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Biochemical and biophysical properties of cytochrome *o* of *Azotobacter vinelandii*

Tsanyen Yang

Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403-0212 (U.S.A.)

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Cytochrome *o*, solubilized from the membrane of *Azotobacter vinelandii*, has been purified to homogeneity as judged by ultracentrifugation and polyacrylamide gel electrophoresis. The detergent-containing cytochrome *o* is composed of one polypeptide chain with a molecular weight of 28000–29000, associated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The enzyme exists as a dimer by gel filtration analysis. The amino analysis which reveals the majority of residues are of hydrophobic nature. The cytochrome *o* oxidase contains protoheme as its prosthetic group and about 20–40% of phospholipids. The phospholipids are identified as phosphatidylethanolamine and phosphatidylglycerol by radioautographic analysis using 2-dimensional thin-layer chromatography. No copper or nonheme iron can be detected in the purified oxidase preparation by atomic absorption and chemical analyses. Oxidation-reduction titration shows this membrane-bound cytochrome *o* to be a low-potential component, and E_m was determined to be −18 mV in the purified form and −30 mV in the membrane-bound form. Both forms bind CO with a reduced absorption peak at 559 and 557–558 nm in the native and solubilized forms, respectively. A high-spin ($g = 6.0$) form is assigned to the oxidized cytochrome *o* by electron paramagnetic resonance analysis, and KCN abolishes this high-spin signal. CO titration of purified cytochrome *o* in the anaerobic conditions shows the enzyme binds one CO per four protohemes and a dissociation constant is estimated to be 3.2 μM for CO. Cyanide reacts with purified cytochrome *o* in both oxidized and CO-bound forms, identified by specific spectral compounds absorbed at the Soret region. Cytochrome *c*, often co-purified with cytochrome *c* from the membrane, cannot serve as a reductant for cytochrome *o* in vitro, due to the apparent potential difference of about 300 mV. Upon separation, both cytochrome *o* and cytochrome c_4 show a great tendency of aggregation. Furthermore, the oxidase activity (measured by tetramethyl-*p*-phenylenediamine oxidation rate) decreases as the cytochrome *c* concentration is decreased by ammonium sulfate fractionation. All these suggest the structural and functional complex nature of cytochrome c_4 and cytochrome *o* in the membrane of *A. vinelandii*.

Introduction

Cytochrome *o* was first observed and characterized in bacteria by Chance and his co-workers [1–3]. Although these workers were unable to identify this bacterial oxidase as a *b*-type cytochrome at the time, it is now established that all bacterial cytochromes *o* characterized so far con-

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tain protoheme as their prosthetic group, as represented by the sources from *Azotobacter vinelandii,* [4,5], *Escherichia coli* [6,7] and *Vitreoscilla* [8,9]. Cytochrome *o* is, therefore, of particular interest in the sense that it is the only bacterial oxidase that possesses protoheme as its prosthetic group. Furthermore, it is becoming increasingly evident that cytochrome *o* is the predominant oxidase in prokaryotes and often accompanied by one or two other oxidases such as cytochrome a_1, *d* and aa_3 [10,11]. At the present time little information is available with respect to the relative physiological significance of these oxidases in most microorganisms. It is quite interesting to note that not all prokaryotes produce multiple oxidase systems. There are organisms, such as *Acetobacter suboxydans, Pseudomonas ovalis, Staphylococcus aureus,* and others, such as those of Enterobacteriaceae, namely, *Enterobacter aeregenes, E. coli,* and *Proteus vulgaris,* that produce cytochrome *o* as the sole oxidase when cells are in their log phase [2,7]. Unlike the eukaryotic mitochondria, the quantity and quality of prokaryotic oxidases found within a species can be greatly influenced by the growth conditions [7,11]. In this context the study of bacterial oxidases bears significant implication with respect to the effect of the environment on the cellular respiration and, as a consequence, cellular metabolism as a whole.

Azotobacter vinelandii is an aerobic, nitrogen-fixing bacterium, which has the most active respiratory system among all the living cells, and its electron-transport chains has been the subject of extensive study for quite some time [11–13]. However, the nature of its terminal oxidase systems remains unknown at the molecular level. Some limited knowledge on the physiological roles of cytochrome *d* and *o* oxidases have been accumulated [11,12,14], despite the fact that the physiological function of cytochrome a_1 remains obscure. Cytochrome *o* appears to be the major oxidase in *A. vinelandii* during the log-phase growth [2,12]. Although all three oxidases have been studied somewhat in the membraneous preparations of *A. vinelandii,* none has been isolated for characterization because of their intrinsically membrane-bound nature. We have recently isolated and purified cytochrome *o* from the membrane particles of this organism [4,5]. This communication deals specifically with the properties and the biological function associated with this oxidase.

