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Cytochrome *c* Oxidase from the Slime Mold *Dictyostelium discoideum*: Purification and Characterization†

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ABSTRACT: Cytochrome *c* oxidase was purified from growing cells of the slime mold *Dictyostelium discoideum* by a procedure based on hydrophobic and affinity chromatography. A highly pure (13.4–15 nmol of heme *a*/mg of protein) and active (turnover number = 280–330 s^{-1}, when assayed polarographically with the slime mold cytochrome *c*) enzyme preparation was obtained. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, under conditions where the 12 polypeptide components of the bovine enzyme are resolved, shows that the amoeba oxidase consists of six subunits with molecular masses of 55, 29.5, 19, 13, 11, and 5.7 kDa. A polypeptide with the characteristics of the eukaryotic subunit III is missing, and *N*,*N*′-dicyclohexylcarbodiimide, a specific reagent for this component, labels subunit I. Under controlled conditions and even at physiological pH, the single subunit present at M_r <10000 can be selectively removed from the complex. Hydrophobic photolabeling suggests that with the mitochondrial subunits I and II only subunit IV among the nuclear coded polypeptides is in contact with lipids.

One of the most intricate aspects of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, concerns the number and function of its subunits. In higher eukaryotes up to 12–13 polypeptides are associated in the protein complex while, in contrast, only one to three of them are found in bacterial organisms where they seem sufficient to carry out the known enzyme functions (Buse et al., 1983; Ludwig, 1980; Yoshida et al., 1984). These observations are used to support growing biochemical evidences indicating that subunits I and II are the catalytic components of oxidase; the two polypeptides, in fact, appear to contain the prosthetic groups and bind the substrate (Bisson et al., 1978, 1982; Winter et al., 1980; Darley-Usmar et al., 1981; Capaldi et al., 1983; Suarez et al., 1984). Subunit III, the third largest enzyme component coded for by mDNA, has been implicated in the proton-pumping process although much controversy still exists concerning its specific function (Casey et al., 1980; Prochaska et al., 1981; Sarti et al., 1985). The role of the remaining nuclear coded polypeptides remains obscure, but their importance has been recently emphasized by the sequence homologies found in oxidases from different unrelated sources (Gregor & Tsugita, 1982; Power et al., 1984a) and by the discovery of tissue-specific isoenzymes (Kadenbach, 1983).

Although many recent studies have been focused on bacterial oxidases because of their structural simplicity, investigations on the enzyme of suitable eukaryotic sources may offer new insights on the possible roles of the cytoplasmic subunits

† This paper is dedicated to the memory of Robert P. Casey.
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as well as a better understanding of the mitochondrial polypeptide functions. A potentially very interesting organism, because of its unique life cycle, is the slime mold *Dictyostelium discoideum*. This lower eukaryote can, in fact, grow as a single amoeboid cell either in suspension or on a suitable substrate. However, under starvation conditions, the cells aggregate, forming multicellular bodies called pseudoplasmodia. Here, an elementary differentiation process takes place that, eventually, leads to the formation of the fruiting body: a cellulose stalk holding on top a balloon-like structure filled with spores. During this process many complex events occur within the cell: several new cellular components are synthesized; others are destroyed or differently regulated to meet the modified cellular needs (Loomis, 1975, 1982). Energy requirements and oxygen metabolism are also affected (Woffendin & Griffiths, 1984), and cytochrome *c* oxidase could be, at least in principle, involved.

Investigations on these possibilities require as a first step the purification and characterization of the enzyme in the growing cells. The procedure should be highly reproducible and flexible enough to adapt to different physiological situations and to other organisms for comparison.

This work utilizes a simple and rapid chromatographic technique that allows the isolation of the amoeba oxidase in a pure and active form from milligrams of mitochondrial protein. The structural and kinetic properties of the enzyme are analyzed and compared with the most studied bovine oxidase.

Experimental Procedures

Cell Culture. Axenic cultures of *Dictyostelium discoideum* (strain AX3) were grown at 22 °C as described elsewhere (Spudich, 1982). Normally, 2 L of cells were grown in 6-L flasks on a gyratory shaker at 120 rpm and harvested in mid-log phase [(3–5) × 10^6 cells/mL] by centrifugation at 850*g* for 5 min. The amoebae were then washed with chilled distilled water and finally resuspended as a thick slurry in 0.25 M mannitol, 20 mM Tris-HCl,[1] and 4 mM EDTA, pH 7.6, supplemented with the following protease inhibitors: 40 μM phenylmethanesulfonyl fluoride (PMSF), 20 μM *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and 10 μM L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) (buffer A).

Preparation of Mitochondria. Mitochondria were obtained by a modification of the method of Stuchell et al. (1975). Cell membranes were disrupted by mechanical homogenization with a Polytron (type PT 10 20 3500, Kinematica Gmbh, Luzern CH), the power control was set to position 6, and two cycles of 20 s each were used, followed by a 1-min interval.

The homogenate was diluted with buffer A and centrifuged at 3000*g* for 1 min. The supernatant was saved, and the homogenization procedure described above was repeated on the pellet. The two supernatants were then pooled and centrifuged for 10 min at 750*g*. The pellet was discarded and the supernatant centrifuged at 7700*g* for 15 min. The mitochondrial pellet was washed once with buffer A, adjusted to 20 mg/mL, and treated with digitonin (3 mg/g of protein) added from a stock solution in Me_2SO, for 20 min. The mitochondrial suspension was then diluted and centrifuged at 8000*g* for 15 min. The pellet was washed again with buffer A, and the mitochondria were stored at −80 °C. All the operations reported above were performed at 2 °C, and the pH was constantly kept at 7.6.

