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PURIFICATION AND CHARACTERIZATION OF CYTOCHROME *O* FROM *AZOTOBACTER VINELANDII*

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Summary

The membrane-bound cytochrome *o* has been solubilized from the *Azotobacter vinelandii* electron transport particle and further purified by use of conventional chromatographic procedures. The spectral characteristics as well as the other properties noted for purified cytochrome *o* are reported herein.

Castor and Chance [1] were the first to recognize that cytochromes *o* and a_1 might function as the terminal oxidase in *Azotobacter* spp. Jones and Redfearn [2] were able to solubilize a "red" particle from the *Azotobacter vinelandii* electron transport fraction which contained relatively high concentrations of the CO-binding cytochrome *o*, although these workers previously indicated there was difficulty in determining cytochrome *o* concentration in an electron transport particle type preparation [3]. Mueller and Jurtshuk [4] and Jurtshuk et al. [5] were able to further establish that cytochrome *o* was part of an enzyme complex which together with *c*-type cytochromes ($c_4 + c_5$) functioned as the major CO-sensitive terminal oxidase in *A. vinelandii* that was responsible for carrying out reduced *N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidation.

The purification of cytochrome *o* was accomplished by first preparing the *A. vinelandii* sonic type electron transport (or R_3) fraction by a differential centrifugation [6]. Triton X-100 was added to R_3 preparation (15 mg/ml) to a final concentration of 0.5 mg Triton per mg protein. This preparation was allowed to stand for 30 min and centrifuged at 105 000 × *g* for 1 h. The resultant pellet was resuspended in 0.025 M phosphate buffer, pH 7.2, and after re-homogenization the pellet was re-washed with the same volume of phosphate buffer. The Triton-R_3 fraction (10 mg/ml) was then treated with sodium deoxycholate (1% w/v), recentrifuged, and subjected to the identical washing procedure. The Triton-deoxycholate pelleted R_3 fraction

still contained the cytochrome *o* associated with TMPD oxidation. The cytochrome *o* was then solubilized by a second Triton X-100 (1.5 mg per mg protein) treatment, now in the presence of 1 M KCl. This latter procedure solubilized both the *c*-type cytochromes and cytochrome *o* that is directly involved with TMPD oxidase activity which previously was associated with the *A. vinelandii* electron transport particle fraction [5, 6]. After centrifugation at 105 000 × *g* for 1 h the Triton-KCl supernatant was carefully removed from the pellet and dialyzed in 0.025 M phosphate buffer, pH 7.2, to remove the KCl. The dialyzed Triton-KCl supernatant fraction was then brought to 27% ammonium sulfate saturation by the slow addition of solid ammonium sulfate. The precipitate after centrifugation at 37 000 × *g* for 20 min was removed and resuspended in a small amount of the same phosphate buffer, and redialyzed until ammonium sulfate was no longer detected by color development using Nessler's reagent. 8 ml of this fraction (3.5 mg protein per ml) was loaded on a Sephadex G-200 column (2.0 cm × 30 cm), that was previously equilibrated overnight with 0.025 M phosphate buffer, pH 7.2, containing 0.1% (v/v) Triton X-100. 3-ml fractions were collected, absorbance being monitored at 412 nm. The fractions having high absorbance values were pooled and further reconcentrated by ammonium sulfate precipitation (0—30% saturation). After recentrifugation the pellet, which floated on the surface was collected and dialyzed exactly as before. The purified cyto-

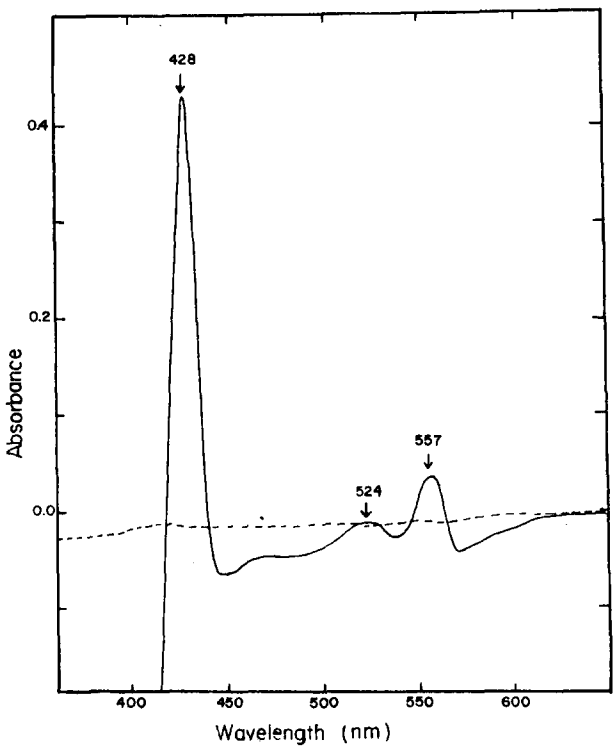

Fig. 1. $Na_2S_2O_4$ reduced-minus-oxidized difference spectrum of the highly purified cytochrome *o* preparation isolated from the electron transfer particle (ETP) fraction of *A. vinelandii*. Protein concentration of the cytochrome *o* is 0.7 mg per ml.