Materials and Methods

Materials

Alcohol dehydrogenase, bovine serum albumin, glyceraldehyde dehydrogenase, lysozyme, myoglobin, pyridine, Tris-base and Triton X-100 were purchased from Sigma. Electrophoresis-grade acrylamide and bisacrylamide were obtained from BioRad; Coomassie brilliant blue R-250 from Mann Research; DEAE-cellulose from Whatman; ultrapure carbon monoxide and argon from Airco; sodium lauryl sulfate (SDS, laboratory grade) and ethylenediaminetetraacetate acid (EDTA) from Fisher; *N,N.N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were from Eastman; Carrier ampholytes, pH 3–10, was from LKB; ascorbic acid, monobasic, and dibasic potassium phosphate, potassium hydroxide, potassium cyanide, sodium acetate and sodium dithionite were from Mallinckrodt.

Ultrafiltration membranes and apparatus were purchased from Aminco Corp. Dialysis tubings from Sigma were boiled in 4% EDTA and washed repeatedly with distilled water before use.

Membrane preparations: isolation and purification of cytochrome o

A. vinelandii strain *o* was grown in N_2-fixing conditions with 1% sodium acetate as the sole carbon source. Cells were harvested at the late log phase and the membrane particles were prepared as previously described [4]. The membrane preparations were washed once and either used fresh or stored at −27°C until used. Protein concentrations in the membrane preparations were determined by the Biuret method of Gornall et al. [15], with bovine serum albumin as standard.

Cytochrome *o* was isolated and purified from the membrane preparations of *A. vinelandii* in a fashion similar to previously published methods [4]. Protein concentrations in the purified preparations were determined by the method of Lowry [16] with 2% (w/v) SDS in the sample preparations.

Molecular-weight determination

Polyacrylamide gel electrophoresis in the presence of SDS was performed by the method of Laemmli [17] using Tris-glycine buffer (pH 8.3). The electrophoreses were run on vertical slab gels, and lysozyme (M_r, 14 300), myoglobin (M_r, 17 200), glyceraldehyde dehydrogenase (M_r, 36 000), alcohol dehydrogenase (M_r, 41 000) and glutamate dehydrogenase (M_r, 53 000) were used as standards. The protein sample was dialyzed against gel buffer for 2 h prior to mixing with a solution of 1% SDS in the presence of 1% 2-mercaptoethanol plus 5% sucrose, and incubated at 100°C for 2 min. The samples were subsequently incubated at 37°C for 1 h before subjected to electrophoresis. Meanwhile, the unloaded gels were pre-electrophoresed for 20 min. After electrophoresis, the gels were stained for protein with Coomassie brilliant blue R-250 at 37°C for 1 h and destained overnight with 7% acetic acid in a shaker bath at 40°C. The destained gels were scanned for protein bands using a Gilford spectrophotometer equipped with a gel scan apparatus.

Polyacrylamide gel electrophoresis and isoelectric focusing

Disc polyacrylamide gel electrophoresis was run essentially by the method of David [18] and isoelectric focusing was performed on polyacrylamide gels according to the published methods [19]. The final acrylamide concentrations was 4.5–5% for disc gels and 6% for isoelectric focusing gels. A carrier ampholyte solution (LKB), pH 3–10 with a final concentration of 2%, was used for isoelectric focusing. After electrophoresis the disc gels were stained for protein, while isoelectric focusing gels were fixed and incubated with 5% trichloroacetic acid for 5 h, prior to 4 h staining and followed by a prolonged period of destaining at 37°C. To determine the pH profile of the gels, a control blank was run and used for sections and pH measurement.

Ion analysis

Ion analysis was performed in a Varian Model AA-575 atomic absorption spectrophotometer, and controls were done with the appropriate buffer containing 0.1% Triton X-100, using double-distilled (deionized) water. Nonheme iron analysis was performed following the procedures according to the established method [20].

