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

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By

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DEDICATION

This dissertation is dedicated to my parents to whom I am indebted to all the love and encouragement that have been extended throughout my graduate studies.

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ABSTRACT

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 cytochrome o, purified as a detergent (Triton X-100) and hemoprotein complex, contained 1.2 nmoles heme per mg of protein. Cold-temperature spectrum showed that no other cytochrome was associated with the purified preparation, and electrophoresis revealed that only one type of hemoprotein was obtained. The purified cytochrome o reacted with both carbon monoxide and cyanide readily. Only in the reduced form did it combine with carbon monoxide, whereas the oxidized form reacted with cyanide. An "oxygenated" form of the cytochrome o was demonstrated to be spectrally distinguishable from both the oxidized and the reduced forms. The prosthetic group for cytochrome o was found to be protoheme IV and the minimum molecular weight, based on protoheme content, was calculated to be 625,000 assuming one mole of heme per mole of protein. The extinction coefficients for cytochrome o were calculated on the basis of the protohemochromogen concentration and the values obtained are reported. The extinction coefficient for the CO:reduced minus reduced cytochrome o, Δ416 nm (peak) - 431 nm (trough) was 235.4 $mM^{-1}cm^{-1}$. Amino acid analyses revealed the purified cytochrome o to be (a) an acid protein, (b) similar to cytochrome p-450 in amino acid composition, and (c) contained high concentrations of hydrophobic residues, indicating a possible structural relationship with membrane phospholipids. Lipid analyses on the cytochrome o revealed the presence of a high concentration

of phospholipid; the amount of phospholipid present was 40.5% by weight. Radioautographic analyses, using two dimensional thin layer chromatography, reveal the presence of phosphatidylethanolamine and phosphatidylglycerol.

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INTRODUCTION

Initial Studies on Cytochrome o

Cytochrome o is a carbon monoxide-binding hemoprotein, mostly found in the bacterial electron transport particles (4,8,15,17,24,29). Like b-type cytochromes, cytochrome o contains protoheme as its prosthetic group (25). However, unlike b-type cytochromes, cytochrome o reacts with carbon monoxide, as well as cyanide, readily (1,29). Cytochrome o is also autooxidizable and reacts with molecular oxygen, while cytochrome b, in general, does not behave as such (17). Most bacterial cytochrome o have been described as membrane-bound hemoproteins; nevertheless, some cytochrome o have been reported to be soluble (22,29).

Chance and his co-workers were the first to observe and to recognize this unique cytochrome as a distinct type of carbon monoxide-binding pigment in Micrococcus pyogenes var. albus (Staphylococcus albus) by a carbon monoxide difference spectrum (8,9,23). The peak and trough of the carbon monoxide absorption band lie at 416 and 432 nm, respectively. The α- and β-bands of the CO-cytochrome o lie roughly at 578 and 540 nm, with less pronounced absorption spectra than the Soret band. It was noted by Chance that the Soret absorption band of the CO-compound of this new cytochrome lies at a wavelength characteristic of protoheme enzyme, instead of green hemin enzymes such as cytochromes of the a-type (9). With this careful observation, he was able to analyze the mixed CO-compounds of Proteus vulgaris and Aerobacter aerogenes (stationary phase), both of which contain, not only cytochrome o, but also d and/or a_1 (9). Using an action-spectrum technique (7), which measures the

monochromatic light relief of carbon monoxide inhibition of oxygen uptake, Chance et al. confirmed that this new type of carbon monoxide-reacting hemoprotein was indeed a respiratory enzyme (7,8). Castor and Chance named this hemoprotein cytochrome o in 1959, advocating that this hemoprotein served as an oxidase in electron transport chains of bacteria (8). There is now ample evidence in the literature to verify their early works. In fact, it seems that cytochrome o might be the most abundantly found cytochrome that reacts with carbon monoxide which can be detected in bacterial systems; therefore, it may serve as an oxidase. However, only in a few instances has the early criterion of action-spectrum analysis been employed to verify the provisionally identified cytochrome o that have been described (8,17).

There is evidence indicating that cytochrome o, in some species of bacteria, is the only cytochrome that is capable of reacting with carbon monoxide, suggesting that cytochrome o might serve as the only oxidase in these organisms (8,15-17). For instance, cytochrome o was the only detectable oxidase in S. albus, Acetobacter suboxydans, and Vitreocilla species by action-spectrum determinations (7,28). Also, cytochrome o was the only oxidase detected in dark, aerobically grown cells of Rhodospirillum rubrum (12,17), and in log-phase Escherichia coli, P. vulgaris, and A. aerogenes (8).

Cytochrome o is generally detected from the characteristic CO-cytochrome o difference spectra (9,15,17). This type of spectral analysis is also used to measure the cytochrome o concentration in either the whole cells or subcellular preparations (10,11,25). Survey studies have shown that CO-reduced difference spectra of cytochrome o derived from various sources have absorption peaks at 578 to 565 nm, 539 to 535 nm, and 410 to 419 nm,

and trough at 560 to 547 nm (17). The position of the α peak in the reduced minus oxidized difference spectra of cytochrome o, which often is used to detect the presence of b-type cytochrome, varies from 565 (in S. albus) to 556 nm (in S. aureus). This, along with biochemical and other spectral characteristics that have been studied in some cytochrome o, suggests that there are several classes of cytochrome o present in bacterial electron transport systems (17,25).

Cytochrome o in Staphylococcus aureus

Taber and Morrison were the first to characterize protoheme as the prosthetic group of cytochrome o by the formation of the pyridine hemochromogen in purified electron transport particles of Staphylococcus aureus (25). This particulate respiratory enzyme system was first shown to contain an a-type cytochrome and two types of cytochrome b. The latter two cytochromes were resolved, by low-temperature spectroscopy, to show absorption peaks at 555 and 557 nm in the visible absorbance region. Ascorbate-dichloroindophenol (DCIP) did not reduce cytochrome b-557, suggesting that it had a lower potential than the other cytochromes. Also, this cytochrome b-557 was sensitive to 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO), and it did not react with carbon monoxide. It was thus concluded that cytochrome b-557 was an intermediate electron carrier of cytochrome b or b_1 type, rather a terminal oxidase. Cytochrome b-555 was found to have a higher potential than cytochrome b-557, as it was not reduced by succinate in a HQNO-inhibited system, but was reduced by ascorbate-DCIP. When reduced by succinate or ascorbate-DCIP and then exposed to carbon monoxide, the preparation showed a spectrum characteristic of the

cytochrome o-CO complex, and it was also identified by carbon monoxide action spectrum. It was concluded that cytohcrome b-555 was a cytochrome o, and that it served as a major oxidase in S. aureus (25). Although cyanide (5×10^{-2}M) inhibited the cytochrome system, the formation of a specific hemoprotein-CN complex could not be detected spectrally.

Cytochrome o in Photosynthetic Bacteria

Rhodospirillum rubrum, when grown aerobically in the dark, like S. aureus, possessed two types of cytochrome b in membrane fractions (26). One b-type cytochrome, at liquid nitrogen temperature, had α and β absorption maxima at 564 and 540 nm, respectively. The CO-difference and CO-action spectra revealed this b-type cytochrome to be cytochrome o, and it was confirmed to be the sole oxidase of R. rubrum. Both ascorbate-DCIP and succinate served equally well as reducing agents. However, when dithionite was used for the reductant, the amount of CO-compound formed was 25% greater than that of the ascorbate-DCIP and succinate-reduced complex (26). The second b-type cytochrome present in the R. rubrum membrane fragment was associated with succinate-dehydrogenase activity. Cytochromes of a-type and cc'-type could not be detected in the aerobically dark grown cells.

In the anaerobic, light-grown cells of Rhodopseudomonas spheroides, cytochrome o was the only oxidase present in the electron transport particles (21). However, when these cells were grown aerobically in the dark, unlike cells of R. rubrum, an a-type cytochrome was the major oxidase, and cytochrome o was found to be a

minor component (21). Cytochrome o was inducible with low oxygen tension and light energy when cytochrome a was so repressed.

