) were grown in synthetic medium as in [8] with the modifications in [9]. Methods for preparation of mitochondria, purification of cytochrome *c* oxidase, counting procedures and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) have also been described [10,11].

2.1. *Measurements of heme* a *synthesis by double-labelling procedure*

Yeast cells, ~2 g, were suspended in 500 ml medium, supplemented with 60 μCi δ-aminolevulinic [^{14}C]acid (δ-aminolevulinic-5-[^{14}C]acid, spec. act. 49 mCi/mmol) and 500 μCi [^{3}H]mevalonic acid (DL-(mevalonic-5-[^{3}H](N))-, spec. act. 5.0 Ci/mmol). The cells were grown in this medium for 12 h, harvested and washed 3 times with distilled water. In all experiments 3% glucose was used as a carbon source.

2.2. *Isolation of hemes and porphyrins*

Heme *a* was isolated from purified cytochrome *c* oxidase as in [12,13]. Hemes and porphyrins were extracted from double-labelled mitochondria with acetone–HCl. The procedure in [12,13] was used for subfractionation of hemes and porphyrins.

2.3. *Immunoprecipitation*

Rabbit antiserum against cytochrome *c* oxidase was prepared as in [14]. Immunoprecipitation was carried out according to a modification of the procedure in [15]. Mitochondria were solubilized in 2% Triton X-100, phosphate buffer 0.02 M (pH 7.4). After 2–3 h incubation with gentle stirring, the mixture was centrifuged at 15 000 rev./min for 20 min. The supernatant was mixed with rabbit serum containing antibodies against cytochrome *c*

oxidase and incubated overnight at 0°C. The white precipitate of antibody/antigen was centrifuged at 5000 rev./min for 5 min. The pellet was then washed twice in phosphate buffer 0.02 M (pH 7.4) containing 1% Triton X-100, and once with phosphate buffer alone.

2.4. *Thin-layer chromatography*

Silica gel coated plates (20 × 20 cm) were prerun in lutidine–water (67.5/32.5) in the presence of ammonia for 6 h. After the plates were dried, thin-layer chromatography of heme *a* and protoheme was done in the same solvent. After chromatography, the plates were dried at 110°C for 10–15 min. After cooling, the plates were sprayed with *O*-tolidine. The spots corresponding to heme *a* and protoheme became brown–red, were scraped carefully and transferred into scintillation vials, and the radioactivity was determined.

2.5. *Mitochondrial delipidation*

Mitochondria and immunoprecipitated cytochrome *c* oxidase were delipidated with acetone/water. Acetone solution, 5 ml, in water were added to 60 μl radioactive mitochondria (17 mg prot./ml), or to the immunoprecipitated cytochrome *c* oxidase. After mixing vigorously, the samples were left at room temperature for 30 min and then centrifuged in a Sorvall SS34 rotor for 10 min at 10 000 rev./min. Supernatants were carefully removed and the amount of radioactivity extracted was determined. The delipidation procedure was repeated until the supernatant collected was devoid of radioacitivity. Usually 3 cycles of washing would give such a result.

3. Results

Table 1 shows the incorporation of radioactive δ-aminolevulinic acid (AmLev) and mevalonic acid (MVA) in the trichloroacetic acid (TCA) precipitable material of various subcellular fractions. High specific radioactivity was found to be associated with mitochondrial fractions for both radioactive AmLev and radioactive MVA. While AmLev, being incorporated into the porphyrin ring, is a specific precursor of all hemes including heme *a* of cytochrome *c* oxidase, MVA would also be incorporated into mitochondrial lipids, e.g., ubiquinone, as well as into heme *a*. The high incorporation of MVA into yeast mitochondria as observed in this work, is consistent with similar observations reported for mitochondria isolated from regenerating rat liver [16]. The ratio of [^{14}C]-AmLev to [^{3}H]MVA is 4.3 in the mitochondrial fraction (table 1), but after delipidation this ratio becomes 10.4 (table 1). About 77% radioactive MVA present in the mitochondria has been removed and so must have been incorporated into lipid. About 23% of the radioactivity still remains associated with the delipidated mitochondria however. When mitochondria were submitted to SDS–PAGE (fig.1), the radioactivity due to AmLev was resolved into 3 bands with app. mol. wt 28 000, 12 500 and 9800 (± 1000), respectively, similar to the pattern obtained from *Candida utilis* yeast cells [17]. The hemes covalently bound to cytochromes c_1 and *c* were resolved into 2 distinct bands at 28 000 and 12 500, respectively. On the other hand, the hemes non-covalently bound to proteins, such as the hemes of cytochrome *b* and cytochrome *c* oxidase, migrate into the SDS–polyacrylamide gels according to their charge only, and are found in the region of low molecular weights (≃ 10 000) [15,17]. The radioactivity due to MVA in the mitochondria, was resolved into a single band (fig.1) with an apparent molecular weight corresponding exactly to that found for the hemes non-covalently bound to proteins. Since heme *a* is

