Oxygen and haem regulate the synthesis of peroxisomal proteins: catalase A, acyl-CoA oxidase and Pex1p in the yeast *Saccharomyces cerevisiae*; the regulation of these proteins by oxygen is not mediated by haem

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Saccharomyces cerevisiae genes related to respiration are typically controlled by oxygen and haem. Usually the regulation by these factors is co-ordinated; haem is indicated as the oxygen sensor. However, the responsiveness of peroxisome functions to these regulatory factors is poorly understood. The expression of *CTA1*, *POX1* and *PEX1* genes encoding the peroxisomal proteins catalase A, acyl-CoA oxidase and Pex1p peroxin respectively was studied under various conditions: in anaerobiosis, in the absence of haem and in respiratory incompetence caused by the lack of a mitochondrial genome (ρ^0). The influence of haem deficiency or ρ^0 on peroxisomal morphology was also investigated. Respiratory incompetence has no effect on the expression of *CTA1* and *POX1*, whereas in the absence of haem their expression is markedly decreased. The synthesis of Pex1p is decreased in ρ^0 cells and is decreased even more in haem-deficient cells. Nevertheless, peroxisomal morphology in both these types of cell does not differ significantly from the morphology of peroxisomes in wild-type cells. The down-regulating effect of anoxia on the expression of *CTA1* and *POX1* is even stronger than the effect of haem deficiency and is not reversed by the addition of exogenous haem or the presence of endogenous haem. Moreover, neither of these genes responds to the known haem-controlled transcriptional factor Hap1p. In contrast with the other two genes studied, *PEX1* is up-regulated in anaerobiosis. The existence of one or more novel mechanisms of regulation of peroxisomal genes by haem and oxygen, different from those already known in *S. cerevisiae*, is postulated.

Key words: anaerobiosis, derepression, haemin, oleate induction, respiratory incompetence.

INTRODUCTION

The yeast *Saccharomyces cerevisiae*, as a facultative anaerobe, is an attractive model organism for studying the regulation of various cellular functions by oxygen availability. On being shifted from an aerobic to an oxygen-depleted environment (and vice versa) many processes within the yeast cell have to adapt to the new conditions. During the transition between fermentative and respiratory modes of energy generation, many genes are switched on, including genes encoding subunits of cytochrome *c* oxidase and cytochrome *c* reductase, whereas many others are switched off. Some oxidases, for instance coproporphyrinogen III oxidase, are induced by hypoxia to compensate for the limitation in electron acceptor supply. Several protein pairs have been identified such that both proteins within a pair have analogous functions but are expressed under either normoxic or hypoxic conditions. Examples are *COX5a* and *COX5b*, encoding variants of subunit V of cytochrome *c* oxidase, and *CYC1* and *CYC7*, encoding iso-1-cytochrome *c* and iso-2-cytochrome *c* respectively. The presence of oxygen induces the synthesis of proteins involved in the protection against oxidative stress, such as catalases and superoxide dismutases. Some results indicate that hypoxic stress might also cause the induction of genes belonging to the global stress response family. There have been several excellent reviews covering these and all other aspects of regulation by oxygen of *S. cerevisiae* cell functions and giving an extensive list of oxygen-regulated genes [1–4]. The system of regulation of cellular processes by oxygen is far from fully understood. Unquestionably, more than one mechanism will be found. Almost all groups of genes mentioned above are regulated by haem in a manner analogous to their regulation by oxygen [1,4]. The intracellular level of haem, whose synthesis requires oxygen [5], seems to be a good candidate for gauging the presence or absence of oxygen. Indeed for many oxygen-responsive genes the addition of exogenous haem to an anaerobic culture reverses the effects of anoxia. Moreover, most of them are sensitive to the transcriptional activator Hap1p [6] and/or Rox1p [7], which itself is induced by Hap1p. So far this has been much the best characterized mechanism of regulation by oxygen. Haem is known for its ability to bind oxygen reversibly in the active centre of haemoglobin or myoglobin. Therefore it could equally well function as a sensor of oxygen concentration, especially in the range that does not affect the rate of haem synthesis. That seems to be true of two hypoxic genes, *CYC7* and *OLE1* [8]. Nevertheless, the induction of global stress response genes by hypoxia is haem-independent [2], hence, another non-haem oxygen sensor must exist in the *S. cerevisiae* cell.

The peroxisomal compartment is yet another candidate target for oxygen regulation. This is the place where oxygen-requiring reactions take place [9]. In addition, metabolic pathways within this compartment, especially β-oxidation, are functionally linked to the respiratory chain. Nevertheless the results on the sensitivity to oxygen of genes encoding peroxisomal proteins, especially oxidases, as well as peroxisome proliferation are scarce. We have shown previously that under anaerobic conditions peroxisomal catalase A and acyl-CoA oxidase are undetectable [10]. The present paper describes our studies on the effect of anaerobiosis, a lack of haem or respiratory incompetence caused by the inactivation of the mitochondrial genome (ρ^0), on the expression of *CTA1*, *POX1* and *PEX1* genes encoding the peroxisomal

Abbreviations used: CPRG, Chlorophenol Red β-D-galactopyranoside; GFP, green fluorescent protein.

