Activation Studies by Phospholipids on the Purified Cytochrome c_4:o Oxidase of *Azotobacter vinelandii*[1]

Tit-Yee Wong[2,3] and Peter Jurtshuk, Jr.[2]

Abstract

A modified procedure is described that was used to solubilize and purify the TMPD-dependent cytochrome c_4:o oxidase from *Azotobacter vinelandii*. Two functional components (Fractions I and V) were obtained after DEAE-cellulose chromatography. Fraction V contained both cytochrome c_4 (3.6 nmol/mg protein) and cytochrome o (1.6 nmol/mg protein). This cytochrome oxidase complex oxidized TMPD at "moderate" rates. Fraction I, a clear greenish-yellow fraction, contained primarily phosphatidylethanolamine with some phosphatidylglycerol. Fraction I itself could not oxidize TMPD, but when it was preincubated with Fraction V, a 2–4-fold stimulation in TMPD oxidase activity occurred. Other "authentic" micellar phospholipids also readily activited TMPD oxidase activity in Fraction V. The *maximum* activation effect obtained with Fraction I was in essence duplicated with purified phosphatidylethanolamine.

Key Words: Cytochrome oxidase; *Azotobacter vinelandii;* phospholipid activation; cytochrome c_4:o oxidase; phosphatidylethanolamine; TMPD oxidase.

Introduction

Much like the mammalian mitochondrial cytochrome $a + a_3$ oxidase, most bacterial cytochrome oxidases are particulate entities, tightly bound to the inner cytoplasmic membrane. The difficulties encountered in studying bacterial oxidases are: (1) the lack of a suitable assay for measuring cytochrome oxidase activity, (2) no established detergent solubilization procedures for isolating such oxidases, and (3) usually two or three multiple types of terminal oxidases are found in a single organism.

The *Azotobacter vinelandii* cytochrome oxidase is of particular interest as this organism has the highest respiratory rate of any known cell (Burk, 1930). Photochemical action spectra have revealed three cytochrome oxidases in *A. vinelandii*, i.e., cytochrome a_1, d, and o (Castor and Chance, 1959; Jones and Redfearn, 1967a). Repske and Josten (1958) isolated an active NADH oxidase from this organism. This complex contained large concentrations of cytochrome b_1, c_4, and d. Subsequently Jones and Redfearn (1967b) solubilized a red particle fraction from *A. vinelandii*. This "red particle" fraction contained "enriched" concentrations of nonheme iron, cytochrome b_1, $c_4 + c_5$, and o, and it oxidized succinate and ascorbate-DCIP fairly rapidly. A concomitantly solubilized "green particle" fraction had low ascorbate-DCIP oxidase activity, but contained high concentrations of cytochrome d and a_1, as well as cytochrome b and ubiquinone. Jurtshuk and associates (Mueller and Jurtshuk, 1972; Jurtshuk *et al.*, 1981) solubilized a highly active membrane-bound TMPD[4] oxidizing enzyme complex from *A. vinelandii*. This solubilized cytochrome oxidase was readily precipitated by ammonium sulfate; it contained predominantly cytochrome c_4 and cytochrome o.

In this report, we present a modified procedure which can be used to purify the cytochrome c_4:o oxidase of *A. vinelandii*. The enzymatic properties and spectral characteristics of this cytochrome oxidase are the main theme of this report. We also show that a specific fraction (Fraction I), isolated from the same purified oxidase complex, was required for maximal TMPD oxidase activity.

Materials and Methods

Preparation of Electron Transport Particle

A. vinelandii strain 0 was grown in a 200-liter capacity fermenter (New Brunswick Scientific Co.) under conditions previously described (Jurtshuk *et al.*, 1967). Late-log phase cells were harvested and washed twice with 0.02 M phosphate buffer, pH 7.5. The electron transport particle, designated R_3, was isolated from sonically disrupted cells by differential centrifugation (Jurtshuk *et al.*, 1967). The R_3 fraction represents the membrane pellet after high-speed centrifugation at $114,000 \times g$, and the resultant supernatant cytosol fraction

was designated the S_3 fraction. Phospholipids extracted from the S_3 fraction were used in some of the reconstitution experiments described.