Isolation of Cytochrome c Oxidase. The thawed mitochondrial membranes were washed in the presence of the protease inhibitors at the concentrations reported above, with (a) distilled water, (b) 50 mM potassium phosphate and 0.2 M KCl, pH 7.6, repeated once, and (c) 20 mM potassium phosphate, pH 7.6. The membranes recovered by centrifugation at 34000*g* were finally resuspended in 0.66 M sucrose, 50 mM Tris-HCl, and 1 mM histidine, pH 7.6 (TSH), at a concentration of 20 mg/mL for the subsequent "green membrane" separation. This step was performed essentially according to Errede et al. (1978). Briefly, to the mitochondrial membranes, 75 mg/mL of KCl and 0.3 mg of KDOC (from a 20% stock solution) per milligram of protein were added. The suspension was stirred for 10 min and then centrifuged at 105000*g* for 30 min. The greenish pellet, which contained more than 90% of the total oxidase, was resuspended in TSH at 20 mg/mL. A total of 75 mg/mL of solid KCl and 0.6 mg of KDOC/mg of protein were then added, and the suspension, after being stirred for 20 min, was centrifuged at 10500*g* for 20 min. The green supernatant was diluted 1:1 with 2% sodium cholate and the crude oxidase precipitated with ammonium sulfate, taking the fraction between 20 and 37% of saturation. The pellet, dissolved in 200 mM Tris–acetate, 0.75% potassium cholate, and 0.5% potassium deoxycholate, pH 8.0 (buffer B), was either frozen or immediately used for the next purification step. At this stage, cytochrome *c* oxidase contributed more than 85% to the visible spectra of the solubilized pellet. This latter aspect is very important as far as the final purity of the preparation is concerned.

The enzyme solution was then applied to a phenyl-Sepharose CL-4B (Pharmacia) column (10 mg of protein/2 mL of resin) previously equilibrated with buffer B containing 3 M KCl. Washings were performed with the same buffer, but the KCl concentration was discontinuously decreased to 1.5 and 0 M before 20 mM Tris–cacodylate and 1% sodium cholate, pH 7.6, were used. Buffers were changed when the UV absorption base line plateaued even though prolonged washings did not appear to give significant loss of enzyme. Cytochrome aa_3 was eluted with 0.1 M $NaHCO_3$, 0.1 M NaCl, and 1% Triton X-100, pH 8.2. Fractions containing the enzyme were immediately pooled and diluted 1:1 with 50 mM Tris-HCl, 2% sodium cholate, and 5% sucrose, pH 7.4. The protein was precipitated at pH 7.4 with 40% ammonium sulfate and dissolved in 25 mM Tris–cacodylate and 0.1% LM, pH 7.4, for storage in small aliquots at −80 °C. Alternatively, the pooled fractions were diluted with 5 volumes of 10 mM Tris–cacodylate and 0.1% LDAO, pH 7.5 (buffer C), and the protein was chromatographed on an affinity matrix of CH-Sepharose 4B–yeast ferrocytochrome *c*. The resin was prepared essentially according to the manufacturer's instructions (Pharmacia), performing the reaction at room temperature for 30 min in a rotatory shaker. Fifteen milligrams of cytochrome *c*/g of activated CH-Sepharose 4B (Pharmacia) were used for covalent binding. Washings and subsequent handling of the resin were performed in a cold room.

After binding to the affinity column, the enzyme was subsequently washed with (a) buffer C and (b) the same buffer plus 25 mM NaCl. Oxidase was eluted by increasing the NaCl

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; TN, turnover number; Tris base, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LDAO, dodecyldimethylamine oxide; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; [^{14}C]PLI, 1-myristoyl-2-[12-[(4-azido-2-nitrophenyl)amino]dodecanoyl]-*sn*-glycero-3-[^{14}C]-phosphocholine; [^{3}H]PLII, 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Me_2SO, dimethyl sulfoxide.

concentration to 100 mM. Fractions containing the enzyme were again concentrated by precipitation with ammonium sulfate, and the protein, dissolved in 25 mM Tris–cacodylate and 0.1% lauryl maltoside, pH 7.4, was stored in small fractions at −80 °C after freezing in liquid nitrogen.

Purification of D. discoideum Cytochrome c. The 50 mM potassium phosphate–0.2 M KCl supernatants used to wash the mitochondrial membranes were pooled and extensively dialyzed (3 days, Sigma dialysis tubing) against distilled water. After centrifugation, the yellow solution was applied to an Amberlite CG50 (−400 mesh) column and washed with distilled water. Cytochrome *c* was then eluted with 1 M NaCl, 5 mM ascorbate, and 10 mM potassium phosphate buffer, pH 7.4. The pink fractions were pooled, dialyzed against distilled water, and, after centrifugation to eliminate turbidity, lyophilized. The protein was then dissolved in a minimum volume of distilled water, again cleared of turbidity by centrifugation, and loaded onto a Sephadex G-50 fine (1 × 50 cm) column equilibrated in 5 mM Tris–cacodylate, pH 7.6. Fractions containing cytochrome *c* were again pooled and lyophilized. Cytochrome *c* was finally dissolved in a minimum volume of distilled water, dialyzed against 5 mM Tris–cacodylate buffer, pH 7.6, and stored at −80 °C. All the steps reported above were carried out at 2 °C.

