Requirements for Unsaturated Fatty Acids for the Induction of Respiration in Saccharomyces cerevisiae*

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Unsaturated fatty acids provided during the release from glucose repression were shown to be essential for derepression of respiration in an unsaturated fatty acid auxotroph of Saccharomyces cerevisiae (KD115). Cells derepressed in the presence of oleic acid contained three to six times as much cytochrome per cell as those derepressed in the absence of unsaturated fatty acid or those derepressed with eicosanoic acid. The Δ9 isomer was the most efficient of the cis-octadecenoic acid isomers in supporting that increase, and eicosanoic acid supported an increase at only 15% the rate observed with oleic acid.

Derepression, even in the presence of oleic acid, proceeded only after a lag of 3 hours. When glucose was removed prior to the addition of oleate, the lag was reduced by the time of the preincubation with glycerol. This result suggests that some processes necessary for increased respiration can proceed in the absence of an added unsaturated fatty acid, but these processes apparently require certain levels of unsaturated acids in the pre-existing lipids, since they occurred in cells whose membranes contained 50 mol % oleate, but not in cells containing only 20 mol %. These processes leading to eventual increased respiration were inhibited by cycloheximide but not chloramphenicol, suggesting that protein synthesis on cytoplasmic ribosomes but not mitochondrial ribosomes was required. Derepression in the absence of oleate for 3 hours lessened the inhibition of respiration induction by ethidium bromide. This result indicates that the transcription of mitochondrial DNA necessary for the induction of respiration may have occurred in the absence of added unsaturated fatty acid, but that some subsequent event required added esterified unsaturated fatty acid.

The yeast Saccharomyces cerevisiae has been used extensively in studies of mitochondrial function and biogenesis (1). The composition and development of the mitochondria in this organism can be controlled by manipulating physiological conditions such as oxygen tension or metabolite levels (1).

When yeasts are grown anaerobically, the synthesis of cytochromes (2), ergosterol, and unsaturated fatty acids (3) are all prevented. If those lipid components are provided, the anaerobic promitochondria appear to have an extensive cristate membrane system (4) and a functional protein-synthesizing system (5, 6). If no lipid supplementation was provided, the folded inner membrane system was absent (4), protein-synthesis was impaired (4), mitochondrial RNA was depleted (7) and the mitochondrial RNA polymerase activity was impaired (8).

The isolation of mutants of yeast which are auxotrophic for unsaturated fatty acids (9) permits an analysis of the role of these compounds in the biogenesis of mitochondria. Using an unsaturated fatty acid auxotroph of yeast, Gordon et al. (10) determined that unsaturated acid availability, either endogenous or exogenous, was essential for development of respiration during aeration of a previously anaerobic culture.

In the present study we have used the unsaturated fatty acid auxotroph of S. cerevisiae KD115 (9) to investigate the role of unsaturated fatty acids, either in pre-existing membranes or provided during derepression, on the increase of respiration after release from glucose repression. The specificity of the requirements for unsaturated fatty acids was investigated by providing the yeast with selected acids shown to differ in their ability to support yeast growth (11).

METHODS

Organism and Media

The mutant strain KD115 (ol 1) of Saccharomyces cerevisiae was the generous gift of Dr. Alex Keith (Department of Biophysics, Pennsylvania State University). This yeast has been shown to be an unsaturated fatty acid auxotroph (9) deficient in Δ9-desaturase (12). Maintenance and growth of this organism was described in the previous paper (11).

Lipid analyses, measurement of respiration, and estimation of cytochrome content were performed by methods described in the previous paper (11).

Induction of Respiration—Cells were grown for 8 hours in medium containing 5% glucose as an energy source. They were then harvested by centrifugation, washed with 0.1 M NaCl, and resuspended in media containing 2% glycerol and 0.05% glucose as the energy source.

Inhibition of Macromolecular Synthesis—Chloramphenicol and cyto-
cloheximide were purchased from Sigma Chemical Co. Ethidium bromide was the generous gift of Dr. W. Folk of this Department. The latter two substances were added to cultures as aqueous solutions while the former was added as a solution in 50% ethanol. Removal of these substances from a culture was effected by centrifugation of the culture and resuspension of cells into fresh medium containing neither inhibitor nor acid. After 5 min these cells were again centrifuged, washed with 0.1 N sodium chloride, and resuspended in the final medium.