Solubilization and Purification of the Cytochrome c_4:o Oxidase

The initial steps used to solubilize the oxidase were identical to the original procedure of Jurtshuk *et al.* (1981). After the cytochrome oxidase was solubilized from the R_3 membrane fraction by Triton/KCl, the supernatant fraction which contained the oxidase was extensively dialyzed with 0.02 M phosphate buffer, pH 7.5, to remove all residual KCl. This sample was then clarified by centrifugation and concentrated 6-fold under N_2 gas (50 lb/in^2) using a XM-50 DIAFLO ultrafiltration membrane (Amico Corp). This preparation was then transferred to a 35 × 1.5 cm diethylaminoethyl (DEAE)-cellulose column, preequilibrated with 0.02 M phosphate buffer, pH 7.5, plus 1% (w/v) Triton X-100. After adsorption to the column, the sample was eluted with the equilibrating buffer–detergent solution at an elution speed of 16 ml/hr. All fractions collected were monitored for protein using a UV-fraction collector (Model 226, ISCO). Fraction I, a yellowish-green component, was the first major peak, of four, eluted from the column. After a subsequent elution interval of 16 hr, a second elution step was carried out using this same buffer–detergent eluent which now contained 0.25 M KCl. This latter step eluted the major hemoprotein component which appeared as a broad peak (Fraction V), and it contained the active TMPD-dependent cytochrome c_4:o oxidase enzyme complex.

Spectral Analyses

A Cary spectrophotometer Model 118C was used for all the spectral analyses. The c-type cytochrome concentration was determined from dithionite-reduced minus oxidized difference spectra (551–538 nm) using an extinction coefficient of 17.3 mM^{-1} · cm^{-1} (Jones and Redfearn, 1966). Carbon monoxide difference spectrum were obtained by bubbling CO for 2 min through a dithionite-reduced enzyme sample. Cytochrome o concentrations were calculated from the CO:dithionite-reduced minus dithionite-reduced difference spectra (417–432 nm) using the extinction coefficient of 170 mM^{-1} · cm^{-1} (Daniel, 1970).

Enzyme Assay

TMPD oxidase activity was measured spectrophotometrically (Lieberman and Lanyi, 1971; Hollander, 1977) using a recording Beckman Model 25 spectrophotometer. Wurster's Blue formation, resulting from the enzymatic oxidation of reduced TMPD, was measured at 610 nm for reaction intervals as long as 5 min. The extinction coefficient for Wurster's Blue used to calculate

specific activities was $12.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Steigman and Cronkright, 1970). The assay mixture contained 0.05 M phosphate buffer (pH 6.0) and usually 10–12 μg of enzyme protein. The final reaction volume was 1.0 ml. Double deionized water was used, which was equilibrated overnight to the assay temperature (25°C) to allow for oxygen saturation by air. All enzyme reactions were initiated by the addition of 10 μl of 0.05 M aqueous solution of purified (Cox and Smith, 1964) TMPD. For all enzymatic activation studies, Fraction V was allowed to interact with Fraction I (or other micellar phospholipids) for 3 min at 25°C, prior to the addition of TMPD. The final concentration of micellar phospholipid in the reaction mixture was usually 100 μg.

Phospholipid Preparation and Analysis

Phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylcholine, and lysophosphatidylcholine were purchased from Avanti Biochemical Co. (Birmingham, Alabama). Stock solutions were kept in chloroform at -20°C. Phosphorus concentrations were determined as described (Marinetti, 1962). Micellar phospholipids were prepared by drying known concentrations of phospholipid stocks under N_2 gas; the residue was resuspended into a known volume of 0.02 M phosphate buffer, pH 7.5, containing 0.1% Triton X-100. Vesicle formation was induced by briefly sonicating for 20 sec just prior to assay. Total lipids were extracted from the S_3 fraction, and from the Fraction I, according to the method of Folch *et al.* (1957). The extracted phospholipids, as well as the authentic phospholipid samples, were then transferred quantitatively to high performance thin-layer chromatographic plates (HPTLC) (Scientific Products, McGraw Park, Illinois). Samples were developed by $CHCl_3:CH_3OH:40\%CH_3NH_2H_2O$ (63:31:5:5, v/v). Phospholipid spots were detected by spraying the phosphorus stain of Vaskovsky and Kostetsky (1968).

