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Negative Control of Yeast Coproporphyrinogen Oxidase Synthesis by Heme and Oxygen*

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Coproporphyrinogen oxidase (EC 1.3.3.3.) catalyzes the sixth enzymic step of the heme biosynthetic pathway. Coproporphyrinogen oxidase activity is increased in mutant cells of *Saccharomyces cerevisiae* deficient in heme synthesis and this effect can be partially reversed by the addition of exogenous hemin. A similar increase is found in wild type yeast cells grown anaerobically. The strain-dependent increase varies between 5- and 40-fold. The activity changes are paralleled by similar changes in 1) the steady-state amounts of coproporphyrinogen oxidase protein determined by immunoblotting and 2) the steady-state concentrations of coproporphyrinogen oxidase mRNA estimated by *in vitro* translation/immunoprecipitation. This demonstrates that coproporphyrinogen oxidase synthesis is regulated by heme and oxygen at a pretranslational level in a negative fashion.

Heme and oxygen have been shown to exert positive control over the synthesis of many (heme)proteins having respiratory functions or involved in oxygen metabolism in the yeast *Saccharomyces cerevisiae*. This regulation operates at the level of transcription in the cases of iso-1-cytochrome *c* (1–6), catalases A and T (1), Mn superoxide dismutase (7, 8), and probably also for subunits V and VII of cytochrome *c* oxidase (9). An additional translational control by heme was described for the catalases (10). Detailed analysis of the iso-1-cytochrome *c* gene *CYC1* revealed the presence of upstream regulatory sites which mediate that control (2, 3). *Trans*-acting mutations which affect the transcriptional control of *CYC1* by heme or oxygen have also been isolated (5, 8). However, a gene, *ANB1*, adjacent to *CYC1*, has been described whose transcript of unknown function is synthesized only in the absence of oxygen. This gene also responds to the regulatory mutation affecting the control of *CYC1* by oxygen (2, 8).

In the course of our studies on heme biosynthesis in *S. cerevisiae* we reported a severalfold increase in the activity of coproporphyrinogen oxidase in mutants deficient in heme synthesis and in wild type strains grown under anaerobic conditions (11–13). Coproporphyrinogen oxidase (EC 1.3.3.3.) is the sixth enzymic step in the heme biosynthetic pathway, catalyzing the sequential oxidative decarboxylations of the two 2- and 4-carboxyethyl side chains in coproporphyrinogen III to yield the two vinyls in protoporphyrinogen. Similar results have also been found in other laboratories (14, 15). The recent isolation of coproporphyrinogen oxidase in our laboratory and the production of a specific antiserum to the protein has provided us with a new tool with which to investigate its regulation. The levels of immunodetectable protein and functional mRNA were measured in heme-deficient and anaerobic cells. The results presented in this communication indicate that a negative control of coproporphyrinogen oxidase synthesis by heme and oxygen occurs at a pretranslational level, placing this enzyme within the group of proteins whose formation is controlled by heme and oxygen.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—The mutant strains G204 (*α,hem1-5,his4*) and G231 (*α,hem15-5,his4*) were isolated from the wild type strain FL200 (*α,his4*) (12). The mutant strains Sm1 (*α,hem12-2,leu1,arg4*) (13) and H11-3D (*a,leu1, ura2,hem13-2*) (16) were derived from the wild type strain SP4 (*α,leu1,arg4*). Cells were grown in a medium containing 1% yeast extract, 1% bactopeptone, and 2% glucose and supplemented as indicated in the table legend. Special air-tight flasks were used for anaerobic growth (17). Cells were harvested in the early logarithmic growth phase and used immediately.

Cytochrome and Heme Contents—Qualitative spectrophotometric analysis of cytochromes was performed on whole cells at liquid nitrogen temperature (12). Heme content of whole cells was determined as described (12) from the spectrum of the pyridine hemochromes.

Coproporphyrinogen Oxidase—The enzyme, purified from yeast, and antisera to it raised in rabbits were prepared in this laboratory.[1] Coproporphyrinogen oxidase activity was measured by a fluorometric coupled assay (18) on cell-free extracts prepared by breaking the cells with glass beads (20). Activity was expressed as nmol of protoporphyrinogen formed/h/mg of protein of the extract.

