Biochimica et Biophysica Acta, 637 (1981) 374–382
Elsevier/North-Holland Biomedical Press

BBA 48127

ISOLATION AND PURIFICATION OF THE CYTOCHROME OXIDASE OF *AZOTOBACTER VINELANDII* *

PETER JURTSHUK, Jr, T.J. MUELLER ** and T.Y. WONG

Department of Biology, University of Houston, Houston, TX 77004 (U.S.A.)

(Received March 3rd, 1981)

Key words: Cytochrome oxidase; Electron transport; (Azotobacter vinelandii)

A membrane-bound cytochrome oxidase from *Azobacter vinelandii* was purified 20-fold using a detergent-solubilization procedure. Activity was monitored using an ascorbate-TMPD oxidation assay. The oxidase was 'solubilized' from a sonic-type electron-transport particle (R_3 fraction) using Triton X-100 and deoxycholate. Low detergent concentrations first solubilized the flavoprotein oxidoreductases, then higher concentrations of Triton X-100 and KCl solubilized the oxidase, which was precipitated at 27–70% $(NH_4)_2SO_4$. The highly purified cytochrome oxidase has a V of 60–78 μgatom O consumed/min per mg protein. TMPD oxidation by the purified enzyme was inhibited by CO, KCN, NaN_3 and NH_2OH; $NaNO_2$ (but not $NaNO_3$) also had a potent inhibitory effect. Spectral analyses revealed two major hemoproteins, the *c*-type cytochrome c_4 and cytochrome *o*; cytochromes a_1 and *d* were not detected. The *Azotobacter* cytochrome oxidase is an integrated cytochrome c_4–*o* complex, TMPD-dependent cytochrome oxidase activity being highest in preparations having a high *c*-type cytochrome content. This TMPD-dependent cytochrome oxidase serves as a major oxygen-activation site for the *A. vinelandii* respiratory chain. It appears functionally analogous to cytochrome $a+a_3$ oxidase of mammalian mitochondria.

Introduction

Photochemical action spectra have revealed three terminal oxidases in *Azotobacter vinelandii*, i.e., cytochromes a_1, *d* and *o* [1,2]. Cytochrome oxidase activity has been studied in *Azotobacter* using artificial electron donor systems like ascorbate-TMPD [3,4] and ascorbate-DCIP [5,6], as well as natural donors like purified *Azotobacter* ferrocytochrome $c_4 + c_5$ [5–7], and reduced horse heart cytochrome *c* [7]. As with most bacterial electron-transport systems [8,9], the *A. vinelandii* respiratory chain oxidizes mammalian cytochrome *c* poorly [3,7,9]. Regardless of such low specific activities, the kinetic analyses of horse heart ferrocytochrome *c* oxidation, by the *A. vinelandii* electron-transport particle (or R_3 fraction), suggested a reaction mechanism typical of an 'oxidase-type' enzyme system [7]. Little is known functionally about any of these terminal oxidase. In contrast, the mitochondrial electron-transport chain contains a single membrane-bound cytochrome oxidase, or the cytochrome $a+a_3$ oxidase, which readily oxidizes soluble ferrocytochrome *c*, its natural substrate.

Bacterial cytochrome oxidases are usually studied by (a) spectral absorbance analyses of (b) with assays employing artificial donors like the indophenol-blue oxidase reaction [9–12]. The latter has led to the development of the microbiological oxidase test [9–11], in which TMPD is commonly used. Keilin and Hartree [12,13] were the first to recognize that artificial donors can measure *c*-type cytochrome-dependent oxidase reactions. Jacobs [14] showed that TMPD-dependent cytochrome oxidase activity was coupled to phosphorylation in mitochondria, and that this oxidation measured cytochrome *c* oxidase activity.

* Preliminary reports of this study have been presented elsewhere [15,16].

** Present Address: Department of Biochemistry, St. Jude's Children's Research Hospital, Memphis, TN 38101, U.S.A.

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol.

0005-2728/81/0000–0000/$02.50

Jurtshuk and co-workers [3,4,7,9,15–19] have carried out extensive TMPD-dependent cytochrome oxidation studies on the *A. vinelandii* R_3 electron-transport fraction, a highly active membrane-bound terminal oxidase that carries out TMPD oxidation as well as oxidation of other *p*-phenylenediamine derivatives. These oxidations were markedly sensitive to the classical cytochrome oxidase inhibitors, i.e., CO, KCN, NaN_3 and NH_2OH [3,4], and these studies, with those of others [2,5,6], have shown that TMPD and DCIP oxidations measure cytochrome oxidase-type activities in this organism. Jones and Redfearn [5] solubilized a 'red particle' which contained enriched amounts of cytochromes c_4+c_5 and *o*; this fraction also possessed high succinate oxidoreductase and ascorbate-DCIP-dependent cytochrome oxidase activities.

This communication describes the successful purification of a membrane-bound cytochrome oxidase from *A. vinelandii.* The enzyme exhibits high TMPD-dpendent cytochrome oxidase activity and consists of two major hemoproteins, the *c*-type cytochrome c_4 and cytochrome *o*. This enzyme complex represents a major terminal oxidase system in *A. vinelandii,* and is analogous to the cytochrome $a+a_3$ oxidase of mitochondria. The *A. vinelandii* cytochrome c_4-*o* oxidase has been referred to as a bacterial alternative oxidase [16,17].

