PORPHYRIN *a* AS A PRECURSOR OF HEME *a* IN *CANDIDA UTILIS*

Jacqueline KEYHANI and Ezzatollah KEYHANI
Department of Biochemistry, St Jude Children's Research Hospital, Memphis, Tenn. 38101, USA

Received 7 September 1976

1. Introduction

The disappearance of cytochrome *c* oxidase from yeast cells after growth in copper-deficient medium was reported nearly four decades ago [1], but despite extensive investigation the mechanism responsible for this phenomenon is not understood. We showed in earlier studies [2], as have others [3–5], that copper deficiency, although leading to the disappearance of cytochrome *c* oxidase, does not significantly affect the level of other hemoproteins. On the other hand, an excess of copper leads to an acceleration of cytochrome biosynthesis [6] and to an increase in mitochondrial volume [6]. Copper-deficient yeast cells therefore offer a reasonable model for studying the biosynthesis and assembly of cytochrome *c* oxidase.

We recently obtained evidence suggesting that the apoprotein of cytochrome *c* oxidase is present in copper-deficient cells [7]. The present study confirms this finding immunochemically and, further, demonstrates that a porphyrin precursor of heme *a*, identified as porphyrin *a*, is synthesized and integrated into the apo-oxidase. This is the first reported evidence implicating porphyrin *a* as a precursor of the heme of cytochrome *c* oxidase.

2. Materials and methods

Candida utilis (ATCC 8205) yeast cells were grown in copper-supplemented and copper-deficient medium as previously described [2]. Methods for extraction of copper from the growth medium, preparation of mitochondria, and purification of cytochrome *c* oxidase have also been described [2,8]. All glassware was washed with acid and rinsed with copper-free medium. The level of copper in our copper-free preparations was always below 2 μg/l, as indicated by atomic absorption measurements.

2.1. *Measurements of protein and heme synthesis by double-labeling procedures*

Approximately 3 g of copper-deficient yeast cells were suspended in each of two flasks containing 4 litre of copper-deficient medium. One group of cells was maintained in copper-deficient medium, and 100 μg of copper per liter was added to the remaining culture. Each culture was supplemented with 1 mCi of uniformly labeled [^{3}H]leucine (sp. act. 40.7 Ci/mmol) and 125 μCi of [δ-^{14}C]aminolevulinic acid (AmLev, sp. act. 29.2 mCi/mmol). In some experiments only heme was labeled. The cells were grown in this medium for 19 h, harvested and washed three times with distilled water.

2.2. *Immunoprecipitation*

Rabbit antiserum against cytochrome *c* oxidase was prepared according to Kraml and Mahler [9]. Mitochondria were solubilized in 2% cholate and subjected to two cycles of ammonium sulfate fractionation at 25 and 45% saturation, respectively. The pellet resulting from 45% saturation was solubilized in 0.02 M phosphate buffer (pH 7.4) containing 1% Triton X-100. The solubilized pellet was mixed with the 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 and then washed twice in phosphate buffer, 0.02 M (pH 7.4), containing 1% Triton X-100 and once with the phosphate buffer only. The immunoprecipitate was dissolved in SDS and electrophoresed as previously reported [7].

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2.3. *Isolation of hemes and porphyrins*

Heme *a* was isolated from purified cytochrome *c* oxidase according to Lemberg et al. [10,11]. Porphyrin *a* was prepared from heme *a* by the iron-extraction procedure of Morell and Stewart [12]. Hemes and porphyrins were extracted from [^{14}C]AmLev-labeled, copper-deficient mitochondria with acetone–HCl, using cold porphyrin *a* as a carrier.

2.4. *Pyridine hemochromogen spectra*

Pyridine hemochromogen spectra analysis was performed according to Horie and Morrison [13], as described by Williams [14]. An Aminco DW-2 spectrophotometer was used to record the spectra.

3. Results

Figure 1 shows the electrophoretic profiles of immunoprecipitated material obtained by incubating double-labeled copper-deficient mitochondria with rabbit serum containing antibody against cytochrome *c* oxidase. The presence of a precipitate indicates that apocytochrome *c* oxidase was synthesized in these copper-deficient cells. The incorporation of [^{3}H] leucine into the immunoprecipitate apo-oxidase was resolved into six polypeptide bands with apparent molecular weights of 46 000, 35 500, 28 000, 16 000, 13 500 and 9700 (fig.1A). [^{14}C]AmLev was incorporated as a single radioactive band with an apparent molecular weight of 10 000 (fig.1B). Our control, an immunoprecipitate obtained with copper-supplemented cells, showed the same number of radioactive bands as the material from copper-deficient mitochondria.