RESULTS

Requirement for Unsaturated Fatty Acid Provided during Derepression

When yeasts are grown in high levels of glucose, many of their mitochondrial functions including respiration are repressed. When transferred to a medium low in glucose these functions can develop. In Fig. 1, the increase in cyanide-sensitive whole cell respiration under various conditions of unsaturated fatty acid supplementation is presented. The cells had been first repressed in 5% glucose in the presence of excess (200 μM) oleic acid before transfer to the derepressing medium. In the absence of a concurrent unsaturated fatty acid supplement, respiration began to increase at about 0.2 nmol of O₂ per min per million cells, but within 2 hours after the start of derepression it leveled and then underwent no further increase. When oleic acid was provided during derepression, a similar initial increase and leveling was observed, but 3.5 hours after the start of derepression, respiration began to increase at a rate of 0.13 nmol per min per million cells per hour until a maximum value of 1.0 nmol per min per million cells was reached. Fig. 1b illustrates that when 20:1 was provided during derepression, respiration increased only slightly more than in the unsupplemented cells.

The increases in respiration which were supported by the positional isomers of cis-octadecenoic acid are tabulated in Table I. The isomers Δ9 through Δ4 and Δ14 through Δ17 did not support an increase in respiration significantly above that seen in unsupplemented cells. The Δ9 isomer supported the maximum increase, 0.83 nmol per min per million cells by 6.5 hours, and isomers with the double bond located nearer either end of the molecule supported less of an increase in respiration than did the Δ9 isomer.

Effects of Unsaturated Fatty Acids Present in Pre-existing Lipids

The unsaturated fatty acid content of the lipids of repressed cells was varied by providing different levels of supplement acid during repression. Table II indicates that cells repressed in the presence of 100 μM or 10 μM oleate contained 50 mol % or 20 mol % unsaturated fatty acids in their phospholipids. These cells had net functionalities of 450 and 80 (11), respectively. For those cells with 50 mol % oleate in their pre-existing lipids, derepression in the absence of oleate for 2 to 4 hours, reduced the subsequent time of the lag before the onset of respiration increased (Fig. 2). When oleate was added immediately after transfer to derepressing medium, the lag was 3.6 hours (Fig. 1), but when added after 2 to 4 hours, the lag was 0.6 hour or less (Fig. 2).

The increase in respiration by cells which contained 20 mol % unsaturated acids in pre-existing lipids is illustrated in Fig. 3. These cells, like those containing 50 mol % oleate, had very little increase in respiration unless oleate was provided during derepression. When oleate was provided, an initial increase and leveling of respiration occurred, followed by an increase at a rate of 0.15 nmol per min per million cells per hour that began 5 hours after transfer to derepressing medium. In contrast to the results with oleate-rich cells (Fig. 2), the lag from the addition of oleate to the onset of maximum rate of increase in respiration remained 5 hours when oleate was added 3 hours after the start of derepression.

Fig. 4 illustrates the increase in respiration in cells which had been repressed in the presence of 100 μM 20:1 (see also Table II). Again, no increase occurred without added oleate, when oleate was provided at the start of derepression, kinetics of respiration increase paralleled those in cells containing 50 mol % oleate: a lag of 3.5 hours before the onset of maximum

![Graph showing increases in respiration under different conditions of unsaturated fatty acid supplementation.](http://www.jbc.org/)
rate of increase and a rate of 0.4 nmol per min per million cells per hour. When oleate was added 3 hours after the start of derepression the observed lag was only 1 hour, but the rate of increase was only 0.07 nmol per min per million cells per hour, one-half that of cells that had been repressed in the presence of oleic acid (Fig. 2). Derepression of the cells for some time without added oleate prior to the addition of oleic acid will subsequently be referred to as “priming.”

