Oxygen-dependent low-temperature $\Delta 12$ (n6)-desaturase induction and alteration of fatty acid composition in *Acanthamoeba castellanii*

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The influence of dissolved oxygen on the synthesis and activity of $\Delta 12$ -desaturase in *Acanthamoeba castellanii* was investigated. A decline in oxygen concentration during batch growth at 30 °C was correlated with a decline in the degree of cellular fatty acid unsaturation. Chilling of early-stationary-phase cultures to 15 °C led to increased dissolved oxygen levels (from < 1 μ M to 305 μ M) and increased fatty acid unsaturation, which has been shown previously [Avery, S. V., Harwood, J. L. & Lloyd, D. (1994) *Microbiology* 140, 2423–2431] to be due mainly to $\Delta 12$ -desaturase induction. In contrast, chilling of mid-exponential-phase cultures, where the dissolved oxygen concentration prior to chilling was high (> 160 μ M), gave no change in cellular fatty acid unsaturation. Measurement of [$1\text{-}^{14}\text{C}$]acetate incorporation by oxygen-limited *A. castellanii* revealed that labelling of the $\Delta 12$ -desaturase product, linoleate (18:2), increased with oxygen concentration. Microsomal levels of the $\Delta 12$ -desaturase enzyme were found to increase by up to 10-fold during aeration of *A. castellanii* cultures; a transient elevation in oxygen was sufficient to induce $\Delta 12$ -desaturase synthesis that was still fully detectable 1 h later. In addition, the activity of pre-existing $\Delta 12$ -desaturase, measured in isolated microsomal membranes, increased by up to fivefold with increases in the oxygen concentration of assay mixtures. These results demonstrate for the first time that (i) oxygen availability alone can regulate *de novo* $\Delta 12$ -desaturase synthesis in *A. castellanii*, and that (ii) oxygen can limit the activity of pre-existing $\Delta 12$ -desaturase. These responses can occur independently of temperature changes.

Keywords: $\Delta 12$ -desaturase, low-temperature adaptation, dissolved oxygen concentration, *Acanthamoeba castellanii*, fatty acid composition

INTRODUCTION

A key adaptation enabling organisms to metabolize and grow at low temperatures is an ability to alter their membrane lipid composition, thus compensating for chill-induced increases in membrane rigidity (Hazel & Williams, 1990; Cossins, 1994). One of the most rapid and widely observed responses to low-temperature shift is the increased synthesis and incorporation of unsaturated fatty acids, although other responses may occur over longer time-scales (Hazel & Williams, 1990; Harwood *et al.*,

1994). Enhanced unsaturated fatty acid synthesis can result from induction of fatty acid desaturases, which catalyse the insertion of double bonds into fatty acyl chains. However, the precise mechanism responsible for low-temperature desaturase induction is still not clearly understood. It is generally considered that there are three possible ways by which desaturase activity can be controlled (Kates *et al.*, 1984; Harwood *et al.*, 1994). These are at the level of: (i) substrate supply, e.g. availability of reductant or oxygen; (ii) activation of pre-existing enzyme activity, e.g. auto-regulation by changes in membrane order and in the conformational state of membrane-bound enzyme; (iii) gene expression and *de novo* enzyme synthesis.

Recent studies have provided further insight into the

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mechanism of desaturase induction in yeasts and cyanobacteria. In *Saccharomyces cerevisiae*, supplementation of growth media with unsaturated fatty acids resulted in a decline in transcription of the desaturase gene (Bossie & Martin, 1989); the specificity of this response to particular fatty acid species suggested that regulation was initiated by fatty acid binding to a transcriptional sensor, rather than by changes in membrane order (McDonough *et al.*, 1992). In the cyanobacterium *Synechocystis* PCC 6803, increases in the degree of plasma-membrane fatty acid saturation, brought about by palladium-catalysed hydrogenation, resulted in enhanced transcription of the *desA* gene encoding the $\Delta 12$ -desaturase in a similar fashion to low-temperature induction (Vigh *et al.*, 1993; Murata, 1994). These results suggested that the physical properties of the cyanobacterial plasma membrane regulated *desA* transcription.

Our studies on low-temperature adaptation in recent years have focused on the soil amoeba *Acanthamoeba castellanii*. This organism displays a very rapid and marked increase in fatty acid desaturation following low-temperature shift, which is primarily a consequence of microsomal $\Delta 12$ -desaturase induction (Jones *et al.*, 1991, 1993; Avery *et al.*, 1994a). Furthermore, this response has now been linked to the activity of an integrated physiological process; using cells acclimated at 30 °C and 15 °C, a clear correlation between desaturase induction, alterations in plasma-membrane order and the cells' capacity to continue phagocytosing at low temperature was demonstrated (Avery *et al.*, 1994a, 1995).

