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Oxygen Influences the Subunit Structure of Cytochrome *c* Oxidase in the Slime Mold *Dictyostelium discoideum**

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The conditions that promote the alternative expression of two nuclear-encoded subunits of cytochrome *c* oxidase in the slime mold *Dictyostelium discoideum* (Bisson, R., and Schiavo, G. (1986) *J. Biol. Chem.* 261, 4373–4376) have been investigated. Oxygen concentration seems to be the only factor able to cause the subunit switching. This result indicates that the polypeptide composition of the mitochondrial enzyme can be influenced by environmental conditions. The significance of this change is discussed.

Cytochrome *c* oxidase is a multisubunit integral membrane protein present in most aerobic organisms, where it catalyzes electron transfer from cytochrome *c* to oxygen coupled to proton translocation across the membrane (1–5). In contrast with the simple subunit composition found in bacteria, up to 13 polypeptides are associated in the enzyme complex isolated from higher eukaryotes (6, 7). Sequence homologies have shown that the bacterial subunits are related to the three largest mtDNA-encoded polypeptides (7–10). Hence, these subunits contain the prosthetic groups and are responsible for the catalytic activity (7, 11–13). For the remaining eukaryotic polypeptides encoded by the nuclear genome, indirect evidence suggests a possible structural and/or regulatory role, based on the following observations: (*a*) tissue-specific subunits are present in higher organisms (6, 14); (*b*) molecular defects appear to be associated with an altered expression, import, or assembly of the nuclear-encoded subunits in some mitochondrial myopathies (15–17); (*c*) sequence homologies persist even among distantly related organisms (18–22); (*d*) cytoplasmic subunits appear to be involved in the interaction with cytochrome *c* and in the enzyme conformational changes induced by ATP (23–24).

Recently, the presence of isoforms for the nuclear-encoded subunits has been discovered also in two lower eukaryotes, the yeast *Saccharomyces cerevisiae* (25) and the cellular slime mold *Dictyostelium discoideum* (26). The latter organism is particularly interesting because "it has many of the attributes and experimental advantages of microorganisms but displays multicellular phenomena similar to those of metazoans" (27). When bacteria or other food supply is available, *D. discoideum* grows as individual amoebae. However, under starvation conditions the cells associate chemotactically to form multicellular bodies (known as pseudoplasmodia) containing up to 10^5 cells. Here, a differentiation process leads to the formation of two different cell types and eventually, to the fruiting body consisting of a balloon-like structure containing spores supported by a thin cellulose stalk. Temperature, light, oxygen, and other environmental parameters appear to influence the developmental stages (27–30).

As isolated from cells in the vegetative stage of growth, the slime mold oxidase contains only six subunits and, at variance with other eukaryotes, a single polypeptide at molecular mass lower than 6 kDa (31). A remarkable difference is found in the subunit composition of the enzyme depending on the phase of growth of the cells. The smallest cytoplasmic subunit is substituted by a larger polypeptide when cells enter the stationary phase of growth (26). The two alternative subunits, which have been termed VIIe and VIIs to distinguish their specific association with the enzyme of cells in the exponential and stationary phase of growth, respectively, appear to be structurally and immunologically unrelated. At variance with yeast, where the presence of two isoforms for one of the nuclear-encoded subunits was deduced by molecular genetics (18, 25, 32), the subunit replacement can be essentially complete in the slime mold oxidase (26).

The above findings have raised the possibility that the slime mold oxidase might respond with limited structural changes to modifications of the environmental conditions (26). In this paper, the cause of the change is analyzed and evidence for a primary role of oxygen in the trigger of the subunit switching is provided.

EXPERIMENTAL PROCEDURES

Cell Culture—D. discoideum (strain AX3) cells were grown axenically at 22 °C, in 5-liter conical flasks containing 1.5 liters of culture medium, on a gyratory shaker (shaking frequency: 120 rpm; amplitude: 40 mm). The growth medium contained (in distilled water) (grams·liter^{-1}): Peptone (Becton Dickinson), 10; yeast extract (Difco), 5; glucose, 10; $Na_2HPO_4 \cdot 2H_2O$, 0.616; KH_2PO_4, 4.86. The pH of the medium was adjusted to 6.7 with NaOH or H_3PO_4 before autoclaving. Modifications in the environmental and growth conditions were as detailed in the figure legends. Always before harvesting, cells were counted to evaluate their growth efficiency (defined as the ratio between the cell density in the sample and in the control) in the different experimental conditions. At the end of each experiment, the cell viability was also checked by comparing the growth efficiency of culture samples with respect to the control under identical conditions; only experiments in which the cell viability was indistinguishable from the control are reported in the present work.