Phospholipid analyses

Total lipid extractions were performed according to the procedure of Folch et al. [21]. All lipid extracts were dried under a stream of purified nitrogen gas; the residues were dissolved in a few drops of $CHCl_3/CH_3OH/H_2O$ (75 : 25 : 2, v/v/v) and transferred quantitatively to the thin-layer plates coated with silica gel H, 250 μm thick. A 2-dimensional separation procedure, similar to that of Getz et al. [22], was used for resolving the individual phospholipid present in the $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (52 : 20 : 7 : 3, v/v/v/v), then air-dried, washed with acetone, and placed in a vacuum desiccator for 20 min. $CHCl_3/CH_3OH/40\%\ CH_3NH_2/H_2O$ (63 : 31 : 5 : 5, v/v/v/v) was used for the secondary development step. The controls, consisting of known radioactive phospholipids, were co-chromatographically resolved by radioautography using X-ray films which were subjected to 48–72 h of exposure in order to locate the radioactive areas and to allow for the identification of the phospholipid present in the cytochrome *o* fraction. The phospholipid components on the thin-layer plates were stained by exposure to iodine vapor.

Phosphorus was determined by the method of Chen et al. [23]. Phospholipid content was estimated by total phosphorus analyses and calculations were made using an average molecular weight (for phospholipid) of 775.

Carbon-monoxide-binding studies

The purified cytochrome *o* preparation (2–5 μM) was first reduced with appropriate amount of sodium dithionite and placed in a cuvette. The cuvette was capped with a rubber stopper provided with two, separated, syringe outlets – one connected to a nitrogen gas tank and the other for gas outlet and CO addition. Saturated CO solution was prepared in phosphate buffer at 25°C and introduced to the reduced oxidase preparation by a microliter syringe. The spectrum was recorded after each addition of CO solution and an equilibration time of 2–3 min. At the end of titration, pure CO was bubbled through the preparation (at this time nitrogen gas was disconnected) for 5 min

to obtain a full CO-saturated spectrum, which was assumed to be a 100% saturation endpoint and later used to calculate the fractional saturation of each titration curve.

Oxidation-reduction titration

Oxidation-reduction titration was performed potentiometrically under anaerobic conditions according to the method described previously [13].

Amino acid analysis

Prior to amino acid analysis, acid hydrolyses were carried out with known amount of samples (1–2 μM) in duplicate for 24, 36 and 72 h, and the hemes (upper fraction) were carefully separated from the lower amino acid fraction; the heme fractions were used for iron analyses, while the soluble fractions were employed for amino acid analyses in a Beckman Model 120C amino acid analyzer. Cysteic acid was determined from a 72 h hydrolysate in 6 M HCl containing 300 mM dimethylsulfoxide [24] and tryptophan was analyzed after hydrolysis carried out in methanesulfonate containing 0.2% 3-(2-aminoethyl)indole [25].

Electron paramagnetic resonance spectral analyses

The EPR spectrum was taken at 5–12 K with a Varian E4 EPR spectrometer (Varian Associates) equipped with a variable temperature cryostat [26]. The samples were prepared as oxidized preparations or oxidized with an addition of 1 mM potassium cyanide preparations.

Results

The majority of cytochrome *o* could not be solubilized from the membrane particles of *A. vinelandii* by either Triton X-100 (2%) or deoxylcholate alone as previously indicated [5]. Only in the presence of high salt (1 M KCl) was most of the cytochrome *o* released from the electron transport particles by the aid of detergent. As expected, the purified cytochrome *o* preparations have a great tendency to aggregate under various conditions: when dialyzed against low ionic strength buffer without salt; when subjected to repeated freezing and thawing; and when extracted with organic solvents such as chloroform or butanol.

Absolute absorption spectra of the purified oxidase are shown in Fig. 1A. The α-absorption peak at 557 nm with a Soret peak at 426 nm are characteristic of reduced *b*-type cytochrome. CO-

