Effect of Mitochondrial Cytochromes and Haem Content on Cytochrome P450 in *Saccharomyces cerevisiae*

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It is well established that the mitochondrial and the microsomal cytochromes in *Saccharomyces cerevisiae* are regulated differently. Mutations affecting the mitochondrial cytochromes *aa₃* or *c* had no effect on the concentration of the microsomal cytochrome P450 even during haem limitation. Moreover, a defect in the cytochrome P450 gene did not affect mitochondrial cytochromes. However, a regulatory mutation present in strain SG1 decreased both mitochondrial and microsomal cytochrome contents. This mutation also affected the intracellular haem concentration. The haem precursor 5-aminolaevulinate increased both mitochondrial and microsomal cytochrome contents. Our results indicate that carbon source and haem concentration are involved in the regulation of cytochrome P450.

INTRODUCTION

In *Saccharomyces cerevisiae* haemoproteins can be divided into two classes by their response to oxygen and glucose. One class, including the mitochondrial respiratory cytochromes, cytochrome oxidase and catalase(s), consists of proteins which attain their full activity only in the absence of glucose and with optimal aeration (Sherman & Steward, 1971; Zitomer *et al.*, 1979; Hoertner *et al.*, 1982). The other class consists of cytochromes which are detectable in high amounts only under semi-anaerobic conditions and in the presence of high glucose concentrations. Cytochrome P450 belongs to this second group (Ishidate *et al.*, 1969*a, b*; Yoshida *et al.*, 1974). Therefore, it has been suggested that repression of mitochondrial cytochromes, especially cytochrome *aa₃*, is required for cytochrome P450 synthesis (Wiseman *et al.*, 1975). This would imply that cytochrome P450 and mitochondrial cytochromes either exert a negative control on each other or are inversely regulated by a common regulator. Haem could function as such a regulator since its availability plays an important regulatory role in the expression and assembly of haemoproteins in yeast (Woods *et al.*, 1975; Haslam & Astin, 1979; Schatz & Butlow, 1983; Guarente & Mason, 1983). However, not all haemoproteins are under this control (Matner & Sherman, 1982) and a regulatory role of haem has so far only been demonstrated for proteins of the first class (oxygen requiring, glucose sensitive). We therefore studied the influence of mitochondrial cytochromes and the haem precursor 5-aminolaevulinate on the microsomal cytochrome P450.

METHODS

Yeast strains. Wild-type strain S288C (*MAT α*) was from Gerald R. Fink (Massachusetts Institute of Technology, Boston, USA). M13-249 (*MAT α oxil*) and PTE12 (*MAT α his3 leu2 petE11*) were from Thomas Fox (Section of Genetics & Development, Cornell University, USA). FY 132 (*MAT α hem1*) originated from a cross of DCZH1-1B (*MAT α leu1 hem1*), provided by Helmut Ruis (Institut für allgemeine Biochemie, University of Vienna, Austria), with the wild-type. SG1 (*MAT α erg11 his1*) was a gift from David B. Sprinson (Rockefeller University, New York, USA). FY 74 (*MAT α his4 trp1 ura3*), FY 155 (*MAT α hem1 cycl leu2 lys2*) and FY 156 (*MAT α hem1 leu2 lys2*) were from this laboratory. Standard genetic techniques were used as described by Sherman *et al.* (1974).

Yeast growth and fractionation. For the preparation of subcellular fractions yeast was grown overnight in 500 ml YEPD (Sherman *et al.*, 1974) which was supplemented for *hem1* mutants with 5-aminolaevulinate (50 mg l⁻¹). Cells were harvested and resuspended in fresh media as indicated in the text. After 4 h or the times indicated in the text, cells were collected by centrifugation and subcellular fractions were prepared according to Kaeppli *et al.* (1982).

Assays. Cytochromes *a*, *b* and *c* were determined in mitochondrial fractions as described by Sanglard *et al.* (1984) for whole cells. Cytochrome P450, cytochrome *b*₅ and cytochrome *c* (P450) reductase activity was measured in microsomal preparations according to Sanglard *et al.* (1984). Protein was assayed with a commercial assay kit (Bio-Rad). Haem was determined in yeast homogenates according to Fuhrhop & Smith (1975).

Experiments were done at least twice. Since absolute data varied considerably between some experiments while relative data (the concentrations of cytochromes proportional to each other) were reproducible, representative data are given.