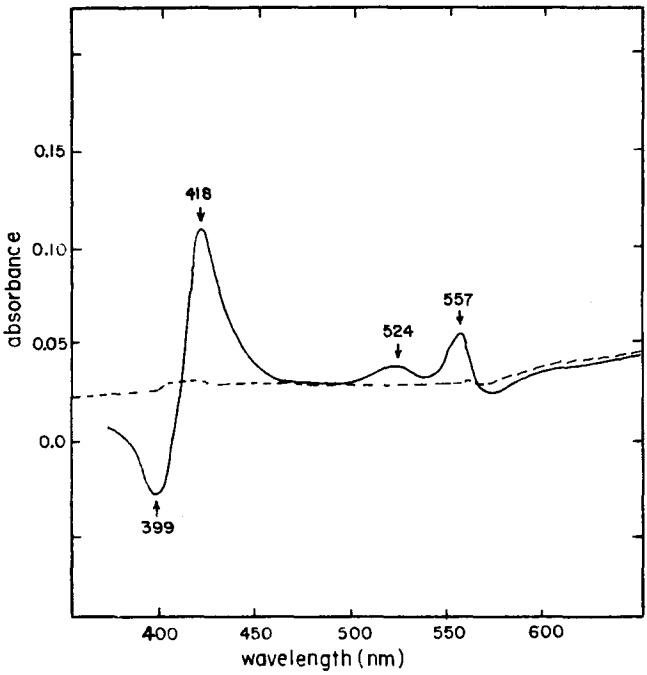

Fig. 2. Pyridine hemochromogen difference spectrum of the purified cytochrome *o* isolated from the ETP of *A. vinelandii*. 2 ml of the purified preparation was used for preparing the pyridine hemochromogen derivative by the method of Pappenheimer and Hendee [8].

chrome *o* appears as clear, reddish solution. Disc gel (5% acrylamide) electrophoresis revealed only trace amounts of a minor protein contaminant (cytochrome c_4) that migrated just above the major band; the latter contained the purified cytochrome *o*. The absorption spectrum (Fig. 1) of the purified preparation contained 1.6 nmol cytochrome *o* per mg protein as calculated from the CO: dithionite reduced-minus-dithionite reduced spectrum, using mM extinction coefficient of 170 measuring the absorbance (416 nm peak minus 431 nm trough) in the Soret region [7]. The prosthetic group of the cytochrome *o* was readily characterized as protoheme by the formation of the pyridine hemochromogen derivative [8]. The pyridine hemochromogen difference spectrum (Fig. 2) showed absorption maxima at 418, 524 and 557 nm. The basic spectral characteristics of *A. vinelandii* cytochrome *o* are summarized in Table I. Cyanide (20 mM) reacts only with the oxidized form of cytochrome *o*; the addition of cyanide to the dithionite-reduced cytochrome *o* does not result in any observable spectral change. Also, no spectral changes were noted when CO was added to the cyanide-complexed cytochrome *o*, which suggests a high degree of stability for the cyanide•cytochrome *o* complex. In contrast, cyanide could markedly alter the CO-dithionite reduced spectrum of cytochrome *o* as shown in Fig. 3.

The cyanide reactivity of the *Azotobacter* cytochrome *o* and its binding stability are very similar to the properties reported for cytochrome *o* in free-living cultures of *Rhizobium japonicum* [9]. Unlike the soluble cytochrome *o* that has been purified from *Vitreoscilla* [10], the *A. vinelandii* cytochrome *o* is tightly bound to the membranous electron transport particle

TABLE I

COMPOSITE SUMMARY OF THE ABSOLUTE AND DIFFERENCE ABSORPTION MAXIMA* OF HIGHLY PURIFIED CYTOCHROME *o* OF *A. VINELANDII*

	α	β	Soret
Absolute spectra			
Oxidized	—	—	412
Reduced**	555—558	523—525	426
CO:reduced	—	—	420
CN:oxidized	550—555	—	417
Difference spectra			
Reduced minus oxidized (see Fig. 1)	557—558	522—525	428
CO:reduced minus reduced	572—573	538—539	416
(see Fig. 3)	559 (trough)	—	431 (trough)
CN-CO:reduced minus reduced	572—573	538—539	435, 412
(see Fig. 3)	559 (trough)	—	427 (trough)
CN:oxidized minus oxidized	545—554	—	430
		518 (trough)	403 (trough)
Pyridine hemochromogen (see Fig. 2)	556—557	522—525	418
Low temperature-reduced (liquid N_2)	555.5	527	423.5

*Absorption maxima are expressed in nm. Except for the low temperature spectrum (77 K), the α and β absorption characteristics appeared as broad peaks, and the values reported are the nm ranges of the broad bands. All Soret (or γ) peaks were sharply defined.