Spectral Measurements. Spectrophotometric determinations were performed on a Perkin-Elmer Lambda 5 UV/vis spectrophotometer. The following extinction coefficients were used to calculate concentrations: cytochrome *c* oxidase, $\Delta\epsilon$(606–630) = 24 mM^{-1} cm^{-1}; cytochrome *b*, $\Delta\epsilon$(560–575) = 23.4 mM^{-1} cm^{-1}. Both were for the difference spectra (dithionite reduced minus air oxidized). The concentration of cytochrome *c* was calculated from $\Delta\epsilon(550\text{–}630)_{reduced}$ = 28 mM cm^{-1}.

Enzyme Assay. Oxygen consumption was measured polarographically with a Clark O_2 electrode at 25 °C in a 1.3-mL water-jacketed chamber. Oxidase samples (0.032 nM aa_3) were assayed in 20 mM Tris–cacodylate, pH 7.6, in the presence of 3 mM ascorbate, 0.69 mM TMPD, and 0.05% lauryl maltoside at 25 °C over a cytochrome *c* concentration range of 0.02–30 μM. Mitochondrial membranes were solubilized before the assay as reported by Thompson & Ferguson-Miller (1983). Oxidase activity was also measured spectrophotometrically at 550 nm in 25 mM Tris–cacodylate, pH 7.6. Egg lysolecithin (0.02%) or lauryl maltoside (0.05%) was used in the assay buffer. Ferrocytochrome *c* was prepared as previously reported (Errede et al., 1978).

Labeling Experiments. Arylazido phospholipids used for hydrophobic photolabeling were prepared according to Bisson & Montecucco (1981). The specific radioactivity of 1-myristoyl-2-[12-[(4-azido-2-nitrophenyl)amino]dodecanoyl]-*sn*-glycero-3-[^{14}C]phosphocholine ([^{14}C]PLI) and 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine ([^{3}H]PLII) was 174 mCi/mol and 2.8 Ci/mmol, respectively. 3-(Trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine ([^{125}I]TID) was from Amersham, and its specific radioactivity was 10 Ci/mmol. For the labeling experiments, [^{14}C]PLI and [^{3}H]PLII were gently dried on the bottom of the reaction vessel with a nitrogen stream. The enzyme (80 μg), solubilized in (200 μL) 10 mM Hepes, 27 mM KCl, 73 mM sucrose, and 0.1% lauryl maltoside, pH 7.2, was then introduced and allowed to equilibrate for 2 h at 0 °C before illumination. [^{125}I]TID (0.5 μCi) was added directly, under stirring, from an ethanol stock solution (1 mCi/mL). Samples were irradiated for 10 min with a 100 long-wave UV lamp (Ultra-Violet Products, San Gabriel, CA), and the protein was recovered by ultracentrifugation, essentially as previously described (Bisson et al., 1979). After SDS–polyacrylamide gel electrophoresis, the radioactivity was detected by fluorography. Labeling experiments were also performed on the enzyme reconstituted in lipid vesicles by the procedure of Casey et al. (1979) by mixing the photoreactive lipids with asolectin. The lipid to protein ratio was 5:1 (w/w).

Labeling with *N,N'*-dicyclohexyl[^{14}C]carbodiimide ([^{14}C]-DCCD specific radioactivity 54 mCi/mol, Amersham) was performed by adding the detergent-solubilized enzyme (60 μg in 150 μL of 10 mM K-Hepes, pH 7.2, buffer containing 30 mM KCl, 79 mM sucrose, and 0.2% LM) to the reagent (0.3–1 μCi) previously dryed on the bottom of the test tube. The reaction was allowed to proceed for 20 h at 2 °C. Protein was recovered as described above for the photolabeling experiments. Radioactivity was detected by autoradiography and by counting the protein bands removed from the gel.

Immunological Procedures. Preparation of cytochrome *c* oxidase antibody was as follows. Antisera against holocytochrome *c* oxidase were raised in rabbits (New Zealand White) essentially as described by Vaitukaitis (1981). The protein (300 μg) dissolved in 50 mM sodium phosphate buffer, pH 7.5, and 1% Triton X-100 (1 mL) was combined with an equal volume of Freund's complete adjuvant previously supplemented with 3 mg/mL of heat-killed tubercle bacillus *Mycobacterium tubercolosis* (H37 RA, Difco) and the homogenized mixture injected at multiple sites on the rabbit back. Blood was collected at weekly intervals from the ear vein, allowed to clot at room temperature, and centrifuged at 27000*g* for 20 min. The IgG fraction was partially purified by adding an equal volume of saturated (at room temperature) ammonium sulfate to the serum. The protein recovered by centrifugation at 27000*g* for 10 min was dissolved in 20 mM Tris-HCl, pH 7.4, and 0.15 M NaCl and dialyzed against frequent changes of the same buffer for several hours. After centrifugation (10 min at 43000*g*) to remove denatured protein, the antibody fraction was frozen in liquid nitrogen and stored at −80 °C. Immunodiffusion tests were performed according to Ouchterlony (1967) on glass slides coated with 0.75% agar in 50 mM potassium phosphate buffer, pH 7.0, 0.1 M KCl, 1% Triton X-100, and 0.02% NaN_3. Normally, precipitation lines were visible 3 weeks after injection.

Immunoprecipitation was obtained both from mitochondrial membranes and purified enzyme. Details of the experiments are reported in Figure 3. For the detection of antigen–antibody complexes after SDS–PAGE, proteins were transferred from slab gels to nitrocellulose (Bio-Rad) (Towbin et al., 1979). The electrode buffer was 25 mM Tris-HCl, 192 mM glycine, and 20% methanol, pH 8.3, and a current of 0.5 A was applied for 4 h. After saturation of the additional protein binding sites with albumin and incubation with the antioxidase antibodies, the washed nitrocellulose sheets were incubated with ^{125}I-labeled goat anti-rabbit IgG (0.5 × 10^6 cpm/sample) at room temperature. The blots were exposed to Kodak X-omat R film for 1–4 days.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed on a vertical slab gel electrophoresis apparatus according to either Kadenbach et al. (1983) or Swank & Munkres (1971). Polypeptides were visualized by Comassie blue or silver staining (Goldman et al., 1981).