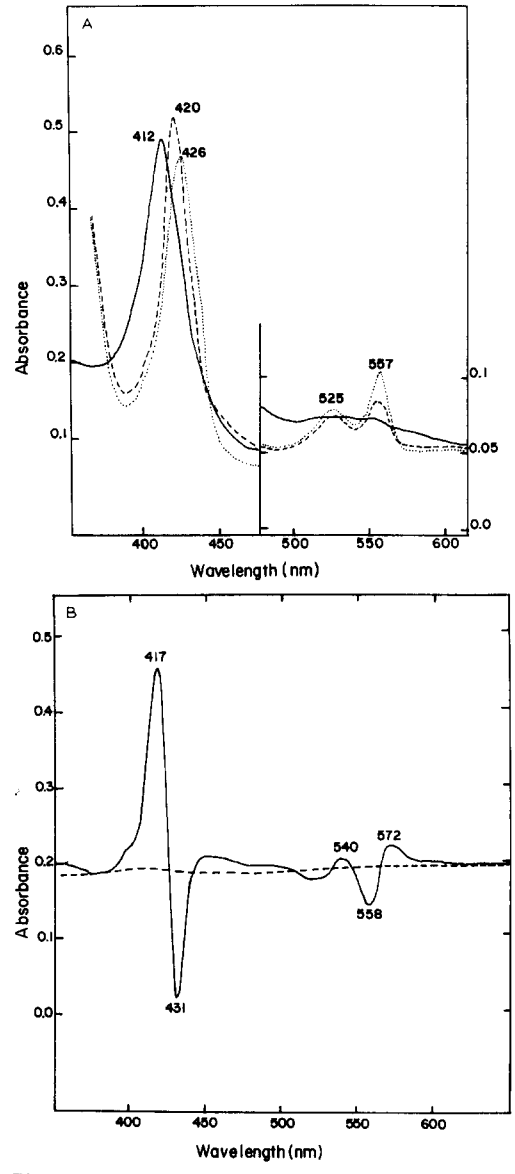

Fig. 1. (A) Absorbance spectra of the purified cytochrome *o* from *A. vinelandii* electron transport particles: the oxidized (———); the reduced, (· · · · · ·), and the carbon monoxide plus reduced spectra (- - - - - -). (B) Carbon monoxide: dithionite reduced-minus-dithionite reduced difference spectra of the purifiec cytochrome *o* preparation of *A*, vinelandii. CO was bubbled for 20 s. In a dithionite reduced oxidase solution (3.1 μM heme) before the spectra were recorded in a Beckman Model 25 dual-beam spectrophotometer.

ligand spectral absorption peaks at 555–556 nm and 420 nm, accompanied by an absorption increase at the Soret peak, are indicative of oxidase. A carbon monoxide difference spectrum as shown in Fig. 1B is normally used to identify the presence of cytochrome *o* oxidase. The concentration of this oxidase in a cell-free extract has been conventionally measured by this type of spectrum. However, in some cases this CO-dependent spectral appearance seems to be unreliable and caution should be exercised in the quantification of this oxidase [27].

The purified cytochrome *o* migrates in an undenatured gel (without SDS and/or urea) as a single protein band with a slight streaking toward the cathode side of the electrode, and this streaking becomes more prominent when the running gel buffer is lower than pH 8.0. When electrophoresis was performed in the presence of 2% SDS and 6 M of urea, a predominant band was again observed. The purity estimated from SDS-gel was about 85–90%. The molecular-weight determination from this gel indicates that the polypeptide has a molecular weight of 28 000 ± 1 000 (result not shown).

The purity of cytochrome *o* was further established by a sedimentation velocity experiment; it sedimented as a single protein species (as evident from a symmetrical Schlieren peak) after 12 h when the centrifugation speed reached 60 000 rpm in a Spinco Model E ultracentrifuge (result not shown).

Gel filtration using Sephadex G-200 column equilibrated with Triton X-100 (0.5%) in phosphate buffer (pH 7.4) indicated cytochrome *o* has a molecular weight of 58 000 (results not shown), suggesting that the purified cytochrome *o* exists perhaps in a dimeric form under the gel filtration conditions. The amino acid composition of the purifed cytochrome *o* preparation was determined (BBA Data Bank). The calculated molecular weight from the amino acid analysis is 25 943, and it approximates well with the value estimated from the gel electrophoresis analyses. The content of the hydrophobic amino acids (about 61%) is compatible to the notion that this oxidase is a transmembrane type protein analogous to mitochondria cytochrome oxidase.