In the experiment depicted in fig.1, cytochrome *c*, purified heme *a* and protoporphyrin IX were used as standards, and electrophoresis was stopped before the marker dye reached 2 cm from the end of the gel. Although heme *a* and protoporphyrin IX migrated to regions corresponding to an apparent molecular weight of 10 000, the heme of cytochrome *c*, covalently bound to protein, remained in the 12 500 region, which corresponds to the actual molecular weight of cytochrome *c*. Since heme *a* and protoporphyrin IX have a molecular weight of about 800 and 563, respectively, their presence in the gel in a low molecular weight region (approximately 10 000) was unexpected.

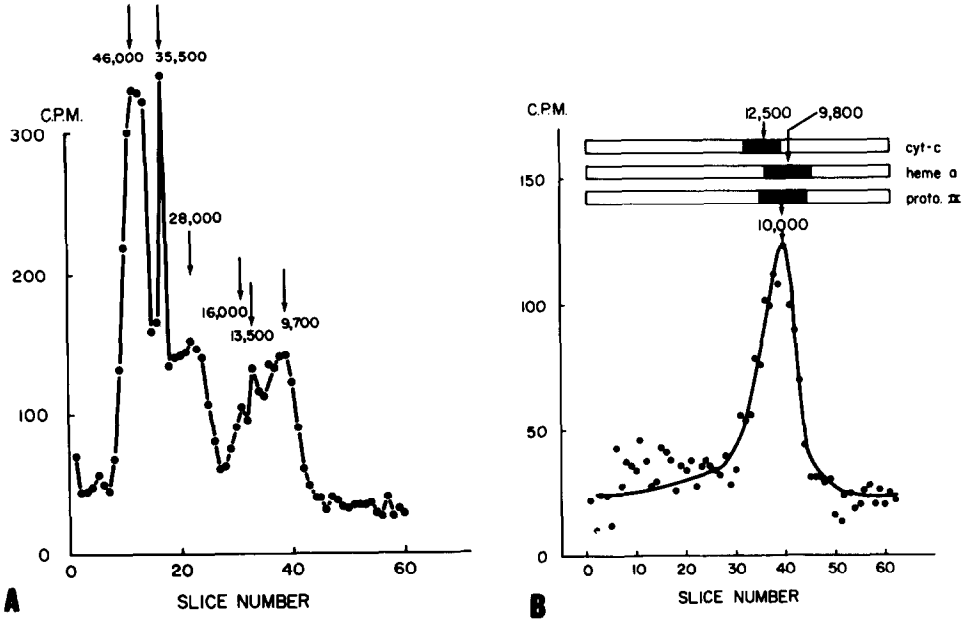


Fig.1. Distribution of radioactivity among electrophoretic fractions of [^{3}H]leucine-labeled apo-oxidase (A) and [^{14}C]AmLev-labeled porphyrin (B), both immunoprecipitated from copper-deficient mitochondria by use of antibodies against cytochrome *c* oxidase.

Possibly, both compounds migrate according to their charges only. In any case, this observation indicates that for a heme noncovalently bound to a protein, SDS–polyacrylamide gel electrophoresis might not be the appropriate method for determining the specific protein subunit to which heme is attached.

Although pyridine hemochromogen difference spectra have shown that heme *a* is absent in copper-deficient cells [15], the association of [^{14}C]AmLev with apo-oxidase suggests that a porphyrin precursor of heme *a* is present in such cells. The precursor might be protoporphyrin IX, since this porphyrin can associate with protein [16] and has been suggested as an intermediary in the formation of all hemes, including cytochrome *c* oxidase [17–20]. To test this supposition, we suspended immunoprecipitated apo-oxidase from copper-deficient cells in pyridine–KOH [14], and recorded the absolute spectra (fig.2A). The spectra of porphyrin *a* (fig.2B), protoporphyrin IX (fig.2C) and heme *a* (fig.2D) in pyridine–KOH are shown for comparison. The porphyrin precursor of heme *a* from copper-deficient cells shows five absorption bands with maxima of 645, 590, 559, 520 and 425 nm. These are distinct from the absorption bands of protoporphyrin IX and heme *a* but identical to that of purified porphyrin *a*. The shift to longer wavelengths at 520 and 425 nm for porphyrin *a* present in copper-deficient cells, as compared to purified porphyrin *a*, was interpreted as the effect of associated lipids and denatured proteins still present in the immunoprecipi-