Materials and Methods

Chemicals and procedures

The chemicals and procedures used are described elsewhere [3,4,7,15–20]. Recrystallized TMPD was purified by vacuum sublimation [21]. Deoxycholate solution (10%, w/v) was prepared by neutralizing (pH 7.5–8.0) enzyme-grade deoxycholic acid (Mann Research Laboratories) with NaOH. Aquous 10% (w/v) Triton X-100 was prepared with deionized water. High-purity CO and N_2 gases were made oxygen free by bubbling through alkaline pyrogallol traps; the gases were rewashed to insure aqueous saturation. Proteins were determined by a modified biuret method of Gornall et al. [22].

Preparation of the R_3 fraction

A. vinelandii strain O (ATCC 12 578) batch cultures were grown on acetate under N_2-fixing conditions [3,4,7]. The R_3 membrane fraction was isolated by differential centrifugation from sonically disrupted resting cells [3,4,7,18,19]. The *Azotobacter* R_3 fraction contained 26 mg phospholipid/100 mg protein [9], with high concentrations of cytochromes, non-heme iron and flavoproteins, and catalyzed the oxidation (by molecular O_2) of NADH, NADPH, L-malate, D-lactate, succinate, L-glutamate, cytochrome *c*, cytochrome c_4+c_5, TMPD and DCIP [3,4,7,9,15–19].

Solubilization of the cytochrome oxidase

The frozen R_3 fraction (15 mg protein/ml) was thawed and homogenized. All subsequent steps were carried out at 4°C. Aqueous Triton X-100 was added dropwise, with constant stirring, to a concentration of 0.5 mg/mg protein. After stirring for 15 min, this preparation was centrifuged at $135\,000 \times g$ for 1 h, and the orange-yellow supernatant was discarded. The brownish-red pellet was resuspended in 0.02 M KH_2PO_4 buffer, pH 7.5, to one-half the original volume, and recentrifuged at $135\,000 \times g$ for 1 h. The supernatant was discarded, the brownish-red pellet was resuspended as above, and the protein adjusted to 10 mg/ml. Deoxycholate solution was added dropwise to a concentration of 0.5 mg/mg protein. After stirring for 15 min, the suspension was centrifuged at $135\,000 \times g$ for 1 h. The pale yellow-orange supernatant was discarded, and the brownish-red pellet was resuspended as above; the protein was again readjusted to 10 mg/ml. Aqueous Triton X-100 was added again slowly to 1.5 mg/mg protein then the enzyme solution was made 1 M by slow addition of solid KCl. After stirring for 40 min, this suspension was centrifuged at $135\,000 \times g$ for 1 h. The yellow-orange Triton/KCl supernatant, containing the solubilized cytochrome oxidase, was carefully decanted and the Triton/KCl pellet discarded. KCl was removed from the Triton/KCl supernatant by overnight dialysis against 0.02 M KH_2PO_4 buffer, pH 7.5. A faint precipitate which appeared was removed by centrifugation at $37\,000 \times g$ for 20 min. The clear supernatant was brought to 27% saturation by the slow addition (with constant stirring) of a saturated $(NH_4)_2SO_4$ solution; the precipitate formed floated when centrifuged at $37\,000 \times g$ for 20 min. The supernatant which contained the oxidase was carefully removed with a Pasteur pipette without disturbing the (0–27%) floating pellet. This lipoprotein

pellet is predominantly cytochrome *o*, which was subsequently used by Yang and Jurtshuk [27,28] to prepare pure cytochrome *o*.

The pipetted (0–27%) supernatant was brought to 70% saturation by the slow addition of solid $(NH_4)_2SO_4$. The precipitate formed was removed after centrifugation for 20 min at 37 000 × *g*. The active cytochrome oxidase was found in the (27–70%) floating pellet; the colorless supernatant was discarded. The 'floating' 27–70% precipitate was resuspended in 0.02 M KH_2PO_4 buffer, pH 7.5, and dialyzed against the same buffer until residual traces of $(NH_4)_2SO_4$ were removed. Following dialysis, the 27–70% fraction was clarified by centrifugation at 37 000 × *g* for 20 min.

Enzyme assays

Spectrophotometric assays. All spectrophotometric assays were performed as previously described [4,7, 16,18–20]. The extinction coefficient used for DCIP-dependent cytochrome reductase assays was 20.5 $mM^{-1} \cdot cm^{-1}$ [23]. 1 mM phenazine methosulfate was used for the mediated DCIP-dependent cytochrome reductase assay with 0.07 mM DCIP. The NADH oxidase activities were calculated using the extinction coefficient 6.22 $mM^{-1} \cdot cm^{-1}$ [24].

Polarographic assays. Polarographic TMPD-dependent cytochrome oxidase assays were performed at 30°C using a Clark-type oxygen electrode (Yellow Spring Instruments) mounted on a Gilson Model KM Oxygraph. The total reaction volume was 1.5 ml and contained 0.648 μgatom O (buffer saturation at 30°C). All reactions were initiated by enzyme addition, and the reactants were 66.7 mM KH_2PO_4 buffer, pH 6.0; 6.67 mM L-ascorbate, pH 6.2; and 0.33–3.33 mM TMPD. TMPD auto-oxidation rates were negligible and no corrections were required for nonenzymatic reactions.

