MEMBRANEOUS CYTOCHROME OXIDASE
PURIFICATION, PROPERTIES AND REACTION CHARACTERISTICS

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The use of the bile salts, sodium cholate and sodium desoxycholate, in the purification of cytochrome oxidase from mitochondria has led to preparations of varying activity, lipid content, stability and physical state (Refs. 1-34). Accordingly, it is not yet possible to characterize this enzyme unambiguously with respect to its form and function either in situ or in ultimate ("solubilized") molecular dispersion. In this and the accompanying communication we wish to describe how the use of the specific non-ionic surfactants, Triton X114 and X100 (t - octylphenoxy polyethoxy ethanols obtained from Rohm and Haas Co., Philadelphia, Pa.) enables preparation of spectrally-pure cytochrome oxidase in both a membraneous polymeric state and a lipid-free monomeric molecular dispersion, and with highly defined and reproducible properties.

The use of various non-ionic surfactants, including an unspecified alkylphenoxy-polyethoxy-ethanol, for purification of the components of the electron transport system of mitochondria was first explored by Wainio and Aronoff (1955), but the key variables were apparently not recognized. Subsequently, Green,

Mii and Kohout (1955) reported the successful use of tert-amyl and isobutyl alcohols for the purification of succinic dehydrogenase, and Yonetani (1959) described the stabilization and activation of cytochrome oxidase, initially purified with sodium cholate, by a variety of non-ionic surfactants. Successful attempts to extract a soluble chlorophyll-protein complex from chloroplasts with non-ionic surfactants were first described by Smith (1941) and more recently by Kahn (1964).

PURIFICATION MATERIALS AND METHODS

Mitochondria were prepared in .25M sucrose from the livers of 100-500 gram Sprague-Dawley male and female rats, washed thoroughly with .25M sucrose to remove liver fat, and then mixed with neutral potassium phosphate to produce a final suspension of exactly 25 mg. mitochondrial (intact) protein/ml. in exactly 0.2M neutral phosphate. The mixture was maintained strictly at 0 C, and .003 ml. of a 20% (V/V) solution of Triton X-114 (7-8 oxyethylene groups) was added with vigorous stirring for each mg. mitochondrial protein. The mixture was maintained strictly at 0 C for another two hours and then centrifuged (Spinco) at 105,000 x g for one hour in a 0 C rotor that was maintained strictly at 0 C during the run. The red supernatant was carefully and cleanly decanted, leaving behind a very dark green filmy residue that quickly slides away from the packing angle to the bottom of the centrifuge tube. The residue is then collected from the bottom of the tube with a pipette, care being taken to leave behind the small red-brown button and white ring that are occasionally seen tightly packed against the wall of the tube and which remain there after the filmy dark green residue slides to the bottom. The collected residue is then washed by

mixing with five volumes of 0.2M neutral potassium phosphate and again centrifuging at 105,000 x g for one hour. The yellow supernatant is carefully and cleanly decanted, leaving behind a dark green filmy residue that appears almost blue from some angles of observation. The washing procedure with 0.2M neutral phosphate is repeated and the final residue is suspended in 0.2M neutral phosphate (or in water for observation under the electron microscope). Recovery of oxidase from mitochondria is essentially quantitative. The final suspension of the membraneous oxidase preparation has a distinct turbid appearance. If the prep is washed 2 times with water instead of the .2M neutral phosphate, the final suspension appears almost transparent but exhibits a marked opalescence, indicating that the physical state of this oxidase is sensitive to salts.

PROPERTIES

The spectral purity of the membraneous oxidase preparation is clearly evident in its reduced-oxidized difference spectrum (Fig. 1). Cytochromes other than a-a_3 appear to be almost completely absent. The lipid content of the preparation is high, but thin layer chromatograms of chloroform-methanol (3:1) extracts show only two main spots, which can be identified with phosphatedylethanolamine and phosphatidyl choline. The lipid phosphorous content measures 4.0ug. P/mg. protein. Total lipid measures .16 mg./mg. protein. When viewed under the electron microscope, negatively stained preparations of the oxidase appear as membraneous sheets derived from broken and unbroken cristae (Fig. 2). In large areas of the sheets, groups of 75-100 A, electron-transparent subunits are seen. Some groups of these subunits appear to be ordered into hexagonal arrays, suggesting the existence of a hexagonal liquid crystalline phase in the