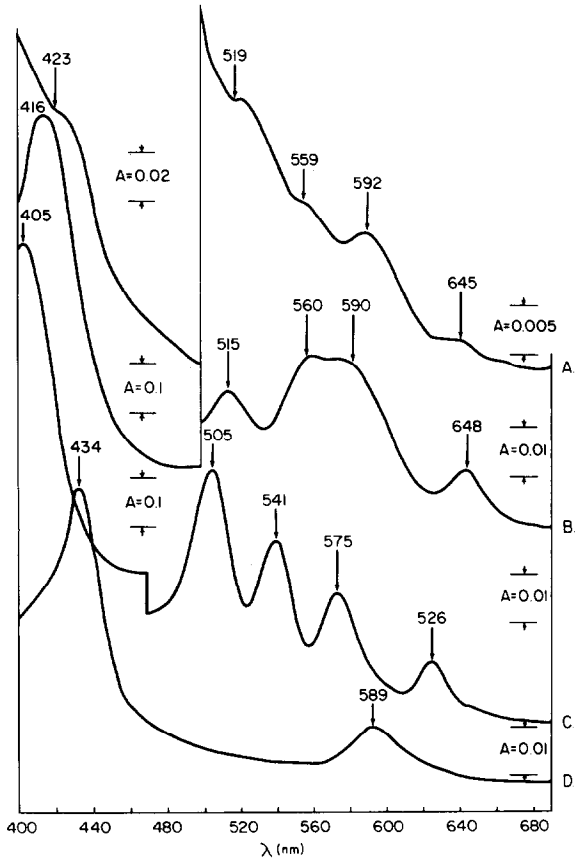


Fig.2. Absolute spectra in pyridine–KOH of (A) porphyrin immunoprecipitated from copper-deficient mitochondria by use of antibodies against cytochrome *c* oxidase, (B) purified porphyrin *a*, (C) protoporphyrin IX, and (D) heme *a*.

Table 1
Distribution of [^{14}C]AmLev among hemes and porphyrins extracted from mitochondria[a] of copper-deficient cells

Expt. No.	Total in washed ether phase	Recovery of radioactivity[b] from washed ether phase using HCl at:[c]			Residual radioactivity[b] in ether phase after HCl extractions
		4%	8%	20%	
1	182 757 (100%)	2808 (1.53%)	3430 (1.87%)	34 990 (19.1%)	141 529 (77.4%)
2	198 214 (100%)	3811 (1.92%)	9570 (4.8%)	47 725 (24%)	137 108 (69.1%)

[a] The extraction procedure of Lemberg et al. [10,11] was used in both experiments.
[b] Expressed as cpm; values in parentheses represent the percentage of total counts present in washed ether phase.
[c] HCl numbers 4, 8 and 20% correspond respectively to protoporphyrin IX, cryptoporphyrin and porphyrin *a* [21].

tated material dissolved in pyridine–KOH. This spectral analysis indicates that porphyrin *a*, but not protoporphyrin IX, is present in the apo-oxidase from copper-deficient cells.

Additional evidence for this finding was obtained by labeling copper-deficient cells with [^{14}C]AmLev and measuring the distribution of radioactivity among hemes and porphyrins extracted from mitochondria according to the procedure of Lemberg et al. [10,11], with purified cold porphyrin *a* used as a carrier. In these experiments, hemes and porphyrins were first extracted by acetone–HCl and then transferred from acetone–HCl to ether. After the ether was washed with 1% HCl, porphyrins were extracted according to their HCl number, while hemes remained in the ether phase. The material extracted from ether phase with 4%, 8%, and 20% HCl corresponds respectively to protoporphyrin IX, cryptoporphyrin, and porphyrin *a* [21]. Table 1 shows the results of two separate experiments. Clearly, at a concentration of 20% HCl, which corresponds to the HCl number of porphyrin *a*, a significant amount of radioactivity was extracted. This radioactivity represented 19 to 24% of the total radioactivity present in washed ether phase. Indeed, the visible spectrum of this fraction is typical of exogenous porphyrin *a* added as carrier in both experiments.