Chemicals. Octyl β-D-glucopyranoside and lauryl β-D-maltopyranoside were synthesized according to the procedure of Rosevar et al. (1980). Other detergents were Triton X-100 and lauryldimethylamine oxide (Serva) and cholic acid and deoxycholic acid (Merk). Protease inhibitors PMSF, TLCK,

Table I: Purification of *D. discoideum* Cytochrome *c* Oxidase

step	protein (mg)	oxidase (nmol)	overall (step) yield (%)	heme *a*/protein (nmol/mg)[a]	turnover number (s^{-1})[b]
washed mitochondrial membranes	100	35.5	100	0.71	25.0
"green membranes"	58	34.1	95.9 (95.9)	1.17	50
deoxycholate extacted "green membranes"	18	31.7	89.1 (92.9)	3.5	134
hydrophobic chromatography	3	15.6	44.0 (49.3)	10.4	150
affinity chromatography	1.7	11.7	32.2 (73.1)	13.8	158

[a] Determined according to Markwell et al. (1981). [b] Measured polarographically in 25 mM Tris–cacodylate, pH 7.6, using horse cytochrome *c* as substrate.

TPCK, and cytochrome *c* (horse heart type VI and yeast type VIII) were purchased from Sigma. *Candida krusey* cytochrome *c* was from Sankyo Co., Tokyo. A sample of *Neurospora* cytochrome *c* and of yeast cytochrome *c* oxidase was a kind gift of Prof. W. Neupert and B. Ludwig. Bacto yeast extract and trypticase peptone were obtained from Difco and Becton & Dickinson, respectively. Other chemicals were the best grade available from the sources indicated: acrylamide (Serva), methylenebis(acrylamide) (Kodak); cacodylic acid (Baker).

Results

Purification of D. discoideum oxidase. Isolation of cytochrome *c* oxidase was performed as detailed under Experimental Procedures; a summary of the results obtained is reported in Table I. The major loss of oxidase occurred in the hydrophobic column during the stepwise lowering of the ionic strength. The final yield, however, was normally around 50%, and this purification step was found to be very effective in removing the small amounts of cytochromes *b* and c_1, which contaminate the crude oxidase extracts from the "green membranes". The eluted enzyme was in fact spectroscopically pure even though traces of contaminant polypeptides were still detectable by SDS–PAGE. These impurities were completely washed out in the affinity column. Here, yeast cytochrome *c*, covalently linked to CH-Sepharose 4B, was used as substrate since (a) Arg replaces Lys-13, which in the horse protein occupies the center of the surface interacting with oxidase and is one of the most reactive amino acid residues (Dickerson, 1972; Koppenol & Margoliash, 1982; Wada & Okunuki, 1969) (this substitution should therefore reduce the possibility of cross-linking the binding site to the resin), and (b) on the evolutionary scale yeast cytochrome *c* appears closer to that of the slime mold.

The binding capacity of the column was in the range of 7–8 mg of oxidase/mL of resin in 0.1% LDAO and 10 mM Tris–cacodylate, pH 7.6, but it decreased to 5 mg/mL in our experimental conditions since different ions and detergents were also present. The importance of the latter factors was evident from control experiments performed on the beef enzyme interacting with horse heart cytochrome *c* bound to CH-Sepharose 4B. We found that the binding capacity decreased more than 3–4 times when 10 mM Tris-HCl and 0.1% Triton X-100 were used as the column buffer [see also Thompson & Ferguson-Miller (1983)].

Approximately 20% of the loaded oxidase was eluted with the void volume, and only a small amount of enzyme was removed when the ionic strength was increased to 25 mM NaCl. Total elution occurred with 100 mM NaCl. As shown in Table I, as much as 70% of the loaded oxidase was recovered in a spectroscopically (average heme to protein ratio of 13.4–15 nmol of heme *a*/mg of protein and 280 nm/480 nm absorption ratio of 1.7) and electrophoretically (see Figure 1) pure form.

Different procedures to prepare the enzyme were also attempted. Ammonium sulfate fractionation as described by Yonetani (1967) resulted in heavy loss of enzyme without

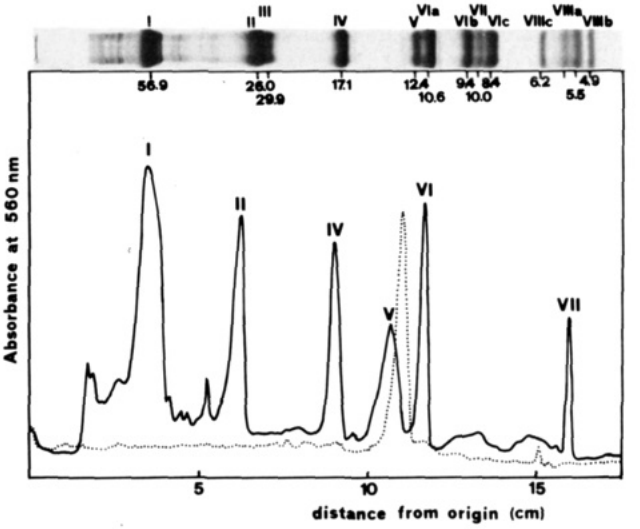

FIGURE 1: Subunit composition of *D. discoideum* cytochrome *c* oxidase. Comassie blue densitometric trace of the amoeba oxidase after SDS–polyacrylamide gel electrophoresis according to Kadenbach et al. (1983). The dotted line is the electrophoretic profile of the *D. discoideum* cytochrome *c* purified as reported under Experimental Procedures. (Top) Photograph of the Comassie blue stained subunits of the beef heart enzyme after electrophoresis under the same conditions; since their sequences are known, here they are used as molecular mass standards (Buse et al., 1983).