Development of D. discoideum—Cell development was induced by washing the amoebae twice in a buffer containing (in distilled water) (grams·liter^{-1}): KH_2PO_4, 0.197; $Na_2HPO_4 \cdot 2H_2O$, 0.356; pH 6.1. The cells, free of nutrients, were then deposited on a 1.5% agar film prepared with the same buffer. Aggregates became visible after 2–3 h, and the differentiation process was completed approximately 20 h later.

Polyacrylamide Gel Electrophoresis—SDS[1]-polyacrylamide gel elec-

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[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

trophoresis was performed according to Kadenbach *et al.* (33) on a vertical slab gel electrophoretic apparatus. Purified cytochrome *c* oxidase samples equivalent to 20 μg of protein were loaded for Coomassie Blue staining. Staining of the enzyme subunits in the mitochondrial membranes was performed by immunodetection on blots as described below. The mitochondrial membranes loaded on gels contained the same amount (0.5 μg) of oxidases, spectrophotometrically determined by dissolving the membranes in 50 mM sodium phosphate, 150 mM NaCl, 2% Triton X-100, pH 7.8, at 4 °C, in the presence of protease inhibitors (31). A series of samples containing a progressively reduced amount of enzyme, obtained from mitochondria of cells in stationary phase of growth, was also present in the gel; they were used for the calculation of the working curve necessary for the standardization of the peak areas obtained by densitometric scanning. Zig-zag scanning was performed with a Shimadzu model CS-930 dual-wavelength scanner.

Immunostaining Techniques—The electrophoretic transfer of proteins from slab gels, after SDS-polyacrylamide gel electrophoresis, to nitrocellulose sheets was carried out essentially as reported by Towbin *et al.* (34) in 25 mM Tris-Cl, 192 mM glycine, 5% (v/v) methanol, pH 8.3, at 0.2 A for 5 h, using a Hoefer TE Transphor apparatus. The nitrocellulose sheets were then soaked for 2 h at room temperature in 3% bovine serum albumin in saline (50 mM Tris-Cl, 2 mM $CaCl_2$, 85 mM NaCl, pH 8.0). The subsequent incubations with antiserum and with either radioactively iodinated or alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) were as described previously (31). In the latter case, antibodies were stained colorimetrically according to Leary *et al.* (35).

RESULTS

A change in the subunit structure of *D. discoideum* oxidase is evident when cells enter the stationary phase of growth (26). In this process the smallest enzyme component (subunit VIIe) present in exponentially growing cells is replaced by a larger polypeptide (subunit VIIs) structurally and immunologically different from any other protein present in the mitochondrial membrane (26).

The observation that the subunit switching begins close to the stationary phase of growth indicates the increasing cell density (and the consequent changes in the culture medium) to be at the origin of the phenomenon. Both a lowering of the concentration of a nutrient(s) and/or the accumulation of a product(s) of the cell metabolism could promote the subunit replacement.

The involvement of a volatile component is suggested by some simple observations. For example, the appearance of the alternative oxidase form defined by the presence of subunit VIIs is influenced by the geometry of the culture flasks. In particular, the synthesis of this polypeptide, normally found in the stationary phase, can also be induced in exponentially growing cells by reducing the opening of the flasks.

This finding demonstrates that a free exchange of volatile components between the culture medium and the external environment is important, although no conclusions about the real origin of the phenomenon can be drawn. Several volatile products, in fact, are produced as result of the slime mold cell metabolism (NH_3, ethane, ethanol, ethylene, acetaldehyde (36)), and their increasing concentration could promote the alternative subunit expression. On the other hand, lack of oxygen close to the stationary phase has been frequently described as one of the most important environmental factors that can influence the expression of terminal oxidases in prokaryotes (37–40).