Jones *et al.* (1993) concluded that $\Delta 12$ -desaturase induction during low-temperature acclimation of *A. castellanii* occurred primarily by new enzyme synthesis, although some increase in enzyme activity was also related to cold-induced changes in the physical properties of the microsomal membrane. However, *A. castellanii* also displays marked changes in $\Delta 12$ -desaturase activity and membrane-lipid composition during growth in batch culture at 30 °C (Avery *et al.*, 1994a, b). The temperature independence of these changes indicates that temperature cannot be the sole trigger for desaturase induction. One alternative possible determinant is oxygen, which acts as the terminal electron acceptor in the aerobic desaturation pathway (Hazel & Williams, 1990). Oxygen concentration decreases with increasing demand during growth; thus progression of *A. castellanii* batch cultures towards stationary phase is associated with a marked decline in dissolved oxygen concentration (Edwards & Lloyd, 1977). When cultures are chilled, oxygen solubility increases. However, the correlation between these changes and those in membrane-lipid composition has yet to be investigated.

The purpose of the present study was, therefore, to examine the role of oxygen in regulating changes in $\Delta 12$ -desaturase activity during temperature acclimation of *A. castellanii*. Our main findings are that oxygen regulates the formation of new $\Delta 12$ -desaturase and the activity of pre-synthesized enzyme. These responses were found to occur independently of temperature changes.

METHODS

Organism and culture conditions. *Acanthamoeba castellanii* was grown in PGY medium, containing (% w/v): Difco proteose peptone, 0.75; Oxoid yeast extract, 0.75; glucose, 1.5. Fresh cultures (200 ml in 500 ml Erlenmeyer flasks) were inoculated from 48-h-old cultures, to give an initial cell concentration of approximately $2 \times 10^5 \text{ ml}^{-1}$, and were incubated at 30 °C with rotary aeration at 200 r.p.m. For temperature-shift experiments using whole cultures, flasks were removed, placed in an ice slurry and agitated using a magnetic stirrer. After 4.5 min, when the temperature of the cultures had decreased to approximately 15 °C, the flasks were returned to a shaking incubator (200 r.p.m.) at 15 °C.

Cell numbers were determined using a Fuchs-Rosenthal haemocytometer slide, after appropriate dilution with distilled water: more than 400 cells were counted in each sample. Membrane dry weights were determined using tared foil cups, dried to a constant weight at approximately 100 °C. Cells for lipid analysis were harvested by centrifugation for 1 min at 750 g.

[1-¹⁴C]Acetate incorporation. For acetate-labelling studies, culture samples (12 ml) were transferred to universal bottles and placed in a water-bath at either 30 °C or 15 °C. Agitation was achieved by sparging suspensions with defined air/nitrogen mixtures. The oxygen concentration of the gas phase was varied by mixing air and nitrogen (containing < 3 p.p.m. oxygen) from separate pure sources. The accuracy of mixing was confirmed by measuring the oxygen concentration of gas-saturated distilled water, using an oxygen electrode (see below). Mixing provided accurate dilution of gases in 5% steps; oxygen concentrations were taken from tables (Wilhelm *et al.*, 1977). After 10 min equilibration, approximately 110 kBq [1-¹⁴C]acetate was added directly to the suspensions. Cells were harvested after 1 h incubation in the presence of labelled acetate.

$\Delta 12$ -Desaturase assay. *A. castellanii* microsomal membranes were harvested from whole-cell homogenates by differential centrifugation, as described previously by Jones *et al.* (1993). Analyses of membrane purity revealed that > 80% of total choline phosphotransferase (a marker enzyme for the endoplasmic reticulum) activity was recovered in microsomal fractions. Microsomal $\Delta 12$ -desaturase activity was determined by measurement of the conversion of oleate to linoleate in isolated microsomal membranes. [1-¹⁴C]Oleoyl-CoA was included as a radiolabelled precursor (which becomes rapidly incorporated into 1-acyl 2-oleoyl phosphatidylcholine, the $\Delta 12$ -desaturase substrate), as described previously by Jones *et al.* (1993). Assays in 1 ml incubation mixtures were carried out in 14 ml glass tubes.

The effect of oxygen concentration on desaturase activity was determined using air and nitrogen mixtures, as described above. Immediately after the addition of radiolabelled substrate to incubation solutions, gas mixtures were blown over the surface of the solution for 30 s. The gas mixture was then bubbled into the solution for a further 10 s, by which time gas bubbles reached the tube rim. Tubes were then rapidly sealed with butyl-rubber-lined caps.