Horio and Taylor conducted photochemical determination and action spectrum studies on the oxidase of R. rubrum, and concluded that cytochrome o was indeed the oxidase of dark-grown cells (12). Although they failed to measure an oxidase action spectrum in light-grown cells because of their photosynthetic activity, they considered cytochrome o too also functioned as an oxidase in these cells (12). Their evidence for believing that cytochrome cc' is the extracted form of cytochrome o was later to be challenged, on the ground that prosthetic groups of the cc'-type and o-type were not the same. Chance et al. also presented evidence to exclude an oxidase role for cytochrome cc' (17).

Cytochrome o in Bacillus species

The electron transport particle of Bacillus megaterium strain KM has been shown to contain two CO-binding pigments, cytochromes a_3 and o, by Broberg and Smith (6). Both hemoproteins were membrane-bound and both were inducible by endogenous substrate in whole cells as well as by NADH as substrate in isolated membrane fragments. The two cytochromes could be separated by treatment with lipase; the cytochrome a_3 component remained bound to the particle, while the cytochrome o was released into the supernatant fraction. In comparing these two pigments, cytochrome o was found to have lower affinity toward CO than cytochrome a_3. The treatment of lipase did not seem to alter the CO-binding capacity of cytochrome o, nor was any spectral change effected by this solubilization procedure (6). The differential solubility, upon lipase treatment, indicated that the binding or association of cyto-

chromes $\underline{a}_3$ and $\underline{o}$ to the membrane was different. A similar conclusion could also be drawn from the work of Brodie and his co-workers (20) in studying the CO-binding pigments of Mycobacterium phlei (see the following paragraph). Inspite of the difference in their linkage, it was thought that the two CO-binding cytochromes must be located on the membrane in such a way that they both can react with the other pigments of the electron transport system; presumably, both were potentially capable of acting as terminal oxidases in this Bacillus species. Although cytochrome $\underline{o}$ was shown to be selectively solubilized from this organism, further purification studies were not attempted. It was shown that the lipase treatment used "to remove" the cytochrome $\underline{o}$ component did not impair the respiratory activity of the particle fraction. If cytochrome $\underline{o}$ was completely removed by the lipase treatment, this implied that the remaining cytochrome $\underline{a}_3$ component was capable of catalyzing respiration maximally. The question of whether the cytochrome $\underline{o}$ could function as a terminal oxidase in the absence of active cytochrome $\underline{a}_3$ might be answered tentatively by the data on carbon monoxide inhibition of respiration. A large proportion of the respiration was inhibited by a low ratio of CO/O_2; from the carbon monoxide affinities observed in spectral studies. The inhibition must be due to cytochrome $\underline{a}_3$-CO complex formation, and, therefore, cytochrome $\underline{a}_3$ must be responsible for a large proportion of the respiration. The small, yet relatively CO-resistant fraction could be due to the cytochrome $\underline{o}$ component (6). It was concluded that, in B. megaterium electron transport chain, although significant quantities of both cytochromes $\underline{a}_3$ and $\underline{o}$ were present cytochrome $\underline{o}$ could only catalyze at most about one-fouth of the cell's total

respiration (6).

Another Bacillus species, B. subtilus,also had been found to synthesize two CO-binding pigments, cytochromes a_3 and o, as does the B. megaterium. The differential solubilization studies have shown essentially the same results as described by Broberg and Smith (27). However, the cytochrome o concentration was found to be highest in dormant spores of B. subtilus strain PC 1219; germinating spores, young vegetative cells, and vegetative cells had only half as much cytochrome o as dormant spores (27). Dormant spores of B. subtilus strain JB 69, which contained cytochrome a_3 as the only CO-binding respiratory pigment in vegetative cells, showed no trace of cytochrome o. Thus cytochrome a_3 was presumably the only oxidase functioning in the dormant spores of this JB 69 strain. Since cytochrome o was not essential for oxidation of NADH in both B. megaterium and B. subtilus cells, the physiological significance of this cytochrome in dormant spores and other cells was uncertain (4, 27).

Cytochrome o in Mycobacterium phlei

Brodie and his co-workers, in studying the electron transport particles of Mycobacterium phlei, found cytochrome aa_3 and o were both present in the respiratory chain that contained the third site of phosphorylation (20). Triton X-100 was used to solubilize a cytochrome aa_3+ o complex. This complex was further digested with trypsin and partially purified by ammonium sulfate fractionation. Although a complete seperation of both cytochromes was never achieved, as in the case described for B. megaterium, a solubilized cytochrome o fraction resulting from a similar lipase treatment contained only small amounts of cytochromes b, c and aa_3. With this supernatant fraction, the CO-cytochrome o peaks absorbed at 416, 538 and 567 nm. In the membrane particle, the

416 nm CO-binding peak of cytochrome o was observed, following reduction by succinate, NADH, or malate-FAD, which utilized the entire respiratory pathway, or by ascorbate-N,N,N',N'-tetramethyl-p-phenylenediamine(TMPD), which utilized a segment of the respiratory chain between cytochrome c and molecular oxygen. It was interesting to note that in the CO-cytochrome difference spectrum, the formation of this CO-cytochrome o peak(s) were time-dependent. In essence, the carbon monoxide peaks of cytochrome o increased in absorption in both the visible and the Soret region of the spectrum. Thus, it would appear that some of the cytochrome o pigment did not combine immediately with carbon monoxide. Increasing in the length of time that carbon monoxide was bubbled through the solution did not affect the final absorption of the CO-cytochrome o complex (20). Again, no further purification of this cytochrome o was reported.

Cytochrome o in Rhizobium japonicum

The presence of cytochrome o, in conjunction with cytochrome a_3, in free living Rhizobium japonicum has been studied by Appleby (2). At air oxygen tension, the respiration of succinate by air-grown Rhizobium was substantially inhibited by carbon monoxide, and a photochemical action spectrum showed that both cytochromes aa_3 and o functioned as terminal oxidases. However, in nitrogen-fixing bacteroids of R. japonicum, both cytochrome oxidases were not present. The oxidase of free living cells was reported to be very sensitive to cyanide, being completely inhibited at level of 10 μM (1-3). Significant spectral changes due to cyanide-cytochrome o formation have not been observed, as was the case of S. aureus. It was speculated that at the concentration of cyanide used, some of the inhibition observed may not entirely be attributed to the formation

of the CN-heme complex, and that another reaction site was involved. At liquid nitrogen temperature, the spectrum of succinate-reduced cultured cells showed the presence of two cytochrome b components, one with α peak at 556 nm and the other at 559.5 nm. When this difference spectrum was recorded in the presence of cyanide the 556 nm peak appeared at undiminished intensity, but the 559.5 nm peak was partially collapsed. It was proposed that cytochrome b-556 represented a cyanide-insensitive component which also was present in bacteroids. The other cyanide-sensitive cytochrome b-559.5, present only in cultured free-living cells, was presumed to be cytochrome o (1,2). The inhibitor studies on the cytochromes of the nitrogen-fixing bacteroids of R. japonicum have suggested the presence of a metalloprotein oxidase other than a cytochrome. Also, several new CO-reactive hemoproteins in bacteroids were detected, and two of them tentatively characterized as cytochromes c-552 and c-554. No definite answer in regard to their function has been offered.

The presence of cytochrome p-450 in bacteroids has been well established by spectral characterization. The inhibition observed by carbon monoxide and N-phenyl-imidazol seemed to suggested that this bacteroid's cytochrome p-450, rather cytochrome c-552 or c-554, may be the terminal oxidase which functioned during efficient respiration yielding a characteristic high ATP production and nitrogenase activity. Both of these c-type cytochromes were soluble and capable of combining with carbon monoxide.. Unlike the cytochrome o components of R. rubrum or S. aureus, which remained oxidized in the presence of HQNO and were thereby distinguishble from other cytochrome b in low-temperature difference spectra, all cytochromes in whole cells or particles from cultured Rhizobium were completely reducible by succinate respiration, following prior equili-

bration with 8 μM HQNO. This observation and also the observation that Rhizobium cytochrome o reduction was inhibited by cyanide meant that,in different microorganisms, the cytochrome o pigments can have different properties and/or be associated with different electron flow systems.