**All reductions were carried out by $Na_2S_2O_4$.

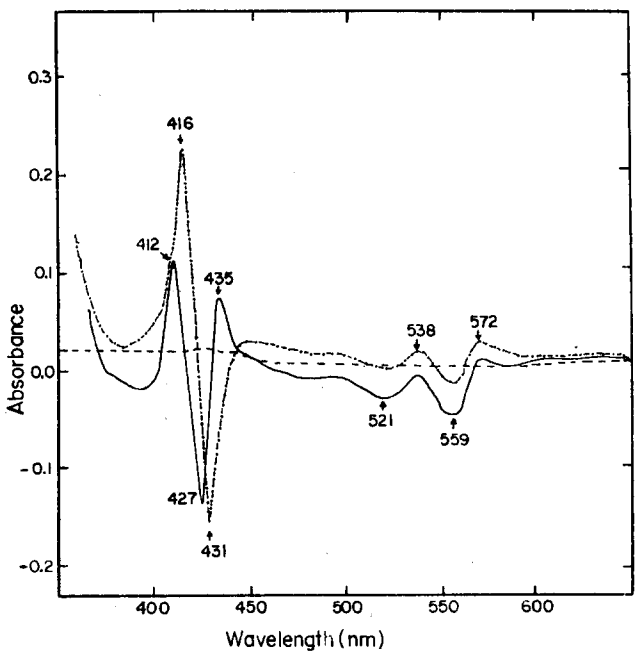

Fig. 3. CO:$Na_2S_2O_4$ reduced-minus-$Na_2S_4O_4$ reduced difference spectra of the purified cytochrome *o* preparation of *A. vinelandii*. CO was bubled for 30 s through a "fully" $Na_2S_2O_4$ reduced cytochrome *o* preparation (0.7 mg/ml protein) and the spectrum obtained is represented by ·····. Cyanide (final concentration of 20 mM) was then added to the CO-containing cuvette and the absorption changes noted are shown (——). — — —, $Na_2S_2O_4$ reduced minus the $Na_2S_2O_4$ reduced difference spectrum or control.

(R_3 fraction) of *A. vinelandii* [5, 6]. No NADH-cytochrome *o* reductase activity could be detected in any of purified or partially purified cytochrome *o* preparations.

The purified cytochrome *o* of *A. vinelandii* is not readily reduced by ascorbate, yet the *c*-type cytochromes, the predominant contaminating proteins are readily reduced by ascorbate, as was noted previously in the *A. vinelandii* electron transport particle [11]. Therefore, it was relatively easy to determine quantitatively the amount of contaminating *c*-type cytochromes that were associated with cytochrome *o* during the course of purification. By reducing the purified cytochrome *o* from *A. vinelandii* with dithionite, it was possible to analyze time sequences spectra of cytochrome *o* reoxidation by molecular O_2. The complete and rapid reduction of this cytochrome component by dithionite, and the difficulty of reducing cytochrome *o* by ascorbate, suggests that this hemoprotein is a low potential oxidase, as previously proposed by Kauffman and Van Gelder [12].

If one examines the distribution of the major cytochrome oxidases in bacteria, cytochrome *o* appears to be the most predominant terminal oxidase found [13]. Cytochrome *o*, for example, can be found in conjunction with cytochrome $a+a_3$ in *Mycobacterium phlei* [14] and *Staphylococcus aureus* [15], and it also appears together with cytochromes a_1 and *d* as in *A. vinelandii* and *Escherichia coli* [13]. There is evidence to suggest that cytochrome *o* is found in most organisms that are "oxidase positive" and have high tetramethyl-*p*-phenylenediamine oxidation rates [5, 16]. Fractionation studies employing detergents and using the tetramethyl-*p*-phenylenediamine oxidase assay, showed that in *A. vinelandii* a membrane-bound terminal oxidase could be solubilized and purified; it contained cytochrome *o* complexed with cytochrome c_4, and both the cytochrome c_4+o appear to be directly involved with tetramethyl-*p*-phenylenediamine oxidase activity [5, 13, 16] as well as with dichloroindophenol oxidase activity [2, 5]. As indicated by the data presented herein, cytochrome *o* appears to be the CO and CN sensitive terminal oxidase in *A. vinelandii*, and its capability to carry tetramethyl-*p*-phenylenediamine oxidations in association with cytochrome c_4, suggests that cytochrome *o* serves as a major cytochrome oxidase as does cytochrome $a+a_3$ for mammalian mitochondria.

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