Inhibition of protein synthesis. Inhibition of protein synthesis, in the presence of anisomycin (0.1 mg ml⁻¹), was measured by [¹⁴C]leucine incorporation as described previously by Jones *et al.* (1993).

Lipid analyses. Lipids were extracted using the method of Bligh & Dyer (1959), as modified by Griffiths & Harwood (1991). For fatty acid analysis, methyl esters were generated by acid-

catalysed methanolysis (2.5%, v/v, H_2SO_4 in methanol) at 70 °C for 2 h. After extraction with light petroleum (b.p. 60–80 °C), aliquots of methyl esters were analysed by radio-gas-liquid chromatography. Pentadecanoate was used as an internal standard. Separations were routinely achieved using 10% SP-2330 on 100/120 Supelcoport (Supelco) packed into a glass column [1.5 m $\times$ 4 mm (internal diameter)]. Fatty acids were identified by comparison with authentic standards and had been fully identified previously (Jones *et al.*, 1991). Fatty acids were quantified using the method of Carroll (1961) or by using integrating software (Rachel, Lab Logic).

Oxygen determination. Changes in oxygen concentration during growth of *A. castellanii* (shown in Fig. 1b) were measured continuously by membrane inlet mass spectrometry. A HAL series quadrupole gas analyser (Hiden Analytical) was used at m/z 32. The inlet of the stainless-steel probe (1.67 mm outer diameter, 2 m long) was a 0.08 mm diameter orifice at 1 cm from the distal tip, covered with silicone rubber tubing (100 μm wall thickness) (Lloyd *et al.*, 1992). The inlet was immersed in the shaking culture.

Dissolved oxygen concentrations in samples used for [$1\text{-}^{14}\text{C}$]-acetate-labelling studies, and in cultures used for examining changes in $\Delta 12$ -desaturase levels (presented in Table 1), were measured using an oxygen electrode covered with a 25- μm -thick Teflon membrane, as described previously by Ellis *et al.* (1991).

Changes in dissolved oxygen during desaturase assays were monitored continuously using a Rank oxygen electrode, connected to a Kipp and Zonen potentiometric recorder.

In all cases, calibration was by means of air- and nitrogen-saturated distilled water; the oxygen concentrations of fully aerated systems were taken to be 230 μM at 30 °C and 305 μM at 15 °C (Jones *et al.*, 1993).

RESULTS

Influence of culture age on low-temperature-induced changes in oxygen concentration and cellular fatty acid unsaturation

Changes in dissolved oxygen concentration and in the degree of cellular fatty acid unsaturation were monitored during growth of *A. castellanii* in batch culture at 30 °C, and following chilling to 15 °C (Fig. 1). Organisms inoculated into fresh medium at 30 °C, from a 48 h culture, displayed exponential growth between 11 h and 30 h, with a doubling time of 7.8 h (Fig. 1a). Culture growth ceased after 48 h, at 10^7 organisms ml^{-1} .

Growth of *A. castellanii* was associated with a marked decline in dissolved oxygen. Little change in oxygen concentration was evident during the first 12 h growth, when the medium was almost fully oxygen-saturated (~ 230 μM oxygen at 30 °C) (Fig. 1b). However, the dissolved oxygen concentration subsequently declined to 168 μM after 24 h and 29 μM after 36 h incubation. After 48 h, oxygen was no longer detectable in the medium (limit of detection ~ 1 μM) despite rotary agitation of the flasks, although a slight increase resulted on further incubation up to 60 h. Chilling of mid-exponential-phase (24 h) cultures to 15 °C resulted in a 1.8-fold increase in the oxygen concentration of the medium (from 168 μM to 305 μM) within 1 h. Changes in oxygen content were much more marked following chilling of early-stationary-phase (48 h) cultures. Here, low-temperature shift was

Fig. 1. Influence of *A. castellanii* culture age on low-temperature-induced changes in dissolved oxygen concentration and cellular fatty acid unsaturation. Changes in (a) cell numbers, (b) dissolved oxygen concentration and (c) fatty acid unsaturation index (mean number of double bonds per fatty acid) were monitored (○) during growth of *A. castellanii* at 30 °C. The effect of chilling to 15 °C was measured after 24 h (●) and 48 h (■) growth at 30 °C. Mean values are shown from three replicate experiments $\pm$ SEM where these exceed the dimensions of the symbols.

accompanied by increases in dissolved oxygen concentration from < 1 μM to 222 μM within 20 min, and to 305 μM within 1 h (Fig. 1b).