Immunodetection of Coproporphyrinogen Oxidase—Published methods were used for preparing extracts from trichloroacetic acid-treated cells (20), and for SDS[2]-polyacrylamide gel electrophoresis (21). Electrophoretic transfer of the proteins to nitrocellulose sheets, incubation with antiserum, visualization by ^{125}I-protein A, and autoradiography were as described by Haid and Suissa (22).

Isolation and Translation of Total RNA—Total RNA was isolated according to Sripati and Warner (23) except that the cell suspension medium was 5% SDS, LiCl was omitted from the medium, and phenol:chloroform:isoamyl alcohol (25:24:1) was present during cell disruption. Total RNA (4 μg) was translated in the presence of [^{35}S] methionine in a nuclease-treated rabbit reticulocyte lysate system (100 μl final volume) according to the recommendations of the manufacturer (Amersham). Immunoprecipitation of labeled coproporphyrinogen oxidase with specific antiserum was carried out as detailed in Ref. 24. Total translation products and immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (21) followed by fluorography (25).

RESULTS AND DISCUSSION

Effect of Heme on the Level of Coproporphyrinogen Oxidase Activity—The level of coproporphyrinogen oxidase activity was much higher in the mutant cells deficient in heme synthesis than in their respective wild type parental strains (Table I). The increase was 6–10-fold for the mutants G204 and G231, which lack 5-aminolevulinic acid synthetase and

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[1] J.-M. Camadro, H. Chambon, J. Jolles, and P. Labbe, manuscript in preparation.

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; l, liter.

TABLE I

Effect of heme deficiency and anaerobiosis on coproporphyrinogen oxidase activity

Strain	Relevant genotype	Growth conditions[a]	Heme content[b]	Coproporphyrinogen oxidase activity[c]
FL200	*HEM*		50–60	1.5–2.5
		Te, N_2	25–35	15–17
		Te, hemin, N_2		15–17
G204	*hem1-5*	Te	0–<1	15–20
		5-Aminolevulinic acid	50–55	6–7
		Hemin		6–8
		Te, N_2	0–<1	15–19
		Te, hemin, N_2		12–14
G231	*hem15-5*	Te	0–<1	16–19
		Hemin		3.5–5
SP4	*HEM*		70–80	0.2–0.4
		Te, N_2	35–45	19–22
		Te, hemin, N_2		19–20
Sm1	*hem12-2*	Te	3–5	18–19
		Te[d]	70–75	0.3–0.5
		Hemin		5–6
		Te, N_2	4–5	18–20

[a] Cells were grown in 1% yeast extract, 1% peptone, and 2% glucose medium supplemented as indicated with Te (0.2% Tween 80 + 30 mg/l ergosterol), 5-aminolevulinic acid (30 mg/l), or hemin (15 mg/l). N_2 represents growth under anaerobiosis.

[b] Determined in whole cells by its pyridine hemochrome (12) and expressed in nmol/g dry weight. The smallest amount of heme detectable was 1 nmol/g dry weight. This method could not be used for the cells grown in the presence of exogenous hemin due to the large amount of hemin remaining bound to the cells despite extensive washing.

[c] The data represent the range of values obtained in 2 or more independent experiments. The activity was measured with an error of 15% and expressed in nmol of protoporphyrinogen/h/mg of protein (18).

[d] 2% ethanol was used in place of glucose.

