LIPID-FREE SOLUBLE CYTOCHROME OXIDASE
PURIFICATION, PROPERTIES AND REACTION CHARACTERISTICS

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Previous attempts by Wainio and Aronoff (1955) and Yonetani (1959) to solubilize cytochrome oxidase with non-ionic surfactants were reported as unsuccessful. Their failure to achieve positive results can be attributed to the remarkably critical structure of the surfactant required to effect the solubilization and the critical conditions under which the process must be carried out. In this communication we describe the procedure by which Triton X-100 (9-10 Oxyethylene groups) in the presence of .2M neutral potassium phosphate effects the complete solubilization of the cytochrome oxidase protein from the membraneous oxidase preparation described in the accompanying communication.

PURIFICATION MATERIALS AND METHODS

The residue collected from the first centrifugation following addition of Triton X-114 to a suspension of intact rat liver mitochondria in .2M neutral potassium phosphate, as described in the accompanying communication, is added with rapid stirring directly to 4 volumes of a solution of 5% (V/V) Triton X-100 in .2M neutral potassium phosphate at 0 C. The clear green solu-

tion that results is allowed to stand at 0 C for 2 hours and then centrifuged at 105,000 x g for 30 min. The clear green supernatant is decanted into a beaker and exactly 3 volumes of water are added with rapid stirring at 0 C. (The residue from this centrifugation usually appears as an extremely small brown ring and is discarded.) A column of DEAE cellulose is equilibrated with a solution of 1% (V/V) Triton X-100 in .05M neutral potassium phosphate. The diluted supernatant is then passed through the column and a dark green band forms at the top. The column is subsequently washed with 10 x holdup volume of a solution of 1% (V/V) Triton X-100 in .05M neutral potassium phosphate, causing the dark green band to spread somewhat. The column is then eluted with a solution of 1% (V/V) Triton X-100 in .2M neutral potassium phosphate. The oxidase elutes as a sharp dark green band and is collected in desired fractions. Phosphate gradient elution has also been applied at this point but no significant separation of the oxidase into fractions of varying activity or composition was achieved by such an elution procedure. The final preparation is optically transparent and extremely stable to storage at 0 C and freezing. Solubility of the oxidase requires the presence of both salt and Triton X-100. Once solubilized, the salt concentration may be reduced to as low as .01M without noticeable effect. Solubility is decreased significantly, however, when the concentration of Triton X-100 is reduced below .1% (V/V). A value of .5% (V/V) should be considered as the minimum concentration of Triton X-100 required to sustain solubility during chromatographic, ultracentrifugal and spectro-photometric operations. Oxygen electrode measurements do not appear to be effected by reduction of Triton X-100 to arbitrarily low levels.

The solubilization of cytochrome oxidase by non-ionic detergents appears to be specific to Triton X-100 applied after exposure of the intact mitochondria to Triton X-114. Octylphenoxy-polyethoxy-ethanols containing other than 9-10 oxyethylene groups (Tritons X-45,102,165,205,305) cannot effect solubilization. Neither can any surfactants of the digitonin, Tween or Emasol classes. Application of very high (10-20% V/V) concentrations of Triton X-100 alone to intact mitochondria in the presence of .2M salt does directly solubilize significant amounts of the oxidase but its subsequent isolation from the original mixture is then extremely difficult.

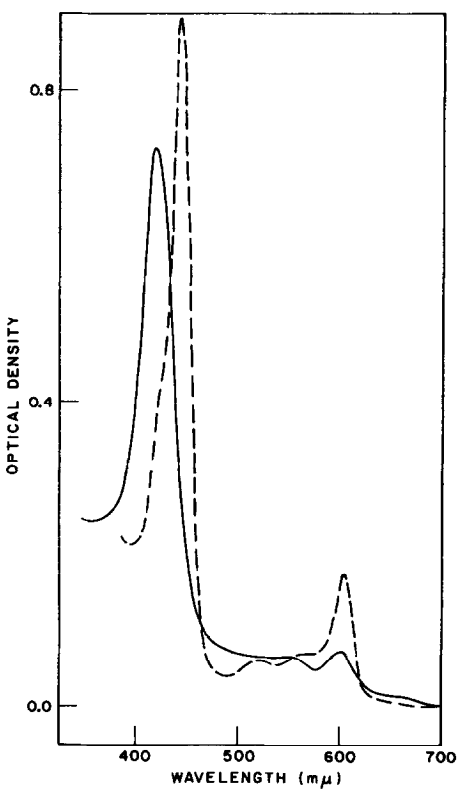

Fig. 1. Absolute absorption spectra of the lipid-free soluble cytochrome oxidase preparation in 0.5% (V/V) Triton X-100 and .2M neutral potassium phosphate. ——— , oxidized; - - - , reduced with dithionite.