The fatty acid unsaturation index (mean number of double bonds per fatty acid) of 48 h *A. castellanii* cultures at 30 °C was low at 1.77 (Fig. 1c). This has been correlated with the low $\Delta 12$ -desaturase activity of these cells (Avery *et al.*, 1994b). However, inoculation of 48 h cells into fresh air-saturated medium at 30 °C was followed by an increase in the relative cellular composition of unsaturated fatty acids (Fig. 1c), particularly linoleate (18:2), eicosatrienoate (20:3) and arachidonate (20:4) (Avery *et al.*, 1994b). Thus, unsaturation indices after 12 h and 24 h growth had increased to 2.09 and 2.17, respectively. Subsequently, a decline in the fatty acid unsaturation index to 2.09 after 36 h, and to 1.77 after 48 h, was evident. Chilling of 24 h cultures had no apparent effect on their unsaturation index, which remained constant at 2.17. However, the unsaturation index of 48 h *A. castellanii* cultures increased to 2.06 within 6 h and to 2.17 within 12 h of chilling. Thus, increased dissolved oxygen concentration during growth and following chilling of 48 h *A. castellanii* cultures appeared to precede increased $\Delta 12$ -desaturase activity

Fig. 2. Influence of dissolved oxygen concentration on labelling of individual *A. castellanii* fatty acids from $[1-^{14}\text{C}]$ acetate. Approximately 110 kBq $[1-^{14}\text{C}]$ acetate was added to 12 ml late-exponential/early-stationary-phase (48 h) culture samples, sparged with differing air/nitrogen mixtures. Dissolved oxygen concentrations were monitored using an oxygen electrode. Cultures were (a) maintained at 30 °C or (b) chilled to 15 °C. (a, b) Labelling of stearate (○), oleate (●) and linoleate (□) as a percentage of total labelled fatty acids after 1 h incubation with $[1-^{14}\text{C}]$ acetate. To enable direct comparison, (c) shows only percentage labelling of linoleate at 30 °C (□) and 15 °C (■). Mean values are shown from three replicate samples $\pm$ SEM where these exceed the dimensions of the symbols.

(Avery *et al.*, 1994a) and increased cellular fatty acid unsaturation (Fig. 1c).

Influence of dissolved oxygen on labelling of individual fatty acids from $[1-^{14}\text{C}]$ acetate

In order to investigate further the influence of oxygen concentration on *in vivo* fatty acid desaturation, *de novo* fatty acid synthesis at fixed oxygen concentrations was measured using $[1-^{14}\text{C}]$ acetate as a precursor. Oxygen was supplied by sparging unchilled and chilled 48 h culture samples with air/nitrogen gas mixtures, at between 5% air ($\sim 1\%$ oxygen) and 100% air ($\sim 20\%$ oxygen). Actual oxygen concentrations measured in solution are shown on the horizontal axis of Fig. 2, and were considerably lower than those supplied. Fig. 2 shows the percentage incorporation of radiolabel into stearate (18:0), oleate (18:1) and linoleate (18:2) after 1 h incubation with $[1-^{14}\text{C}]$ acetate. Some labelling of myristate (14:0) and palmitate (16:0) was also evident,

but these results were of less relevance to temperature-adaptive changes and are omitted for clarity. Rates of total $[1-^{14}\text{C}]$ acetate incorporation were approximately linear over the 1 h time course.

The relative labelling of stearate, oleate and linoleate was dependent on dissolved oxygen concentration. At oxygen concentrations below 10 μM , oleate accounted for approximately 32% of the total incorporated radiolabel at 30 °C, whereas 25% of the radiolabel was incorporated into stearate and only 3% into linoleate (Fig. 2a). However, increases in the dissolved oxygen concentration to 16 μM and then to 36 μM were accompanied, respectively, by 4.5-fold and then 1.7-fold increases in the percentage incorporation values of radiolabel into linoleate. At 75 μM oxygen, labelling of linoleate accounted for approximately 25% of total incorporated radiolabel; this proportion did not change appreciably at higher dissolved oxygen concentrations. Interestingly, an increase in oxygen concentration from 8 μM to 16 μM at 30 °C was also accompanied by a marked decline in the relative labelling of stearate measured after 1 h (to 13%) and a concomitant increase in labelling of oleate (to 39%). (It should be noted that $[1-^{14}\text{C}]$ acetate incorporation progresses through the pathway 18:0 $\rightarrow$ 18:1 $\rightarrow$ 18:2. Thus, a decline in stearate labelling can be due to increased desaturation as well as decreased stearate synthesis.) A further increase in dissolved oxygen concentration to 36 μM was associated with a decline in the relative labelling of oleate (coincident with an increase in linoleate labelling), but the proportion of label present in stearate was comparatively unaffected. No additional major changes in relative labelling of stearate or oleate were evident at higher dissolved oxygen concentrations (Fig. 2a). It should be noted that the rate of total incorporation of radioactivity from $[1-^{14}\text{C}]$ acetate increased with oxygen availability. Thus, oxygen-dependent increases in absolute rates of linoleate synthesis could be even more marked than the increases in relative rates presented here. However, the former give no information relating to changes in membrane fatty acid composition.