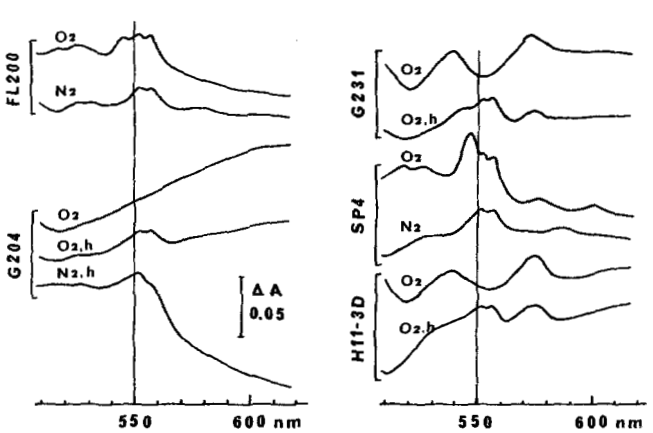

FIG. 1. **Low temperature spectrophotometric analysis of cytochromes in wild type yeast strains grown anaerobically and in heme-deficient mutant strains grown with exogenous hemin.** The cells were grown under conditions described in Table I and its legend. Cells were harvested in the early logarithmic growth phase and their absorption spectra were recorded at liquid nitrogen temperature (12). Reduction was achieved by endogenous substrates. Cells grown in the presence of hemin (*h*) were washed extensively with 1% Tween 80. Despite this washing, a large amount of hemin remained bound to the cells, making the recording of some spectra difficult, if not impossible.

ferrochelatase activity, respectively (the first and last enzymes of the heme biosynthesis pathway). Since G231 cells accumulate protoporphyrin (40–50 nmol/g dry weight; Fig. 1), it is likely that heme itself, and not its precursor protoporphyrin, is involved in the effect observed. A similar high level of coproporphyrinogen oxidase activity was also measured in the mutant Sm1, which carries the mutation *hem12-2*. This mutation leads to a modified uroporphyrinogen decarboxylase activity and to residual heme synthesis in glucose grown cells. Because of the low level of the activity in its parental strain SP4, this represents a 50–100-fold increase in the mutant Sm1. The reason for the 10-fold difference in the activities of the two wild type strains FL200 and SP4 is unclear at present. Glucose catabolite repression, which affects the respiratory functions to different extents in the two strains, does not appear to modulate the level of coproporphyrinogen oxidase. The activity was practically unchanged whether the cells are grown on 2% glucose, 10% glucose, or 2% ethanol for the SP4 strain, whereas, for the FL200 strain, the growth under glucose derepressing conditions led to a 2-fold decrease of the activity.

A more or less complete return to the wild type situation was observed when the heme-deficient mutants were grown under conditions of heme sufficiency (Table I). This reversion was only partial in G204, G231, and Sm1 grown in the presence of exogenous hemin, probably due to uptake problems. However, their low temperature spectra, which show a partial recovery of their cytochromes (Fig. 1), indicate that hemin did enter the cells. Surprisingly, as previously observed with other *hem1* mutants (11), 5-aminolevulinic acid supplementation of G204 did not cause recovery of the basal level of activity, although heme synthesis was apparently fully restored. On the other hand, total reversion to wild type occurred in Sm1 cells grown respiratively on ethanol, although heme synthesis was still 3 times lower than in ethanol-grown SP4 cells.

Steady-state Levels of Coproporphyrinogen Oxidase Protein—Whole cell extracts were probed by immunoblotting using an antiserum raised against yeast coproporphyrinogen oxidase. The results, illustrated in Fig. 2, were quantified by scanning densitometry of the autoradiograms. Heme deficiency increased the relative concentration of coproporphyrinogen oxidase protein by a factor 4–6 and 20–30 in the FL200 and SP4 genetic backgrounds, respectively. This is about half the increase found in enzymic activity. This discrepancy was not explored further, owing to the many pitfalls of quantitative immune blotting (22) and to the fact that the lipids in the assay medium can cause a 2-fold increase in enzymic activity under certain circumstances (18).[1] The presence of exogenous hemin in the cultures of the mutants reduced the amount of immunodetectable protein by 60–70%, whereas a full recovery of the wild type level was seen in the mutant Sm1 grown on ethanol. All these results are consistent with the changes in enzyme activity, indicating that coproporphyrinogen oxidase activity is primarily determined by the steady-state concentration of the protein. Interestingly, the amount of inactive protein made by the mutant H11-3D lacking coproporphyrinogen oxidase activity was also increased 30–40-fold and the addition of hemin to the medium restored the level to that of the parental strain (Fig. 2*b*, *lanes 9* and *10*).