A dramatic decrease in oxygen concentration is in fact observed in the culture medium of *D. discoideum* cells approaching the stationary phase (Fig. 1). The data are particularly interesting when directly compared with the relative amount of the two oxidase forms present in the mitochondrial membrane. As shown by Fig. 1, there is a clear correlation between the decrease in oxygen concentration and expression

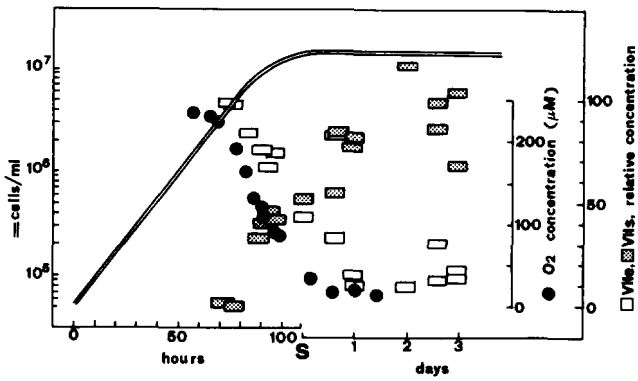

FIG. 1. **Oxygen concentration decreases dramatically during the transition from exponential to stationary phase of growth.** The oxygen concentration (●) of culture samples, measured polarographically with a Clark-type oxygen electrode, is reported as function of the cell density from middle exponential to late stationary phase of growth. For a better comparison, the result is overlapped with previous data (26) which show the subunit switching as it appears by monitoring the relative amount of subunit VIIe (□) and VIIs (▩) in several purified cytochrome oxidase samples, after SDS-polyacrylamide gel electrophoresis, Coomassie Blue staining, and quantitative densitometric scanning.

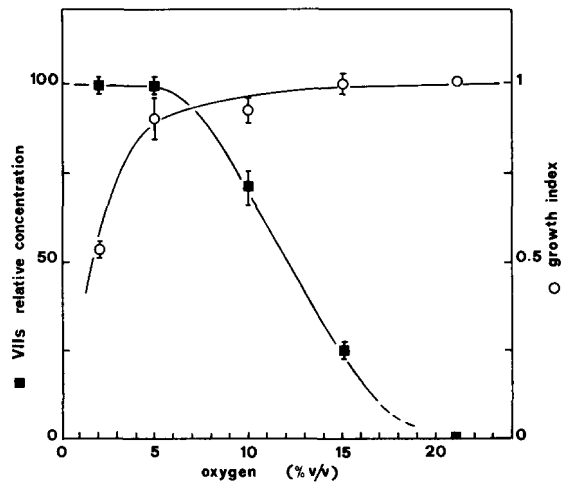

FIG. 2. **Subunit VIIs expression under reduced oxygen tension.** Culture flasks containing vegetatively growing cells in mid log phase (0.5×10^6 cells/ml) were fluxed with nitrogen/oxygen mixtures (1.5 liters/min) containing an oxygen concentration variable from 2 to 21% (v/v). After 24 h of exponential growth (final cell concentration in the control: 4×10^6 cells/ml), cells were harvested, the mitochondrial membranes recovered, and the content of VIIs (■) analyzed by quantitative immunoblotting using alkaline phosphatase-conjugated goat anti-rabbit IgG, essentially as described under "Experimental Procedures." The amount of subunit VIIs is reported as the percentage of its maximal value (normally found in cells in stationary phase of growth). At the end of the experiment, the cell density in the different samples was also monitored: the growth index (○) is the ratio between these values and the cell concentration in the control (growth under normal oxygen tension).

of the alternative oxidase form.

This observation suggests an involvement of oxygen in the subunit change, but at the same time it does not exclude other possibilities mentioned above.

The effect of oxygen can be investigated directly. Cells are grown in an atmosphere containing defined oxygen concentrations, obtained by fluxing the culture flasks with mixtures of N_2/O_2 in different proportions, under the experimental conditions described in Fig. 2. Despite the exponential cell growth, a small decrease in environmental oxygen concentration is sufficient to induce the expression of subunit VIIs.

The amount of the alternative subunit reaches its maximal value when the atmosphere contains 5% oxygen and does not change by further decreasing the oxygen concentration. As shown in Fig. 2, cell growth is unaffected by a decrease of oxygen in the range of values higher than 5%, while below this threshold small fluctuations in oxygen tension dramatically affect growth.

Hence, it would appear that cells can cope with a lowering of the environmental oxygen concentration as long as they can replace the oxidase form normally present in the exponential phase of growth with the enzyme typical of the stationary phase; when this process is completed, a further decrease in oxygen tension leads to a reduced growth efficiency.