At 15 °C, the rate of *de novo* linoleate synthesis was lower than that of the other 18-C fatty acids at 10 μM oxygen (Fig. 2b). However, an increase in dissolved oxygen concentration to 24 μM was coincident with a very marked decline in the relative proportion of label present in stearate (from 22% to 4%) and increases in labelling of linoleate (from 5% to 9%) and oleate (from 23% to 39%). Further increases in dissolved oxygen concentration at 15 °C were associated with increases in linoleate labelling, but little alteration in labelling of stearate or oleate. Thus, linoleate accounted for approximately 14% and 16% of total labelled fatty acids at 74 μM and 232 μM oxygen, respectively (Fig. 2b).

For ease of comparison, data for ^{14}C -labelling of linoleate at 30 °C and 15 °C are presented together in Fig. 2(c). The dependence of *de novo* linoleate synthesis on dissolved oxygen concentration was influenced by the incubation temperature of the cells. At 8–10 μM oxygen, relative incorporation of $[1-^{14}\text{C}]$ acetate into linoleate was

Table 1. Influence of culture aeration on microsomal levels of $\Delta 12$ -desaturase

Microsomal membranes were isolated from cells grown for 48 h at 30 °C. Prior to membrane isolation, cultures were sparged with either air or nitrogen for 1 h at 30 °C or 15 °C. $\Delta 12$ -Desaturase activity of isolated microsomal membranes was assayed under optimal conditions at 30 °C (see Methods). Activities are not expressed as (mg protein)⁻¹ because the protein content of *A. castellanii* microsomal membranes shows temperature- and growth-stage-dependent variations (Avery *et al.*, 1994b). Rates of desaturation (calculated from percentage desaturation during 1 h incubation) are presented as means of three replicate determinations $\pm$ SEM.

Culture incubation temperature	Gas used for sparging	Anisomycin (0.1 mg ml ⁻¹)	$\Delta 12$ -Desaturase activity [pmol 18:2 formed (mg membrane) ⁻¹ h ⁻¹]
30 °C	Air	—	1150 $\pm$ 75
30 °C	Nitrogen	—	117 $\pm$ 20
15 °C	Air	—	1832 $\pm$ 68
15 °C	Air	+	1310 $\pm$ 155
15 °C	Nitrogen	—	260 $\pm$ 28
15 °C	Nitrogen*	—	1814 $\pm$ 33

* Cells chilled (and thereby transiently oxygenated) 5 min prior to bubbling with nitrogen.

approximately twofold higher in cells incubated at 15 °C than in those incubated at 30 °C. However, oxygen-dependent increases in linoleate synthesis were more marked in cells incubated at 30 °C. Thus, at approximately 75 μ M oxygen, percentage labelling of linoleate was almost twofold higher in cells incubated at 30 °C than in those at 15 °C (Fig. 2c).

Influence of culture aeration on microsomal levels of $\Delta 12$ -desaturase

Increases in the degree of fatty acid unsaturation at low temperature in *A. castellanii* reflect increased activity of the microsomal $\Delta 12$ -desaturase protein (the activities of other microsomal membrane components which could affect measured desaturation, e.g. cytochrome *b₅* or acyltransferase, are not altered) (Jones *et al.*, 1993; Avery *et al.*, 1994a; A. L. Jones, D. Lloyd & J. L. Harwood, unpublished results). The above results show that *in vivo* synthesis of the $\Delta 12$ -desaturase product, linoleate, is markedly oxygen-dependent. This may be a result of changes in (i) the synthesis of new $\Delta 12$ -desaturase protein and/or (ii) the activity of pre-existing $\Delta 12$ -desaturase. In order to examine the effect of oxygen availability on induction of $\Delta 12$ -desaturase synthesis, 48-h-old oxygen-limited *A. castellanii* cultures were sparged with either air or nitrogen for 1 h prior to cell disruption and isolation of microsomal membranes. Subsequent measurements of microsomal $\Delta 12$ -desaturase activity were performed under optimal assay conditions (Jones *et al.*, 1993), which included saturating oxygen levels. These measurements are, therefore, related to the maximum potential *in vivo* enzyme activity.