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proteins catalase A, acyl-CoA oxidase and Pex1p peroxin, which are important in the following ways. (1) Acyl-CoA oxidase is the key enzyme of peroxisomal β-oxidation conducting the first step of this pathway, namely the oxidation of acyl-CoA in the 2,3 position of the fatty acid moiety. (2) Catalase A is the peroxisomal enzyme co-operating with β-oxidation by removing H_2O_2, the by-product of the reaction performed by acyl-CoA oxidase, and thus protecting the cell against potential damage caused by this compound. (3) Pex1p is a protein involved in peroxisome biogenesis [9,11]. The effect of a lack of haem or of respiratory incompetence on peroxisome proliferation in a *S. cerevisiae* cell was also studied. The presented data clearly show that the absence of haem or oxygen, in contrast with respiratory incompetence, strongly affects the synthesis of the peroxisomal proteins studied. We also addressed the question of the interdependence of these two regulatory factors and provide some evidence that the regulation of *CTA1*, *POX1* and *PEX1* by oxygen is not mediated by haem. This indicates that the expression of peroxisomal genes might be regulated by haem and oxygen by a novel mechanism, different from those known so far. Finally, we show that under oleate-induction conditions in the absence of haem, as well as in the absence of mitochondrial DNA, the peroxisomes proliferate normally.

MATERIALS AND METHODS

Yeast strains and plasmids

The *S. cerevisiae* strains used in this study are listed in Table 1. Cells were made respiratorily deficient (ρ^0) through treatment with ethidium bromide [12]. To obtain a *hem1*Δ strain, the internal 1 kbp fragment of the genomic copy of the *HEM1* gene was removed with a single-step disruption technique [13] by using the *hem1*Δ*::LEU2* gene-disruption construct provided by R. Labbe-Bois (Institut Jacques Monod, Université Paris, Paris, France). A *CTA1–lacZ* fusion cassette containing the promoter region of the *CTA1* gene (-1550 to $+18$ bp relative to ATG) fused in frame to the β-galactosidase coding sequence was constructed and integrated into the *CTT1* locus, as described [14]. A *PEX1–lacZ* fusion containing the *PEX1* gene promoter (-1702 to $+110$ bp relative to ATG) fused in frame to the β-galactosidase coding sequence [15] was integrated into the *URA3* locus. A multicopy plasmid YEp-AOx-GFP expressing the peroxisomal marker acyl-CoA oxidase–green fluorescent protein (improved version, with an S65T mutation [16]) was constructed by cloning the *POX1* gene (complete with its promoter) fused in frame with a DNA fragment containing the gene for green fluorescent protein into the multicopy YEp352 shuttle vector. Plasmid MSEp1 overexpressing catalase A was made by cloning the *CTA1* open reading frame and 819 bp of its upstream promoter region into the multicopy YEp352 shuttle vector. Yeast transformations were performed with the improved lithium acetate procedure [17].

Table 1 ***S. cerevisiae*** **strains used in the study**

Strain	Genotype	Source
WS17-5D	*MAT*α *leu2-3,112 trp1 ura3-52 arg1*	[33]
WS17-5Dρ^0	*MAT*α *leu2-3,112 trp1 ura3-52 arg1* ρ^0	This study
WS17-5D *hem1*Δ	*MAT*α *leu2-3,112 trp1 ura3-52 arg1 hem1*Δ*::LEU2*	This study
MSCTA1ρ^{+*}	*ctt1*Δ*::CTA1-lacZ*(*URA3*) ρ^+	This study
MSCTA1ρ^{0*}	*ctt1*Δ*::CTA1-lacZ*(*URA3*) ρ^0	This study
MSCTA2ρ^{0*}	*ctt1*Δ*::CTA1-lacZ*(*URA3*) *hem1*Δ*::LEU2* ρ^0	This study
MSPEX1ρ^{+*}	*URA3::PEX1-lacZ* ρ^+	This study
MSPEX1ρ^{0*}	*URA3::PEX1-lacZ* ρ^0	This study
MSPEX2ρ^{0*}	*URA3::PEX1-lacZ hem1*Δ*::LEU2* ρ^0	This study

* All these strains are WS17-5D derivatives and have the same basic genotype: *MAT*α *leu2-3,112 trp1-1 ura3-52 arg1.*