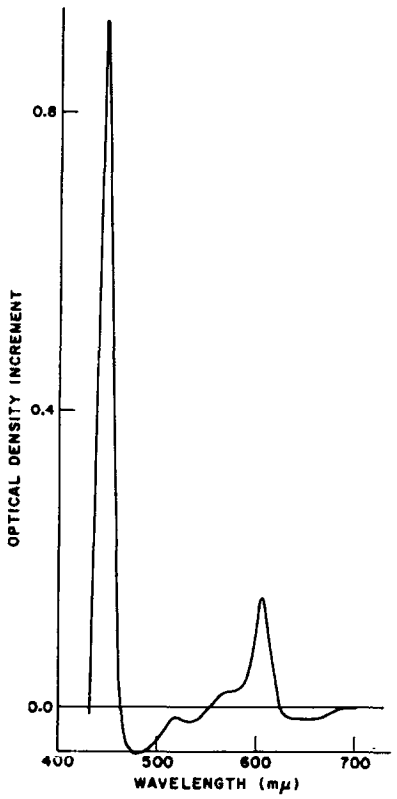

Fig. 1. Reduced minus oxidized difference spectrum of the membraneous oxidase preparation. The samples are suspended in .2M neutral potassium phosphate and reduction was effected with a minimum quantity of dithionite.

membrane. The existence of a hexagonal liquid crystalline phase in phospholipid-water systems has been established by Luzzati and Husson (1962). The possibility that these balls indeed represent lipid and not oxidase protein, as McConnell et al. (1966) contend, is supported by our failure to observe the truly lipid-free soluble oxidase derived from the membraneous oxidase under high magnification electron microscopy (refer to accompanying communication). A detailed description of these observations will be presented elsewhere.

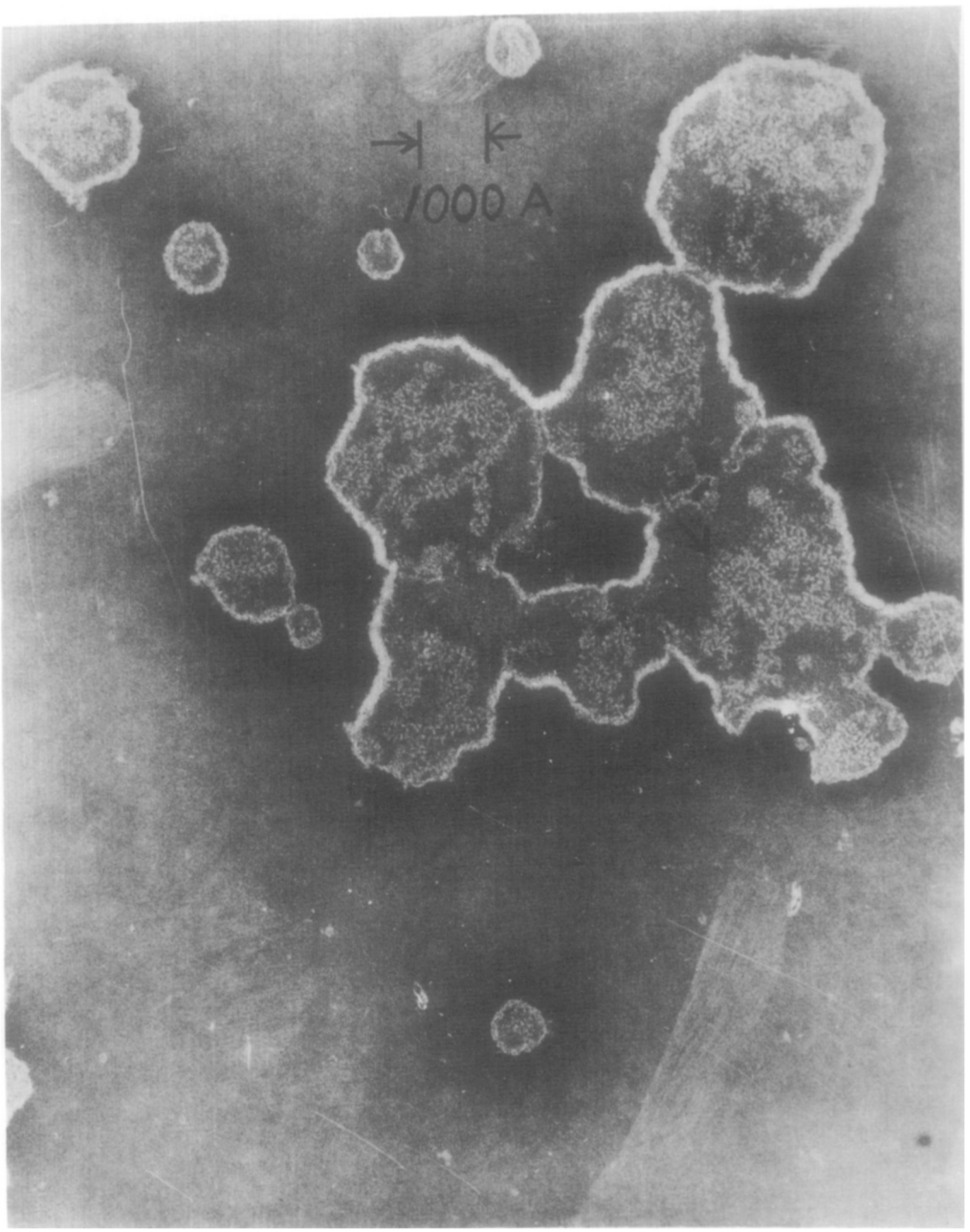

Fig. 2. Negatively stained (PTA) electron micrograph of membraneous oxidase. Arrow points to subunits ordered in hexogonal array.