Table 1
Subcellular distribution of radioactive AmLev and MVA in *Saccharomyces cerevisiae*

Fraction[a]	[^{14}C]AmLev[b]	[^{3}H]MVA[b]	[^{14}C]AmLev / [^{3}H]MVA
Homogenate	16 959	3598	4.7
Microsomes	18 198	3607	5.05
Mitochondria	47 705	10 911	4.37
Delipidated mitochondria	26 470	2539	10.4
Immuno precipitate[c]	1822	252	7.2

[a] TCA precipitable
[b] Expressed as cpm/mg protein
[c] Delipidated immunoprecipitate of cytochrome *c* oxidase

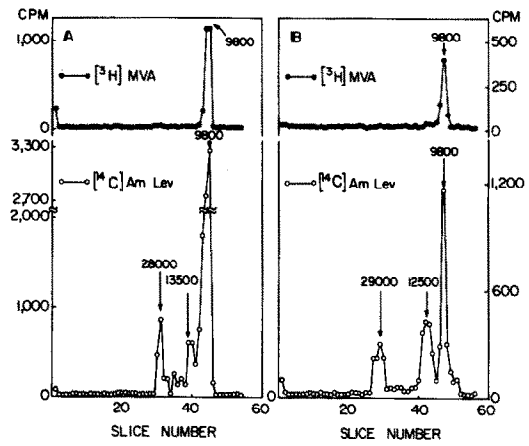

Fig.1. Distribution of radioactivity among electrophoretic fractions of [^{14}C]AmLev- and [^{3}H]MVA-labelled normal mitochondria (A) and delipidated mitochondria (B).

the only heme containing an alkyl side chain, the radioactivity due to MVA resolved in the low molecular weight region, in delipidated mitochondria, can be attributed to heme *a* rather than protoheme.

Further experiments support this finding:

1. When [^{14}C]AmLev, [^{3}H]MVA-labelled mitochondria were solubilized with Triton X-100, as described in section 2, and incubated with rabbit serum containing antibodies against cytochrome *c* oxidase, a precipitate was formed. This precipitate, after washing as indicated in section 2, was subjected to 3 cycles of delipidation. The radioactivity due to MVA was still present in delipidated immunoprecipitate of cytochrome *c* oxidase with a ratio of [^{14}C]AmLev to [^{3}H]MVA of 7.2 (table 1).
2. The distribution of radioactivity among hemes and porphyrins extracted from mitochondria by the method in [12,13] is shown in table 2. At 20% HCl, which corresponds to the HCl number of porphyrin *a* [18], a significant amount of radioactivity was extracted. The ratio of [^{14}C]AmLev to [^{3}H]MVA was 7.1, identical to the value of 7.2 obtained with the immunoprecipitated cytochrome *c* oxidase.
3. When cold heme *a* and protoheme were added as carriers to the ether phase containing radioactive heme *a* and protoheme, and the ether phase was evaporated to dryness, transferred to acetone and subjected to thin-layer chromatography in lutidine-water as described in section 2, all the radioactive MVA was found in the spot corresponding to heme *a*.

Table 2
Distribution of [^{14}C]AmLev and [^{3}H]MVA among porphyrins extracted from mitochondria[a] of *Saccharomyces cerevisiae*

[HCl][b]	[^{14}C]AmLev[c]	[^{3}H]MVA[c]	[^{14}C]AmLev / [^{3}H]MVA
4%	3682	98	37.5
8%	4600	570	8.0
20%	48 282	6746	7.1

[a] The extraction procedure [12,13] was used in these experiments
[b] HCl numbers 4%, 8%, 20% correspond, respectively, to protoporphyrin IX, cryptoporphyrin and porphyrin *a* [18]
[c] Expressed as total cpm present in the fraction

4. Discussion

These three results show clearly that MVA is incorporated into the alkyl side chain of heme *a*. MVA enters the biosynthetic pathway of the terpenoids by transformation to isopentyl pyrophosphate (IPP). The conversion of IPP to dimethylallyl pyrophosphate, acts as a starter for condensation with further molecules of IPP [7] to give the oligomeric isoprenoid pyrophosphates, of which the side chain of ubiquinone is one such derivative [7,19]. Farnesyl pyrophosphate is the trimer of IPP and so is derived directly from MVA. The results of this work imply that farnesyl pyrophosphate reacts directly with protoporphyrin IX, or protoheme as suggested [5], which offers a new approach to the investigation of the biosynthetic pathway of heme *a* of cytochrome *c* oxidase. Such work is in progress.

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