Growth conditions

Yeast cells were cultured either in rich YP medium [1% (w/v) yeast extract/1% (w/v) bactopeptone; Difco] or, whenever the maintenance of plasmids introduced into yeast cells was required, in synthetic complete SC medium (0.67% yeast nitrogen base, supplemented with amino acids and adenine but lacking uracil). As a carbon source the media contained 2% (w/v) dextrose (YPD and SCD, preculture media), 0.1% glucose and 1% (w/v) raffinose (YPR and SCR; derepression media) or 0.1% glucose, 1% (w/v) raffinose, 0.25% (v/v) oleic acid and 0.5% (v/v) Tween 80 (YPRO; oleate induction medium). The media for growing haem-deficient strains and for anaerobic cultures were supplemented with 0.5% (v/v) Tween 80, 0.0025% ergosterol and 0.002% methionine. To anaerobic cultures the redox indicators 0.0005% Methylene Blue and 0.0005% Resazurin were also added. Where indicated, 0.0015% haemin was added to anaerobic cultures [100 μl of 1.5% (w/v) haemin in 0.1 M NaOH per 100 ml of culture]. Cells were inoculated to a density of 10^5 cells/ml and harvested at a density of approx. 2×10^7/ml unless otherwise mentioned. Before harvesting, anaerobic cultures were cooled to 0 °C in an ice bath and opened; cycloheximide was then added to a final concentration of 0.005%. The cells were harvested by centrifugation at 4 °C.

Growth of yeast cells under anaerobic conditions

Cells were cultured in glass vessels with rubber enclosures. After inoculation the vessels were closed and the air from inside was evacuated with the aid of a sterile needle that punctured the enclosure and was connected to a vacuum pump. After 15 min of evacuation, 1 ml of freshly made 1% (w/v) dithionite solution per 100 ml of medium was injected through the enclosure into the medium to remove the traces of oxygen. Both needles were taken out and the punctured enclosure was covered with paraffin wax to assure air tightness. The content of the flask was shaken thoroughly to facilitate the reaction of dithionite with the remaining oxygen.

Induction with oleate under anaerobic conditions

A culture pregrown anaerobically (as described above) for 24 h was injected through the rubber enclosure with an additional 1 ml of 1% (w/v) dithionite solution and 2.5 ml of 10% (v/v) oleic acid/20% (v/v) Tween 80 solution in sterile distilled water, per 100 ml of medium [to bring the final concentration of oleic acid and Tween 80 to 0.25% (v/v) and 0.5% (v/v) respectively]. Growth was continued for a further 24 h.

Time course of induction with oleate in anaerobiosis

Yeast cells were precultured in the presence of oxygen in YPR medium for 24 h to a density of 2×10^7 cells/ml; 2.5 ml of 10% (v/v) oleic acid/20% (v/v) Tween 80 solution per 100 ml of medium was then added to this culture and oxygen was removed from it as described above. Separate cultures were prepared for each time point. Three other series of cultures were made at the same time: growth under derepressing conditions in anaerobiosis, prepared as above but without the addition of oleic acid/Tween 80 solution, and growth under inducing or derepressing con-

ditions in the presence of oxygen, with and without the addition of oleic acid/Tween 80 solution.

Enzyme assays

Yeast cells were disrupted by shaking the cell suspension in 0.05 M potassium phosphate buffer, pH 7.0, with 0.5 mm glass beads in a Braun homogenizer. The suspension was then centrifuged for 5 min at 15000 ***g*** and 4 °C; the supernatant was taken for the assays.

Acyl-CoA oxidase activity was measured spectrophotometrically at 500 nm by using an indirect assay of palmitoyl-CoA-dependent H_2O_2 formation with 4-aminoantipyrin, phenol and horseradish peroxidase [10,18]. The activity is expressed in nmol of H_2O_2 generated/min per mg of protein.

Catalase activity was assayed by measuring the decomposition of H_2O_2 at 240 nm. The activity is expressed in μmol of H_2O_2 decomposed/min per mg of protein [19].

β-Galactosidase activity was assayed by using *o*-nitrophenyl-β-D-galactopyranoside as the substrate [20]. The activity was expressed in nmol of *o*-nitrophenol/min per mg of protein. The molar absorption coefficient of *o*-nitrophenol was taken as ϵ_{420} 4500 $M^{-1} \cdot cm^{-1}$. To measure low levels of β-galactosidase activity a more sensitive spectrophotometric assay with Chlorophenol Red β-D-galactopyranoside (CPRG; Boehringer) as a substrate was developed. The reaction mixture (final volume 130 μl) contained 2.5 mM CPRG and 1–10 μl of cell extract in Z-buffer [60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$ (pH 7.0)] [20]. The formation of Chlorophenol Red was followed spectrophotometrically at 575 nm at 30 °C for 1–10 min. The activity is expressed in pmol or nmol of Chlorophenol Red/min per mg of protein. The molar absorption coefficient of Chlorophenol Red at 575 nm was taken as ϵ_{575} 75000 $M^{-1} \cdot cm^{-1}$. Protein was measured by the method of Lowry et al. [21].

Microscopy

A Nikon Microphot-SA microscope equipped with filters for Nomarski optics and an FITC filter for epifluorescence was used. Cells were viewed at $\times$600 magnification. Images were digitized with a video camera attached to the microscope; they were then finished with Adobe Photoshop, version 4.0.