RESULTS

In order to study whether a direct, mutual influence exists between mitochondrial and microsomal cytochromes we compared the cytochrome P450 concentrations in mutants lacking one of the mitochondrial cytochromes with those from a wild-type strain grown under identical conditions (3% glucose or 2% galactose as carbon source). Mutants lacking cytochrome *aa*₃ due to either a nuclear mutation (*petE11*) or a mitochondrial mutation (*oxi1*) exhibited similar levels of cytochrome P450 to the wild-type strain S288C under conditions of catabolite repression (glucose) or derepression (galactose) (Table 1). The cytochrome P450 concentration was lower on galactose than on glucose, indicating that the carbon source was the predominant factor in the regulation of cytochrome P450 levels. Similar results were obtained with double mutants carrying defects in the aminolaevulinate synthase (*hem1*) and in the cytochrome *c* (*cyc1*) genes (Table 2). Since the *hem1* mutation blocks haem biosynthesis, haem levels in *hem1* mutants can

Table 1. *Cytochrome P450 levels in the microsomal fraction of cytochrome aa₃ mitochondrial and nuclear mutants and wild-type S. cerevisiae under glucose repressed and derepressed conditions*

Cells from a 500 ml culture were resuspended in YEP medium containing 3% glucose or 2% galactose and maintained for 4 h with agitation before fractionation.

Strain	Substrate	Cytochrome P450 (pmol mg ⁻¹)*
PTE12 (<i>petE11</i>)	Glucose	72.0
	Galactose	36.5
M13-249 (<i>oxi1</i>)	Glucose	72.0
	Galactose	40.0
S288C	Glucose	78.0
	Galactose	37.0

* Per mg microsomal or mitochondrial protein respectively.

Table 2. *Effect of 5-aminolaevulinate on levels of mitochondrial and microsomal cytochromes in strains FY 156 (hem1) and FY 155 (cyc1 hem1)*

Cells were incubated with shaking in YEPD medium supplemented with 5-aminolaevulinate for 4 h before fractionation. ND, Not detectable.

Strain	5-Aminolaevulinate (mg l ⁻¹)	Cytochrome content [pmol (mg subcellular protein) ⁻¹]			
		<i>a</i>	<i>b</i>	<i>c</i>	P450
FY 155	13	123	290	ND	38.0
FY 155	80	150	430	ND	93.0
FY 156	13	140	320	490	58.0
FY 156	80	180	440	690	110.0

Table 3. Segregation of nystatin resistance and haem and cytochrome content in parents and a tetrad from cross SG1 (*Nys^r his1*) × FY 74 (*his4 trp5 ura3*)

Cells were incubated with shaking in YEPD for 4 h before fractionation.

Spore	Nys ^r	Haem [pmol (g wet wt cells) ⁻¹]	Cytochrome content [pmol (mg subcellular protein) ⁻¹]			
			<i>a</i>	<i>b</i>	<i>c</i>	P450
A	+	0.33	ND	56	71	ND
B	+	5.80	117	187	228	161
C	—	0.53	ND	20	44	20
D	—	3.50	98	390	475	143
Parents						
FY 74	—		65	173	230	52
SG1 (— Als)*	+		21	28	50	18
SG1 (+100 µg Als ml ⁻¹)*	+		21	23	50	17

ND, Not detectable.

* Als, 5-aminolaevulinate.

be adjusted by the amount of 5-aminolaevulinate supplied in the medium. The absence of cytochrome *c* does not influence the levels of other cytochromes, including cytochrome P450, under aminolaevulinate limiting (13 mg l⁻¹) and non-limiting conditions (83 mg l⁻¹). Thus, even under haem limitation the absence of one major mitochondrial cytochrome does not elevate the cytochrome P450 concentration.

However, we obtained evidence for a regulatory effect of haem on both mitochondrial and microsomal cytochromes in yeast from genetic studies of mutant SG1. This strain carries the *erg11* mutation (Gollub *et al.* 1974, 1977) which affects demethylation of lanosterol (Trocha *et al.* 1977) due to low levels of an altered cytochrome P450 which does not bind the cytochrome P450 inhibitor buthioate [3-pyridinylcarbonimidodithioic acid butyl(4-(1,1-dimethylethyl)phenyl)-methyl ester; Aoyama *et al.*, 1983]. Due to the absence of ergosterol SG1 is resistant to nystatin but does not require sterol supplementation since it can accumulate other sterols. We further observed that SG1 contained low levels not only of cytochrome P450 but also of cytochromes *aa*₃, *b* and *c* (Table 3). Addition of aminolaevulinate, which stimulates mitochondrial cytochrome synthesis in yeast (Sanders *et al.*, 1973; Haslam & Astin, 1979), did not enhance cytochrome levels in SG1 (Table 3). Tetrad analysis was used to determine whether it is the lesion in the cytochrome P450 gene which affects the other cytochromes. A 2:2 segregation of nystatin resistance was observed in eight tetrads from a cross of SG1 × wild-type. A 2:2 segregation was also observed for the outside markers *trp5* and *ura3*. The mutation causing low cytochrome levels also segregated 2:2 but it was not linked to the mutation conferring nystatin resistance. Moreover, spores with low cytochrome levels also contained decreased haem concentrations (Table 3). It might be assumed that low cytochrome levels are a consequence of the reduced haem concentration. Since the spores with low haem (and cytochrome) levels grew like the wild-type on YEPD and minimal media, and even grew on glycerol (YPG), the low haem and cytochrome concentrations seem not to impair any vital function. The different segregation also shows that the failure of the C-32 demethylation and the resulting nystatin resistance cannot be simply a consequence of the low cytochrome P450 levels. Moreover, the defect in the cytochrome P450 has no impact on the mitochondrial cytochromes.