Cytochrome o in Micrococcus denitrificans

Scholes and Smith reported on the similarity of the respiratory chain systems of the mammalian mitochondria to that of Micrococcus (Paracoccus) denitrificans (22). The carbon monoxide spectra of (a) anaerobically grown cells reduced with substrate, or of (b) dithionite-reduced membranes from cells grown either aerobically or anaerobically at any stage of growth, showed peaks of carbon monoxide compounds of both cytochromes a_3 and o. The intensity of the absorption peak at 415 nm was increased by treating the membrane with either Triton X-100 or deoxycholate, a result similar to that reported earlier by Porra and Lascelles (19). The carbon monoxide spectrum of cytochrome o was not seen in membranes of 12-hour aerobically grown cells reduced anaerobically with substrate; this pigment was not considered to have a function in the aerobically grown cells. Based on some unpublished observations that cytochrome o had been detected in the soluble fraction of anaerobically grown cells, and on the results which showed that deoxycholate increased the absorption due to the carbon monoxide compound of cytochrome o, Smith and her co-worker suggested that this cytochrome o may be an altered form of some pigment (22), but they presented no evidence of its functional role in the electron transport system of M. denitrificans.

Cytochrome o in Acetobacter suboxydans

A cytochrome b-558, identified as the effective cytochrome o of Acetobacter suboxydans strain IAM 1828 grown on lactate, was isolated and reported to be crystallized (13,14). Oxidation of lactate was stimulated when partially purified cytochrome b-558 was added to A. suboxydans membrane fragments which contained cytochrome a and another cytochrome b; but no lactate oxidase activity occurred without added cytochrome. However, there had been no report that the crystallized b-558 possessed cytochrome oxidase activity. Another A. suboxydans (ATCC 621), studied by Daniel, was unique in that it contained cytochrome o in the absence of a-type cytochromes (11). The low-temperature carbon monoxide difference spectrum demonstrated that two CO-binding pigments were present in this organism, when cells were grown with glycerol as carbon source. The carbon monoxide inhibition of oxidase activity and the relief of this inhibition with light showed that at least one of these CO-reactive cytochromes was a terminal oxidase (11). The Lineweaver-Burk plot of the oxygen affinity of the NADH oxidase system, studied in the presence and absence of carbon monoxide, was interpreted as indicating two terminal oxidases. Daniel, therefore, suggested that both CO-binding cytochromes b may serve as terminal oxidases of this A. suboxydans. The two Co-binding cytochromes were then designated as cytochrome o-565 and cytochrome o-558. Evidence had also been presented to show that these two cytochrome o had different affinities toward carbon monoxide ; cytochrome o-565 having a high carbon monoxide affinity and cytochrome o-558 being a low carbon monoxide affinity pigment (11)

Cytochrome o in Vitreoscilla species

Up to date, the most extensively studied case of cytochrome o has been that of Vitreoscilla,a species of obligately aerobic gliding bacteria which were previously classified as members of the Flexibacteriaceae and now classified as Beggiatoaceae (5,18). Webster and Hackett first examined the respiratory chain of these colorless "algae" and found that, at room temperature, the difference spectrum of the whole cells showed no evidence of the presence of a- or c-type cytochromes (28). A CO-binding pigment having absorption peaks at 570 to 535 nm and at 416 nm was found to be present in large amounts. The CO-difference spectra of this organism, with α and β absorption peaks at 570 and 535 nm and a Soret peak at 416 nm, was revealed to be characteristic of cytochrome o. The action spectrum for light reversal of carbon monoxide inhibition of Vitreoscilla respiration showed absorption maxima at 568 and 534 nm with a monochromatic light source, and 416 nm was found to be the most effective wavelength for reversal of the carbon monoxide inhibition. The carbon monoxide difference spectrum and action spectrum of this cytochrome o supported the evidence that this cytochrome o functioned as a respiratory enzyme in Vitreoscilla species. This hemoprotein was highly purified and characterized substantially by Webster and his co-workers(28-30).

Two types of cytochorme o preparations were obtained originally from the early work of Webster and Hackett (28). The purification involved freezing and thawing of Vitreoscilla cells in the presence of 1 % sodium deoxycholate, followed by protamine sulfate and ammonium sulfate fractionation. These procedures were followed by Sephadex and TEAE-cellulose chromatographic steps to obtain two highly purified cytochrome

o fractions, designated as Fraction I and II. Fraction I had two α bands and two Soret bands in liquid nitrogen difference spectra, indicating that there may be two heme groups involved. Using a gel filtration technique, a molecular weight of 27,000 was determined for this fraction, and 22,000 for Fraction II. Fraction I had CO-difference spectra maxima at 570,534 and 419 nm, with a trough at 436 nm, while Fraction II had absorption maxima at 566, 532 and 418 nm, with a trough at 436 nm. Fraction I had a redox potential of 1 Volt and was slowly autooxidizable, yet Fraction II had a redox potential of -0.09 Volt and was rapidly autooxidizable. Because of its extreme autooxidizability, Fraction II was considered to be a soluble form of the membrane-bound cytochrome o. Even though the redox potential of this fraction was too low to be considered an oxidase, Webster and Hackett argued that its true redox potential could be much higher in the membrane milieu (29). This arguement could also be equally applied to explain the low autooxidizability of FractionI which had a much higher redox potential. One suggested possibility was that a combination of both Fraction I and Fraction II could serve as functional cytochrome o of Vitreoscilla. However, in these earlier studies, no NADH oxidation activity, which was found to be high in whole cells as well as membrane particles of Vitreoscilla, was detected in these two cytochrome o preparations. No explanation was offered at that time. In 1974 Webster and Liu, while reinvestigating a purified cytochrome o preparation from another Vitreoscilla species(Murray strain 389), reported the reduction of this purified cytochrome o preparation by NADH, catalyzing by the enzyme NADH reductase, which was co-purified with the cytochrome o (30). Using NADH as the reducing agent, the difference spectra obtained for this cyotchrome o preparation showed absorption

maxima at 577, 544 and 420 nm. This was believed to be an oxygenated form of reduced cytochrome o. Dithionite-reduced difference spectra of this cytochrome o had absorption maxima at 560 and 435 nm. The CO-difference spectra of this preparation resembled the Fraction I of the earlier studies. However, the dithionite-reduced difference spectra showed absorption characteristics of both Fraction I and II. These closely related, but not identical spectral properties of the cytochrome o that have been purified from Vitreoscilla species seem to indicate that more than one type of cytochrome o may actually be present in these organisms. Oxygen uptake exhibited by the various purified fractions from Vitreoscilla species(Murray strain 389) preparation was found to be associated with NADH-cytochrome o reductase activity. It was concluded from the oxygen uptake experiments that cytochrome o and the reductase(s) constituted an electron path for the oxidation of NADH by molecular oxygen. However, the attempts to separate cytochrome o from NADH-cytochrome o reductase were unsucessful. Therefore, the nature of the reaction mechanism was not resolved. The oxygenated form of this soluble cytochrome o was identified to be the predominant steady state species during turn-over of the cytochrome in vivo. This was based on the observation of the spectra of actively respiring cells, and from comparing directly with the puirfied oxygenated cytochrome o preparation. Evidence also indicated that the cyclic changes of the oxidized, reduced and oxygenated cytochrome o were all involved in the terminal respiration of Vitreoscilla. The oxygenated form was undoubtly an active intermediate species of this cytochrome o (30,31). The results, presented by Webster and Liu, represented the first evidence for a functional oxygenated compound.