RESULTS

S. cerevisiae cells incapable of respiratory metabolism (haem-deficient cells, ρ^0 cells or cells grown in anaerobiosis) require a fermentable carbon source. Glucose cannot be used for this purpose because the synthesis of peroxisomal proteins analysed here and the proliferation of peroxisomes are sensitive to glucose repression [10,22]. Moreover, no induction by oleate could be observed when the medium contained glucose in addition to oleate [10,22]. Therefore in all experiments reported here the derepression medium contained raffinose, a fermentable but not repressing carbon source, and the oleate induction medium contained raffinose and oleic acid. The expression of the *POX1* gene was monitored by measuring the enzymic activity of acyl-CoA oxidase. Because the activity of the haemoprotein catalase A cannot be measured in anaerobiosis or in haem-deficient strains, and the activity of Pex1p is unknown, we used the *PEX1–lacZ* and *CTA1–lacZ* reporter genes integrated into the genomic DNA to examine the regulation of expression of the *PEX1* and *CTA1* genes. In our experiments with derepressed and oleate-induced ρ^+ cells the activities of the *PEX1* and *CTA1* promoters, measured as the levels of β-galactosidase, are well correlated with the published results of Northern blot analyses of *PEX1* and *CTA1* expression under the same growth conditions [23,24], validating the *lacZ* reporter approach. The use of a high-absorption-coefficient substrate for β-galactosidase, CPRG, permitted the straightforward and reliable quantification of promoter activities even at the extremely low expression levels of *PEX1* and *CTA1* observed in haem deficiency or anaerobiosis.

Expression of the *POX1, CTA1* and *PEX1* genes in ρ^0 cells

Four strains (MSCTA1ρ^+, its derivative MSCTA1ρ^0 with a non-functional respiratory chain, both of which contained the *CTA1–lacZ* construct, and MSPEX1ρ^+ and MSPEX1ρ^0, both of which contained the *PEX1–lacZ* construct) were grown in YPR or in YPRO medium and the enzymic activities in the cells were measured as described in the Materials and methods section. The activities of acyl-CoA oxidase and β-galactosidase expressed from either the *CTA1–lacZ* or the *PEX1–lacZ* fusion genes are shown in Table 2. As can be seen, the respiratory incompetence caused by a lack of mitochondrial DNA had no influence on the induced level of acyl-CoA oxidase, although it did induce approx. 4-fold the derepressed level of this enzyme. A similar but weaker inductive effect of ρ^0 in derepression medium can be seen for *CTA1–lacZ* expression. However, as for acyl-CoA oxidase, there also seems to be no significant effect of ρ^0 on the induction of *CTA1–lacZ* by oleate. In contrast, the expression of the *PEX1–lacZ* fusion gene both in derepressing conditions and in inducing conditions with oleate is markedly decreased in ρ^0 as compared with ρ^+ cells, indicating that the *PEX1* gene might be regulated differently from the other two genes under study.

Lack of haem strongly decreases the expression of the *POX1, CTA1* and *PEX1* genes

MSCTA2ρ^0 and MSPEX2ρ^0 strains with *hem1*Δ gene disruption and, respectively, the *CTA1–lacZ* or *PEX1–lacZ* fusion constructs were grown in YPR or YPRO medium and the enzymic activities in the cells were measured as described in the Materials and methods section. The activities of acyl-CoA oxidase and β-galactosidase are shown in Table 2. In *hem1*$\Delta\rho^0$ cells the expression of *CTA1–lacZ* in YPR derepression medium decreased to 1/25 compared with *HEM1*ρ^0 cells. Under inducing conditions with oleate (YPRO) the absence of haem caused an 81-fold decrease in the level of expression of this fusion. Catalase A encoded by the *CTA1* gene is a haemoprotein and its synthesis has previously been shown to be regulated by the level of haem in the cell [25]. Interestingly, the levels of acyl-CoA oxidase in *hem1*Δ cells are also strongly (at least 10-fold) decreased under both derepressing and inducing conditions. Also decreased (3-fold or 6-fold depending on the carbon source) was the expression of the *PEX1–lacZ* fusion gene. Despite this marked effect of the absence of haem, induction by oleate was still observed. The use of oleate as an energy source requires a functional respiratory chain, but its use as a source of acetate residues and other compounds for syntheses does not, which is in line with the results for ρ^0 strains presented above. Nevertheless, the catabolism of oleate requires oxygen as an electron acceptor in the first step of β-oxidation performed by acyl-CoA oxidase. Because haem is known to be a mediator of the signal of the presence or absence of oxygen [1], it is conceivable that the regulation of *CTA1*, *POX1* and *PEX1* genes by haem is in fact the result of a haem-mediated sensitivity to oxygen.