In order to investigate the impact of haem on cytochrome P450 and mitochondrial cytochromes in greater detail, we cultivated *hem1* mutant FY 132 at different concentrations of 5-aminolaevulinate in YEPD medium. The results (Table 4) showed that cytochrome P450 levels, like those of the mitochondrial cytochromes, increased with increasing extracellular concentrations of 5-aminolaevulinate. Apparently haem or one of its precursors enhances cytochrome P450 levels as well as those of mitochondrial cytochromes. When ethanol was used

Fig. 1. Time course of the induction of mitochondrial and microsomal cytochromes in the absence (□, ○, △) or presence (■, ●, ▲) of cycloheximide. (a) Mitochondrial cytochromes: △, ▲, cytochrome *aa*₃; ○, ●, cytochrome *b*₅; □, ■, cytochrome *c*. (b) Microsomal proteins: ○, ●, cytochrome *b*₅; △, ▲, cytochrome P450; □, ■, cytochrome *c* (P450) reductase.

as carbon source, a further increase in mitochondrial cytochromes was observed in the presence of 5-aminolaevulinate, but no cytochrome P450 could be detected. Since we separated mitochondria from microsomes, this could not have been due to interference of cytochrome *aa*₃ with the spectral determination of cytochrome P450.

In order to determine whether the increase in cytochrome P450 after treatment with 5-aminolaevulinate was due to new protein synthesis, we studied the time course of induction of microsomal and mitochondrial cytochromes in the presence or absence of cycloheximide. Yeast strain FY 132, grown on YEPD medium containing ergosterol, oleic acid and 5-aminolaevulinate (10 µg ml⁻¹), was transferred to YEPD medium containing 250 µg 5-aminolaevulinate ml⁻¹ with or without cycloheximide (15 µg ml⁻¹) and incubated under vigorous shaking for different times. The rate of overall porphyrin synthesis in homogenates measured according to Labbe-Bois & Volland (1977) was in the same range at the beginning of the experiment (112 pmol h⁻¹ mg⁻¹) as in cells incubated with 5-aminolaevulinate for 4 h without cycloheximide (118 pmol h⁻¹ mg⁻¹), indicating that all enzymes required for haem biosynthesis were already present at the beginning of the experiment. As shown in Fig. 1, the levels of the mitochondrial cytochromes doubled within 2 h and increased severalfold within 4 h of transfer to the 5-aminolaevulinate containing medium in the absence but not in the presence of cycloheximide. In the latter case the (low) levels present before the transfer remained constant or increased only slightly. The microsomal cytochrome *b*₅ was not detected in the cells before the transfer and remained undetectable in cells treated with cycloheximide. In the absence of cycloheximide this cytochrome was induced in a similar manner to mitochondrial cytochromes. Cytochrome P450 levels increased significantly in the culture without cycloheximide, although lagging behind the other cytochromes. In the presence of cycloheximide cytochrome P450 became undetectable within 2 h. It seems that a cessation of cell growth leads to a quick decline

Table 4. *Effect of 5-aminolaevulinate on mitochondrial and microsomal cytochrome contents*

Activities were measured in microsomal or mitochondrial fractions from strain FY 132 (*hem1*) incubated on YEP medium with either 3% glucose or 3% ethanol containing ergosterol, oleic acid and the concentration of 5-aminolaevulinate indicated. ND, Not detectable.

Carbon source	5-Aminolaevulinate concn (mg l ⁻¹)	Cytochrome content [pmol (mg subcellular protein) ⁻¹]			
		<i>a</i>	<i>b</i>	<i>c</i>	P450
Glucose	0	15	< 35	< 35	< 26
	40	54	76	62	130
	250	68	140	190	167
Ethanol	40	100	280	450	ND
	300	130	280	520	ND

in cytochrome P450 levels, since a rapid loss of cytochrome P450 was also observed when protein synthesis was blocked in a leucine auxotroph by leucine starvation (not shown). These results are not in agreement with the assumption of a pre-existing cytochrome P450 apoprotein which takes up haem as soon as it is supplied.

The activity of another enzyme of the microsomal monooxygenase system, cytochrome *c* (P450) reductase, was not affected by 5-aminolaevulinate. The apparent increase in the reductase activity in cycloheximide treated cells might be due to the significant decrease in the total microsomal protein at constant levels of the reductase rather than to a post-translational activation of the enzyme.