If oxygen concentration is the only factor influencing the subunit switching, it should be possible to prevent the expression of subunit VIIs, in cells entering the stationary phase, simply by increasing the relative amount of oxygen well above the value normally found in the atmosphere. Fig. 3 shows that, even after 2 days in the stationary phase, cells grown under an atmosphere with 40% oxygen do not contain in their mitochondrial membranes any significant amount of subunit VIIs, while the same polypeptide approaches its maximal level in the control.

From these data, subunit switching appears to be a process activated in response to environmental stress. This interpretation poses several questions concerning the rate of the process, its reversibility, and ultimately its function.

An answer to the first question is reported in Fig. 4. Exponentially growing cells are suddenly exposed to an atmosphere containing 2% oxygen. Under this condition and at the particular cell density (3×10^6 cells/ml) used in the experiment, cells stop growing but remain viable. The synthesis of the alternative subunit form, triggered by low oxygen tension, is now monitored as a function of time. After 2 h, a relevant amount of subunit VIIs is already present in the mitochon-

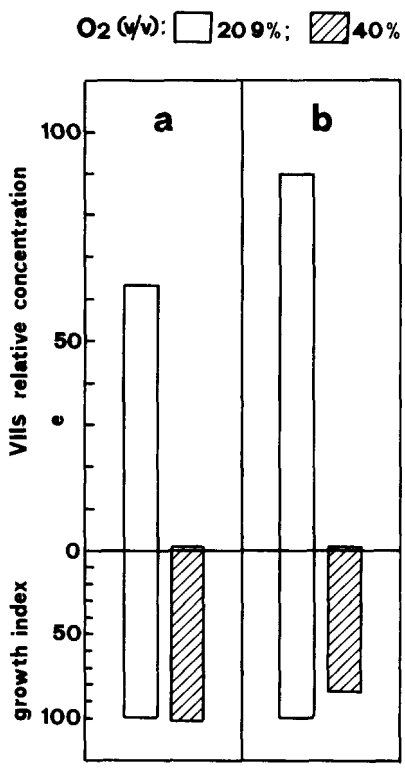

FIG. 3. **High oxygen concentration prevents the subunit change.** Culture flasks containing cells in the middle log phase (4×10^6 cells/ml) were fluxed with a nitrogen/oxygen mixture containing 40% oxygen, for either 24 (*a*) or 48 (*b*) h; thus, harvesting was performed at early stationary or late stationary phase of growth, respectively. Mitochondrial membranes were recovered and analyzed as described in Fig. 2 and under "Experimental Procedures." Despite the long time spent in the stationary phase, cells grown in an oxygen-enriched atmosphere (▨) do not show the presence of subunit VIIs which, as shown by the controls (□), is characteristic of this phase of growth. As indicated by the growth index (*bottom panel*), cells double normally in both conditions in the first 24 h. A slight decrease in the cell density is noticeable in the oxygen-enriched culture after 2 days, possibly because of toxic effects of a prolonged exposure to high oxygen concentration.

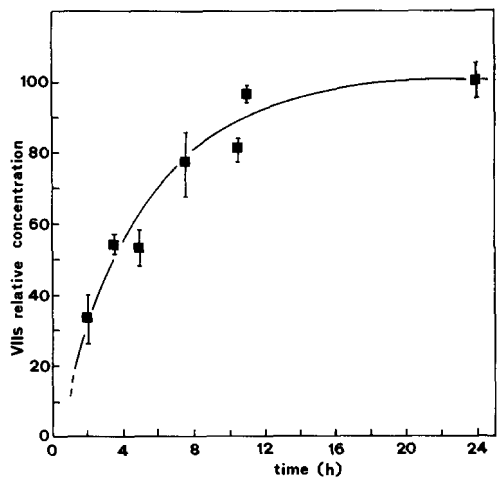

FIG. 4. **Time course for subunit VIIs biosynthesis.** Exponentially growing slime mold amoebae (3×10^6 cells/ml) were suddenly fluxed with a nitrogen/oxygen gas mixture containing 2% (v/v) oxygen. Cells were harvested at different times and the presence of subunit VIIs monitored by quantitative immunoblotting as reported in Fig. 2 and under "Experimental Procedures."