Involvement of Unsaturated Fatty Acid in Macromolecular Syntheses during Induction

Cytochrome Synthesis—The cytochrome content of cells derepressed under various conditions of unsaturated fatty acid supplementation is illustrated in Fig. 5 and Table III. When cells were derepressed in the presence of oleic acid, the cytochrome content per cell was similar regardless of the fatty acid supplementation is illustrated in Fig. 5 and Table III. When cells were derepressed in the presence of oleic acid, the cytochrome content per cell was similar regardless of the fatty acid supplementation during the earlier repression. If oleate was not present during derepression, the cytochrome content was one-third or less than in cells which were concurrently supplemented, regardless of whether the pre-existing lipids contained 50 or 20 mol % unsaturated fatty acid.

Mitochondrial Synthesis of Proteins—Chloramphenicol has been shown to inhibit protein synthesis in yeast cytoplasmic ribosomes without directly inhibiting synthesis on mitochondrial ribosomes (13). Fig. 6 illustrates that when 4 mg per ml of chloramphenicol were added along with oleate to cells derepressed in 200 μM oleate at the start of derepression, essentially no increase in respiration occurred over 10 hours. When, after 3 hours of priming, chloramphenicol was added along with oleate, the increase in respiration was again inhibited. However, when that amount of chloramphenicol was added at the start of derepression, then removed after 3 hours, and oleic acid then provided, respiration increase commenced with almost no lag and at a rate of 0.9 nmol per min per million cells per hour. Apparently, the presence of chloramphenicol during priming did not increase the lag and it permitted a subsequent respiratory increase at 70% the uninhibited rate observed in Fig. 2.

Cytoplasmic Synthesis of Proteins—Cycloheximide has been shown to inhibit protein synthesis in yeast cytoplasmic ribosomes. Cells were grown in repressing medium (5% glucose) with the indicated fatty acid supplement for 8 to 9 hours. At that time cells were harvested and washed with 0.1 N sodium chloride and lyophilized. Lipids were extracted, phospholipids isolated, and fatty acid composition determined as described under “Methods.” The data presented are the mean values of two separate experiments.

Table II

| Fatty acid composition of phospholipids from cells | Phospholipid | Mole % in Phospholipid |
|---------------------------------------------------|--------------|------------------------|
| Cells in derepressed medium | 12:0 | 14:0 | 16:0 | 16:1 | 18:1 | 20:1 | 6 |
| 100 μM oleate | 0.42 | 2.5 | 27 | 16 | 1.96 | 50 | - | 450 |
| 10 μM oleate | 2.00 | 4.7 | 63 | 11 | 0.16 | 17 | - | 80 |
| 100 μM eicosa-enoate | 0.00 | 4.2 | 68 | 5 | 0.00 | 13 | 9 | 20 |

Fig. 2 (left). Effects of supplementation with oleate at different times on increase in respiration. Cells were repressed for 8 hours in medium containing 5% glucose and 200 μM oleic acid. At zero time, cells were transferred to derepressing medium to which 50 μM oleate was added at 2 (O), 3 ( ), or 4 ( ) hours. The dashed lines represent the increase in respiration when oleate was added at zero time or not at all from Fig. 1. Arrows indicate lag from time of addition of oleate to onset of increase in respiration. All values represent means of at least two experiments.

Fig. 3 (center). Increase of respiration of cells repressed in limited levels of oleate. Cells were treated as in Fig. 1 except that the repressing medium contained only 10 μM oleic acid. The cultures were made 50 μM in oleate at zero time ( ) or 3 hours ( ) after transfer to derepressing medium. One culture (O) was not supplemented with any acid. The values represent the means of three experiments. Arrows represent lag from time of addition of oleate (indicated by first number) to time of beginning of maximal increase in respiration (indicated by final number).

Fig. 4 (right). Increase of respiration of KD115 cells previously repressed in eicosanotic acid. Cells were treated as in Fig. 1 except that the repressing medium was supplemented with 200 μM 20:1. Cells were supplemented with 50 μM oleic acid at zero time ( ) or 3 hours ( ). One culture was not supplemented with any acid (O). The values represent the means of two experiments. Arrows indicate lag from time of addition of oleate to onset of respiration increase.
Table IV.