PROPERTIES

The optical absorption spectra of the oxidized and reduced (dithionite) states of the soluble oxidase are shown in Fig.1. The spectral changes induced by adding the electron donor, tetramethylphenylenediamine, in the absence of cytochrome c are shown in Fig.2. An immediate 50% reduction of the α band at 603 mu occurs, followed by collapse of the latter and concomitant build up of a shoulder band at 580 mu. Similar

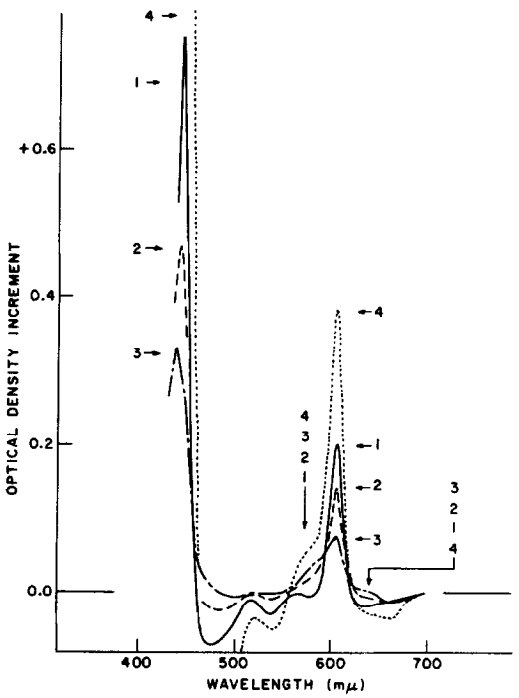

Fig. 2. Effect of ascorbate-tetramethylphenyl-enediamine (TMPD) on the spectrum of soluble cytochrome oxidase. Reference cell contains oxidized oxidase. Ascorbate (25 umoles) plus TMPD (0.5 umoles) is added to sample cell. Curve 1 (———), spectrum scanned from 700 400 mu at 50 mu/sec. immediately after addition of asc.-TMPD. Curve 2 (- - -), spectrum scanned again, in same way, 15 seconds after addition. Curve 3 (- · -), scan repeated 3 min. after addition. Curve 4 (. . . .), spectrum scanned after addition of dithionite to oxidase in state characterized by curve 3 (ΔO.D. at Soret peak on curve 4 is 2.4). Reaction carried out in 0.5% (V/V) Triton X-100 in .2M neutral potassium phosphate at 20 C.

anomalies can be noted at 630 mu and 670 mu. Reduction by dithionite or removal of oxygen on a high vacuum line lead to the fully reduced spectrum. The persistence of the shoulder at 580 mu in the absence of oxygen, and even when cytochrome c is also present (not shown), argue against this being due to an oxygenated form of the oxidase described by Orii and Okunuki (1965). Our data suggest a differential response of two components in the oxidase to the reducing agent. One possible explanation consistent with the data is that TMPD rapidly reduces only a component absorbing at 603 mu (cytochrome a ?), and whose electronic state is strongly influenced by the presence of oxygen and by the electronic state of the other component, absorbing positively at 580 mu and 630 mu, negatively at 670 mu (cytochrome a_3 ?). Subsequent reduction of the latter by the former in the presence of oxygen and in the absence of the polycation function of cytochrome c might then conceivably convert the 603 mu component to a difficulty-reduced form. If electronic interaction between the two components is through an oxygen bridge, then removal of the latter would allow eventual complete reduction of both the components, as the data indicate. Anomalous behavior of spectral absorption between 565-620 mu was previously reported by Gibson and Greenwood (1965). Effects exactly similar to the above were also observed to characterize the membraneous oxidase. An anomalous behavior of the oxidase in the Soret region can also be seen in Fig.2. Lemberg and Mansley (1965) have earlier detected a differential response of two components in cytochrome oxidase to reduction by dithionite by examining spectral changes in the Soret region.

There is essentially no measurable lipid associated with

this oxidase preparation. Chloroform-methanol (3:1) extraction of preparations that have been dialyzed against water to remove Triton X-100 and salt, and collected and washed with water by centrifugation at 144,000 x g, failed to reveal the presence of lipids detectable by thin layer chromatography or by lipid phosphorous analysis.