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Running Title: CYTOCHROME OXIDASE FROM *AZOTOBACTER VINELANDII*

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ABSTRACT

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.

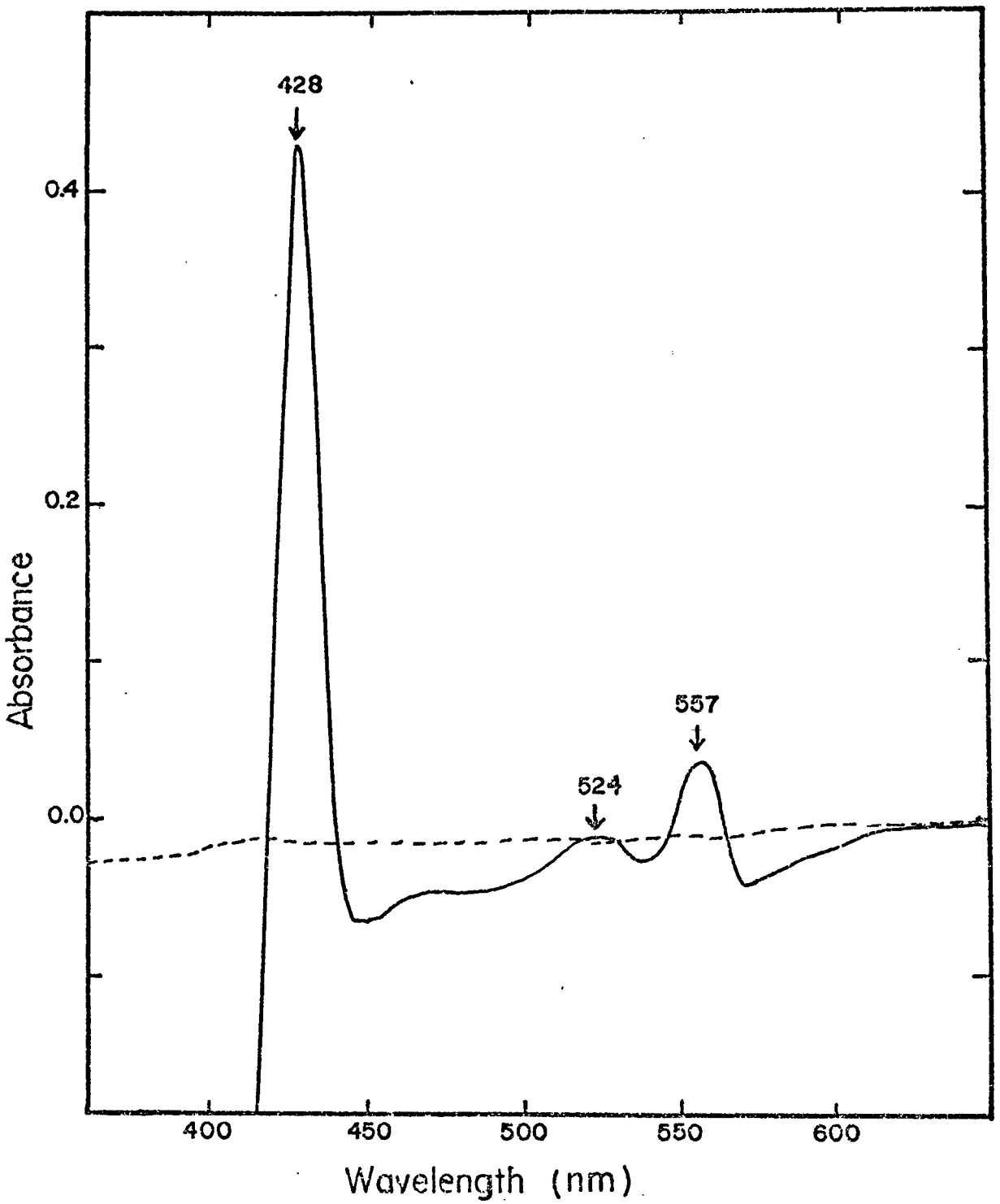
INTRODUCTION

Castor and Chance (2) were the first to recognize that cytochromes *o* and a_1 might function as the terminal oxidases in *Azotobacter* species. Jones and Redfearn (5) 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 (4). Mueller and Jurtshuk (12) and Jurtshuk *et al.* (10) 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.

METHODS AND RESULTS

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 the 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 minutes and centrifuged at 105,000 x g for one hour. The resultant pellet was resuspended in 0.025M 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 1M KCl. This latter procedure solubilized both the c-type cytochromes and cytochrome o that is directly involved with the TMPD oxidase activity which previously was associated with the A. vinelandii electron transport particle fraction (6,10). After centrifugation at 105,000 x g for one hour, the Triton-KCl supernatant was carefully removed from the pellet and dialyzed in 0.025M 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 x g for 20 minutes, was removed and resuspended in a small amount of the same phosphate buffer, and redialyzed until ammonium sulfate

Figure 1. Sodium dithionite-reduced minus oxidized difference spectrum of the highly purified cytochrome o preparation isolated from the electron transport particle fraction of Azotobacter vinelandii. Protein concentration of the cytochrome o was 0.7 mg per ml.


428
524
557
0.4
0.2
0.0
Absorbance
400
450
500
550
600
Wavelength (nm)

was no longer detected by color development using Nessler's reagent. Eight ml of this fraction (3.5 mg protein per ml) was loaded on a Sephadex G-200 column (2.0 cm x 30 cm), that was previously equilibrated overnight with 0.025M phosphate buffer, pH 7.2, containing 0.1% (v/v) Triton X-100. Three-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 cytochrome o appeared as a 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 (Figure 1) of the purified preparation contained 1.6 nmoles cytochrome o per mg protein as calculated from the CO:dithionite-reduced minus dithionite-reduced spectrum, using a mM extinction coefficient of 170 and measuring the absorbance (416 nm peak minus 431 nm trough) in the Soret region (3). The prosthetic group of the cytochrome o was readily characterized as protoheme by the formation of the pyridine hemochromogen derivative (13). The pyridine hemochromogen difference spectrum (Figure 2) showed absorption maxima at 418, 524, and 557 nm. The basic spectral characteristics of A. vinelandii cytochrome o are summarized in Table 1. Cyanide (20 mM) reacted only with the oxidized form of cytochrome o; the addition of cyanide to the dithionite-reduced cytochrome o did not result in any observable spectral change. Also, no spectral changes were noted when carbon monoxide was added to the cyanide-complexed cytochrome o, which suggests a high degree of stability for the cyanide-cytochrome o

Figure 2. Pyridine hemochromogen difference spectrum of the purified cytochrome o isolated from the electron transport particle of Azotobacter vinelandii. Two ml of the purified preparation was used for preparing the pyridine hemochromogen derivative by the method of Pappenheimer and Hendee (13).