Manometric assays. Manometric assays were employed for CO inhibition studies [3,4]. One Warburg flask side-arm was sealed with a rubber serum stopper. 1 ml quantities of varying CO/air mixtures were injected through this serum stopper. For controls, N_2 gas (in place of CO) was injected into a comparable flask to allow for the calculation of the TMPD oxidation rate at reduced oxygen concentrations.

Spectral analyses. All spectral analyses were performed with a Backman Model 25 recording spectrophotometer using quartz curvettes having a 1 cm light path. A few crystals of sodium dithionite were used for chemical reductions. For (a) CO reduced minus oxidized spectra or (b) CO reduced minus reduced absorbance analyses, oxygen-free CO was bubbled for 60–90 s, immediately after reducing with dithionite. The concentrations (*c*-type cytochrome Δ 551–538 nm) were calculated using the extinction coefficient 17.3 $mM^{-1} \cdot cm^{-1}$ [25]. Cytochrome *o* concentrations (Δ417–432 nm) were calculated using 170 $mM^{-1} \cdot cm^{-1}$ [26] as the extinction coefficient.

Results

Distribution of protein, activity, and recovery of units during purification of the cytochrome oxidase

Table I shows the protein distribution, specific activities and the recovery of activity units for the various stages of cytochrome oxidase purification. Exposure of the R_3 fraction to Triton X-100 stimulated TMPD-dependent cytochrome oxidase activity. As noted, very little TMPD-dependent cytochrome oxidase activity was removed by the initial Triton X-100- or deoxycholate-solubilization steps. The second Triton X-100 solubilization, with 1 M KCl, released the cytochrome oxidase from the membrane. The solubilized oxidase in the Triton-KCl supernatant had a 6-fold higher TMPD-dependent cytochrome oxidase activity than did the original R_3 fraction (31.8 as against 5.3). Precipitation with $(NH_4)_2SO_4$ results in a further 2-fold purification, the highest activity being found in the 27–70% $(NH_4)_2SO_4$-precipitated fraction.

The purified cytochrome oxidase in the 27–70% fraction is completely free of detectable flavoprotein-dependent oxidoreductase activities. Activities for the phenazine methosulfate-mediated DCIP-dependent cytochrome oxidoreductases (for L-malate, D-lactate and succinate), as well as the NADH-DCIP-dependent cytochrome reductase activity were undetected in the 27–70% fraction.

Oxidation of electron donors by the purified cytochrome oxidase

Other electron donors also were oxidized by the highly purified TMPD-dependent cytochrome oxidase. The capability of DCIP, phenazine methosulfate,

TABLE I

DISTRIBUTION OF PROTEIN, SPECIFIC ACTIVITIES, AND RECOVERY OF ACTIVITY UNITS DURING THE ISOLATION OF THE CYTOCHROME OXIDASE OF *AZOTOBACTER VINELANDII*

V values expressed as μgatom ($2e^-$) O consumed/min per mg protein at 30°C.

Fraction	Protein concentration (%)	Specific activities (*V*)	Recovery activity units (% of R_3)
R_3	100	5.3	100
Triton R_3	100	6.9	–
Triton supernatant	63	1.2	18
Triton wash	4.5	–	–
Deoxycholate	5.0	7.6	9
Triton/KCl supernatant	6.0	31.8	47
Triton/KCl pellet	11.5	1.9	4
0–27% $(NH_4)_2SO_4$	3.5	4.0	3.5
27–70% $(NH_4)_2SO_4$ (purified oxidase)	1.1	67.0 [a]	18

[a] Highest specific activity recorded was 76.9 on a *V* basis.

menadione and cytochrome *c* in serving as donors, relative to TMPD, is shown in Table II. Specific activities for the active donors are reported as apparent *V* values. The highest activity was obtained with TMPD as donor; the specific activity with DCIP was approx. one-half that of TMPD. This observation is in accord with the study of Ackrell and Jones [6], who reported *V* values for TMPD oxidation were 3–4-fold higher than for DCIP oxidation, with an *A. vinelandii* phosphorylating-type preparation. Phenazine methosulfate also served as a donor for cytochrome

TABLE II

THE CAPABILITY OF VARIOUS ARTIFICIAL REDOX CARRIERS IN SERVING AS ELECTRON DONORS FOR THE PURIFIED CYTOCHROME OXIDASE

All assays were performed at pH 6.0, at 6.67 mM ascorbate. Specific activities are expressed as μgatom O consumed/min per mg protein at 30°C.

Electron donor	Specific activity	K_m (mM)
Ascorbate + TMPD	46.5 [a]	1.9
Ascorbate + DCIP	23.3 [a]	0.36
Ascorbate + phenazine methosulfate	6.8 [a]	0.01
Ascorbate + menadione (7.6 μM)	0.09	–
Ascorbate + cytochrome *c* (5.4 μM)	0	–
Ascorbate + cytochrome *c* (14.4 μM)	0	–

[a] Represents *V* values at infinite electron donor concentration.

oxidase activity, but menadione was a poor electron donor. Although vitamin K is not found in *Azotobacter* spp., a menadione oxidase [29] as well as L-malate and D-lactate-menadione reductase activities [18,19] has been reported in studies on the *Azotobacter* electron-transport-type preparations.