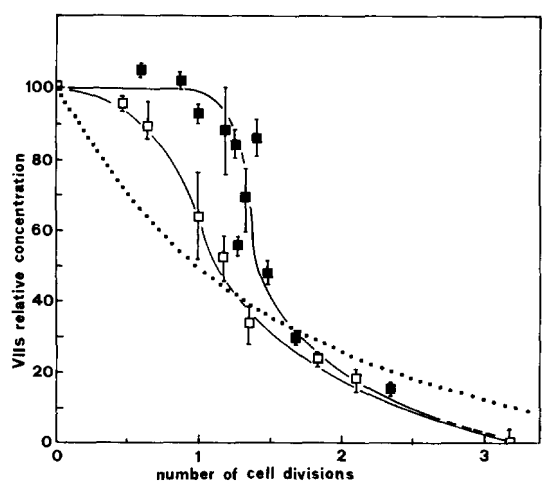

FIG. 5. **The cytochrome *c* oxidase subunit change is reversible.** Cells containing only the oxidase form that is synthesized in a poorly oxygenated environment were inoculated into a fresh culture medium (initial concentration 0.5×10^6 cells/ml) where they grew exponentially. The patterns shown in the figure refer to cells previously left in the stationary phase of growth for 2 days (■) and to cells grown exponentially for 1 day but in culture flasks where the diameter of the neck was reduced from 45 to 11 mm (□). In the fresh culture medium, the presence of a normal concentration of oxygen induces the replacement of subunit VIIs by VIIe. The rate of decreasing of subunit VIIs, however, cannot be explained as a simple dilution of the polypeptide as a function of the cell growth (*dotted line*); rather, there is an initial lag phase whose length seems to be dependent on the time spent by the cells in the oxygen-limited environment. Nevertheless, the amount of subunit VIIs is always negligible at the end of the second doubling cycle.

drial membranes. Subunit replacement is completed in about 10 h. This apparent slowing of the process could be due to limitations of cell metabolism at very low oxygen concentration, as suggested by the fact that, under these experimental conditions, cells cannot grow.

A different pattern is found when the reverse process is analyzed. In this case, the cells are first grown under an oxygen-limited supply (either exponentially or in the stationary phase) and then diluted in a fresh culture medium where they can grow exponentially, under normal oxygen concentration. These latter conditions favor the replacement of the oxidase form prevailing at low oxygen tension, monitored by following the disappearance of subunit VIIs. As shown in Fig. 5, the synthesis of subunit VIIs does not stop immediately, but continues for a number of hours apparently correlated with the time spent by the cells under low oxygen tension. Then, the replacement takes place in a time scale comparable to that of the opposite process shown by Fig. 4, and it is essentially complete at the end of the second doubling cycle. Hence, the removal of subunit VIIs is an active and reversible process that cannot be explained simply as a dilution of a preexisting product, represented by the *dotted line* in Fig. 5.

DISCUSSION

A highly complex picture is emerging from the most recent studies on the oxidase nuclear genes of higher eukaryotes. Pseudogenes, in addition to "isogenes," have been found; some of them resemble processed genes but with typical features of functional genes (20, 41, 42). Earlier observations suggesting that tissue specificity overrides species specificity are now confirmed by sequence homologies (6, 14, 20), but new, complicated aspects begin to be disclosed. Amino acid substitutions in the primary structure of two subunit isoforms can involve more than 50% of the sequence (20, 43). Different isoforms of the same subunit are simultaneously expressed in certain tissues (43). Moreover, some oxidase isoenzymes of higher organisms might be a mosaic of subunit isoforms specific to different tissues (44).

This structural heterogeneity of the enzyme[2] is difficult to explain, even admitting a functional role for some of the nuclear-encoded subunits. Indeed, functional studies on various isolated eukaryotic isoenzymes have not shown differences that can be attributed univocally to a particular subunit set (45, 46).

The recent discovery of cytochrome *c* oxidase isoenzymes in lower eukaryotes (25, 26) may offer alternative approaches to the problem and facilitate the study of the molecular mechanisms that control the differential expression of the oxidase genes.

In this paper, the possible factors that can be responsible for the subunit switching of *D. discoideum* cytochrome *c* oxidase have been analyzed. All evidence specifically links the phenomenon to the oxygen tension in the culture medium. Changes in the concentration of the carbon source and other nutrients were found to affect the cell doubling time and concentration at the stationary phase of growth, but never the expression of the two alternative subunit forms (data not shown).