satisfactory purification. Only 20–25% of the enzyme, originally present in the mitochondrial membranes, was recovered after three fractionation steps. The heme content of different preparations ranged from 2.6 to 4.2 nmol of heme *a*/mg of protein, and the low purity was evident by SDS–PAGE. The additional hydrophobic chromatography purification step did not sufficiently improve the quality of the preparations (8.2 nmol of heme *a*/mg of protein). This latter result further emphasizes the importance of the green membrane separation (see Experimental Procedures).

Polypeptide Composition. The polypeptide composition of *D. discoideum* oxidase was analyzed by two SDS–PAGE systems [conditions according to Kadenbach et al. (1983) and Swank & Munkres (1971)] and compared with the bovine enzyme (Figures 1 and 2). In both cases the protein complex showed six polypeptide components with molecular masses of 55, 29.5, 19, 13, 11, and 5.7 kDa, calculated by using as standards the known molecular weights of the bovine oxidase subunits and averaging the values on the two different gel systems.

At variance with other characterized eukaryotic oxidases (Power et al., 1984b; Kuhn-Nentwig & Kadenbach, 1984), a single polypeptide is present at a molecular mass lower than 10 kDa. Moreover, subunit III is apparently missing in these preparations since at the expected molecular mass range only the polypeptides corresponding to subunit II and IV can be identified.

The possibility that subunit III could not be visible merely because it was poorly stained by Comassie blue or overlapped

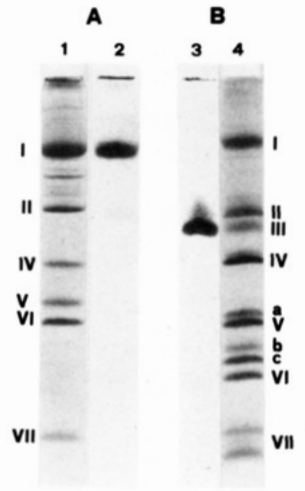

FIGURE 2: Labeling of *D. discoideum* oxidase with DCCD. After incubation with the probe under the conditions reported under Experimental Procedures, the reaction mixture was centrifuged through 10% sucrose and potassium phosphate 10 mM buffer, pH 7.4, and the enzyme, recovered as pellet, was dissolved in a SDS–urea buffer for electrophoresis according to Swank & Munkres (1971). (A) Comassie blue stained gel (1) and the corresponding autoradiography (2). (B) Result obtained under identical conditions with the beef enzyme [nomenclature is according to Downer et al. (1976)].

with subunit II was probed by silver staining and labeling with DCCD. This reagent has been shown to be highly specific for this polypeptide in eukaryotes (Casey et al., 1980; Prochaska et al., 1981; Puttner et al., 1983). However, rather surprisingly, the only polypeptide that was selectively modified in *D. discoideum* oxidase was subunit I (Figure 2); no radioactivity was found in the molecular mass range expected for subunit III (35–20 kDa). Moreover, the number of counts associated with subunit I was comparable to that of subunit III of the beef heart oxidase, labeled under the same conditions.

Proteolytic degradation of the enzyme during its isolation, despite the presence of different protease inhibitors, was probed indirectly by dissolving samples of the bovine oxidase and of the *D. discoideum* membranes together, in 2% Triton X-100, 50 mM potassium phosphate, and 150 mM NaCl, pH 7.4. Even after 100 h at 2 °C, the beef enzyme could be recovered by immunoprecipitation without an appreciable loss of subunit III or other protease-sensitive polypeptides (data not shown).

Immunoprecipitation with antibodies raised against the purified protein was also utilized to detect possible artifacts due to purification. Samples of the immunoprecipitated enzyme from mitochondrial membranes solubilized with three different detergents (Triton X-100, octyl glucoside, lauryl maltoside) in a pH range from 7 to 9 did show a polypeptide composition identical with that of the purified oxidase. Despite the various detergent and pH conditions used, subunit III was never found associated to the complex. Only in the immunoprecipitates formed at pH 6 were significative differences with respect to the purified enzyme found. Subunit V was lost while a new polypeptide appeared at lower molecular mass. This result was clearly due to proteolysis since the loss of the subunit could not be reproduced with the purified enzyme. We also found that antibody binding strongly increased the efficiency of proteolytic digestion since solubilized membranes maintained for 20 h at pH 6 and brought to pH 7.5 *before* adding the antibodies did not show modifications in the electrophoretic pattern of the immunoprecipitated enzyme.