Table 2 Activities of acyl-CoA oxidase and β-galactosidase expressed from *CTA1–LacZ* or *PEX1–LacZ* gene fusions in respiratorily incompetent ρ^0, *hem1*Δ and anoxic cells, grown under derepressing (YPR medium) or oleate inducing (YPRO medium) conditions

Enzyme assays were performed as described in the Materials and methods section. Measurements of the activity of β-galactosidase expressed from the *CTA1–LacZ* fusion gene under conditions of haem deficiency or anoxia and from the *PEX1–LacZ* fusion gene under any conditions were made with the more sensitive assay with CPRG as a substrate (see the Materials and methods section). The results from the *CTA1–LacZ* and *PEX1–LacZ* fusions come from measurements made on separate sets of strains (MSCTA and MSPEX respectively) but are shown side by side for easier comparison. The values are means ± S.D. for three (β-galactosidase) or six (acyl-CoA oxidase) separate experiments. Abbreviation: n.d., not detected (the detection limit of acyl-CoA oxidase was approx. 0.2 nmol/min per mg of protein; 'n.d.' denotes that the enzymic activity was below that limit).

			Enzyme activities		
				β-Galactosidase	
Genotype	Growth conditions		Acyl-CoA oxidase (nmol/min per mg of protein)	*CTA1–LacZ* (nmol/min per mg of protein)	*PEX1–LacZ* (pmol/min per mg of protein)
HEM1 ρ^+	$+O_2$	YPR	1 ± 0.5	7 ± 0.3	35 ± 6
		YPRO	44 ± 3	111 ± 8	443 ± 41
HEM1ρ^0	$+O_2$	YPR	4 ± 0.3	12 ± 1	18 ± 2
		YPRO	50 ± 4	77 ± 5	85 ± 5
*hem1*Δρ^0	$+O_2$	YPR	n.d.	0.47 ± 0.5	7 ± 1
		YPRO	4 ± 0.7	0.95 ± 0.15	15 ± 3
HEM1ρ^0	$-O_2$	YPR	0	0.09 ± 0.01	27 ± 2
		YPRO	0	0.17 ± 0.01	29 ± 7

Table 3 Activities of catalase and acyl-CoA oxidase in wild-type cells grown anaerobically in SCR derepression medium supplemented with haemin

Haemin was added to a final concentration of 0.0015%. Enzyme assays were performed as described in the Materials and methods section.

Genotype	Growth conditions	Catalase activity (units per mg of protein)	Acyl-CoA oxidase (nmol/min per mg of protein)
Wild type		0	0
	+ Haemin	0	0
Wild type + MSEp1		0	0
	+ Haemin	9 ± 0.9	0

Effect of anaerobiosis on the expression of *POX1*, *CTA1* and *PEX1* genes is similar to, but not identical with, the effect of lack of haem

MSCTA1ρ^0 and MSPEX1ρ^0 strains containing the *CTA1–lacZ* or *PEX1–lacZ* constructs respectively were grown anaerobically in YPR or in YPRO medium and the enzymic activities in the cells were measured as described in the Materials and methods section. The results are shown in Table 2. The lack of oxygen had an even stronger effect on the expression of the *CTA1–lacZ* fusion gene than the lack of haem. In both cases the levels of β-galactosidase were very low, but the use of the very sensitive β-galactosidase assay revealed that the activity of the *CTA1* promoter in anaerobically grown cells was approximately one-fifth of that in haem-deficient cells. Acyl-CoA oxidase activity was undetectable in anaerobiosis in both derepressed and induced cells. Because haem-deficient oleate-induced cells showed measurable levels of acyl-CoA oxidase, it also seems that the synthesis of this protein was down-regulated more by a lack of oxygen than by a lack of haem. The effect of anaerobiosis on the expression of the *PEX1–lacZ* fusion gene seemed similar to the effect of haem deficiency but in this case there was no difference between the derepressed and the induced levels of β-galactosidase.

These results suggested that the regulation of the genes under study by oxygen might not employ haem as a mediator, unlike most known oxygen-regulated genes. To test this hypothesis, WS17-5D cells were grown in anaerobiosis under derepressing conditions in the presence of 0.0015% haemin. The activities of catalase and acyl-CoA oxidase in the protein extracts from these cells were measured. In this particular experiment it was not necessary to use the strain containing the *CTA1–lacZ* construct to measure catalase A promoter expression. If haemin were able to reverse the effect of anaerobiosis, it would also serve as an exogenous source of haem to form the catalase holoenzyme. The results of this experiment are shown in Table 3. The activities of catalase and acyl-CoA oxidase were undetectable in both the absence and the presence of haemin in the medium. Apparently the exogenous haemin could not reverse the regulatory effect of anaerobiosis on the synthesis of these two proteins. Also included in this experiment was a WS17-5D strain transformed with multicopy plasmid MSEp1, containing the *CTA1* gene, overexpressing catalase A. These cells grown in anaerobiosis under derepressing conditions in the presence of haemin did contain catalase but not acyl-CoA oxidase activity, proving that haemin was taken up and could be used by the cells under the experimental conditions (Table 3).