Protein Determination

Protein concentration were determined by a modified Biuret method as described by Yonetani (1961).

Results

Solubilization and Purification

Specific activities and protein recovery values obtained during the purification of the *A. vinelandii* cytochrome c_4:*o* oxidase are shown in Table I. The R_3 is the original "sonic" ETP fraction; the Triton/KCl represents the

Table I. Specific Activities and Protein Recovery Values Obtained During the Isolation and Purification of the Cytochrome c_4:o Oxidase of *Azotobacter vinelandii*

Fraction	Protein (mg)	Specific activity[a] (μmol/min/mg)	Total activity (μmol/min)	Recovery (%)
R_3[b]	1290	0.47	606	100
Triton/KCl	284	1.4 ($\times$ 3)[c]	539	89
Fraction I	0	0.0	0	0
Fraction V	72	1.6 ($\times$ 3.4)[c]	115	19
Fractions I + V	72[d]	4.4 ($\times$ 9.4)[c]	324	53

[a] The concentration of TMPD used in the assay was 0.5 mM.
[b] Sonic ETP-type preparation.
[c] Purification factor from the R_3 fraction.
[d] Represents 5% protein recovery from the R_3 fraction.

"crude" solubilized cytochrome oxidase fraction. The purified cytochrome c_4:o oxidase is designated Fraction V and the cytochrome oxidase activating factor is found in Fraction I. The TMPD oxidase specific activity in the original R_3 fraction was 0.47 μmol TMPD oxidized per minute per milligram protein. The solubilized oxidase purified in the Triton/KCl supernatant fraction exhibited a 3-fold higher specific activity. Fraction V essentially exhibited the same specific activity as the solubilized cytochrome oxidase found in the Triton-KCl fraction. However, by reconstituting Fraction V with Fraction I, a 2.8-fold stimulation of TMPD oxidase activity occurred above that noted for Fraction V alone. This represents a 9.4-fold purification for TMPD oxidase activity as noted from the R_3 fraction, and similarly the protein and activity recoveries for the activated Fraction V were 5 and 53%, respectively. No protein was detected in Fraction I; this fraction itself exhibited no TMPD oxidase activity.

Spectral Characteristics of Cytochrome c_4:o Oxidase

A difference spectrum of the purified *A. vinelandii* cytochrome oxidase (Fraction V) is shown in Fig. 1. The dithionite-reduced sample (dotted line) showed a sharp alpha peak for cytochrome c_4 at 552 nm. The concentration of the c-type cytochrome present in this preparation was 3.6 nmol/mg protein. This value represented an approximate 4-fold increase in cytochrome c concentration from that found in the R_3 fraction (Jurtshuk *et al.*, 1981). The shoulder at 557 nm represents the b-type(s) cytochrome found in Fraction V, and the predominant component most probably is the functional oxidase, cytochrome o. Bubbling CO into the dithionite-reduced sample of Fraction V caused pronounced spectral changes (dashed line) in the cytochrome oxidase. Absorption at 557 nm is notably reduced. This bleaching at 557 nm by the addition of CO indicates that the b-type cytochrome in this complex is cytochrome o (Castor and Chance, 1959). Figure 1 also shows that the
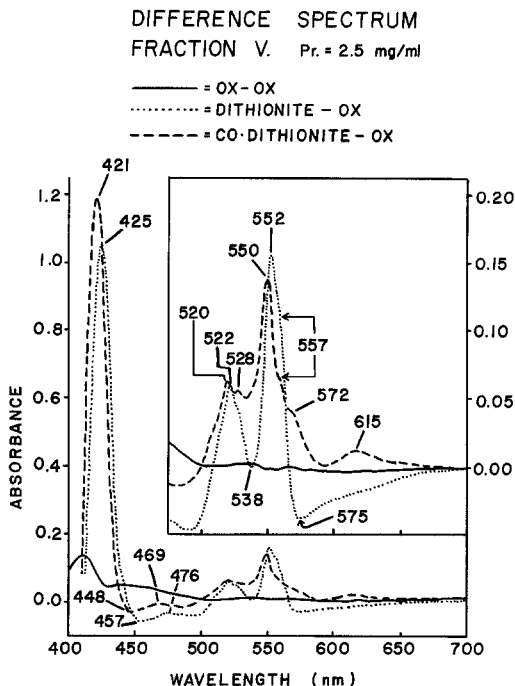
Fig. 1. Difference spectra of Fraction V, at 2.5 mg protein/ml, showing the dithionite-reduced minus oxidized (.....) and CO-reduced minus oxidized (———) absorbancy changes. The insert shows the prominent alpha and beta peaks of the reduced (.....) and CO-reduced (———) cytochrome c_4:o oxidase. The solid line represents the oxidized minus oxidized control spectrum.