Proteolysis appeared to be affected also by the nature of the detergent. As shown in Figure 3 (lanes 1–3), immunoprecipitation in LDAO-solubilized mitochondrial membranes, at

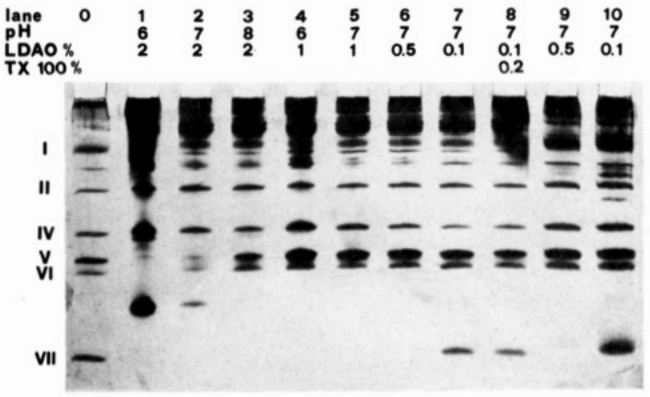

FIGURE 3: Effect of the detergent lauryldimethylamine oxide (LDAO) on the apparent polypeptide composition of *D. discoideum* cytochrome *c* oxidase. Washed mitochondrial membranes (1.8 mg, 0.65 nmol of aa_3) and samples of the enzyme obtained from the hydrophobic column were solubilized in 50 mM sodium phosphate, 0.5 mM EDTA, and 150 mM NaCl at a pH ranging between 6 and 9 and at the detergent concentration reported on the top of the figure. Immunoprecipitation was started by adding 4 mg of antibodies against the amoeba oxidase. After 20 h, the immunoprecipitates were recovered by centrifugation, washed twice with the solubilizing buffer, and finally washed with distilled water. All operations were performed at 2 °C. Samples were dissolved in a SDS–urea buffer (in the absence of β-mercaptoethanol to limit the penetration of the antibody subunits in the gel) for electrophoresis according to Swank & Munkres (1971); one-fifth of the protein was loaded. Silver staining was used for polypeptide detection. (Lane 0) *D. discoideum* oxidase after the hydrophobic column purification step. (Lanes 1–3) Immunoprecipitates from solubilized membranes at pH 6, 7, and 8, respectively. Samples immunoprecipitated from the isolated enzyme at variable pH and detergent conditions are shown in lanes 4–8. (Lanes 9 and 10) Results obtained by dissolving the isolated oxidase under the same conditions used for the samples shown in lanes 6 and 7. In this latter case, however, no antibodies were added, and the enzyme was recovered by ultracentrifugation (200000*g*, 5 h through 10% sucrose and 10 mM potassium phosphate buffer, pH 7.4.

acidic pH, gave rise to dramatic changes in the electrophoretic profile, which involved almost all oxidase subunits. Traces of proteolytic degradation were in this case noticeable even at neutral pH but not in the immunoprecipitates obtained from the purified enzyme in all conditions (Figure 3, lanes 4–8, see also below).

An interesting aspect of this detergent, however, is that its concentration seems to affect the association of the smallest subunit to the rest of the complex. This polypeptide in fact did not coprecipitate with the enzyme when the detergent concentration was equal to or higher than 0.5% (Figure 3, lanes 5–8) regardless of the method used to recover the complex (compare lanes 6 and 7 with lanes 9 and 10).

Analysis of the polyclonal antibodies used for the above experiments shows that they are essentially directed against subunits IV, VI, and to a lesser extent V and I (Figure 4a). Therefore, it appears rather unlikely that the smallest polypeptide might be a contaminant of the enzyme.

Attempts were also made to establish an immunological relationship between the *D. discoideum* and the bovine oxidase subunits by using cross-reactivity with antibodies directed specifically against single subunits of the enzymes. However, as shown in Figure 4b, the degree of cross-reactivity on a blot of antibodies against the bovine enzyme with *D. discoideum* and even yeast oxidase subunits was extremely low. This result is rather surprising in light of the considerable sequence homologies found in the nuclear-encoded subunits of the bovine and yeast enzymes (Power et al., 1984a).

Interaction with Lipids. The topology of the enzyme within the membrane was studied with three different photoreactive probes. Two of them were phospholipid derivatives bearing

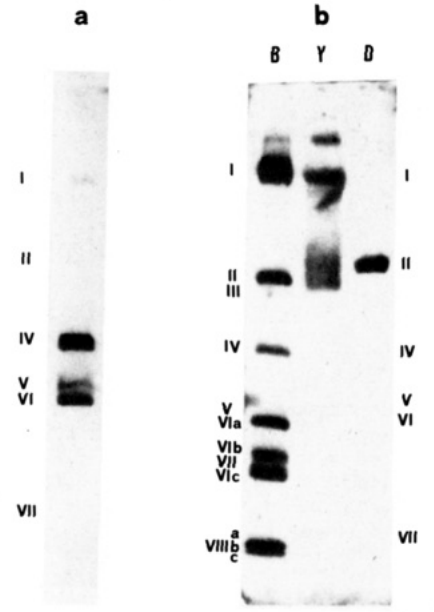

FIGURE 4: Binding of *D. discoideum* and beef heart antioxidase antibodies to purified oxidases. SDS–PAGE (Kadenbach et al., 1983) and immunoblotting were performed as reported under Experimental Procedures. (a) Blot of purified *D. discoideum* oxidase stained with ^{125}I-labeled goat anti-rabbit IgG after binding of the *D. discoideum* oxidase antibodies. (b) Immunological relationship between cytochrome *c* oxidase from beef (B), yeast (Y), and *D. discoideum* (D) detected with anti-bovine oxidase antibodies. Enzyme concentration in lanes Y and D was 50 times higher than that in lane B.