To study further the effect of anaerobiosis on the *POX1*, *CTA1* and *PEX1* genes, their expression levels were followed during simultaneous induction with oleate and adaptation to anaerobiosis of *S. cerevisiae* cells pregrown to mid-exponential phase in derepressing conditions in the presence of oxygen. The aim of this experiment was to test whether the absence of oxygen

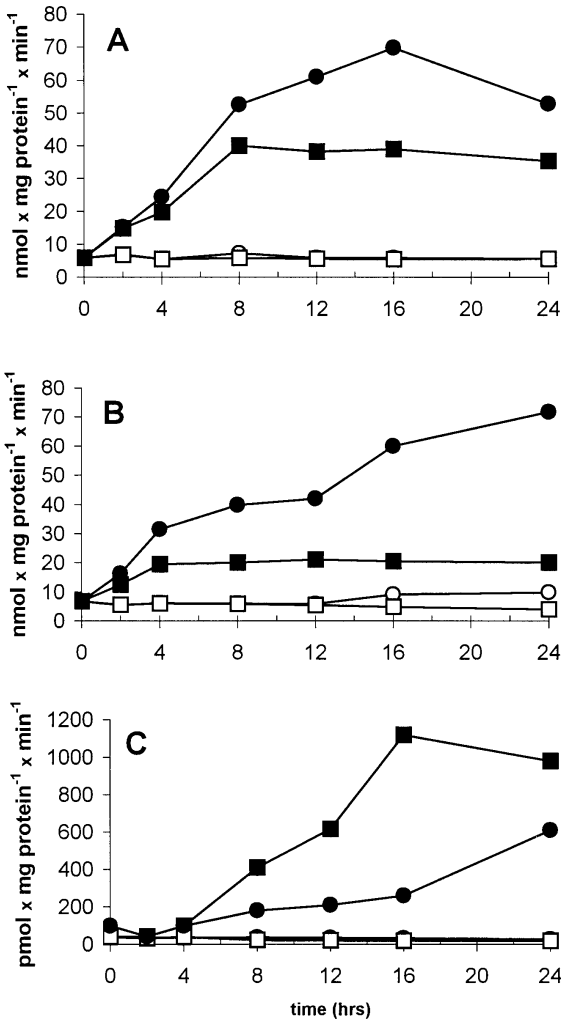

Figure 1 Expression of *POX1*, *CTA1* and *PEX1* during induction with oleate in anaerobiosis

The graphs depict changes in the levels of: (**A**) acyl-CoA oxidase, (**B**) β-galactosidase, representing the activity of the *CTA1* promoter, and (**C**) β-galactosidase, representing the activity of the *PEX1* promoter, after the transfer of aerobically pregrown yeast cells to conditions of derepression in the presence of oxygen (○), derepression in anaerobiosis (□), induction in the presence of oxygen (●) or induction in anaerobiosis (■). Cells were pregrown for 24 h in the derepression YPR medium to a cell density of 2×10^7 cells/ml. Cultures were then transferred to anaerobic flasks, oleic acid/Tween 80 mixture was added and cultures were made anaerobic where indicated (see the Materials and methods section for details). For anaerobic conditions, a separate culture was prepared for each time point. At defined intervals, cells were harvested and enzymes assayed as described in the Materials and methods section. The results from one representative experiment of three are shown.

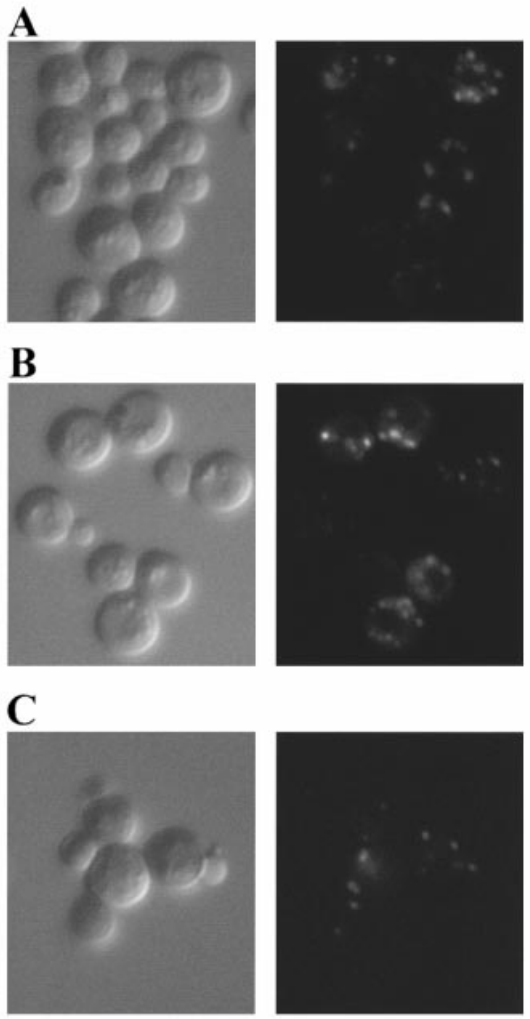

Figure 2 Peroxisomal compartment in wild-type, respiratorily incompetent and haem-deficient yeast cells