addition of CO to the dithionite-reduced sample also shifts the Soret peak from 425 to 421 nm. This effect of CO on the Soret peak of the reduced cytochrome oxidase is very pronounced in the difference absorption spectrum shown in Fig. 2. The CO-reduced minus reduced difference spectrum shows prominent absorption peaks at 572, 542, and 416 nm. Troughs were observed at 557, 523, and 430 nm. These peaks and troughs were similar to the CO-reduced spectra reported previously for cytochrome o (Daniel, 1970; Jurtshuk and Yang, 1980). The concentration of cytochrome o calculated in this sample of Fraction V was 1.6 nmol/mg protein. The R_3 fraction usually contained approximately 0.3 nmol of cytochrome o per milligram protein (Jurtshuk *et al.*, 1981).

Reconstitution Studies on the Cytochrome c_4:o Oxidase

The data shown in Table II again demonstrates the activation effect of Fraction I on the purified Fraction V cytochrome oxidase. This study shows
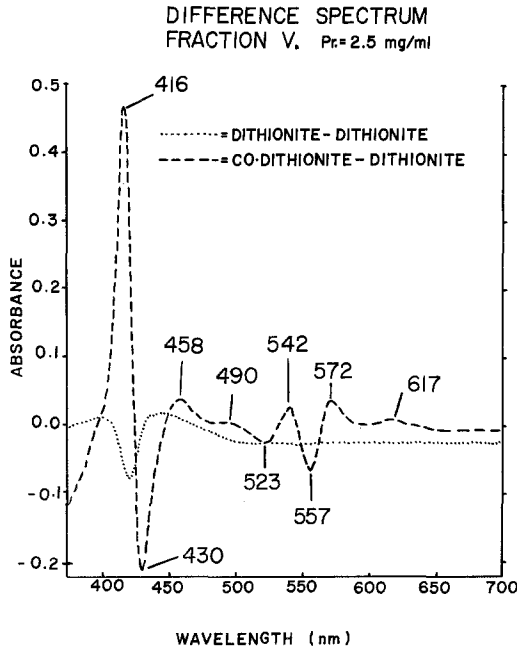
Fig. 2. The CO:dithionite-reduced minus dithionite-reduced difference spectrum of Fraction V, containing the *A. vinelandii* cytochrome c_4:o oxidase. The (.....) line represents the dithionite-reduced minus dithionite-reduced control spectrum; the (———) line represents the CO-reduced minus reduced spectrum. The trough at 557 nm, and the Soret peak at 416 nm with its concomitant trough at 430 nm, are the spectral characteristics most commonly associated with cytochrome o.