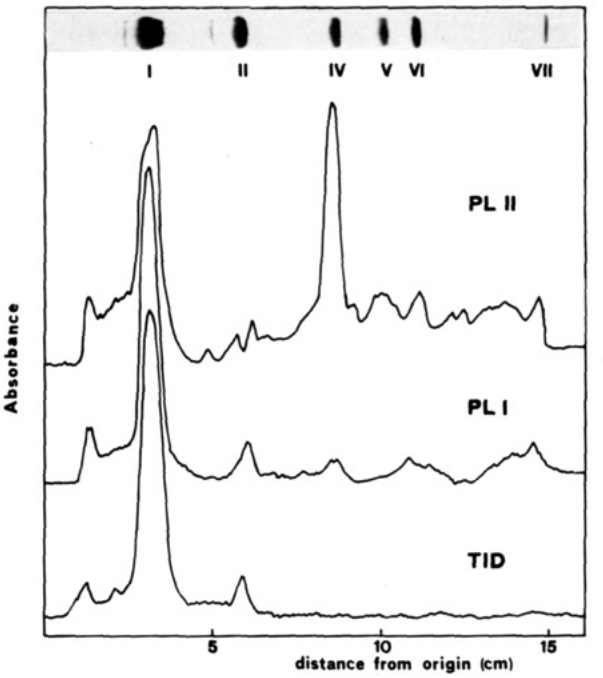

FIGURE 5: Hydrophobic photolabeling of *D. discoideum* cytochrome *c* oxidase. (Top) Photograph of the Comassie blue stained gel of the enzyme analyzed by SDS–PAGE according to Kadenbach et al. (1983). (Bottom) Autoradiographic labeling profiles of cytochrome *c* oxidase labeled with the two photoreactive arylazido phospholipids PLI and PLII and with the small lipophilic molecule TID. Details of the labeling experiments are reported under Experimental Procedures.

the photoactivatable nitroarylazido group either at the methyl terminus of one fatty acid chain (PLI) or close to the lipid polar head region (PLII) (Bisson & Montecucco, 1981). The third (TID) was a small lipophilic molecule (Brunner & Semenza, 1981). The probes were made highly radioactive to allow their use in minute amounts in the labeling experiments.

Upon illumination, these reagents generate highly reactive intermediates (nitrene and carbene in the case of the phospholipid derivatives and TID, respectively) that are able to cross-link neighboring molecules, thereby labeling them radioactively. The comparison of the labeling profiles obtained with the three probes is useful since it can reduce some pitfalls of this technique due to, for example, the different reactivity of the protein residues, their position and orientation within the membrane, and other problems discussed in detail elsewhere (Bisson & Montecucco, 1985). Our results (Figure 5) suggest that subunit I contributes mostly to lipid–protein interaction in the membraneous sector of *D. discoideum* oxidase. Subunits II and IV, though to a much lower extent, are also significantly labeled and therefore in contact with lipids.

Kinetics. The kinetic properties of oxidase were followed during the different purification steps by measuring oxygen consumption as a function of substrate concentration in the presence of TMPD and ascorbate. At low ionic strength (25 mM Tris–cacodylate, pH 7.6), there was a continuous change in both the apparent K_m and V_{max} of the enzyme. The activity of the purified oxidase (TN = 150 s^{-1}, $K_{m,1} = 6.1 \times 10^{-8}$ M) was approximately 10 times higher with respect to the membrane-bound form [TN = 12–25 s^{-1}, $K_{m,1} = (5–7) \times 10^{-7}$ M], and multiphasic kinetics with horse heart cytochrome *c* as substrate were found. This difference was only qualitatively maintained in the spectrophotometric assay. In fact, under the conditions reported under Experimental Procedures, the rate of electron transfer of the isolated oxidase was closer to that of the membranous form, although still 2–3 times higher. Moreover, this divergency could be further reduced by using lauryl maltoside both to solubilize the membranes and to replace lysolecithin in the assay buffer (data not shown).

Table II: Kinetic Parameters of *D. discoideum* Cytochrome *c* Oxidase as a Function of Cytochrome *c* from Different Eukaryotic Sources

source	$K_{m,1}$ (μM)	$K_{m,2}$ (μM)	TN (s^{-1})[a]
horse heart	0.061	0.55	144
N. crassa	0.069	0.97	271
S. cerevisiae	0.058	0.92	239
C. krusey	0.033	1.17	329
D. discoideum		1.26	300

[a] Maximal turnover number [mol of cytochrome *c* s^{-1} (mol of aa_3)$^{-1}$] measured polarographically according to Vik & Capaldi (1980) in 25 mM Tris–cacodylate, pH 7.6.

The activity of the purified enzyme was also studied as a function of different types of cytochrome *c*, including the *D. discoideum* substrate purified as reported under Experimental Procedures. As can be seen from Table II, the amoeba cytochrome *c* gives, as opposed to the various eukaryotic substrates tested, monophasic kinetics with an apparent K_m (1.26×10^{-6} M) comparable to that found for the so-called "low-affinity binding site" in the other cases. Noteworthy, even though the endogenous substrate appears to bind the enzyme with lower strength, it transfers electrons to the oxidase very efficiently.

DISCUSSION

Our understanding of the role of the nuclear DNA coded subunits of oxidase depends, among other things, on the structural characterization and comparison of homologous enzymes from different suitable sources. *D. discoideum* may be one of these interesting sources since it can develop multicellular bodies and give rise to two different cell types (Loomis, 1975, 1982).

The purification of oxidase from such an organism, using conventional procedures based on ammonium sulfate frac-

tionation in the presence of salts and detergents, has been proved to be inefficient for several reasons. Some technical aspects such as reproducibility (in our case important for the comparison of preparations from the different stages of growth and development), applicability to other sources, and yields are seriously affected by this isolation technique.