Surprisingly, horse heart cytochrome *c* oxidase activity was completely absent in purified cytochrome oxidase preparations. Since cytochrome *c* oxidase activity is readily detected in the R_3 fraction, but is absent in the purified oxidase, this suggests that perhaps another oxidase pathway of the branched electron-transport chain might be responsible for the enzymatic oxidation of mammalian cytochrome *c*. Thus, cytochrome *c* oxidation by the *Azotobacter* respiratory chain might actually be carried out by pathways in which cytochrome a_1 or *d* is an integral part. It is also possible that the detergent-solubilization procedure used herein may have altered the purified cytochrome oxidase complex, so that it can no longer oxidize horse heart ferrocytochrome *c*.

Effects of inhibitors on the purified cytochrome oxidase

The effects of cytochrome oxidase inhibitors on the purified *Azotobacter* oxidase are shown in Fig. 1 and Table III. Fig. 1 shows the effects of KCN, NaN_3, $NaNO_2$ and NH_2OH on TMPD-dependent cytochrome oxidase activity. The percent inhibi-

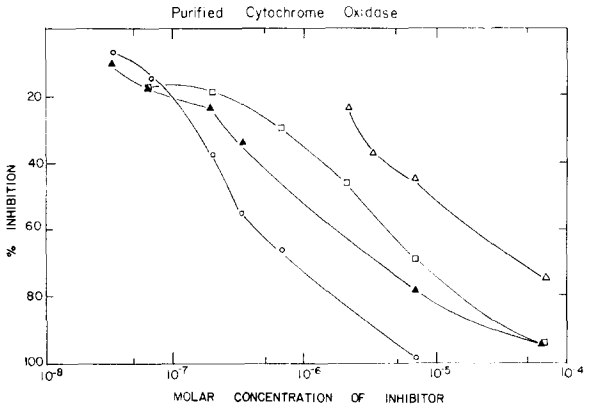


Fig. 1. The effect of the cytochrome oxidase inhibitors and $NaNO_2$ on TMPD oxidation by the purified cytochrome oxidase of *A. vinelandii*. The purified oxidase (0.02 mg protein in 1.5 ml) was preincubated for 1 min with ascorbate, in the presence of inhibitor, and the reaction was initiated with 2.65 mM TMPD. The interpolative 50% inhibition values for the inhibitors were $2.8 \cdot 10^{-7}$ M for KCN (○———○), $8.1 \cdot 10^{-7}$ M for NaN_3 (▲———▲), $2.5 \cdot 10^{-6}$ M for $NaNO_2$ (□———□) and $1 \cdot 10^{-5}$ M for NH_2OH (△———△).

tion of TMPD-dependent cytochrome oxidase activity was plotted as a function of the molar concentration of inhibitor present in the polarographic assay. The interpolative 50% inhibition values were $2.8 \cdot 10^{-7}$ M for KCN, $8.1 \cdot 10^{-7}$ M for NaN_3 and $1 \cdot 10^{-5}$ M for NH_2OH. These inhibitory values are quite similar to those previously reported for TMPD oxidation by the *Azotobacter* R_3 fraction [3]. The purified cytochrome oxidase also was inhibited by $NaNO_2$, 50% inhibition occurring at $2.5 \cdot 10^{-6}$ M. The latter effect was surprising, since $NaNO_2$ is not usually considered an inhibitor of the mitochondrial cytochrome oxidase activity. TMPD oxidation by the particulate R_3 fraction also was sensitive to nitrite, 50% inhibition occurring at $6 \cdot 10^{-5}$ M. Nitrate, at $1.33 \cdot 10^{-3}$ M, had no effect on TMPD oxidation in either the R_3 fraction or the purified cytochrome oxidase.

TABLE III

CO INHIBITION STUDY WITH THE PURIFIED CYTOCHROME OXIDASE

Specific activity expressed as μgatom O consumed/min per mg protein, at 30°C. Assays were performed using the manometric technique. Gas mixtures expressed as v/v ratio (in parenthesis).

Gas phase	Specific activity	% activity
Air	18.3	100
N_2/air (9 : 91)	19.2	105
CO/air (2 : 98)	11.0	60
CO/air (4 : 96)	9.9	54
CO/air (9 : 91)	7.6	41

The data in Table III show the inhibitory effects of CO on TMPD oxidation. A 59% inhibition occurred at a 9% CO/air mixture when it was used for the gas phase in a manometric assay. A control vessel, which contained 9% N_2 (in air) as the gas phase, exhibited 105% of the activity recorded for the control vessel, in which solely air was used. Lower CO concentrations also induced inhibitory effects; 46% inhibition occurred at a 4% CO/air mixture, and a 40% inhibition level was noted at a 2% CO/air mixture.

Spectral analyses

An absolute spectrum of the highly purified cytochrome oxidase is shown in Fig. 2. The solid line is the oxidized spectrum which has a sharp Soret peak at 408 nm. The dithionite-reduced spectrum (dotted line) shows prominent peaks at 549, 520 and a Soret peak at 416 nm. The absorbance changes induced by bubbling CO were minor as shown by the dashed-line spectrum. Only slight absorbance changes were noted in the α- and β-regions after CO addition. However, a new Soret peak for the CO/dithionite-reduced enzyme was formed at 415 nm.