Patterns of fluorescence seen under epifluorescence (FITC filter, right column) or light microscopic images (Nomarski optics, left column) of living, oleate-induced *S. cerevisiae* cells transformed with a multicopy plasmid encoding the peroxisomal marker, acyl-CoA oxidase–GFP fusion protein: (**A**) wild-type WS17-5Dρ^+ cells; (**B**) respiratorily incompetent WS17-5Dρ^0 cells; (**C**) haem-deficient WS17-5D*hem1*Δ cells.

has any influence on the expression of genes under study when endogenous haem was still present in the cell. MSCTA1ρ^0 and MSPEX1ρ^0 cell cultures were pregrown, made anaerobic and harvested at various time points as described in the Materials and methods section. The activities of acyl-CoA oxidase and β-galactosidase were measured in cellular extracts and the results are shown in Figure 1. A comparison of oleate induction profiles of acyl-CoA oxidase and *CTA1–lacZ* in cells kept in the presence or the absence of oxygen clearly show that the induction process with oleate is oxygen dependent. In anaerobiosis the induction of both proteins was weaker and, moreover, took place during the initial few hours of incubation only, whereas in the presence of oxygen the increase in the activities continued for 16 h (acyl-CoA oxidase) or 24 h (*CTA1–lacZ*) (Figures 1A and 1B). More puzzling is the effect of oxygen on the induction of the expression of *PEX1–lacZ*. During the first 16 h of incubation the induction of this fusion gene by oleate under anaerobic conditions was 2–3-fold the induction in the presence of oxygen (Figure 1C). In addition, its induction profiles in both the presence and the absence of oxygen showed a characteristic lag period of approx. 4 h (Figure 1C).

Peroxisome morphology of oleate-induced ρ^0 cells and *hem1*Δ cells

To investigate the effect of respiratory incompetence due to a lack of mitochondrial DNA or haem deficiency on peroxisome morphology, WS17-5Dρ^+, WS17-5Dρ^0 and WS17-5D*hem1*Δ strains transformed with multicopy plasmid YEp-AOx–GFP

(green fluorescent protein) were grown under inducing conditions with oleate and examined under a fluorescence microscope equipped with an FITC filter. The plasmid present in yeast cells encodes an acyl-CoA oxidase–GFP fusion protein that is efficiently imported into peroxisomes [26] and can be used to reveal these organelles in living cells. The results of this experiment are shown in Figure 2. A particulate pattern of green fluorescence was visible and looked similar in all three types of cell, although the organellar particles were less numerous in WS17-5D*hem1*Δ cells. This indicates that the morphology of the peroxisomal compartment was not significantly affected by the conditions tested.

DISCUSSION

Yeast *S. cerevisiae*, being a facultative anaerobe that is able to utilize fermentation as a sole means of generating energy, is a unique model organism for studying all kinds of cellular process related to respiration. The functioning of major peroxisomal metabolic pathways depends on the presence of oxygen and is connected to the respiratory functions of mitochondria. Nevertheless, the regulatory routes linking peroxisomes and mitochondria are far from fully understood. In particular, little is known about the regulation by oxygen of the synthesis of peroxisomal proteins and of peroxisome proliferation. In our previous study we demonstrated that catalase A and acyl-CoA oxidase are absent from *S. cerevisiae* cells grown in anaerobiosis in derepressing medium [10]. We have also shown that, in ρ^0 cells grown either under derepressing conditions or under inducing conditions with oleate, both proteins are present in an organellar pellet and they co-sediment when the pellet is separated on the gradient. These facts indicate a peroxisomal localization of catalase A and acyl-CoA oxidase in ρ^0 cells [10]. In the present study we have analysed the effects of three kinds of respiratory deficiency [an inactive respiratory chain owing to the deletion of mitochondrial DNA (ρ^0), the absence of haem due to the deletion of the *HEM1* gene, or the absence of oxygen] on the functioning of the peroxisomal compartment. We also tested the effects of ρ^0 and *hem1*Δ on the morphology of peroxisomes. As markers of the peroxisome function we chose three proteins, acyl-CoA oxidase, catalase A and the peroxin Pex1p, the products of the genes *POX1*, *CTA1* and *PEX1* respectively.

Functional dependences of peroxisomes and mitochondria had been substantiated by the identification of a number of transcriptional elements (encoded by the genes *RTG1*, *RTG2* and *RTG3*) participating in the so-called retrograde induction of peroxisomal citrate synthase (encoded by the gene *CIT2*) in response to respiratory incompetence of the *S. cerevisiae* cell [27]. It was also shown that the deletion of these genes impairs the induction of citrate synthase by oleate, and also acyl-CoA oxidase and catalase A [24]. However, the possibility of the retrograde response of *POX1* and *CTA1* to respiratory incompetence has never been tested. The existence of such regulatory links prompted us to use in our experiments respiratorily incompetent ρ^0 cells in addition to haem-deficient *hem1*Δ and anaerobically grown cells, which are also incapable of respiration. In this way we were able to distinguish the regulatory effects of haem or oxygen from retrograde or other effects (if any) caused by compromised respiration. As our results show clearly, under inducing conditions with oleate, a lack of mitochondrial DNA has no effect on these two genes. It also has no effect on peroxisome morphology. However, ρ^0 caused some increase in *POX1* and *CTA1* expression in derepressed cells. Interestingly, ρ^0 causes a significant decrease in *PEX1* expression in derepressing as well as inducing conditions with oleate, a phenomenon for which we cannot give an explanation at present.