$\Delta 12$ -Desaturase levels were approximately 10-fold higher in cells sparged with air than in cells sparged with nitrogen at 30 °C for 1 h prior to isolation of microsomal membranes (Table 1). Dissolved oxygen concentrations

during sparging were found to be 93 μ M and undetectable (i.e. < 1 μ M) in air and nitrogen, respectively (results not shown). A further increase in dissolved oxygen concentration to 202 μ M, brought about by chilling air-sparged cultures to 15 °C, was correlated with a further 1.6-fold increase in $\Delta 12$ -desaturase activity of isolated microsomal membranes. This further increase was confirmed to be essentially attributable to *de novo* enzyme synthesis. Cultures supplemented with anisomycin (0.1 mg ml⁻¹), in which protein synthesis ([¹⁴C]leucine incorporation) was inhibited by approximately 60% (present study, results not shown, and Jones *et al.*, 1993), showed only a small and statistically non-significant ($P > 5.0\%$) increase in desaturase levels over cells incubated at 30 °C in the absence of anisomycin (Table 1).

Microsomal membranes of nitrogen-sparged cultures shifted to 15 °C also displayed very low $\Delta 12$ -desaturase levels. In fact, desaturase activity was more than fourfold greater in microsomal membranes from cells aerated at 30 °C than in those from cells sparged with nitrogen at 15 °C. However, when cultures were shifted to 15 °C 5 min prior to sparging with nitrogen, allowing a transient increase in dissolved oxygen concentration (see Fig. 1b), the desaturase level of microsomal membranes isolated 1 h later was approximately sevenfold higher than that of cultures sparged with nitrogen prior to chilling, and similar to that of cultures aerated at 15 °C for 1 h (Table 1).

Influence of dissolved oxygen concentration on the activity of pre-existing $\Delta 12$ -desaturase in isolated microsomal membranes

The oxygen dependence of pre-existing $\Delta 12$ -desaturase activity was determined in microsomal membranes, isolated from cells previously chilled for 1 h at 15 °C to induce $\Delta 12$ -desaturase, at assay temperatures of 15 °C and

Fig. 3. Influence of dissolved oxygen concentration on the activity of pre-synthesized $\Delta 12$ -desaturase in microsomal membranes from *A. castellanii*. $\Delta 12$ -Desaturase activity was measured following equilibration of microsomal membrane suspensions with differing air/nitrogen mixtures. Dissolved oxygen concentrations were monitored in parallel assays. Desaturation of [1- 14 C]oleoyl-CoA was measured after 1 h incubation at 30 °C (○) or 15 °C (●). [It should be stressed that this determination of desaturase activity underestimates total activity of the enzyme because of the desaturation of endogenous substrate, which occurs at approximately 10–12 times the rate found for exogenously added substrate (Jones *et al.*, 1993).] Mean values are shown from three replicate determinations $\pm$ SEM where these exceed the dimensions of the symbols.

30 °C (Fig. 3). Desaturase incubations were initially sparged with differing air/nitrogen mixtures; initial dissolved oxygen concentrations supplied are presented on the horizontal axis of Fig. 3. Measurements of changes in dissolved oxygen in parallel assays revealed a depletion of oxygen with time (results not shown). For example, during assays conducted at 30 °C, oxygen became completely depleted ($< 1 \mu\text{M}$) within approximately 3 min, 7 min and 12 min at initial oxygen concentrations of 22 μM , 68 μM and 113 μM , respectively. Oxygen depletion occurred more slowly at 15 °C, and was complete within approximately 12 min, 18 min and 37 min at initial oxygen concentrations of 37 μM , 137 μM and 195 μM . Thus, it should be emphasized that the data presented in Table 1 cannot be taken to indicate continuous rates of desaturation during 1 h incubation, but strictly refer to total levels of desaturation after 1 h.