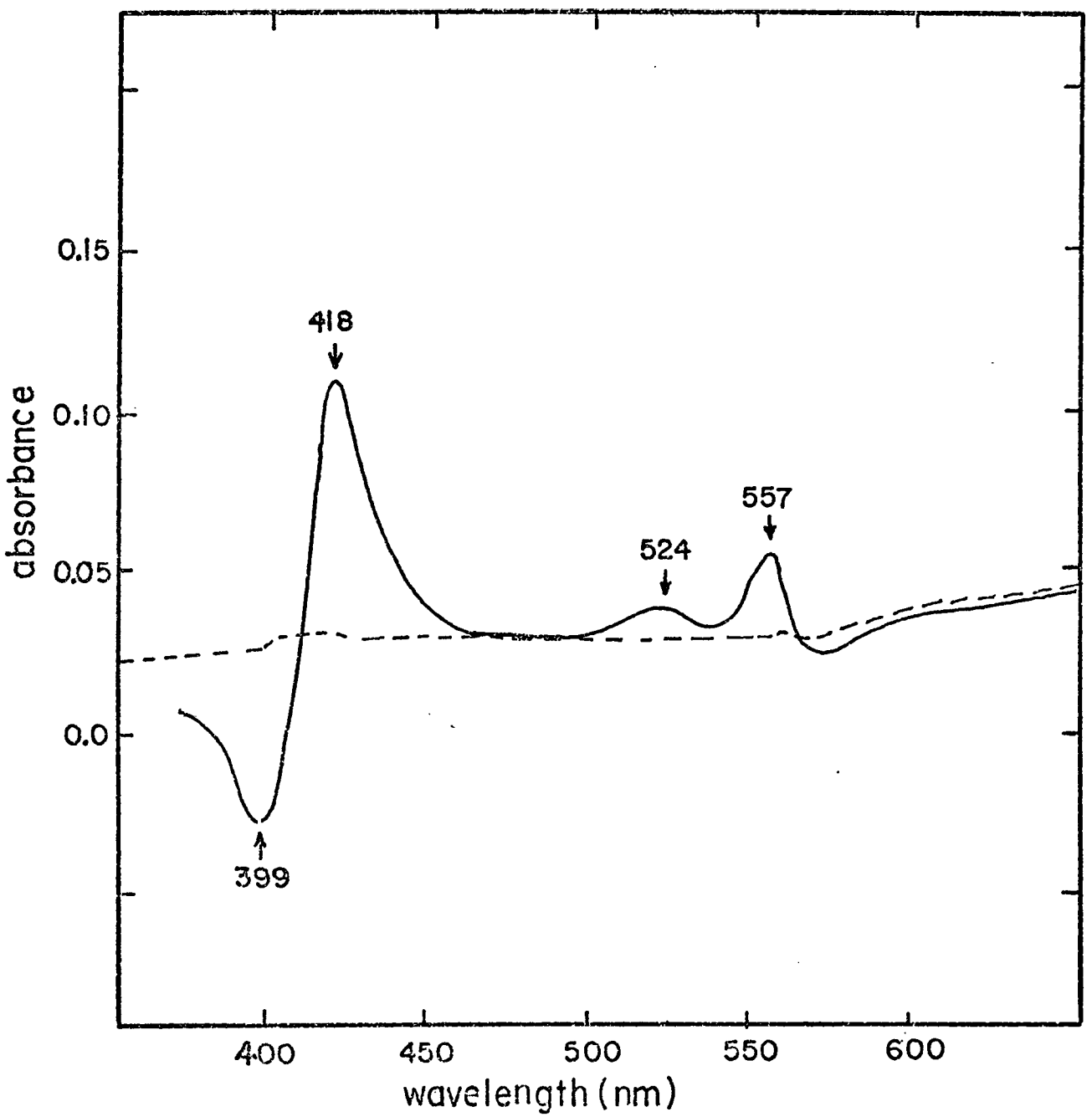

0.15
0.10
0.05
0.0
absorbance
418
524
557
399
400
450
500
550
600
wavelength (nm)

TABLE 1

Composite Summary of the Absolute and Difference Absorption Maxima*
of Highly Purified Cytochrome o of Azotobacter 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 Figure 1)	557-558	522-525	428
CO:reduced minus reduced	572-573	538-539	416
(see Figure 3)	559 (trough)	—	431 (trough)
CN-CO:reduced minus reduced	572-573	538-539	435, 412
(see Figure 3)	559 (trough)	—	427 (trough)
CN:oxidized minus oxidized	545-554	—	430
		518 (trough)	403 (trough)
Pyridine hemochromogen (see Figure 2)	556-557	522-525	418
Low-temperature reduced (liquid nitrogen)	555.5	527	423.5

*Absorption maxima are expressed in mn. 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.

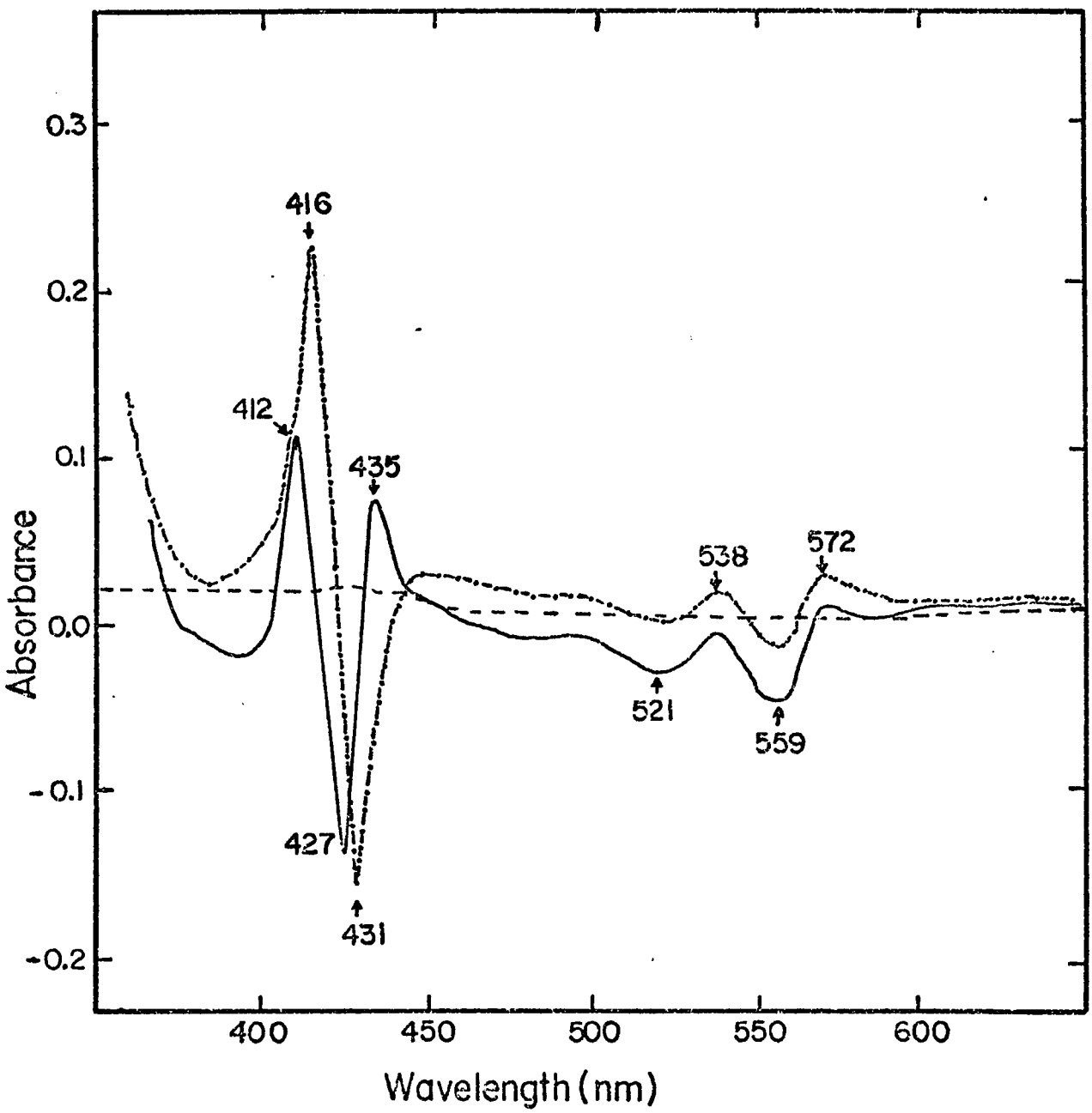
**All reductions were carried out by sodium dithionite.

complex. In contrast, cyanide markedly altered the CO:dithionite-reduced spectrum of cytochrome o, as shown in Figure 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 (1). Unlike the soluble cytochrome o that has been purified from Vitreoscilla (16), the A. vinelandii cytochrome o is tightly bound to the membranous electron transport particle (R_3 fraction) of A. vinelandii (6,10). No NADH-cytochrome o reductase activity could be detected in any of the purified or partially purified cytochrome o preparations.