Table III

Cytochrome content of cells derepressed under various conditions of oleate supplementation

Cytochrome content was estimated from spectra of homogenates of cells grown as described in Fig. 5.

| Oleate Concentrations | Cytochrome Content |
|-----------------------|--------------------|
|                      | c+c₁    | b    | a+a₃ |
| (µM)                 | (µM)    |      |      |
| 200                   | 0.155   | 0.075| 0.029|
| 10                    | 0.213   | 0.061| 0.031|
| 200                   | 0.060   | 0.023| -    |
| 10                    | 0.051   | -    | -    |

To investigate the effect on the content of respiratory chain components of unsaturated fatty acids that were either pre-existing in membrane lipids or provided during derepression, the induction of respiration was studied after transfer of the yeast from a glucose- to a glycerol-based medium. Our results show that at least one essential step in the development of respiration requires added unsaturated fatty acid, and that at least one other essential step is sensitive to the levels of unsaturated fatty acids in pre-existing membranes.

Requirement for Unsaturated Fatty Acid during Derepression—The exogenous unsaturated fatty acid appeared to be required for the accumulation of the cytochromes since cells derepressed in the absence of oleate had less than one-third as much cytochrome c + c₁, b, or a + a₃ as cells derepressed in the presence of oleate, after 8 hours of derepression.

Kadenbach (20, 21) has suggested that the cytochrome c apoenzyme is synthesized by ribosomes on the endoplasmic reticulum, where it then associates with newly synthesized phospholipids. The lipoprotein complex is then transported into the mitochondrial membrane and finally is inserted into the mitochondrial membrane. If the new phospholipid with which the cytochrome c associates must contain an unsaturated acid, then the association of apoenzyme with new phospholipid might not take place without unsaturated fatty acid provided during derepression. Cytochromes a + a₃ (22) and b (23) appear to contain polypeptide components synthesized on cytoplasmic ribosomes and these may also require association with newly synthesized unsaturated phospholipids for insertion into the mitochondrial membrane. Recent findings of Janki et al. (24) suggest that there is a specific association of newly synthesized cytochrome c oxidase and ATPase subunits with newly synthesized, unsaturated lipids and subsequent incorporation of this lipoprotein complex into preformed membranes, after transfer from a nitrogen to an oxygen atmosphere.

The process(es) which require a free unsaturated fatty acid were sensitive to both cycloheximide (Fig. 7) and chloramphenicol (Fig. 6) added either at the time of transfer from glucose to glycerol media or later. This result does not necessarily imply that unsaturated fatty acids are necessary for protein synthesis on both cytoplasmic and mitochondrial ribosomes. The synthesis of some proteins of mitochondrial origin has been shown to be controlled by products of the cytoplasmic protein synthetic system (25, 26).

The requirement for added unsaturated fatty acid during derepression appears to be somewhat specific. All other positional isomers of cis-octadecenoate were less effective than the

FIG. 5. Difference spectra of KD115 derepressed under different conditions of oleate supplementation. Cells were repressed in media containing 5% glucose and the indicated oleate concentration for 8 hours. Then 2 to 2.5 x 10⁹ cells were transferred in 500 ml of derepressing medium containing 0.05% glucose and 2% glycerol and the indicated oleate concentration for 5 hours. Cells were then harvested and spheroplasts were homogenized and reduced minus oxidized spectra generated as described under "Methods." Spheroplasts were homogenized and reduced minus oxidized spectra generated as described under "Methods." a, cells repressed in presence of 100 µM oleate; derepressed in presence of 50 µM oleate. b, cells repressed in presence of 10 µM oleate; derepressed in presence of 50 µM oleate. c, cells repressed in presence of 200 µM oleate; derepressed in absence of oleate. d, cells repressed in presence of 10 µM oleate; derepressed in absence of oleate.

repressed in 200 µM oleate, at the start of derepression, both the respiration and the increase in respiration were inhibited. When this concentration of inhibitor was added alone with oleate after 3 hours of priming, respiration increased for 1 hour at a rate equal to that of uninhibited cells observed in Fig. 2 and no further increase in respiration was observed after this time. Three hours of priming appeared to decrease the inhibition of respiration increase by ethidium bromide. The effect of the various inhibitors on macromolecular synthesis on the kinetic parameters of respiration increase are summarized in Table IV.