A difference spectrum of the same purified cytocrhrome oxidase preparation is shown in Fig. 3. The following absorbance changes were readily noted: (a) the sharp absorbance at 549 and 520 nm represents the α- and β-peaks of the *c*-type cytochrome c_4, (b) the α- and β-absorbance shoulders at 557 and 522 nm can be attributed to cytochrome *o*, (c) the Soret peak of the dithionite-reduced enzyme was at 422 nm, (d) bubbling CO into the dithionite-reduced enzyme resulted in the formation of new prominent troughs at 568, 538, 472 and 450 nm, and (e) CO also induced a shift in the Soret peak from 422 to 420 nm. The addition of CO to the dithionite-reduced oxidase alters those spectral characteristics that are attributed to cytochrome *o*. The spectral characteristics of the *c*-type cytochrome, which is

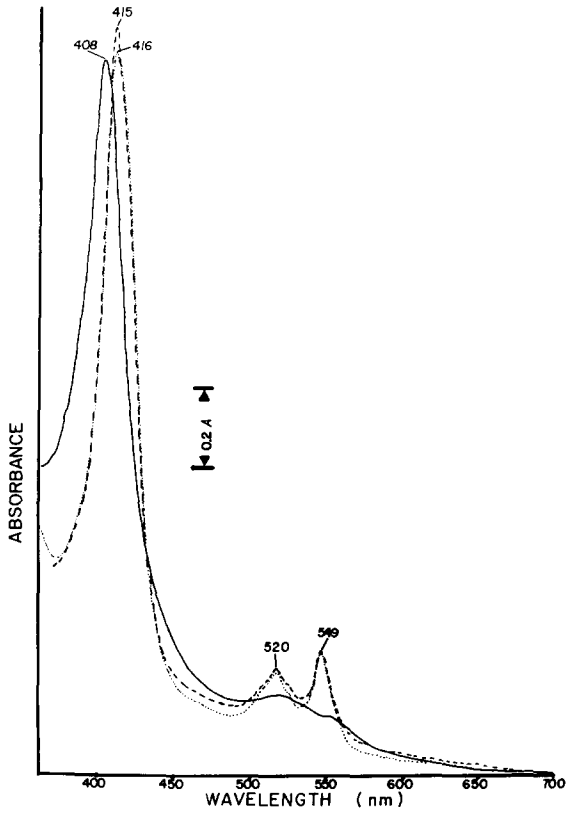


Fig. 2. Absorbance spectra of the purified cytochrome oxidase of *A. vinelandii*. The protein concentration was 1.36 mg/ml. (———) Oxidized enzyme, (· · · · ·) dithionite-reduced enzyme, (– – – –) CO/dithionite-reduced enzyme. The high concentration of *c*-type cytochrome found in all active oxidase preparations masks the absorbance changes due to cytochrome *o*, the CO-reactive hemoprotein.

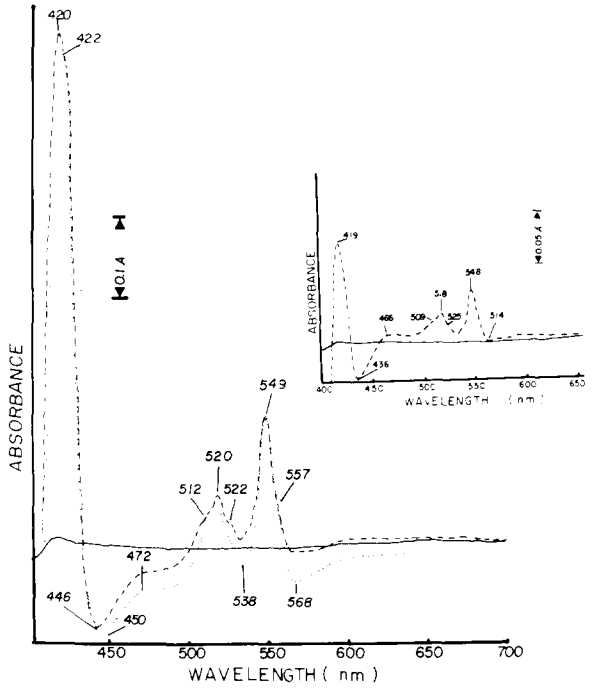


Fig. 3. Difference spectrum of the purified cytochrome oxidase of *A. vinelandii*. The protein concentration was 1.36 mg/ml. (———) Oxidized minus oxidized control (in main figure and inset). (· · · · ·) Dithionite-reduced minus oxidized spectrum, from which the *c*-type cytochrome concentration was calculated to be 7.9 nmol/mg protein. (– – – – –) CO/dithionite-reduced minus oxidized spectrum. (Inset) (– · – · – ·) Ascorbate-reduced minus oxidized spectrum which primarily shows the absorbance contributed by the *c*-type cytochrome c_4 present in the oxidase.

found in large concentrations in this type of purified cytochrome oxidase, appear to be unaffected by CO. Fig. 3, inset, shows that ascorbate also reduced the *c*-type cytochrome of the purified oxidase. Prominent α- and β-peaks are noted at 548 and 518 nm, respectively with absorbance shoulders at 525, 509 and 466 nm. A major trough is observed at 436 nm, and the Soret peak at 419 nm. Ascorbate is not oxidized by the purified cytochrome oxidase; oxygen uptake occurs catalytically only upon the addition of TMPD. Attempts at analyzing the spectral shifts induced by the addition of TMPD, to an ascorbate-reduced enzyme, were unsuccessful because of the highly reactive oxidation reaction which resulted in Wurster's blue formation. The latter reaction was so rapid that it masked all spectral changes in the visible region. Little is known about the absorbance trough at 436 nm, since CO was not bubbled into the ascorbate-reduced oxidase. This trough suggests that the purified cytochrome oxidase may contain a new high-potential redox component, one that is capable of undergoing significant redox changes upon the addition of ascorbate (or E'_0 = +80 mV).