The molecular weight of the oxidase was determined by the Archibald technique as modified by Ehrenberg (1957) and extrapolated to infinite dilution as prescribed by Mueller (1964). A value of 15-20,000 has been found and should be assumed as tentative until corroborated. This work will be described in detail elsewhere.

Examination of the oxidase under the electron microscope at high magnification has failed to reveal a structure distinguishable from background. The upper limit of particle size may tentatively be placed at about 20 A, the realistic limit of resolution. This negative finding is consistent with the unusually low molecular weight noted above, even though both observations are in serious conflict with the findings of Tzagoloff et al., 1965 (M.W. 230,000), and McConnell et al., 1966 (50-100A subunits). Another unexpected finding is our failure to detect any structural protein, described by Criddle et al., (1962), in the original solution of oxidase, obtained when the membraneous oxidase is solubilized by mixing with Triton X-100. In fact, recovery of the protein of the membraneous oxidase as soluble oxidase protein was almost quantitative. This phenomenon remains to be explained, if the so-called membraneous oxidase is indeed truly membraneous.

REACTION CHARACTERISTICS

The catalytic activity of the lipid-free, soluble cytochrome oxidase is shown in Fig.3. The Q_{O_2} values calculated from these tracings are shown in Table I. The data clearly demonstrate that cytochrome c oxidase activity can be restored to a high value with added phospholipid but that tetrachloroquinol-polylysine activity has been markedly and irreversibly decreased by the solubilization process. Thus we may reasonably conclude that the reaction of tetrachoroquinol-polylysine, but not that of cytochrome c, with cytochrome oxidase is indeed sensitive to some critical and labile feature of the native membraneous, polymeric or lipid-bound structure of cytochrome oxidase in situ that is not being reconstructed in solubilized cytochrome oxidase by added phospholipid.

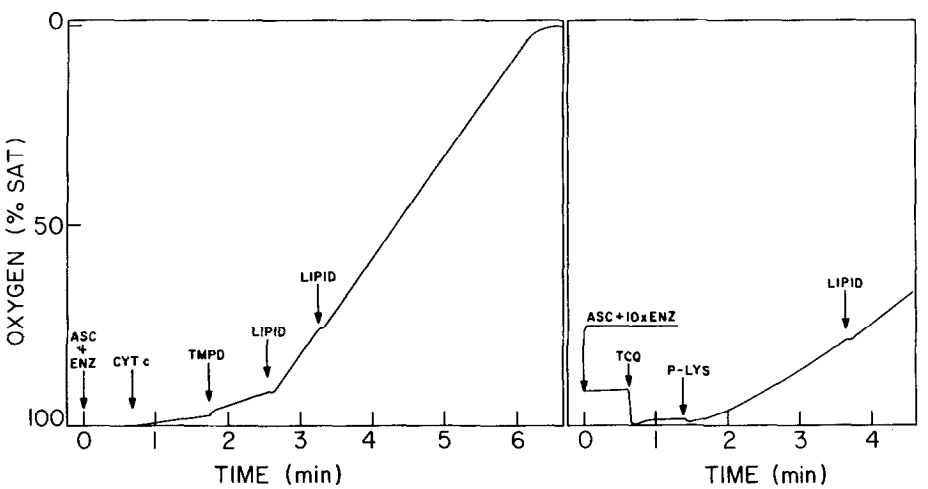

Fig. 3. Oxygen consumption initiated by cytochrome c and by tetrachloroquinol (TCQ). Temperature of reaction, 20 C; Clark electrode with .5 mil Teflon membrane; rapid rotary stirring. Additions where indicated are: Lipid-free, soluble oxidase enzyme, .055 mg. protein; potassium ascorbate, 75 umoles; cytochrome c, 1 mg; TMPD, 2 umoles; phospholipid, 5 mg. of water dispersion of lecithin cephalin; TCQ, .15 ml of .1M ethanol solution; polylysine, molecular weight 15,000, .05 umoles. Basic reaction mixture, 10 ml of .01M neutral potassium phosphate.

TABLE I

VALUES OF Q_{O2} FOR REACTIONS CATALYZED BY LIPID-FREE SOLUBLE CYTOCHROME OXIDASE

ELECTRON DONOR	ADDITIONS	Q_{O2}
Cytochrome c		1400
Cytochrome c	TMPD	3500
Cytochrome c	Lipid	15,000
TCQ		
TCQ	Polylysine	750
TCQ	Polylysine, Lipid	750

Q_{O2} of intact mitochondria measured with asc-TMPD-cyt.c is 635.

Reaction conditions described in Fig.2.

Acknowledgements

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