DISCUSSION

In the presence of high levels of glucose, mitochondrial function is repressed and many of the mitochondrial enzymes are present in very low quantities (15, 16). After a transfer to glycerol medium, morphogenesis of mitochondrial structure occurs accompanied by a large increase in the activities of many of those proteins associated with mitochondrial energy metabolism. As mitochondrial membranes proliferate (16), lipid content (17), particularly cardiolipin, increases (18) and the content of unsaturated fatty acids increases (19).

To investigate the effect on the content of respiratory chain components of unsaturated fatty acids that were either pre-existing in membrane lipids or provided during derepression, the induction of respiration was studied after transfer of the yeast from a glucose- to a glycerol-based medium. Our results show that at least one essential step in the development of respiration requires added unsaturated fatty acid, and that at least one other essential step is sensitive to the levels of unsaturated fatty acids in pre-existing membranes.

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The requirement for added unsaturated fatty acid during derepression appears to be somewhat specific. All other positional isomers of cis-octadecenoate were less effective than the
shorter lag times after priming and presumably had perceived in excess 20:1 (with a calculated functionality of 20) did show decrease in lag after a 3-hour priming and may not have even % oleate (and a calculated net functionality of 80) exhibited no content of pre-existing lipids (Fig. 3). Cells containing 20 mol fatty acid appears to be affected by the unsaturated fatty acid.

respiration which proceed in the absence of added unsaturated acid. The presence of inhibitor is indicated by □ and □, and its absence by ● and ●. The values represent the means of three experiments. The arrow indicates the lag from the time of addition of oleate to onset of increase in respiration in Experiment 3.

Fig. 7 (center). Effect of cycloheximide on increase of respiration and cell growth of KD115 cells. Cells were treated as in Fig. 1 with the following differences: in Experiment 1, 0.02 mM cycloheximide was added at zero time along with 50 μM oleic acid; in Experiment 2, 0.02 mM cycloheximide was added along with 50 μM oleic acid 3 hours after the transfer to derepressing medium; in Experiment 3, 0.03 mM cycloheximide was added at zero time and removed by harvesting and washing cells after 3 hours, at which time 50 μM oleic acid was added. The presence of the inhibitor is indicated by ○ and □ and its absence by ● and ●. Respiration of aliquots removed at various times is indicated in a; the number of generations of growth in b. The values represent the means of three experiments. The arrow indicates the lag from time of addition of oleate to onset of respiration increase in Experiment 3.

Fig. 8 (right). Effect of ethidium bromide on increase in respiration and growth of KD115 cells. Cells were treated as in Fig. 1 except that 0.2 mg per ml of ethidium bromide was added, along with 50 μM oleate at zero time (○) or 3 hours (□) after transfer to derepressing medium. Cellular respiration values represent the means of two experiments. The presence of inhibitor is indicated by ● and □ and its absence by ■.

Δ9 isomer (Table II), and 20:1 was only 15% as effective as oleate (Fig. 1b) in supporting a sustained increase in respiration. Our results do not indicate whether this apparent specificity results from interactions of phospholipid acyl chains with newly synthesized proteins, the suitability of the different acids as substrates for phospholipid synthesis, or the fluidity of the newly synthesized lipid. It is also possible that an acid such as 20:1 which leads to a low level of respiratory control (11) and supports very little growth with glycerol as the carbon source may not allow the generation of sufficient energy to support the continued development of cytochromes and mitochondria.

Requirement for Unsaturated Fatty Acids in Pre-existing Lipids—Derepression in the absence of added unsaturated acid for 2 to 4 hours produced cells which were primed, ready to commence respiration increase with little or no lag when oleate was subsequently added (Fig. 2). Thus, some processes necessary for increasing the respiration can occur even in the absence of added unsaturated fatty acid, and the presumed initiation signal for the entire processes of induction of respiration may not be dependent on the presence of added unsaturated fatty acid.