The results of CO binding studies of the oxidase are presented in Figs. 2 and 3. The numbers of

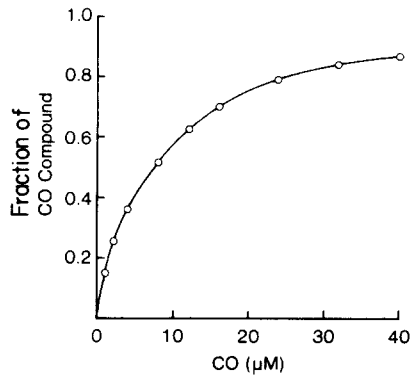

Fig. 2. CO-titration of the purified cytochrome *o*. The experimental procedure follows the details described under the Materials and Methods. The total heme was 3.8 nmol contained in 0.92 ml of well-buffered, air-tight solution. The cuvette was purged with N_2 gas during titration to ensure anaerobiosis. Aliquots of CO-saturated double-distilled H_2O previously made anaerobically were introduced into the cuvette for titration.

hemes which bind CO and the dissociation constant for CO of the enzyme could be determined by a Hill plot. Fig. 2 represents the actual experimental data with an assumption that the end point of titration was accomplished by saturation of CO

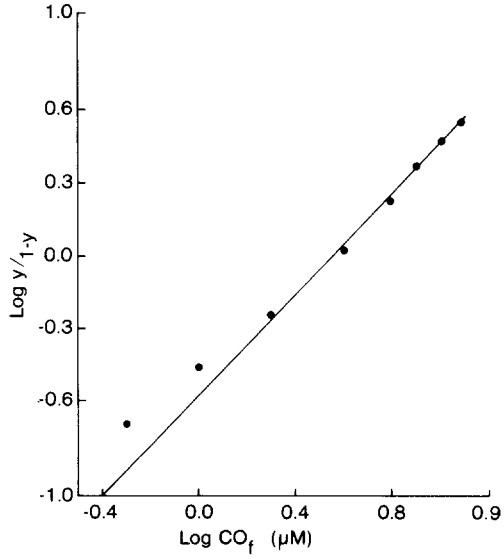

Fig. 3. Hill-plot of the data obtained from CO-titration of cytochrome *o*. CO_f represents the free CO concentration obtained after equilibration and spectral scanning followed each addition of CO solution. *Y* is the total liganded cytochrome *o* concentration, and $1 - Y$ indicates the unbounded cytochrome *o* concentration.

as described under Materials and Methods. The fractional saturation ($1 - Y$) at each titration point was calculated from the spectra of the reduced and CO-saturated, known enzyme concentrations. The line drawn in Fig. 3 is considered a satisfactory fit and it approximates a slope of 1.0. Apparent deviation at the end of saturation, as evident in Fig. 3, may suggest another site of binding, however. The slope of 1.0 corresponds to a dissociation constant of 3.2 μM. The fraction of hemes binding CO as calculated from Fig. 3 is 0.24, suggesting that as many as four protohemes bind one CO under the conditions described. This low fraction binding may suggest either actual loss of heme proteins or point to possible conformational heterogeneity in the preparation studied.

The concentration of phospholipids have been measured in several preparations; however, the contents vary considerably over a wide range (12–40.5% by weight). The significance of this high content of phospholipid that associates with the purified cytochrome *o* is presently unknown. However, a phospholipid depletion study using partially purified TMPD-oxidase preparations preliminarily indicated that oxidase activities depend on the amount of phospholipids that are associated with the oxidase (Table I). A detailed reconstitution study is needed to prove this point.

Two of the phospholipids, phosphatidylethanolamine and phosphatidylglycerol, were readily established by using an autoradiographic technique, as described under Materials and Methods. Cardiolipin could not be detected. The result of the autoradiographic analysis is shown in Fig. 4.

The EPR spectrum of the air-oxidized cytochrome preparations shows a suspected copper-like signal with a *g*-value of 2.2, as shown in Fig. 5. However, atomic-absorption analyses repeatedly fail to detect any significant amount of copper associated with purified oxidase preparations. Atomic-absorption analyses did reveal that cytochrome *o*, based on its minimum iron content, contains two protoheme as its prosthetic group. Taken into consideration of its polypeptide composition, this would suggest that the dimeric oxidase consists of one protoheme per peptide as its physical unit. However, no difference in the redox potential of the heme components can be demonstrated. Hemochromogen analyses showed

TABLE I

PHOSPHOLIPID-DEPENDENT TMPD-OXIDASE ACTIVITIES OF THE PARTIALLY PURIFIED CYTOCHROMES $c_4 + o$ OXIDASE PREPARATIONS FROM *AZOTOBACTER VINELANDII*