The purified cytochrome o of A. vinelandii was not readily reduced by ascorbate, yet the c-type cytochromes, the predominant contaminant proteins,were readily reduced by ascorbate, as was noted previously in the A. vinelandii electron transport particle (7). Therefore, it was relatively easy to quantitatively determine 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-sequence spectra of cytochrome o reoxidation by molecular oxygen. 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 (11).

Figure 3. CO:dithionite-reduced minus dithionite reduced difference spectra of the purified cytochrome o preparation from the electron transport particle of Azotobacter vinelandii. Carbon monoxide was bubbled for 30 seconds through a "fully" dithionite-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 resulting absorption changes noted are shown by (———). (-----) represents the dithionite-reduced minus dithionite-reduced difference spectrum, or the control.


0.3
0.2
0.1
0.0
-0.1
-0.2
Absorbance
416
412
435
538
572
521
559
427
431
400
450
500
550
600
Wavelength (nm)

DISCUSSION

If one examines the distribution of the major cytochrome oxidases in bacteria, cytochrome *o* appears to be the most predominant terminal oxidase found (9). Cytochrome *o*, for example, can be found in conjunction with cytochrome *a* + a_3 in *Mycobacterium phlei* and in *Staphylococcus aureus* (15), and it also appears together with cytochromes a_1 and *d* in both *A. vinelandii* and *Escherichia coli* (9). 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 (8,10). 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 cytochromes c_4 and *o* appeared to be directly involved with tetramethyl-p-phenylenediamine oxidase activity (8-10), as well as with dichloroindophenol oxidase activity (5,10). As indicated by the data presented herein, cytochrome *o* appears to be the carbon monoxide- and cyanide-sensitive terminal oxidase in *A. vinelandii*, and its capability to carry out 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.

ACKNOWLEDGEMENTS

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Title: STUDIES ON THE RED OXIDASE (CYTOCHROME *o*) OF *AZOTOBACTER VINELANDII*

by

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and
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Houston, Texas 77004

ABSTRACT

In an attempt to isolate and to study the electron transport system of Azotobacter vinelandii, we have isolated and purified a membrane-bound cytochrome o. The cytochrome o, purified as a detergent (Triton X-100) and hemoprotein complex, contained 1.6 nmoles heme per mg of protein. Cold-temperature spectrum showed that no other cytochrome was associated with the purified preparation, and electrophoresis revealed that only one type of hemoprotein was obtained. The purified cytochrome o reacted with both carbon monoxide and cyanide readily. Only in the reduced form did it combine with carbon monoxide, whereas the oxidized form reacted with cyanide. An "oxygenated" form of the cytochrome o was demonstrated to be spectrally distinguishable from both the oxidized and the reduced forms.

INTRODUCTION

Cytochrome o was first implicated as a terminal oxidase in bacterial cytochrome systems by Castor and Chance (1). Most bacterial cytochrome oxidases are tightly bound to the intracellular membrane. The consequent difficulty encountered in solubilizing the cytochrome components that are associated with the oxidases has long delayed the understanding of these oxidase systems in bacteria (7,9,13). Jones and Redfearn (5) were the first to solubilize, from the membrane of Azotobacter vinelandii, a red particle that contained an enriched amount of cytochrome o. Jurtshuk et al. (8,10) solubilized and purified a TMPD oxidase from the same bacterium, and tentatively identified it as a complex containing c-type cytochrome(s) and cytochrome o. By using a similar procedure, we have isolated and purified the cytochrome o from Azotobacter vinelandii. Due to its clear reddish appearance in the final preparation, we have named this cytochrome the "red oxidase." The purification and spectral properties of this red oxidase are reported in this communication.

MATERIALS AND METHODS

Preparation of electron transport particles. Azotobacter vinelandii strain O was grown in a 200-liter capacity fermenter (New Brunswick Scientific Company) under conditions previously described (6). Late log phase cells were harvested and washed twice with 0.02M phosphate buffer, pH 7.5. The elctron transport particle, designated R_3, was isolated from sonically disrupted cells by differential centrifugation (6).

Solubilization and purification of the red oxidase. A procedure similar to that of Mueller (11) was employed to solubilize the red oxidase from the membrane R_3 fraction. Ammonium sulfate fractionation and diethylaminoethyl (DEAE) cellulose column chromatography were used to further purify the hemoprotein. The purification procedure and the fractionation scheme are presented in Figure 1 and Table 1, respectively.

Spectral analysis. A Beckman Model 25 spectrophotometer was used for all of the room-temperature spectral studies. A Cary Model 17, equipped with a liquid nitrogen chamber, was used for cold-temperature analysis.

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed in a 5% acrylamide gel, according to the method of Davis (3), using Coomossie brilliant blue for protein staining.

RESULTS

Figure 1 shows the diagramatic procedures for solubilization and purification of the red oxidase from the membrane fraction (R_3) of Azotobacter vinelandii. The ammonium sulfate fraction (27% saturation) contained the majority of cytochrome o in the original membrane fraction, along with minor amounts of c-type cytochrome. This fraction showed some TMPD oxidase activity, possibly due to the presence of a c-type cytochrome that was complexed with cytochrome o. The c-type was removed from this fraction by the DEAE cellulose chromatographic step. The purified red oxidase was eluted as Fraction I from the column by a NaCl gradient (0 - 0.5M) in 0.02M phosphate buffer, pH 7.5, containing 0.1% Triton X-100 (w/v). Table 1 shows a typical fractionation scheme for the purification of the red oxidase. The final preparation represented about a twelve-fold purification, in comparison with that of the Triton-treated membrane fraction. The purified hemoprotein contained 1.6 nmoles heme per mg protein. This value was calculated from the CO:dithionite-reduced minus dithionite-reduced spectrum, using a mM extinction coefficient of 170 (2). Electrophoresis of the purified preparation in polyacrylamide gel showed only one protein band, suggesting that a pure cytochrome was obtained. SDS gel electrophoresis of the purified cytochrome has not been accomplished at present.

As shown in Figure 2, the absolute spectrum of oxidized cytochrome o had an absorption maximum at 412 nm; maxima for the reduced form appeared at 426, 525, and 557 nm. Addition of carbon monoxide to the reduced form shifted the Soret peak to 420 nm and the absorbance increased,

Figure 1. Scheme for purification of the red oxidase (cytochrome o) from Azotobacter vinelandii electron transport particle, R_3 fraction. All procedures shown were carried out at temperatures below 5° C, and 0.02M phosphate buffer, pH 7.5, was used.

AZOTOBACTER R_3 FRACTION

15 mg/ml
0.5 mg Triton X-100 per mg protein
Centrifuge 105,000 xg - 1 hour.

Triton supernatant (Discard)

Triton pellet

Resuspend in buffer.
Centrifuge 105,000 xg - 1 hour.

Triton wash (Discard)

Washed Triton pellet

Resuspend in buffer.
To 8-10 mg/ml protein, add 0.4-0.5 mg deoxycholate per mg protein.
Centrifuge 105,000 xg - 1 hour.

Deoxycholate supernatant (Discard)

Deoxycholate pellet

Resuspend in buffer.
Centrifuge 105,000 xg - 1 hour.

Deoxycholate wash (Discard)

Washed Deoxycholate pellet

Resuspend in buffer.
To 8-10 mg/ml protein, add 1.5 mg Triton X-100 per mg protein and KCl to 1M.
Centrifuge 105,000 xg - 1 hour.

Triton-KCl pellet (Discard)

Triton-KCl supernatant

Dialyze overnight.
Add ammonium sulfate to 27% saturation.
Centrifuge 37,000 xg - 20 minutes.

Ammonium sulfate supernatant (Discard)

Ammonium sulfate pellet

Resuspend in buffer.
Dialyze and concentrate by ultrafiltration to 6-8 mg/ml protein.
DEAE cellulose column (34 x 2.5 cm), equilibrated with buffer, 0.1% Triton X-100.
Elute with 0-0.5M NaCl gradient.