Table II. Activation Studies on the *Azotobacter vinelandii* Cytochrome c_4:o Oxidase by Fraction I

TMPD oxidase assay system	Specific activity (nmol/min/mg)
1. Test	
Fraction V	1.6
Fraction V + I	3.5
Fraction V + I (heated)[a]	4.4
2. Controls	
Fraction I	0.04
Fraction I (heated)[a]	0.0
Fraction V (heated)[a]	0.04
Fraction V (heated) + I (heated)[a]	0.03

[a] Heated to 70°C 10 min, then clarified by centrifugation.

that the cytochrome c_4:o oxidase, in Fraction V, oxidized TMPD at a moderate specific activity of 1.6. TMPD oxidation by Fraction V was totally enzymatic, as heating this fraction to 70°C for 10 min results in almost complete loss of activity. When Fraction I was added to Fraction V, a 2.2-fold increase in TMPD oxidation occurred. Heat-treated preparations of Fraction I activated TMPD oxidation to an even higher degree (2.8-fold), which suggests that this activation effect is most probably due to micellar phospholipids (Jurtshuk *et al.*, 1963). Fraction I itself exhibited a very low or residual TMPD oxidation rate. This small degree of activity disappeared completely after heat treatment, which suggests that Fraction I may have been contaminated to a small extent with Fraction V.

Although data are not presented in this study, TMPD oxidase activity, both with Fraction V alone and reconstituted with Fraction I, showed a marked sensitivity for KCN. Cyanide, at a 50 μM concentration level, completely inhibited TMPD oxidation in Fraction V alone and Fraction V activity that was reconstituted with Fraction I.
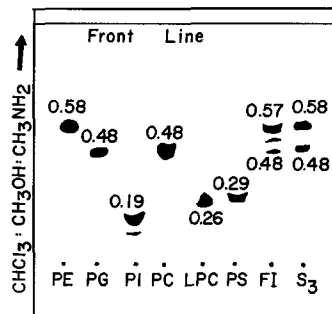

Fig. 3. A one-dimensional thin-layer chromatogram showing the R_f values of the phospholipid components found in the Fraction I (FI) and the centrifically clarified sonic supernatant (S₃) fraction. These are compared to the known authentic phospholipid samples that were obtained from commercial sources. The abbreviations used for the phospholipid samples analyzed are PE for phosphatidylethanolamine, PG for phosphatidylglycerol, PI for phosphatidylinositol, PC for phosphatidylcholine, LPC for lysophosphatidylcholine, and PS for phosphatidylserine. (Data of Wong, Eichberg, and Jurtshuk.)

Studies on the Activation Factor(s) in Fraction I

The fact that the activation factor(s) in Fraction I was heat stable suggested that phospholipids might be the cause of this activation. Phospholipids were detected readily in Fraction I when samples were analyzed by HPTLC (Fig. 3). Thin-layer chromatographic analyses revealed a major component (R_f = 0.57) that was identified as phosphatidylethanolamine. Another phospholipid, phosphatidylglycerol (R_f = 0.48) was also detected, but it was present at a much lower concentration level. Phospholipids extracted from the S_3 fraction (a clarified supernatant which remains after sedimenting the sonic ETP R_3 fraction) also contained measurable amounts of phosphatidylethanolamine and phosphatidylglycerol (Fig. 3), which also activated TMPD oxidation substantially with Fraction V.

Activation of TMPD Oxidase Activity by Authentic Phospholipids

Attempts were also made to activate TMPD oxidation with the Fraction V cytochrome c_4:o oxidase using authentic micellar phospholipids. These preparations were obtained from commercial sources, and chromatographic analyses revealed these phospholipids to be pure components (Fig. 3). Figure 4 shows the spectrophotometric assays in which absorbance increases at 610 nm are plotted as a function of time in minutes. Wurster's Blue formation, which would result from TMPD oxidation, is essentially negative for all the controls shown. Autoxidation of TMPD (without adding Fraction V) does not occur. Similarly no TMPD oxidation is noted from solely adding authentic micellar phospholipid fractions, or Fraction I or the S_3 fraction. TMPD oxidation only occurs in the presence of Fraction V, the cytochrome c_4:o oxidase. The activity of Fraction V is stimulated to varying degrees by the addition of authentic phospholipids, or Fraction I or the S_3 lipid fraction. Phosphatidylethanolamine (PE) maximally activated TMPD oxidation by the cytochrome c_4:o oxidase, and the rate of Wurster's Blue formed was slightly higher than that obtained by a boiled preparation of Fraction I. Other pure commercial phospholipids, including phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidylcholine, and lysophosphatidylcholine also activated the cytochrome c_4:o oxidase significantly, as did the phospholipid extracts obtained from the *A. vinelandii* S_3 fraction (S_3-lipid). Of interest is that phosphatidylcholine is not found in *A. vinelandii,* although phosphatidylethanolamine is a major phospholipid component in this bacterium.