Chromatographic procedures have been recently introduced in different laboratories (Ozawa et al., 1975; Azzi et al., 1982; Thompson et al., 1983), and although variable in their performances, they appear to be more suitable to the needs of this type of investigation. The method described here takes advantage of the strong interaction existing between the hydrophobic resin phenyl-Sepharose and mitochondrial oxidases (Ozawa et al., 1982; Goto et al., 1982). Only a preliminary step, the "green membrane" separation, is required before loading the protein onto the hydrophobic column.

For the affinity chromatography, several technical solutions have been proposed by different laboratories. Most of them emphasize the importance of the correct orientation of the substrate binding site, the nature of the resin, and the density of cytochrome *c* to avoid steric hindrance (Weiss et al., 1978; Azzi et al., 1982; Thompson et al., 1983). Although at first we preferred to attach the substrate to the support via a long spacer (a six-carbon arm) using yeast cytochrome *c* to make the binding site more available to the enzyme, we became convinced that the nature of the ions and detergents is the governing factors for enzyme–substrate interaction (see results).

At the end of the purification procedure and despite the extensive washings with different detergents, the protein still had a phospholipid content ranging between 15 and 20% [50–70% after the hydrophobic chromatography step, determined by the method of Bartlett (1959)]. Because of this, the enzyme was not activated by the addition of exogenous phospholipids (Vik & Capaldi, 1980) unless it was previously frozen. Nevertheless, the kinetic properties of the complex appeared to change during its isolation. This became immediately evident after the membrane was partially solubilized, even before the two chromatographic steps were carried out. The oxygen reduction velocity, in fact, appeared to increase during purification when monitored polarographically. However, since these measurements take into consideration only part of the catalytic process and might be affected by the presence of the artificial electron donors ascorbate and TMPD (Ferguson-Miller et al., 1978), the enzyme activity was also probed spectrophotometrically. In this assay, where binding, electron transfer, and release of the oxidized substrate contribute to the measured activity, the isolated enzyme exhibited a behavior much closer to its membranous form. Whether the remaining difference is the result of changes in the bilayer phase of a consequence of modifications in the oxidase structure is difficult to assess. Perturbation on the enzyme subunit composition due to different detergents, salts, and pH conditions cannot be excluded a priori. Therefore, immunoprecipitation of the membranous enzyme, solubilized in different detergents and in a wide pH range, was used as a tool to gain information on this problem. Proteolysis, which is the most reasonable explanation to the modifications of the electrophoretic pattern at acidic pH, does not appear to be a serious source of artifacts in the experimental conditions used for the enzyme isolation. This is mainly due to two reasons: first, polypeptides were degraded at a detectable rate only at a pH lower than 7 and, noteworthy, upon antibody binding while all purification steps were carried out in the pH range 7.6–8.2. These observations were further supported by experiments with the bovine enzyme, which, dissolved with the *D. discoideum* membranes at pH 7.6, could be recovered unaltered after 3–4 days at 2 °C. Second, the incubation time for immunoprecipitation was rather long (approximately 20 h) compared with the 5–6 h required to go from membranes to the end of the hydrophobic column purification step, where, as shown by the experiments reported in Figure 3, proteolytic effects were virtually absent even at acidic pH.

Besides proteolysis another explanation for the loss of subunit III is also possible. It is well-known that in the case of the bovine enzyme this polypeptide is rather loosely bound to the protein complex; a moderate change of pH in the presence of the detergent Triton X-100 is sufficient to promote the dissociation of the subunit (together with two-to-three additional smaller components) (Saraste et al., 1981; Penttila, 1983). A similar event, even at physiological pH, does occur in the *D. discoideum* oxidase solubilized with LDAO at a concentration equal to or higher than 0.5% and affects the smallest subunit. The latter result is particularly interesting since, as suggested by the hydrophobic photolabeling experiments, subunit VII does not appear to be in contact with lipids; this would indicate that a relatively small perturbation on the membranous sector can be transmitted along the enzyme structure to alter polypeptide interactions. Whether or not the "loose association" of certain subunits has a physiological significance, for example, on regulating enzyme functions, remains to be established. In this particular case, however, the easy removal of subunit VII may be useful to probe its function. Furthermore, the presence of this component appears to be linked to the cell growth in the log phase; preliminary experiments show that when cells enter the stationary phase, it is drastically reduced and replaced by a larger polypeptide (Bisson et al., 1984). Another aspect that requires further investigations is the unusual reactivity of subunit I toward DCCD. In beef heart oxidase this reagent inhibits proton pumping, selectively labeling a single carboxyl group located in subunit III. This polypeptide, however, does not appear to carry this function since the subunit III depleted beef enzyme and the two subunits of paracoccus oxidase are still able to transfer protons across the membrane (Sarti et al., 1985; Solioz et al., 1982). Attempts to investigate on these aspects indicate that the *D. discoideum* oxidase can be incorporated in phospholipids vesicles even though, to date, only rather low respiratory control indexes (2–2.5 at 15 °C) have been obtained.

Finally, it should be stressed that the amoeba enzyme, on the basis of its polypeptide compositions, appears to be the simplest eukaryotic oxidase so far isolated. This observation is supported by the highly resolving electrophoretic conditions used in this study and is demonstrated by the contemporary separation of the 12 components of beef heart oxidase. This comparison is especially important and hence desirable in light of the disputes existing on the number of polypeptides associated to eukaryotic oxidases.

The procedure described above should now allow one to investigate the enzyme structure and properties in the different developmental stages of the organism; hopefully, these studies will shed some light on the elusive problem of a "nuclear control" of this motichondrial enzyme.

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Registry No. Cytochrome *c* oxidase, 9001-16-5; cytochrome *c*, 9007-43-6.

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