Haem deficiency markedly affects the expression of the genes under study. Both derepressed and induced expression levels were lowered by as much as two orders of magnitude for *CTA1*, at least one order of magnitude for *POX1* and for *PEX1* 3–6-fold, relative to the levels in ρ^0 cells. Anaerobiosis affects the synthesis of acyl-CoA oxidase more than haem deficiency under inducing conditions with oleate, and at least as much as haem deficiency under derepressing conditions. In both these circumstances *CTA1* expression is 5-fold lower in anoxia than in the absence of haem. Because the synthesis of haem requires oxygen, anaerobic conditions also cause haem deficiency. If the presence or absence of haem is the only signal of the presence or absence of oxygen, there should be no difference between the regulatory effects of lack of haem and anoxia. Therefore the observed differences in the expression levels of *CTA1* and *POX1* suggested that their mechanisms of regulation by haem and oxygen are not linked, unlike most oxygen-regulated genes. The addition of an exogenous source of haem to the anaerobic growth medium or the presence of the endogenous haem in anaerobic culture does not overcome the effect of the absence of oxygen on the synthesis of acyl-CoA oxidase and *CTA1* expression. This provided additional support for our hypothesis, because it was shown for some genes that are haem-responsive and whose oyxgen response is haem-mediated, that the addition of haemin to the anaerobic culture did reverse the regulatory effects of lack of oxygen [28,29]. The only haem-controlled transcriptional regulatory protein that has been well characterized and shown to regulate many genes sensitive to haem and/or oxygen is Hap1p (Cyp1p) [1,2,4,6,30,31]. To investigate whether *POX1*, *CTA1* and *PEX1* regulation by haem is mediated by Hap1p, we tested the levels of acyl-CoA oxidase and β-galactosidase expressed from *CTA1–lacZ* or *PEX1–lacZ* fusions in the strain carrying *cyp1*Δ*::LEU2* disruption and its parental strain grown in YPR and YPRO media. In addition, the expression of *POX1* and *CTA1* was studied in the other two mutants *CYP1-18* and *cyp1-23* (all three strains were provided by Dr J. Verdiére, CNRS, Gif-sur-Yvette, France). The *CYP1-18* mutation leads to the overproduction of Hap1 protein and, in consequence, to the increased expression of regulated genes, whereas the two mutations *cyp1-23* and *cyp1*Δ*::LEU2*, causing a lack of Hap1p in the cell, lead to the decreased expression of a number of Hap1p-dependent genes [6,32]. We found that neither overexpression nor the absence of Hap1p significantly affected the levels of expression of *POX1*, *CTA1* or *PEX1* (results not shown). If these results are taken together it seems that these three genes are regulated by haem by a novel non-Hap1p regulatory route. Moreover, *CTA1* and *POX1* are regulated by oxygen via a mechanism that does not rely on haem, either as a gauge of the presence or absence of oxygen or as a sensor of its concentration.

In contrast with the marked effect of *HEM1* deletion on the expression of *CTA1* and *POX1*, the consequences of haem deficiency on the morphology of peroxisomes under induction with oleate are negligible. This indicates that haem is not a general regulator of peroxisome biogenesis but is probably an agent specific for only some peroxisomal proteins. In future studies it would be interesting to test the regulation by haem of other genes encoding peroxisomal proteins.

Even more interesting is the regulation of the expression of *PEX1* by oxygen. As mentioned previously, derepressed as well as induced levels of *PEX1* expression are lower in ρ^0 cells and decreased even further in the absence of haem, whereas the results of its oxygen regulation are ambiguous. In derepressed or oleate-induced cells grown in the absence of oxygen for many

generations, the expression of *PEX1* is very low. However, in cells subject to simultaneous induction with oleate and adaptation to anoxia its expression reaches levels 300-fold higher. Further, it is 2–3-fold higher than in cells subject to induction with oleate in the presence of oxygen. An additional interesting feature of *PEX1* is its oleate induction profile, which differs from those of the other two genes by a characteristic lag period of approx. 4 h (Figure 1). Pex1p peroxin is a protein involved in peroxisome biogenesis that belongs to the family of AAA ATPases [15]. It is a low-abundance protein to which no specific function has yet been assigned. We hope that the observed patterns of *PEX1* expression not only will help to identify novel, peroxisome-specific mechanisms of regulation by haem and oxygen but might also contribute to uncovering the function of this mysterious protein.

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