Discussion

All of our previous efforts to *further* purify the *A. vinelandii* cytochrome c_4:o oxidase, solubilized into the Triton/KCl supernatant fraction, proved
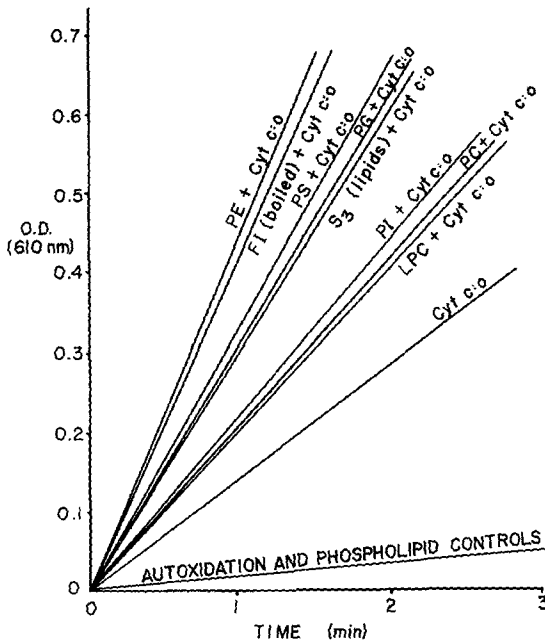
Fig. 4. A spectrophotometric assay showing the increase in cytochrome oxidase activity induced by the addition of phospholipids to the assay system. Wurster's blue formation, resulting from reduced TMPD oxidation, is plotted as a function of time. The purified cytochrome c_4:o oxidase used in this study is Fraction V (cty c:o), which is active even when assayed for in the absence of phospholipid(s). Reconstituted activities with Fraction V are shown with specific phospholipids that were either obtained from commercial sources (as authentic components) or extracted directly from two *Azotobacter vinelandii* fractions (FI and S_3). See Fig. 3 for the specific phospholipids found in both FI and S_3 fractions, and for the abbreviations used for the various micellar phospholipid preparations tested in this study.

futile. Ammonium sulfate precipitation of this fraction, with extensive dialyses, led to the isolation of a highly active TMPD oxidase complex (Mueller and Jurtshuk, 1972; Jurtshuk *et al.*, 1981), but further purifications could not be attained. However, by use of DEAE-chromatography it was possible to resolve this cytochrome c_4:o oxidase into multiple components, neither individual component being more active than the original enzyme fraction. However, reconstituting these various DEAE fractions and testing

the reconstituted oxidase complex for activity revealed that highly active TMPD-oxidizing preparations could be obtained. The DEAE-treated cytochrome c_4:o oxidase complex, represented by Fraction V, was *very active* when assayed either in the presence of heated Fraction I or purified micellar phospholipids. All purified phospholipid preparations tested to date were active in stimulating TMPD oxidation; the activation effect was nonspecific. However, the highest activation effect was obtained with chromatographically pure phosphatidylethanolamine. The degree of activation by phosphatidylethanolamine was slightly higher than that obtained using a heated preparation of Fraction I, a DEAE-chromatographed fraction originally associated with the active oxidase (see Fig. 4). In this manner the solubilized DEAE-treated *A. vinelandii* cytochrome c_4:o oxidase mimics the phospholipid activation effects previously reported for the highly purified mammalian mitochondrial cytochrome $a + a_3$ oxidase (Wharton and Griffiths, 1962; Green and Fry, 1980).