REACTION CHARACTERISTICS

The catalytic activity of the membraneous oxidase, as measured with a Clark oxygen electrode using a .5 mil teflon membrane and rapid stirring, is shown in Fig. 3. The Q_{O_2} values calculated from these tracings are shown in Table I. The most interesting feature of these results is the close parallel between tetrachloroquinol-polylysine oxidase activity and cytochrome c oxidase activity in the absence of added phospholipid. Note that addition of phospholipid enhances the latter 3-fold but is without effect on the former. Attention is drawn to the importance of the tetrachloroquinol-polylysine reaction (Jacobs et al., 1965) as a measure of the influence of membrane structure on electron transport processes, a phenomenon which is not manifest by measurement of cytochrome c oxidase activity. Jacobs et al. (1965) demonstrated that mitochondria depleted of

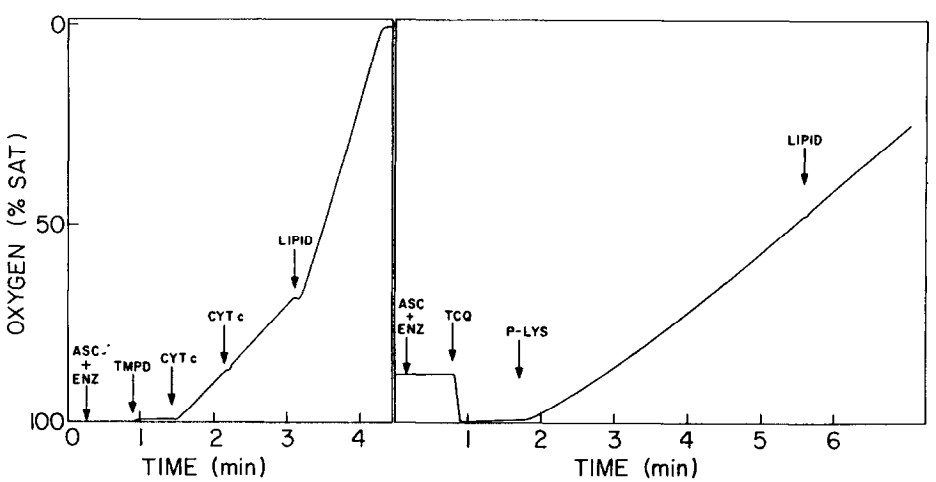

Fig. 3. Oxygen consumption initiated by cytochrome c and by tetrachloro-p-benzoquinol. Temperature of reaction, 20°C; Clark electrode with .5 mil Teflon membrane; rapid rotary stirring. Additions where indicated are: membraneous oxidase enzyme, .12 mg. protein; potassium ascorbate, 75 umoles; cytochrome c, 1 mg; tetramethylphenylenediamine (TMPD), 2 umoles; phospholipid, 5 mg. of water dispersion of lecithin plus cephalin; tetrachlorobenzoquinol (TCQ), .15 ml. of .1M ethanol solution; polylysine, molecular weight 15,000, .05 umoles. Basic reaction mixture, 10 ml. of .01 M neutral potassium phosphate.

Table I

VALUES OF Q_{O_2} FOR REACTIONS CATALYZED BY MEMBRANEOUS CYTOCHROME OXIDASE

ELECTRON DONOR	ADDITIONS	Q_{O_2}
TMPD		0
Cytochrome c		5400
Cytochrome c	Lipid	16,200
TCQ		0
TCQ	Polylysine	4200
TCQ	Polylysine, Lipid	4200

Reaction conditions described in Fig. 2.

endogenous cytochrome c by the mildest possible treatment (hypotonic exposure followed by saline washing) possess high tetrachloroquinol-polylysine oxidase activity, thereby establishing the capacity of nearly undamaged mitochondrial membranes to effect this reaction. The finding that membraneous cytochrome oxidase can also effectively catalyze this reaction, whereas the lipid-free soluble oxidase can not, attests to the relatively intact state of the membrane structure associated with the membraneous oxidase. Finally, the finding that the two oxidase reactions do not respond commensurately to added phospholipid strongly implies that the latter is not reversibly reconstituting a lipid-depleted oxidase, as most investigators tend to assume. This effect is even more dramatically emphasized with the lipid-free soluble oxidase described in the accompanying communication.

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