The understanding of the molecular mechanisms which allow oxygen to modulate the subunit composition of cytochrome *c* oxidase certainly requires further investigation. Our studies, however, do not support the possibility that the synthesis of subunit VIIs, the polypeptide present at low oxygen tension, is a consequence of a reduced oxidase activity or an artifact due to cell damage. The first conclusion is suggested by experiments where the enzyme is inhibited by cyanide. Because of the presence of a branched respiratory chain containing an alternative, cyanide-insensitive oxidase, *D. discoideum* cells can survive in the presence of 200 μM cyanide, an amount of inhibitor sufficient to block the cytochrome *c* oxidase activity (data not shown). Although they are unable to grow under these conditions, cells remain viable, but no trace of subunit VIIs is found in mitochondria. Moreover, most of the different experimental conditions used to promote the subunit switching do not have any effect on the cell doubling time, viability, and morphology, as expected in the case of cell damage.

As shown in Figs. 4 and 5, the need for the two alternative subunits does not appear to be "symmetric." The presence of subunit VIIs seems an absolute requirement under low oxygen tension. This is suggested by the rapid triggering of its synthesis (Fig. 4) and is confirmed further by the data of Fig. 2 (see comment under "Results"). With the same polypeptide, however, cells can grow normally for several hours under normal oxygen tension; as shown in Fig. 6, subunit VIIs continues to be synthesized even after the removal of the environmental stress. Although the present data do not allow a clear interpretation, it seems that the environmental danger signal can be *memorized* by the cell for a limited amount of time. The presence of an intermediate able to influence the subunit VIIs expression, whose concentration depends on the time spent by the cells under oxygen-limited conditions, is a possible explanation. Accordingly, the absence of cell division

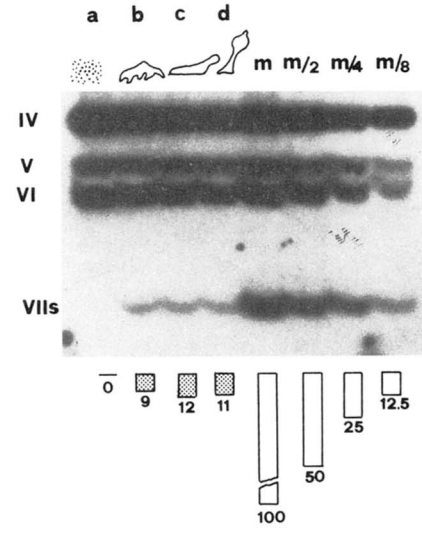

FIG. 6. **Cytochrome *c* oxidase subunit change in the developmental stages of the slime mold.** Mid-log phase cells (4×10^6 cells/ml) were induced to develop and differentiate as described under "Experimental Procedures." At different times, corresponding to different steps of the developmental process (shown by simple schemes at the *top*), the aggregates were collected and the mitochondrial membranes analyzed. *Lane a* refers to cells just after harvesting (*control*). *Lanes b–d* show the incorporation of VIIs in the mitochondrial membranes of amoebae from early aggregates (3–5 h), migrating slugs (8–10 h), and at the beginning of the culmination (14–16 h), respectively. Samples containing progressively diluted mitochondrial membranes (*m*), taken from cells in stationary phase, were also included for standardization of the peak areas, as described in Fig. 2 and under "Experimental Procedures." *Shaded* and *open bars* refer to the relative amount of subunit VIIs in the samples and in the controls, respectively. Antigen-antibody complexes were detected by autoradiography after binding of radioactively iodinated goat anti-rabbit IgG (31).

[2] It should be noticed, however, that the large number of different isoenzymes suggested by the above findings is at the moment limited by the fact that isoforms for only 4 of the 10 nuclear-encoded polypeptides have been found (14, 15, 20).

in the stationary phase accentuates this effect (Fig. 5).

That the influence of oxygen is probably exerted through an "oxygen sensor" is suggested by the chemotactic and morphogenetic response of *D. discoideum* cell clumps to oxygen gradients (30). The possibility that the structural change in the mitochondrial enzyme might be part of these more general processes is at the moment only a fascinating speculation which, however, raises the problem of the function of this subunit change.