The *A. vinelandii* cytochrome c_4:o oxidase characterized herein most closely resembles the purified cytochrome bc_1 complex of the mitochondrial electron transport chain. Cytochome c_4 is most intimately associated (or integrated) with the terminal oxidase component, cytochrome o; both complexes can be solubilized as functional membrane fractions, the active entities containing phospholipids. Cytochrome o contains protoheme as the prosthetic group, as do all cytochrome b preparations regardless of whether or not they are present in mitochondrial particles. But the difference between these two complexes is that the cytochrome o in *A. vinelandii* functions as a terminal oxidase, capable of reacting with oxygen and carbon monoxide, while the mitochondrial cytochrome b cannot function in this manner. One point of interest here is that cytochrome o is more commonly found as a terminal oxidase component in bacteria than any oxidase studied to date, including cytochromes $a + a_3$, a_1, and d (Jurtshuk and Yang, 1980). In addition, as indicated by the absorption spectra shown in Figs. 1 and 2, there may well be other functional components present in our cytochrome c_4:o oxidase complex. For example, one notes a prominent dithionite-reduced CO-reacting component at 615 nm (Fig. 1) or 617 nm (Fig. 2). This may well represent another oxidation–reduction component that has yet to be identified in this organism. Analyses of the CO-reduced minus reduced difference spectra of our previously isolated cytochrome c_4:o oxidase (Jurtshuk *et al.,* 1981) showed a prominent trough at 551 nm, in addition to a "shoulder trough" at 556 nm. The 551 nm trough suggests that a CO-reacting c-type cytochrome was present in the oxidase preparation, and the shoulder trough at 556 nm demonstrated the presence of the CO-reactive cytochrome o component. The absence of this CO-reacting c-type cytochrome in our DEAE-purified cyto-

chrome c_4:o oxidase characterized herein (see Fig. 2) most strongly suggests that this component is not required for TMPD oxidase activity. Although the previously isolated cytochrome c_4:o oxidase preparation oxidized TMPD at high specific activities, the enzyme had no requirement for phospholipid. The phospholipid requirement can only be demonstrated in enzyme preparations passed through a DEAE-column. Cytochrome o was readily purified earlier by using a similar type of DEAE-column (Yang and Jurtshuk, 1978), and such cytochrome o preparations, which were completely free of c-type cytochrome, possessed no TMPD oxidation capabilities.

The phospholipid activation effect described herein is reminiscent of that previously noted for the D-β-hydroxybutyrate dehydrogenase (Jurtshuk *et al.,* 1963; Sekuzu *et al.,* 1963). A heated mitochondrial lipid extract containing micellar phosphatidylcholine activated this unusual detergent-solubilized mitochondrial dehydrogenase. Unlike other NAD$^+$-dependent dehydrogenases, the D-β-hydroxybutyrate dehydrogenase is tightly bound to the inner mitochondrial membrane and can only be solubilized after extensive detergent treatment, much like the TMPD-dependent cytochrome c_4:o oxidase described in this communication. The fact that micellar phosphatidylethanolamine activated the cytochrome c_4:o oxidase to the same extent as a heated preparation of Fraction I (Fig. 4) suggests that the phosphatidylethanolamine component in Fraction I (Fig. 3) was primarily responsible for this activation effect.

Further studies will be attempted to resolve the *Azotobacter vinelandii* cytochrome c_4:o complex into its constitutive functional components, e.g., cytochrome c_4 and cytochrome o. Reconstitution of TMPD oxidase activity will be attempted by mixing these two components in the presence of micellar phospholipid. It appears that the *A. vinelandii* cytochrome c_4:o oxidase will exhibit the functionally analogous phospholipid activation responses previously noted for the mitochondrial cytochrome c oxidase (Wharton and Griffiths, 1962). More current studies by Green and Fry (1980) indicate that by delipidating the purified mitochondrial cytochrome c oxidase complex with Triton X-100 in glycerol, oxidase activity can only be fully recovered after the addition of micellar phospholipids.

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