Steady-state Levels of Coproporphyrinogen Oxidase mRNA—Coproporphyrinogen oxidase mRNA activity was assayed by cell-free translation of total RNA and immunoprecipitation with anti-coproporphyrinogen oxidase serum (Fig. 3). There were no significant quantitative or qualitative differences in total translation products with RNA isolated from

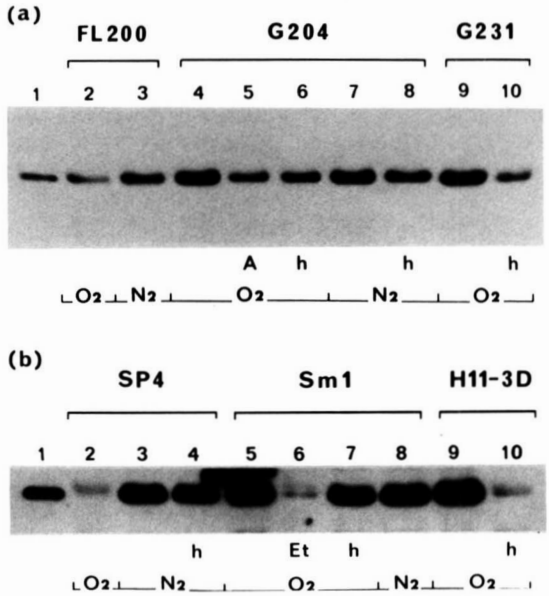

FIG. 2. **Immunoblotting analysis of coproporphyrinogen oxidase in wild type and heme-deficient mutant yeast strains grown under different conditions.** Total cell proteins (approximately 50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. The replica were reacted first with coproporphyrinogen oxidase antiserum and subsequently with ^{125}I-protein A. The strains and growth conditions are described in Table I. Cells were grown aerobically (O_2) or anaerobically (N_2) in medium supplemented where indicated with 5-aminolevulinic acid (*A*) or hemin (*h*). *b*, *lane 6*, Sm1 grown on 2% ethanol (*Et*) instead of glucose. *a* and *b*, *lane 1*, pure coproporphyrinogen oxidase (0.1 μg). The fluorogram (*b*) was overexposed to reveal the coproporphyrinogen oxidase bands in *lanes 2*, *6*, and 10.

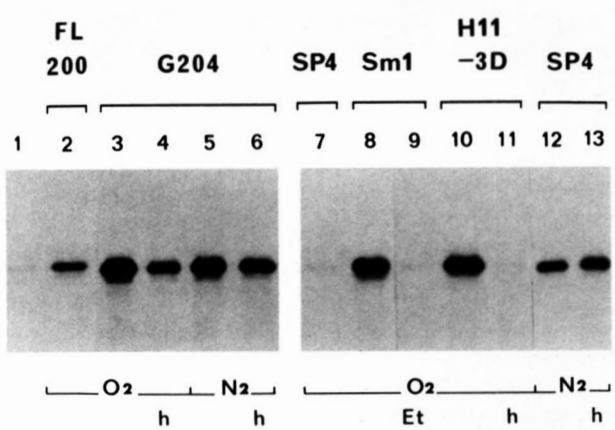

FIG. 3. **Analysis of coproporphyrinogen oxidase active mRNA in wild type and heme-deficient strains grown under varying conditions.** Total RNA (4 μg, except in *lanes 12* and *13* (2 μg)) was translated in a rabbit reticulocyte lysate system (100 μl). The [^{35}S]methionine-labeled coproporphyrinogen oxidase synthesized was immunoprecipitated from 95 μl of the translation mixture with specific antiserum. Immunoprecipitates were electrophoresed on a 10% SDS-polyacrylamide gel and fluorographed. The strains and growth conditions used are described in Table I. O_2, N_2, *h*, and *Et* as in Fig. 2. *Lane 1*, coproporphyrinogen oxidase labeled *in vivo*.