Fig. 4 shows a spectrum of the CO/dithionite-reduced minus dithionite-reduced cytochrome oxidase preparation. The spectral changes induced by CO are shown by the dashed line. The inset shows the prominent α- and β-absorbance changes that occurred when a more sensitive (5-fold) spectrophotometric setting was used. CO-induced peaks are noted at 570

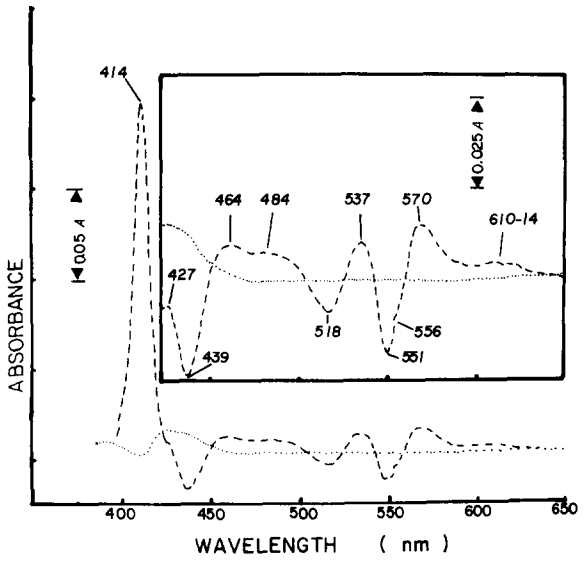


Fig. 4. A CO/dithionite difference spectrum of the purified cytochrome oxidase of *A. vinelandii*. (- - - - -) CO/dithionite-reduced minus dithionite-reduced spectrum, (· · · · ·) dithionite-reduced minus dithionite-reduced control. The protein concentration was 1.36 mg/ml. The concentration of cytochrome *o* calculated from the Soret absorbance was 0.92 nmol/mg protein. The inset shows the CO-induced changes in the α- and β-regions at a 5-fold increase in absorbance sensitivity; these absorbance changes are readily noted in the main spectrum, which shows the Soret peak at 414 nm.

and 537 nm, and CO-induced absorbance shoulders were recorded at 610–614, 484 and 464 nm. Troughs were noted at 551, 518 and 439 nm, with absorbance shoulders at 556 and 427 nm. A sharp CO/dithionite-reduced minus dithionite-reduced Soret peak was found at 414 nm. The CO-reduced spectral changes above are typical of all highly active cytochrome oxidase preparations, and represent those attributed to cytochrome *o*. Cytochrome *o* is the only CO-reacting hemoprotein component that has been detected consistently in purified *Azotobacter* cytochrome oxidase preparations. It is undoubtedly the hemoprotein that reacts with molecular O_2. Table III shows the CO inhibition data for TMPD-depdendent cytochrome oxidase activity by this highly active *A. vinelandii* cytochrome oxidase.

Discussion

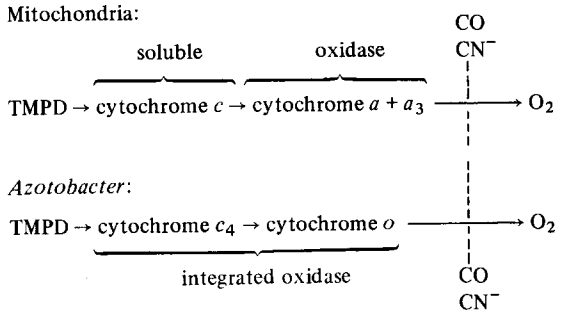
At least two hemoproteins are consistently found in all purified TMPD-dependent cytochrome oxidase preparations, namely cytochromes c_4 and *o*. Cytochrome c_4 is always found in highest concentrations ranging from 10 to 12 nmol/mg protein. In the R_3 fraction the concentrations of *c*-type cytochromes are usually 0.8–1.2 nmol/mg protein. In the Triton/KCl supernatant, TMPD-dependent cytochrome oxidase activity is usually 5–8-fold higher than that of the R_3 fraction; the *c*-type cytochrome concentration ranges from 4.3 to 5.2 nmol/mg protein. TMPD-dependent cytochrome oxidase activity in the purified cytochrome oxidase preparation is usually 2-fold higher than that found in the Triton/KCl supernatant and the *c*-type cytochrome concentration is also increased 2-fold. All indications are that TMPD-dependent cytochrome oxidase activity is directly proportional to the concentration of *c*-type cytochrome found in all highly active cytochrome oxidase preparations.