Oxidase activity was measured polarographically with TMPD-ascorbate as substrate at 37°C, using partially purified cytochromes $c_4 + o$ preparations. The activity unit was defined as μmol oxygen consumed per min per mg protein. The molar ratio of cytochromes c_4/cytochrome *o* was measured by the method described previously [26]. The Roman numerals (I, II and III) in the parentheses designate the number of times that a given ammonium sulfate fractionation (25–45%) procedure has been performed. Thus, the C (III) preparation indicates that fractionations were done three consecutive times; dialysis against buffer was repeated in between fractionation steps. A(I) preparation was the originally isolated oxidase sample and was used as the initial materials to obtain B and C.

Preparations Cyt $c_4 + o$	Phospholipid content (w/w)	Oxidase activity (TMPD-dependent)	Molar ratio (c_4/o)
A(I)	27.3%	5.3	8.3
B(II)	10.1%	4.1	7.9
C(III)	7.4%	1.8	8.1

the heme absorbed at 557 nm as a protoheme IX component [4]. No split of α- or β-absorption in the cold temperature (77 K) could be demonstrated.

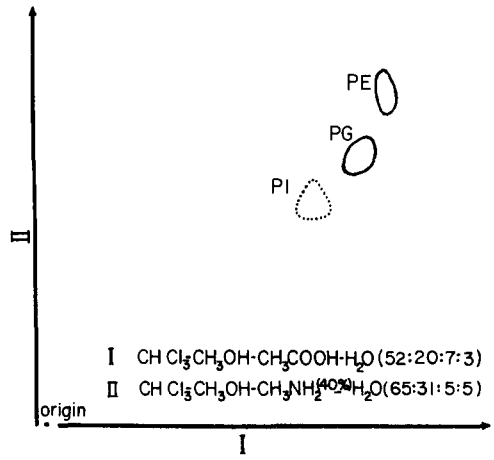

Fig. 4. Two-dimensional thin-layer chromatogram used for identifying the phospholipids present in cytochrome *o* of *A. vinelandii*. Known radioactive phospholipids were used for identifying the presence of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), shown in solid line circles, as the two major phospholipid components in the purified cytochrome *o* preparation. Phosphatidylinositol (PI) was one of the radioactive markers used and is shown in the chromatogram as a dotted circle.

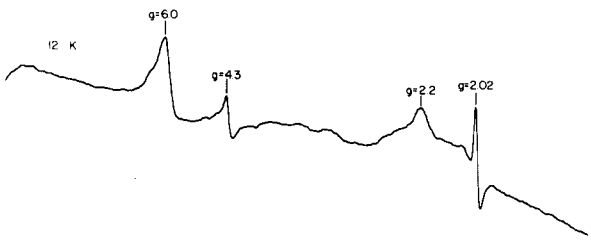

Fig. 5. An electron paramagnetic resonance spectrum of the purified cytochrome *o* isolated from *Azotobacter vinelandii*. The spectrum was taken with a Varian E4 EPR Spectrometer equipped with a variable temperature cryostat. The spectrum was taken at 12 K with the magnetic-field strength increasing to the right of the figure. The ordinate represents the first derivative of the microwave absorption in the arbitrary units. The oxidase preparation contained about 2.1 μM of heme in 25 mM phosphate buffer (pH 7.4) in 1% Triton X-100 solution. The g value of the major peaks are shown.

Cytochrome *o*, as purified, exhibits a high spin form at low temperature (5–12 K) with $g = 6.0$ in neutral phosphate buffer solution (Fig. 5), although its room temperature optical absorbance spectra suggest it contains a typical low spin heme. The fractions of hemes giving rise to the $g = 6.0$ signal in the oxidized preparations have been quantitated to be about 75–87%, suggesting that the bulk of the hemes is responsible for this sign. In addition, when cyanide with final concentrations of 0.1 mM were added, the high spin form ($g = 6.0$) of the oxidase was completely abolished. The disappearance of this signal corresponding to an appearance of an optical absorbance peak at 434 nm at room temperature (results not shown) suggests a formation of cyanide-liganded protoheme compound which no longer is in a high-spin form. It is apparent that the ferric hemes are quite readily accessible to the ionized cyanide and the perturbation of the heme molecules immediately takes place. Furthermore, we have previously shown that the CN-binding reaction of the oxidized form of cytochrome *o* is biphasic, possibly indicating that the oxidase may offer two binding sites per molecule [5]. This could mean that the two hemes may react with KCN differently, although there is not sufficient evidence to verify this.