Microsomal $\Delta 12$ -desaturase activity was markedly dependent on initial dissolved oxygen concentration. This was particularly so at initial oxygen concentrations of between 0 and 50 μM , over which range approximate 3-fold and 4.5-fold increases in desaturation were evident at 30 °C and 15 °C, respectively (Fig. 3). At an initial oxygen concentration of approximately 12 μM , desaturation after 1 h was approximately 1.7-fold higher in microsomal membranes incubated at 15 °C than in membranes incubated at 30 °C. These results appear to be consistent with

a possible role of the membrane physical environment in the control of $\Delta 12$ -desaturase activity (Jones *et al.*, 1993), although it should be noted that oxygen depletion was more rapid at 30 °C. Despite the fact that oxygen depletion occurred within 1 h in all incubations, at initial oxygen concentrations higher than 50 μM , $\Delta 12$ -desaturation showed less oxygen dependency. Thus, only an approximate 10% increase in desaturation was evident between 100 μM and 300 μM oxygen. Desaturation was similar at 15 °C and 30 °C at initial oxygen concentrations greater than 50 μM (Fig. 3).

DISCUSSION

The results presented here indicate that synthesis of unsaturated fatty acids in *A. castellanii* after growth under conditions of oxygen deficiency is markedly oxygen-dependent. These observations support the early study of Harris & James (1969), who reported increased unsaturated fatty acid synthesis in intact castor oil and sunflower seeds at elevated oxygen partial pressures. However, whereas oxygen-dependent desaturase activation or fatty acid desaturation has been inferred in certain other organisms (Rebeillé *et al.*, 1980; Bligny *et al.*, 1985; Arneborg *et al.*, 1993; Gille *et al.*, 1993), the temperature dependence of oxygen solubility is relatively slight and observed effects have thus been considered to be of little importance in the natural environment (McKeon & Stumpf, 1982). In certain organisms, oxygen over a limited range has been found to have a negligible effect on desaturation rates (Skriver & Thompson, 1976; Browse & Slack, 1983; Jones *et al.*, 1993).

In the present study, decreased oxygen concentration during growth was accompanied by diminished cellular fatty acid unsaturation, and by the marked decline in $\Delta 12$ -desaturase activity previously reported to occur between 24 and 36 h growth (Avery *et al.*, 1994b). It appears that oxygen availability in mid-exponential-phase cultures at 30 °C was sufficient for maximal $\Delta 12$ -desaturase activity. The dependency on limited oxygen availability that restricts desaturation in older cultures was alleviated following chilling because of (i) increased oxygen solubility and (ii) decreased respiratory demand (Lloyd *et al.*, 1983a).

The temperature dependence of cellular respiration appears to have been overlooked in a number of studies on fatty acid desaturation. For example, because the rate of air input was kept constant during low-temperature shift of *Escherichia coli*, Arneborg *et al.* (1993) concluded that the observed induction of desaturation was a true temperature effect. However, these workers did not measure dissolved oxygen. The importance of these considerations was highlighted by [1- 14 C]acetate-labelling experiments in the present study, where gaseous partial pressures always exceeded dissolved oxygen levels. Moreover, except at low ($\leq 10 \mu\text{M}$) oxygen concentrations, relative incorporation of radiolabel into linoleate was markedly higher at 30 °C than at 15 °C, when determined at the same dissolved oxygen concentration. Similarly, an

elevated temperature in plant leaves caused an increase in linoleate labelling at the expense of oleate, which was interpreted as being partly due to increased flux of carbon through the $\Delta 12$ -desaturase substrate, phosphatidylcholine (Harwood, 1995). Thus, in contrast to the widely accepted relationship between temperature and fatty acid desaturation (Hazel & Williams, 1990; Cossins, 1994), under conditions of oxygen sufficiency and at constant dissolved oxygen concentration, the relative rate of unsaturated fatty acid (linoleate) synthesis decreases following low-temperature shift in *A. castellanii*.

It was noted that at both 30 °C and 15 °C, an increase in oxygen concentration from approximately 8–10 μM to 15–25 μM was also associated with a marked increase in labelling of oleate, at the apparent expense of stearate. Small increases in oleate have also been observed immediately following (within 5 min) low-temperature shift of stationary-phase *A. castellanii*, prior to the onset of $\Delta 12$ -desaturation (S. V. Avery, J. L. Harwood & D. Lloyd, unpublished results). Thus, both the $\Delta 9$ -desaturase (which also uses oxygen as substrate) and $\Delta 12$ -desaturase activities of *A. castellanii* are influenced by oxygen availability. Because the effects of oxygen on $\Delta 9$ -desaturation occur at lower concentrations than on $\Delta 12$ -desaturation, one might infer that the K_m for oxygen of the former enzyme is lower.