This observation was not true for cytochrome *o*, the oxygen-reactive hemoprotein. The cytochrome *o* component reacts with CO, and it was previously shown that the *A. vinelandii* cytochrome *o* also reacted with KCN [27,28]. KCN addition induced major spectral changes only in ferricytochrome *o*, and not with dithionite-reduced hemoprotein. The cytochrome *o* concentration in the R_3 fraction varied from 0.25 to 0.35 nmol/mg protein. The cytochrome *o* concentration was always highest in the Triton/KCl supernatant where its concentration ranged from 1.0 to 2.0 nmol/mg protein. In highly active cytochrome oxidase preparations, the cytochrome *o* concentrations varied markedly from 0.2 to 1.4 nmol/mg protein. Of interest is that the previously purified cytochrome *o* preparation [27,28], which was free of all *c*-type cytochromes, was not able to oxidize ascorbate-TMPD. The same purified cytochrome *o* also (a) could not be reduced by ascorbate like the *c*-type cytochrome of the cytochrome c_4-*o* enzyme complex, and (b) it exhibited an oxygenated form spectrally [28].

Subsequent studies on our purified cytochrome c_4-*o* oxidase have shown it to be a phospholipid-containing enzyme complex, and low-temperature spectroscopy studies demonstrate that cytochrome c_4 was the sole *c*-type cytochrome present; cytochrome c_5, if present, is found only in trace amounts [30,31].

Both TMPD and DCIP with ascorbate have been

used for studying the terminal oxidase reactions in *A. vinelandii,* The early studies of Tissieres et al. [32] as well as those of Jones and Redfearn [2,5] and Jurtshuk et al. [3,4] indicated that the site of entry of electrons from TMPD (and DCIP) into the *A. vinelandii* respiratory chain was at the cytochrome *c* level. However, unlike the mitochondrial electron-transport system, no soluble *c*-type cytochrome component(s) is available for use in studying the *Azotobacter* (or other bacterial) cytochrome oxidase system. Thus, for most 'oxidase-positive' bacteria, the natural electron donor for oxidase activity appears to be a membrane-bound (or insoluble) *c*-type cytochrome, which by analogy is more similar to cytochromes *b* and c_1 of mitochondria [9]. There is evidence that in the *Azotobacter* electron-transport chain, the *b*-type cytochrome serves as a donor for the 'cyanide-insensitive' oxidase pathway, or the cytochrome $b \rightarrow d \rightarrow O_2$ complex pathway [33]. The latter pathway would thus function in the absence of a *c*-type cytochrome oxidase, but would still contain the CO-reactive hemoprotein, cytochrome *d*. For the highly active cytochrome oxidase purified herein, it appears that cytochrome *o* is intimately associated with cytochrome c_4 in carrying out TMPD oxidation. Thus, in many ways the TMPD-dependent cytochrome oxidase of *A. vinelandii* can be functionally compared to the mitochondrial cytochrome $a+a_3$ oxidase. A cytochrome *c* is required for TMPD-dependent cytochrome oxidase activity, and when cytochrome *c* is reduced it donates electrons to the terminal cytochrome oxidase cytochrome *o*, and these electrons are then passed on to molecular O_2 as shown schematically below:



In mitochondria it has been shown that complete removal of cytochrome *c*, in a cytochrome $a+a_3$ oxidase preparation, results in the loss of all TMPD-dependent cytochrome oxidase activity [34,35]. This is true even though the cytochrome *c*-free cytochrome $a+a_3$ oxidase can be readily reduced by exogenously added TMPD [34,35]. The latter finding may also be true for bacterial *c*-type cytochrome oxidase. For example, in one large bacterial survey study [36] in which *Pseudomonas* spp. were examined, only the 'oxidase-negative' cultures, which lacked *c*-type cytochromes, failed to oxidize dimethyl-*p*-phenylenediamine. Thus, it appears that bacterial *c*-type cytochrome must integrate with a terminal oxidase, like cytochrome $a+a_3$ or *o* which are the mandatory reactants for catalytic TMPD oxidation by molecular O_2. To date, no exception to this has been reported (see Refs. 37 and 38).

Role of cytochrome o as an oxidase

Recently, Kauffman and Van Gelder [33] have questioned whether or not cytochromes a_1 and *o* can function as terminal oxidase in *A. vinelandii.* The redox potentials of these two hemoprotein components were found to be lower than those of cytochromes c_4, c_5 and *d.* It was suggested that since no low-potential oxidase exists, the roles of cytochromes a_1 and *o*, as oxidases in *A. vinelandii,* had to be questioned. The wavelength used for the redox potential measurements for cytochrome *o* was 560 nm, but the major component which absorbs at this wavelength is cytochrome b_1. The purified *A. vinelandii* cytochrome *o* has its absorption peak at 557 nm [28]. Other studies have shown that the concentration of cytochrome b_1 is several-fold higher than that of cytochrome *o* in the *A. vinelandii* respiratory particle [39] and it may well be that the redox potential calculations of Kauffman and Van Gelder [33] may more closely reflect that of cytochrome b_1 rather than that of cytochrome *o*. Ackrell and Jones [6] also have reported on an oxidative phosphorylation site on the cytochrome $c_4 + c_5 \rightarrow o \rightarrow O_2$ branch of the *A. vinelandii* electron-transport chain. This pathway was responsible for both TMPD and DCIP oxidation. It is more likely that the highly active cytochrome c_4-*o* oxidase purified herein represents the major terminal oxidase of this branched pathway. There are, however, reports which consider the cytochrome c_4-*o* oxidase pathway to be an alternative to the major branch, the components of which are represented by the cytochrome $b \rightarrow d$ complex pathway. This major branch primarily carries out NADH

oxidase activity in the *Azotobacter* electron-transport chain [2,5,9,16,17,25].