DEAE Fractions II and III (Discard)

DEAE Fraction I

Concentrate by ultrafiltration.
Dialyze in 0.02M phosphate buffer, pH 7.5, 1% Triton X-100.

PURIFIED CYTOCHROME o

TABLE 1

Fractionation Scheme for the Purification of Cytochrome o from the Azotobacter vinelandii Membrane

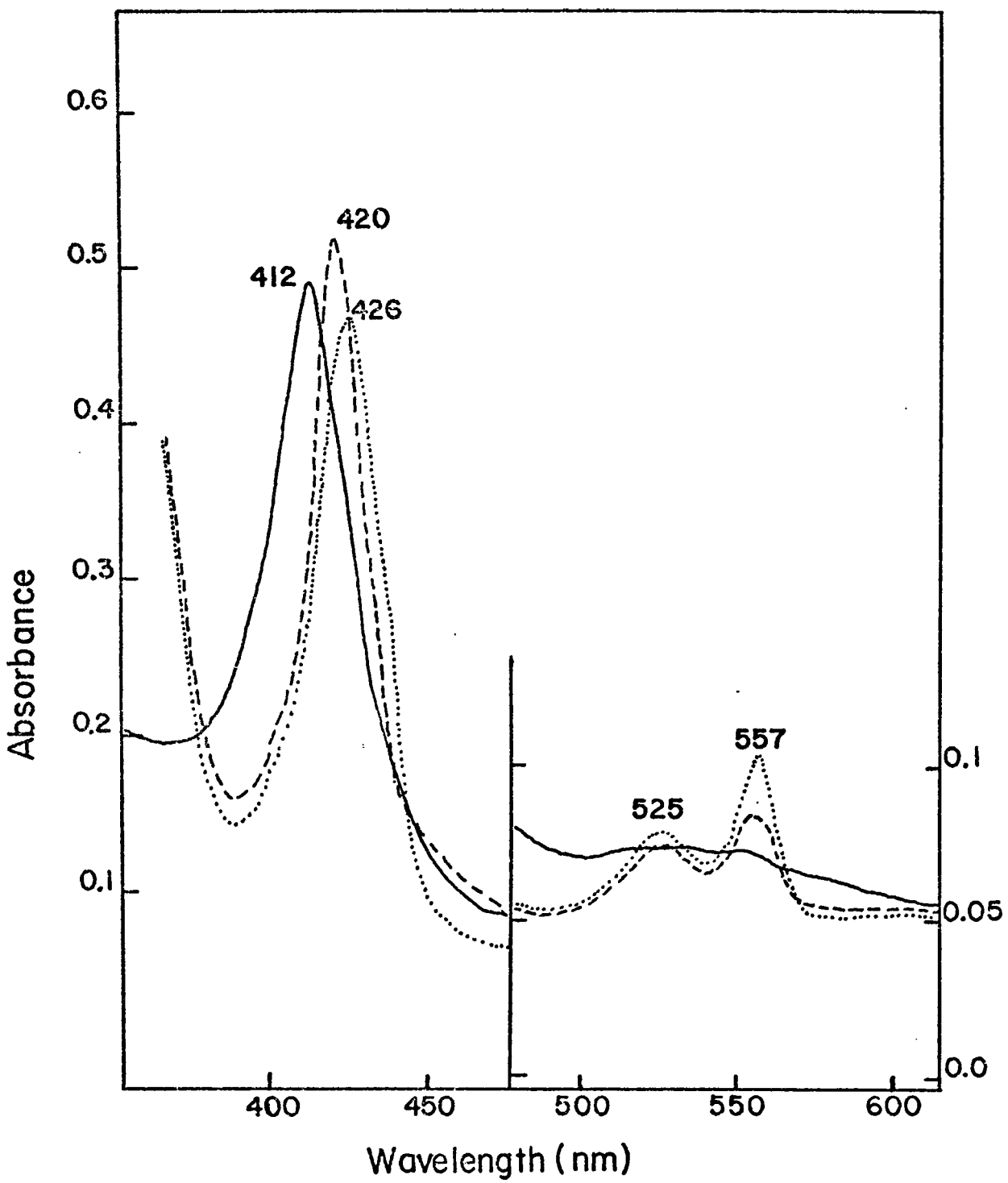
Fraction	Volume (ml)	Total protein (mg)	Cytochrome o concentration* (nmole/mg protein)	Yield (%)
Triton-R_3	176	2640	0.13**	100
Triton-KCl	125	325	0.58	54
Ammonium Sulfate 0-27% saturation	29	116	1.3	44
DEAE Cellulose Column Fraction I***	20	48	1.6	22

*Calculated as nmole heme per milligram protein, using a mM extinction coefficient of 170 (2).

**Estimated value, due to some interference from Triton.

***See Figure 1 for details.

Figure 2. Absorbance spectra of the purified red oxidase (cytochrome o) from Azotobacter vinelandii membrane fraction. Solid line (———) represents the oxidized cytochrome; dotted line (·····) indicates the reduced form; dashed line (-----) shows the CO-reduced form.


0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
420
412
426
557
525
0.1
0.05
0.0
400
450
500
550
600
Wavelength (nm)

while absorbances of both α and β peaks decreased somewhat. The low-temperature spectrum of the purified red oxidase showed the absorption maxima to be at 527 and 555 nm in the visible spectrum (Figure 3). The low-temperature spectrum of the partially purified preparation (ammonium sulfate fraction, as shown in Figure 3 inset, clearly demonstrated the presence of a c-type cytochrome, associated with cytochrome o, which had absorption maxima at 520 and 549 nm. The absorption maxima of the c-type cytochrome were undetectable after column chromatography. The greater purification achieved with this step was also reflected in the slight increase in cytochrome o content, as shown in Table 1.

The oxidized form of the red oxidase was found to react with cyanide quite readily; however, the reduced form showed no reactivity upon spectral analysis. The reaction of cyanide with the red oxidase revealed that the spectral alteration was dependent on the final concentration of cyanide. A small amount of dithionite could completely reduce the cytochrome o-cyanide complex, and the spectrum changed to that of reduced cytochrome o. This is shown in Figure 4. After reduction with dithionite, the spectrum had absorption maxima at 429, 526, and 557 nm, which resembled the reduced difference spectrum of the non-cyanide treated preparation.

The red oxidase was found to be highly autooxidizable in a freshly prepared preparation. The steady-state of autooxidation could be achieved in 15 minutes after complete reduction had been carried out in a preparation containing 0.5 mg/ml hemoprotein. A so-called "oxygenated" form was obtained by reducing the cytochrome o with a small amount of dithionite, and subsequently aerating the reduced form for about 30 seconds. Alternatively, this "oxygenated" form could also be approached

Figure 3. Low-temperature (77 °K) spectrum of the purified red oxidase (cytochrome o) from Azotobacter vinelandii membrane fraction. Inset shows the partially purified preparation, containing some c-type cytochrome, with absorption maxima at 520 and 549 nm.