Two possible mechanisms may account for oxygen-dependent induction of $\Delta 12$ -desaturation: (i) oxygen-dependent induction of enzyme synthesis; (ii) the oxygen requirement of the pre-existing enzyme. A dependence of only the latter on oxygen cannot, however, account for the growth-stage-dependent changes in specific activity observed previously (Avery *et al.*, 1994b). Microsomal desaturase was assayed under optimal conditions in the latter study; thus, measured changes could be attributed to the amount, and not activity, of enzyme present. Furthermore, using anisomycin as an inhibitor of protein synthesis, our present results confirmed those of Jones *et al.* (1993), who demonstrated that low-temperature desaturase induction in oxygen-deficient *A. castellanii* is largely attributable to *de novo* enzyme synthesis. Thus, the large increase in desaturase activity of microsomal membranes from cells supplied with air, compared to those supplied with nitrogen, indicates an oxygen requirement for the synthesis of $\Delta 12$ -desaturase protein in *A. castellanii*. The alternative possibility that oxygen- or nitrogen-induced covalent modification altered the relative proportion of 'active' enzyme can be discounted here. Firstly, no such mechanism has been reported for desaturases, where regulation is generally considered to occur at the transcriptional or translational level (Bossie & Martin, 1989; McDonough *et al.*, 1992; Vigh *et al.*, 1993). Moreover, it was found that cells which were transiently exposed to elevated oxygen, by chilling cultures just prior to sparging with nitrogen, still displayed considerably higher desaturase activity 1 h later than cells chilled after exposure to nitrogen. Whereas enhancement of the *de novo* synthesis of $\Delta 12$ -desaturase protein within a few minutes of chilling is quite feasible (Jones *et al.*, 1993), rapid covalent modification, in

response to altered dissolved oxygen or nitrogen, would be expected to result in similar desaturase activities of membranes from cells treated in both ways.

This is the first report that implicates oxygen in the regulation of desaturase synthesis. Oxygen-dependent regulation of gene transcription is well documented for other enzymes. For example, the abundance of mRNA transcripts, encoding enzymes involved in oxygen-radical scavenging, increases markedly during exposure of micro-organisms to elevated oxygen (Matsumura *et al.*, 1993; Unden *et al.*, 1994; Li *et al.*, 1995). Indeed, in common with the present work, inoculation to fresh (oxygen-replete) medium alone can induce synthesis of these enzymes (Chamnonngpol *et al.*, 1995). An oxygen-controlled transcriptional sensor-regulator, the FNR protein, has been characterized in *E. coli* (Unden *et al.*, 1994). It seems reasonable to speculate that a similar mechanism may mediate oxygen-controlled synthesis of $\Delta 12$ -desaturase in *A. castellanii*.

The activity of pre-existing $\Delta 12$ -desaturase, assayed using isolated microsomal membranes, was also oxygen-dependent. Substrate limitation would account for restricted desaturase activity at low oxygen concentrations, as molecular oxygen acts as the terminal electron acceptor in the aerobic desaturation pathway (Hazel & Williams, 1990). McKeon & Stumpf (1982) also observed a marked oxygen-concentration dependence of stearoyl-ACP desaturase activity from immature safflower seeds, but their calculated K_m for oxygen ($\sim 56 \mu\text{M}$) was considered to be too low to limit the activity of the enzyme *in vivo*. The rapid utilization of available oxygen during desaturase assays here may have led to rate limitation and also precluded estimation of a K_m value. Jones *et al.* (1993) reported no clear difference in desaturase activities of *A. castellanii* microsomal membranes maintained at constant oxygen concentrations of 3.5 μM and 305 μM . However, previous evidence indicates the existence of a steep oxygen-concentration gradient, of up to one order of magnitude, between the plasma membrane and internal organellar membranes of *A. castellanii* (Lloyd *et al.*, 1983b). It is therefore likely that oxygen effects on *A. castellanii* $\Delta 12$ -desaturase activity do occur, even at relatively high extracellular oxygen concentrations prevalent in laboratory culture and in the natural environment. Gradient effects may partly account for many of the reported discrepancies concerning the oxygen dependency of desaturase activity.

The oxygen dependence of desaturase activity and particularly desaturase synthesis in *A. castellanii* complements the recently described membrane-order- and fatty-acid-controlled desaturase-gene transcription in cyanobacteria (Vigh *et al.*, 1993) and yeast (McDonough *et al.*, 1992), respectively; evidently, diverse signals can invoke desaturase responses in different organisms. As in other organisms (McKeon & Stumpf, 1982; Browse & Slack, 1983), increased oxygen availability following low-temperature shift of *A. castellanii* might not be expected to stimulate desaturase activity in oxygen-sufficient environments. However, where intense biological activity leads

to oxygen depletion, oxygen-dependent desaturase induction may occur during low-temperature acclimation in the natural environment.

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