Acknowledgements

This research was supported in part by a former U.S. Public Health Service Grant awarded by the National Institute of General Medical Sciences, GM 17607-02, and more recently by the financial support provided by the University of Houston, New Research Opportunities Program.

References

1 Castor, L.N. and Chance, B. (1959) J. Biol. Chem. 234, 1587–1592
2 Jones, C.W. and Redfearn, E.R. (1967) Biochim. Biophys. Acta 143, 340–353
3 Jurtshuk, P., Jr, Aston, P.R. and Old, L. (1967) J. Bacteriol. 93, 1069–1078
4 Jurtshuk, P., Jr, May, A.K., Pope, L.M. and Aston, P.R. (1969) Can. J. Microbiol. 15, 797–807
5 Jones, C.W. and Redfearn, E.R. (1967) Biocheim. Biophys. Acta 143, 354–362
6 Ackrell, B.A.C. and Jones, C.W. (1971) Eur. J. Biochem. 20, 22–28
7 Jurtshuk, P., Jr, and Old, L. (1968) J. Bacteriol. 95, 1790–1797
8 Smith, L. (1968) in Biological Oxidation (Singer, T.P., ed.), pp. 55–122, Interscience, New York
9 Jurtshuk, P., Jr, Mueller, T.J. and Acord, W.C. (1975) CRC Crit. Rev. Microbiol. 3, 399–468
10 Gordon, J. and McLeod, J.W. (1928) J. Pathol. Bacteriol. 31, 185–190
11 Ellingsworth, S., McLeod, J.W. and Gordon, J. (1929) J. Pathol. Bacteriol. 32, 173–183
12 Keilin, D. (1966) The History of Cell Respiration and Cytochromes, pp. 176–185, Cambridge University Press, London
13 Keilin, D. and Hartree, E.F. (1938) Proc. R. Soc. B. 125, 171–186.
14 Jacobs, E.E. (1960) Biochem. Biophys. Res. Commun. 3, 536–539
15 Mueller, T.J. and Jurtshuk, P., Jr (1972) Fed. Proc. 31, 888
16 Jurtshuk, P., Jr, Mueller, T.J., McQuitty, D.N. and Riley, W.H. (1978) in Functions of Alternate Terminal Oxidases (Degn, H., Lloyd, D. and Hill, G.C., eds.), pp. 99–121, Proc. 11th FEBS Meet., Pergamon Press, Oxford
17 Jurtshuk, P., Jr and Yang, T.Y. (1980) in Diversity of Bacterial Electron Transport Systems, (Knowles, C., ed.), vol. 1, CRC Press, Boca Raton, Florida
18 Jurtshuk, P., Jr, Bednarz, A.J., Zey, P. and Denton, C.H. (1969) J. Bacteriol. 98, 1120–1127
19 Jurtshuk, P., Jr and Harper, L. (1968) J. Bacteriol. 96, 678–686
20 Cardini, G. and Jurtshuk P., Jr (1970) J. Biol. Chem. 245, 2789–2796
21 Cox, J.R., Jr and Smith, B.D. (1964) J. Org. Chem. 29, 448
22 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766
23 Ziegler, D.M. and Doeg, K.A. (1962) Arch. Biochem. Biophys. 97, 41–50
24 Horecker, B.L. and Kornberg, A. (1948) J. Biol. Chem. 175, 385–390
25 Jones, C.W. and Redfearn, E.R. (1966) Biochim. Biophys. Acta 113, 467–481
26 Daniel, R.M. (1970) Biochim. Biophys. Acta 216, 328–355
27 Yang, T.Y. and Jurtshuk, P., Jr (1978) Biochim. Biophys. Acta 502, 543–548
28 Yang, T.Y. and Jurtshuk, P., Jr (1978) Biochem. Biophys. Res. Commun. 81, 1032–1039
29 Schils, D.J.J., Hovenkamp, H.G. and Colpa-Boonstra, J.P. (1960) Biochim. Biophys. Acta 43, 129–131
30 Jurtshuk, P., Jr and Yang, T.Y. (1979) Abstr. Ann. Meet. Am. Soc. Microbiol., Los Angeles, CA, p. 158
31 Jurtshuk, P., Jr, McQuitty, D.N. and Riley, W.H., (1979) Curr. Microbiol. 2, 349–354
32 Tissieres, A., Hovenkamp, H.G. and Slater, E.C. (1957) Biochim. Biophys. Acta 25, 336–347
33 Kauffman, H.F. and Van Gelder, B.F. (1973) Biochim. Biophys. Acta 305, 260–267
34 Sekuzu, I., Takemore, S., Orii, Y. and Okunuki, K. (1960) Biochim. Biophys. Acta 37, 64–71
35 Kimelberg, H.K. and Nicholls, P. (1969) Arch. Biochem. Biophys. 133, 327–335
36 Stanier, R.Y., Palleroni, N.J. and Doudoroff, M. (1966) J. Gen. Microbiol. 43, 159–271
37 Jurtshuk, P., Jr and McQuitty, D.N. (1976) Int. J. Syst. Bacteriol. 26, 127–135
38 Jurtshuk, P., Jr and McQuitty, D.N. (1976) Appl. Environ. Microbiol. 31, 668–679
39 Ackrell, B.A.C. and Jones, C.W. (1971) Eur. J. Biochem. 20, 29–35