different strains and under different growth conditions (not shown). Quantification by densitometric scanning of the fluorograms showed that the relative amount of active coproporphyrinogen oxidase mRNA was greatly elevated in heme-deficient cells: 4–6-fold and 20–40-fold in the FL200 and SP4 genetic backgrounds, respectively. The growth of the mutants under heme sufficiency led to a decrease in active coproporphyrinogen oxidase mRNA which paralleled that observed with the enzymic activity and protein. These results clearly show that the increases in steady-state levels of protein and active mRNA were similar, indicating that the concentration of its mRNA is likely to be the only step determining the level of coproporphyrinogen oxidase. However, the direct demonstration that heme regulation operates at the level of the rate of mRNA synthesis must await the isolation of the *HEM13* gene encoding coproporphyrinogen oxidase.

Effect of Anaerobiosis on Coproporphyrinogen Oxidase Synthesis—As shown in Table I, Fig. 2 (*a*, *lane 3*; *b*, *lane 3*) and Fig. 3 (*lane 12*), the growth of the wild type strains under anaerobic conditions caused a concomitant increase in the steady-state levels of coproporphyrinogen oxidase activity, protein, and translatable mRNA comparable to that observed in the heme-deficient cells. No further increase in coproporphyrinogen oxidase occurred when the heme-deficient mutants were grown anaerobically (Fig. 2*a*, *lane 7*; Fig. 2*b*, *lane 8*; and Fig. 3, *lane 5*). These results, *in toto*, indicate that the effects of heme and oxygen deficiency are not additive. The question remains as to whether the oxygen and heme act independently, as discussed by Hortner *et al.* (1) and Laz *et al.* (6) or whether the regulation by oxygen may be explained by the lack of heme in anaerobically grown cells (3). The addition of exogenous hemin did not reverse the effect of anaerobiosis in wild type cells and caused only a modest reduction in coproporphyrinogen oxidase in G204 cells. However, these experiments can hardly be considered to be conclusive, since cells grown in the presence of Tween 80, which is required for anaerobic growth, take up hemin very poorly, if at all. But, we have also shown that the product of the anaerobic gene *ANB1* (2, 8) is not involved in the regulation of coproporphyrinogen oxidase. A strain carrying the deletion *cyc1-1*, which eliminates part of *ANB1*, shows a 35-fold increase in coproporphyrinogen oxidase when grown anaerobically (data not shwon).

In fact, a limited amount of heme is synthesized *in vivo* by anaerobically grown cells (this work) (19, 26), although both yeast coproporphyrinogen oxidase and protoporphyrinogen oxidase, the next enzyme in the pathway, require oxygen for their activity *in vitro* (18).[1] There is no satisfactory answer to these contradictory findings at the present time, except to state that it is virtually impossible to avoid all traces of oxygen in the anaerobic system despite the care taken and that these traces are sufficient for the functioning of the two oxidases, which have an exceptionally high affinity for oxygen.[3] This "anaerobic" heme has been found associated with the microsomal cytochromes b_5 (see Fig. 1) and P-450 and with a soluble pigment absorbing at 420–422 nm in the presence of carbon monoxide (27–29). But, if heme is present in anaerobic cells, then coproporphyrinogen oxidase should be repressed. Nothing is known at present about the fate of newly synthesized heme and the presence of a small cytosolic pool of "regulatory" heme in yeast, although they have been documented in hepatocytes (30, 31). It is possible that heme is sequestered or compartmentalized in anaerobic yeast cells in such a way that it is not accessible for metabolic or regulatory functions. Such a compartmentalization would explain why the apoproteins and not the hemoproteins are present in anaerobic cells, as is the case for cytochrome *c* peroxidase (32) and cytochrome c_1 (33). This explanation implies that the oxygen regulation of coproporphyrinogen oxidase synthesis is mediated by heme. Further work combining physiological and genetic approaches and using the cloned *HEM13* gene is obviously needed to answer these questions and to reveal the physiological signif-

[3] P. Labbe, unpublished results.

icance of the regulation of coproporphyrinogen oxidase by heme and oxygen.

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