DISCUSSION

Haem is an essential cofactor for the functioning of haemoproteins and is also involved in the regulation of the biosynthesis of several such proteins (Guarente & Mason 1983; Guarente *et al.*, 1984). Since important steps of haem biosynthesis itself are subjected to catabolite repression and require oxygen (Mahler & Lin, 1978), it might be that the low concentrations of several mitochondrial and cytoplasmic haemoproteins observed in the presence of glucose or low oxygen tension are the consequence of the low haem concentration. However, some microsomal cytochromes, including cytochrome P450, are detectable in significant levels only in the presence of high amounts of glucose and at a low oxygen tension. Our results show that this inverse regulation is not due to a direct effect of one group of cytochromes on the other. Accordingly, the absence of either cytochrome *aa*₃ or cytochrome *c* has no direct effect on the other cytochromes, including cytochrome P450. Although the lack of cytochrome *aa*₃ or *c* renders the yeast cell unable to metabolize a carbon source through the tricarboxylic acid cycle and prevents respiration, it did not affect cytochrome P450 levels. This implies that the increase of cytochrome P450 levels after oxygen limitation (Ishidate *et al.*, 1969*b*) is not directly coupled to mitochondrial respiration. The predominant regulator of cytochrome P450 and mitochondrial cytochromes seems to be the carbon source (Tables 1 and 4). Also, the presence of an altered cytochrome P450 in strain SG1 (Gollub *et al.*, 1977; Aoyama *et al.*, 1983) did not affect the concentration or function of the mitochondrial cytochromes (Table 3). Accordingly, all spores from the cross SG1 × wild-type grew on glycerol.

Our results also show that the haem levels themselves may play an important role in the regulation of all cytochromes. The haem precursor 5-aminolaevulinate stimulated synthesis of both microsomal cytochromes P450 and *b*₅ and the mitochondrial cytochromes. This stimulation was directly proportional to the amount of 5-aminolaevulinate provided to *hem1* mutants. Under haem limitation low levels of all cytochromes were observed (Tables 2 and 3). The absence of a major mitochondrial cytochrome, cytochrome *c*, did not enhance the concentrations of the other cytochromes under haem limitation. Therefore the idea of

competition for haem between cytochromes is rather unlikely. Further evidence for the effect of haem on the concentration of cytochromes comes from genetic analysis of strain SG1, which carries the defect in the cytochrome P450 gene and a mutation which causes low concentrations of mitochondrial as well as microsomal cytochromes. This mutation segregates differently from the cytochrome P450 mutation and spores carrying this mutation have low haem levels. We assume for two reasons that this mutation does not affect catabolite repression but is rather a mutation in a gene affecting the regulation of haem concentration. First, a mutation in catabolite repression should affect mitochondrial and microsomal cytochromes inversely (see above). However, the mutation described here results in low concentrations of all cytochromes. Second, spores from an SG1 $\times$ wild-type cross grown on glucose or glycerol respectively showed the same segregation pattern with respect to mitochondrial cytochrome concentrations (high:low, not shown). The idea of a haem-regulatory mutation in SG1 is further supported by the finding that 5-aminolaevulinate, which usually increases the concentrations of mitochondrial and microsomal cytochromes in glucose- and ethanol-grown cells (Table 4) did not increase cytochrome concentrations in SG1.

In the presence of cycloheximide, an inhibitor of protein biosynthesis, no increase in cytochrome P450 content after 5-aminolaevulinate addition was observed. On the contrary cytochrome P450 levels declined rapidly. This indicates that the increase in cytochrome P450 content in the absence of cycloheximide might not be simply due to the insertion of haem into an existing apoprotein. In contrast to cytochrome P450, the levels of other (mitochondrial) cytochromes increased slightly even in the presence of cycloheximide, in agreement with previous studies (Charalampous & Chen, 1974; Schatz & Butlow, 1983). The rapid disappearance of cytochrome P450 in cycloheximide treated cells could also reflect an accelerated degradation of its haem moiety under conditions where protein synthesis is blocked. For mammalian cells it has been demonstrated that the haem moiety may have a turnover rate different from the protein part of cytochrome P450 (Gasser *et al.*, 1982). Interestingly, in the presence of cycloheximide no decrease in the specific activity of NADP-cytochrome *c* (P450) reductase was observed. Besides its interaction with cytochrome P450 this enzyme has also been shown to be involved in haem degradation in higher eukaryotes (Yoshinaga *et al.*, 1982). NADPH-cytochrome *c* (P450) reductase also remained unaffected by 5-aminolaevulinate. This might indicate that the two components of the cytochrome P450 system are not coordinately regulated.

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