At least one of those processes involved in the induction of respiration which proceed in the absence of added unsaturated fatty acid appears to be affected by the unsaturated fatty acid content of pre-existing lipids (Fig. 3). Cells containing 20 mol % oleate (and a calculated net functionality of 80) exhibited no decrease in lag after a 3-hour priming and may not have even transmitted the initiation signal. Nevertheless, cells repressed in excess 20:1 (with a calculated functionality of 20) did show shorter lag times after priming and presumably had perceived some initiation signal. They appeared, however, to be less effective in supporting those processes which occur during priming, since their increase of respiration proceeded at only one-half the rate observed in cells that contained 50 mol % oleate during priming.

What is the nature of the processes which require a certain level of unsaturated fatty acid in pre-existing membranes but not the concurrent presence of an unsaturated acid? The mitochondrial protein synthesizing system appears to be required. The presence of cycloheximide during priming of cells containing 50 mol % oleate (Fig. 7) prevented a decrease in the lag observed before the start of increase in respiration. The cytoplasmic protein synthetic system appears to provide several products crucial to the morphogenesis of mitochondria. It is the probable site of synthesis of many of the components of the mitochondrial transcription and translation machinery: the mtDNA-dependent RNA polymerase (27), the mitochondrial ribosomal proteins, and the initiation and elongation factors of mitochondrial protein synthesis all appear to be designated by nuclear genes (28, 29) and synthesized on the cytoplasmic ribosomes (6, 29, 30). When ethidium bromide, which has been shown to inhibit both mtDNA transcription (31) and translation (14) was added to primed cells, the inhibition of increase in respiration by ethidium bromide was considerably less than when it was added to unprimed cells (Fig. 8). For 1 hour, the increase in respiration proceeded at a rate equal to that in uninhibited cells. These results suggest that during priming, products whose formation is inhibited by ethidium bromide (e.g. mitochondrial nucleic acids) had accumulated in a form which permitted their function subse-
The absence of newly synthesized unsaturated lipids. The mitochondrial ribosomal RNA produced by this enzyme could then combine with the ribosomal proteins synthesized in the cytoplasm (6, 29, 30) to form functional mitochondrial ribosomes, and mitochondrial protein synthesis could begin. These events may account for the observed priming effect which can occur without additional non-esterified unsaturated fatty acids. The demonstrated need for non-esterified acids to provide increased cytochrome and increased respiration after the priming event suggests that some lipoprotein complex necessary for assembling fully functioning mitochondria must be synthesized concomitant with that assembly. Apparently, pre-existing lipids, even though of adequate acyl chain composition, cannot be utilized for this assembly.

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**Table IV**

**Effect of inhibitors on kinetic parameters of respiration increase**

| Additions | Inhibitor | Olate | Rate (hr) | Lag Rate (nmoles/min/10^6 cells/hr) |
|-----------|-----------|-------|----------|-----------------------------------|
| CAP at t = 0 | t = 0 | 8+ | 0 | |
| CAP at t = 3 | t = 3 | 7+ | 0.06* | |
| CAP at t = 0; removed at t=3 | t = 3 | 0.2 | 0.90 | |
| CHI at t = 0 | 8+ | 0 | | |
| CHI at t = 3 | 7+ | negative | | |
| CHI at t = 0; removed at t=3 | t = 3 | 3.5 | 0.14 | |
| EBr at t = 0 | t = 0 | 7+ | negative | |
| EBr at t = 3 | t = 3 | 0 | 0.11* | |
| None | t = 0 | 3.6 | 0.13 | |
| None | t = 3 | 0.5 | 0.16 | |

* The indicated rate was sustained for no more than one hour.

**NOTE:**

The table shows the effect of various inhibitors on the kinetic parameters of respiration increase. The results indicate that the presence of oleate has a significant effect on the rate of respiration, with the addition of CAP at t = 0 and CHI at t = 0 showing the most pronounced effects. The table provides a basis for understanding how different inhibitors affect the mitochondrial respiratory process.
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