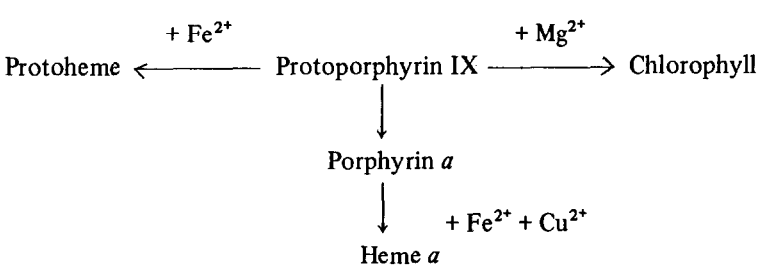
When copper-deficient cells were grown in copper-supplemented medium and [^{14}C]AmLev, hemes and porphyrins extracted from mitochondria as described in the preceding paragraph, the radioactivity recovered in 20% HCl was approximately 2% of the total radioactivity present in the washed ether phase. This result indicates that the porphyrin *a* present in copper-deficient cells is converted to heme *a* and, further, that a small amount of porphyrin *a* is present even in yeast grown in copper-supplemented medium.

4. Discussion

These immunochemical studies confirm our previous finding [7] that the apoprotein of cytochrome *c* oxidase is synthesized in copper-deficient cells. The comparable subunit structures of the apo-oxidase from copper-deficient cells versus the cytochrome *c* oxidase from copper-supplemented cells suggest that copper is not required for apoprotein biosynthesis or its integration into the inner mitochondrial membrane.

An unexpected finding was the presence of porphyrin in the apo-oxidase of copper-deficient mitochondria immunoprecipitated by antibodies against cytochrome *c* oxidase. Using two different methods, we found that this porphyrin has the same properties as porphyrin *a*, which indicates that copper is not necessary for the synthesis of porphyrin *a* or its integration into the apo-oxidase. This observation suggests further that porphyrin *a* is an intermediary in heme *a* biosynthesis.

It is generally accepted that the synthesis of hemes and chlorophyll follows a common pathway up to the formation of protoporphyrin IX [17–20]. The insertion of iron by ferrochelatase into protoporphyrin IX leads to heme formation, while the integration of Mg into protoporphyrin IX gives rise to chlorophyll. Thus, if porphyrin *a* is indeed a precursor of heme *a*, the final step of heme *a* biosynthesis would have a different pathway from that of other hemes. Specifically, porphyrin *a* would be present as an intermediary between protoporphyrin IX and heme *a*, and iron would be incorporated into porphyrin *a* when copper is present. The following scheme is suggested for the final step of heme *a* biosynthesis.



The transformation of protoporphyrin IX to porphyrin *a* requires that the formyl group replace the methyl group at position 8 and that the vinyl group at position 2 be replaced by the α-hydroxyfarnesylethyl group [22]. Our data suggest that copper has no function in the transformation of protoporphyrin IX to porphyrin *a*, but is necessary for the incorporation of iron into porphyrin *a*, by a mechanism that remains to be elucidated. In vitro experiments [23] have shown that ferrochelatase cannot incorporate iron into porphyrin *a*, and the incubation of copper-deficient cells in a medium containing iron and copper (non-growing medium) does not induce cytochrome *c* oxidase formation [2]. It is reasonable to assume, therefore, that the integration of Fe into porphyrin *a* requires a copper-dependent enzyme that is absent in copper-deficient cells. Indeed, our previous studies show that a cytoplasmic protein sensitive to cycloheximide is necessary for transport or integration of copper into cytochrome *c* oxidase [7]. It is possible that copper is first integrated into a complex of porphyrin *a* and apo-oxidase and that this incorporation, by reducing steric hindrance due to long alkyal side chains, allows the incorporation of Fe catalyzed by ferrochelatase.

Acknowledgements

This investigation was supported by ALSAC. One of us (J.K.) was supported by a post-doctoral fellowship from NCI, grant CA 05176.

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