We have previously reported that partially purified cytochrome *o* was autooxidizable and the ferric heme reacted with oxygen to form an 'oxygenated' species [5]. This unique property, characteristic of being an oxidase, was later found to be observable only with the partially purified, freshly prepared samples (Yang, T., unpublished results), and it lends support to the view that this oxidase is indeed an important functional enzyme in the log phase cells of *A. vinelandii*. The final purified oxidase showed little or no reactivity with molecular oxygen. Subsequent analysis indicated that partially purified cytochrome *o* contained some *c*-type cytochrome (presumably c_4 by cold-temperature spectral characteristics) which not only contributed to the activity as assayed with *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as a substrate [4], but also showed an active reaction with oxygen to form the 'oxygenated' form. However, neither purified cytochrome c_4 nor bovine heart cytochrome *c* could serve as a substrate for the pure cytochrome *o* in vitro.

The isoelectric point of the purified oxidase was found to be about 8.1. Very little nonheme iron or flavin could be detected in oxidase preparations.

Table II summarizes some molecular properties that have been accumulated so far for the purified cytochrome *o* oxidase of *A. vinelandii*. The number of the polypeptide shown are proposed with respect to the dimeric and possibly the functional form of the enzyme in vitro. The midpoint redox potential in the solubilized state was determined in

TABLE II

MOLECULAR PROPERTIES OF CYTOCHROME *o*-OXIDASE PURIFIED FROM *A. VINELANDII*

Molecular weight	28000–29000
Prosthetic group	protoheme IX
no. of heme	1
polypeptide	1
Possible functional form	dimer (?)
Isoelectric point	8.1 ± 0.6
Copper	0.0
Nonheme iron	trace
Flavin	trace
Midpoint potentials	
membranous	$-25(\pm 5)$ mV
purified	$-18(\pm 2)$ mV
Spin state	high spin (ferric state)
Phospholipids	Phosphatidylethanolamine Phosphatidylglycerol
Absorption spectra	557–558, 525 and 426 nm (reduced)

a preparation where no *c*-type cytochromes could be detected either by gel electrophoresis or heme analysis, and this value is somewhat higher (12 mV) than has been previously reported [28].

Discussion

There are some extensive studies that have been attempted in other bacterial cytochrome *o* such as *E. coli* and *Pseudomonas aeruginosa* [26,29–32]. The biochemical properties of cytochrome *o* from *Vitreoscilla* and *Azotobacter vinelandii* are quite similar in many respects. However, unlike the different redox potentials of *Vitreoscilla* diheme oxidase, cytochrome *o* of *Azotobacter* apparently has two identical protohemes as their potentials were raportedly being −18 mV in vitro [28]. In addition, in *Azotobacter,* cytochrome *o* has been demonstrated to be different from another membrane-bound cytochrome *b* electron transport component in that cytochrome *o* reacts with CO and has much lower mid-point potential than cytochrome b_1 (*b*-561). This low mid-point potential cytochrome *o* was speculated to play a limited role in energy conservation when participated in the coupling of oxidative phosphorylation [33].

A second cytochrome *o* was recently described in the membrane particles of *Vitreoscilla* by Webster and his co-workers using spectrophotometric methods [34]. This cytochrome *o* was described as being similar to that of *E. coli* because of their similarities in spectral and kinetical characteristics at low temperatures. We have also suggested previously that a second *o*-type oxidase might have been present in the electron-transport chain of *A. vinelandii* cells when cells were grown under N_2 fixing conditions, based on some preliminary observations in cyanide-binding properties [11]. Although those observations have yet to be substantiated with kinetic analyses and spectral characterization, it seems that evidences that have been accumulated so far suggest the possibility that cytochrome *o* oxidase might be multiple-species oxidase system. If this proves to be the case in other bacteria which contain only cytochrome *o* as an oxidase, it may imply that it has a much more versatile physiological role. Thus, it is interesting to note that cytochrome *o*, in addition to being an oxidase, was identified quite recently as the aerotaxis receptor in anaerobically grown *Salmonella typhimurium* [35]. It is conceivable, therefore, that gene(s) for this oxidase may be much less conservative than others such as cytochrome aa_3.