Previous studies on the isolated, detergent-solubilized enzymes, using conventional spectroscopic and polarographic techniques, do not show any significant difference between the two slime mold oxidase forms (26). These observations, of course, cannot rule out the possibility of a different behavior of the two isoenzymes *in vivo* where, for example, specific interactions with intracellular components could modulate the enzyme activities (24, 45, 47, 48).

A selective advantage for the organism would be evident if the two oxidase forms have a different affinity for oxygen. In this context an apparent contradiction is offered by the data of Fig. 1, with the oxidase alternative form beginning to assemble at oxygen concentrations (100–200 μM) well above the K_m value for oxygen of a eukaryotic oxidase (0.3–3 μM) (49). However, a response given too late, namely at very low oxygen tension, could be too dangerous for a strictly aerobic organism such as the slime mold.[3] On the other hand, a different oxygen affinity is not to be taken as the sole possibility. In fact, the subunit change could influence other characteristics of the enzyme such as, for example, its proton pumping efficiency or, as mentioned above, its interactions with intracellular components; this in turn could affect other little known properties of cytochrome *c* oxidase. It has been reported, for example, that the enzyme can also exhibit catalase, peroxidase, superoxide dismutase, and carbomonoxy oxygenase activities, in addition to the oxygen reduction function (53).

The low K_m value for oxygen, typical of the eukaryotic oxidases, does not support the possibility that the concentration of this substrate might ever be limiting for the slime mold. However, an indication that oxygen concentration is important for this strictly aerobic organism comes from a careful consideration of its lifestyle. *D. discoideum* is found in nature as soil amoeba in forest detritus. The cells feed on bacteria of decaying fallen leaves and other decomposing matter. In this environment, competition for oxygen may be important. The possibility of surviving at low oxygen concentration could also enable the slime mold cells to reach additional sources of food such as anaerobic bacteria. Moreover, during the developmental stage, the amoebae aggregate in multicellular bodies (pseudoplasmodia) which include 10,000 cells at least and have a diameter between 0.2 and 0.4 mm (27–29). These developing cells exhibit chemotactic and morphogenetic responses to oxygen, light, and heat; these remarkable properties seem necessary for the migrating slugs to move out of soil so that fruiting can take place on the surface. When the cells are forced to aggregate in the laboratory, the oxygen concentration is certainly higher than in their natural environment. Nevertheless, when the mitochondrial membranes of aggregating cells in the different stages of the development were analyzed, the results shown in Fig. 6 were obtained. Subunit VIIs is now a sensitive "oxygen probe." The fact that this subunit appears as soon as the amoebae aggregate suggests that part of the cells (presumably those located in the center of the aggregate) are under a limited supply of oxygen. Hence, it is probable that in the more hostile environment of the forest soil the presence of the oxidase form found at low oxygen tension might be more the rule than the exception.

In conclusion, these data offer an example of environmental influence on the subunit composition of a mitochondrial enzyme. For the reasons mentioned above, it is particularly intriguing that this enzyme is cytochrome *c* oxidase and that the regulating parameter is the concentration of one of its substrates.

These observations recall a situation found frequently in prokaryotes where structurally and functionally different terminal oxidases, present in highly branched respiratory chains, are expressed in response to changes in the environmental conditions (37, 38). Eukaryotes appear to have evolved a different strategy based on adaptation of the same catalytic core, formed by the three largest subunits of cytochrome *c* oxidase, to the increasing complexity of the organisms. The presence of interchangeable nuclear-encoded subunits, as found in *Dictyostelium* and suggested by the existence of isoenzymes, may represent a mechanism for "fine tuning" the enzyme. In this context, an important question concerns the extent of the functional differences that would result. Presumably, they should be rather small if, for example, two completely different oxidases have been developed in *Escherichia coli* to cover a change of 1 order of magnitude in the affinity for oxygen (38). This raises the problem of measuring such differences particularly when technically difficult and when using enzymes isolated from their natural environment. In this situation, useful approaches can be offered by molecular genetics as recently shown in the yeast *S. cerevisiae* (18, 54, 55).

The characterization at the molecular level of the process described in this work seems now to be one of the possible useful steps toward an understanding of the complex problem concerning the presence of isoforms of cytochrome *c* oxidase in higher organisms.

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[3] Very recently, a dependency of mitochondrial oxidative phosphorylation on oxygen concentrations up to at least 20 μM has been reported (50). Moreover, a major controversy concerns the intracellular oxygen gradients and diffusion (51, 52).

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