The purified cytochrome *o* oxidase has a mid-point oxidation-reduction potential (E_m) of −18 mV and an E_m value of 6 mV in its membrane bound state [28]. Furthermore, it has been determined that in the pH range 6.2–9.8, the value of cytochrome *o* varied little, suggesting that in this pH range cytochrome *o* redox reactions do not involve a proton-binding reaction. Whether this is the case in vivo remains to be seen. Although the results from kinetic studies done by Kaufman and Van Gelder seem to support the cytochrome $c_4 \rightarrow o$ and $b_1 \rightarrow d$ pathways as originally proposed from the inhibitor studies [12], however, quinone and other flavoproteins are also possible candidates for serving as the electron sources for cytochrome *o* oxidase. Furthermore, we have previously reported that cytochromes c_4 and *o* complex have a difference of E_m about 300 mV that imposes a problem for electron-transfer schemes, as proposed by Jones and Redfearn, in which cytochrome *c* with an E_m of +260 mV is to reduce cytochrome *o* at −18 mV [28]. Indeed, pure cytochrome c_4 could not serve as a substrate for cytochrome *o* in vitro. Therefore, the true physiological substrates of cytochrome *o* remain unclear in *A. vinelandii* electron-transfer system. Other *b*-type cytochromes with a reported E_m value of 100 mV [13] are quite capable, and perhaps thermodynamically more suitable, of serving as an electron source. In fact, other proposed schemes have indicated that this might very well be the case, even though little supporting evidence is available [7].

It seems that purified cytochrome *o* contains phospholipids, almost similar in make-up to that found for the electron-transport particle in *A. vinelandii.* The presence of phospholipids in association with the purified cytochrome *o*, per se, does not warrant any functional relationship between these two components. But one is tempted to speculate that these phospholipid components might be essential for oxidase activity as is the case for the mitochondria cytochrome oxidase. The fact that cytochrome *o* aggregates when the purified preparation is delipidated, or passed

through a Sephadex (G-50) column to decrease its detergent content, suggests that both phospholipids and detergent (Triton X-100) are essential for maintaining the native or 'solubilzied' state of cytochrome *o*. This apparent structural integrity of the cytochrome *o* in the membrane environment is undoubtedly maintained in part by the phospholipid components reported here. This appears to be in agreement with the finding that the composition of the amino acids of the purified cytochrome *o* contained a high percentage of similar hydrophobic residues when compared to other membrane-bound protoheme-containing protein, such as cytochrome *P*-450 of liver microsomes [36].

The functional requirement of phospholipids on the electron transfer of mitochondrial oxidase has been well-established [37,38]. The latest evidence suggests that phospholipids are functionally involved in modifying the 'molecular environemnt' of cytochrome *c* oxidase to facilitate oxygen binding and thus make possible the fast electron transfer. At the present time, no direct evidence is available to support the same functional role of the phospholipid components for the activity of cytochrome *o*.

More recently, cytochrome *o* oxidase has been solubilized and purified as a cytochrome *b*-562-*o* complex from *E. coli* [32]. It contains equimolar amounts of two polypeptides, with M_r 33000 and 55000 [32]. Unfortunately, the identity of cytochrome *o* component could not be assigned to the specific polypeptide due to the denaturation of the complex with SDS [32]. Nonetheless, the spectral data have confirmed that the heme absorbed at 555 nm (77 K) was the oxidase [32]. Cytochrome *o* from *E. coli* was characterized to have an E_m value of 125 mV. This is, of course, higher than the oxidase found in *A. vinelandii* [28]. A second report showed cytochrome *o* of *E. coli* consists of four polypeptides, with molecular weight of 66000, 35000, 22000 and 17000, and behaves as a monomer in octyl-β-D-glucopyranoside solution [27]. It contains two *b*-type cytochromes and 2 mol of heme per mole of enzyme. It uses ubiquinol-1 and other artificial electron donors as substrates; however, the activities are highly dependent on exogenous phospholipids and other detergents. Reconstitution studies reveal that a proton electrochemical gradient was generated by the oxidase, and it does not appear to catalyze vectorial proton translocation. However, it is not clear that both hemes are reactive with CO. In any event, the studies of *E. coli* cytochrome *o* so far have shown great promise in providing insights into this interesting oxidase [27,29,39].

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