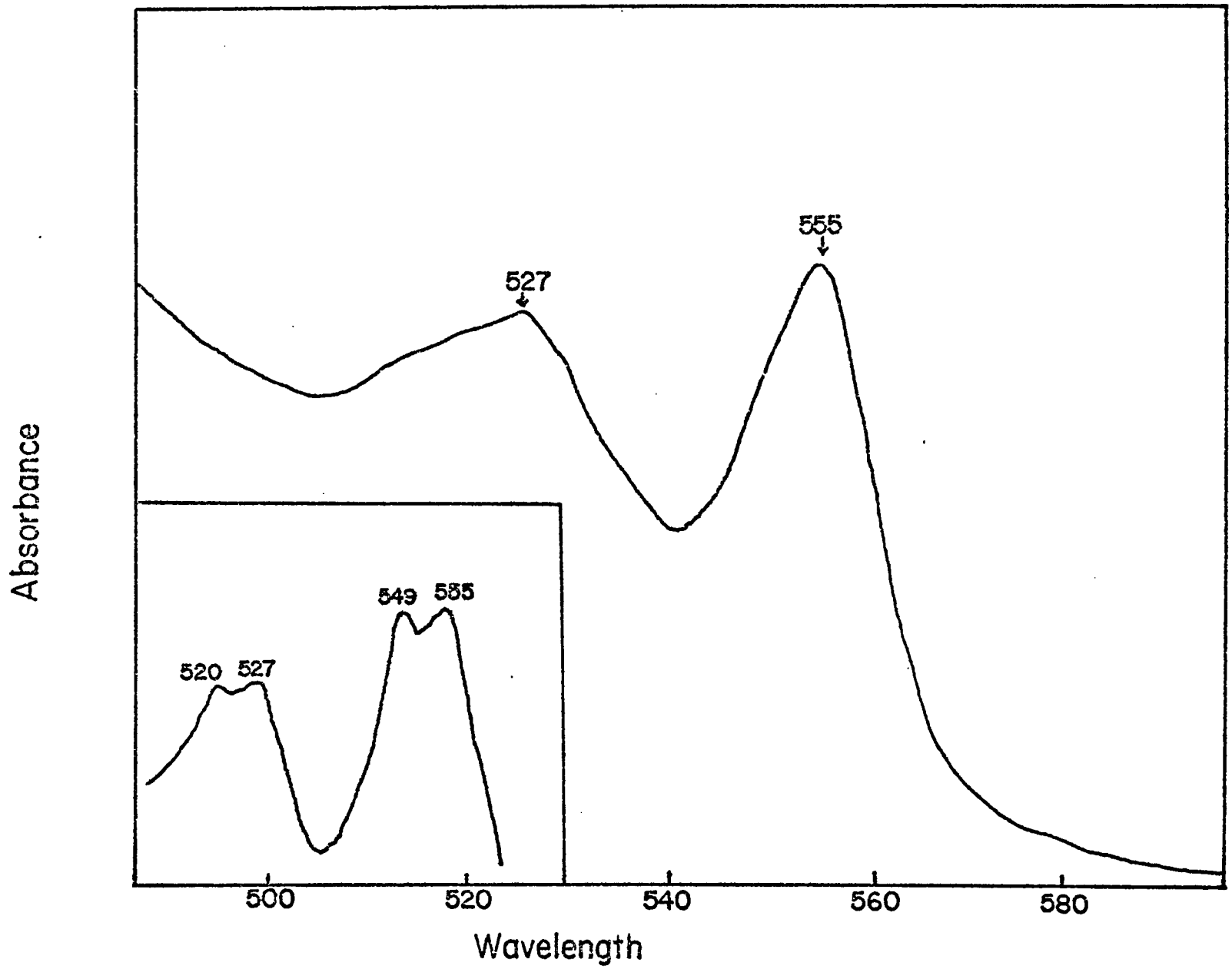
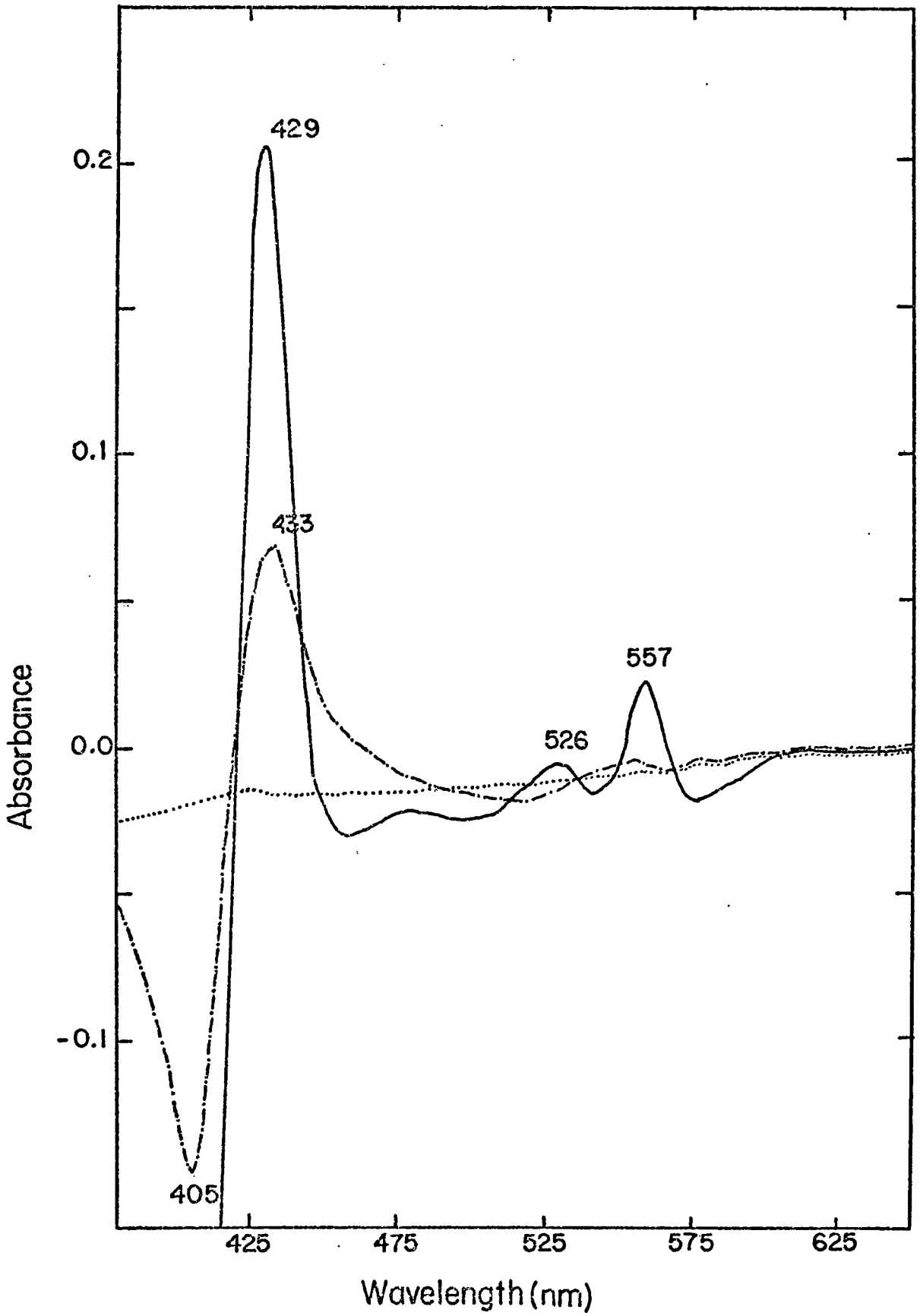

Absorbance
555
527
549
555
520
527
500
520
540
560
580
Wavelength

Figure 4. Cyanide and reduced difference spectra of the red oxidase (cytochrome o). Dotted line (·····) represents the base line (oxidized minus oxidized); dashed line (-·-·-) shows the cyanide plus oxidized spectrum; solid line (———) represents cyanide plus reduced spectrum.


429
433
557
526
405
0.2
0.1
0.0
-0.1
Absorbance
425
475
525
575
625
Wavelength (nm)

gradually by exposing the reduced form to the air after a brief shaking. As shown in Figure 5, the untreated preparation had its maximum at 412 nm, and the reduced form at 425 nm in the Soret region. The effect of "oxygenation" shifted the reduced peak back to 414 nm, with a substantial decrease in absorbance. Thus, the form that had a Soret maximum at 414 nm is considered as an "oxygenated" form of the cytochrome o. Table 2 shows that the spectral shift during "oxygenation" was a function of time after reduction. Interestingly, the untreated preparation had much more absorbance, in both visible and Soret regions, in comparison with that of the "oxygenated" form, possibly indicating that a partially reduced cytochrome o was obtained after purification. This "oxygenated" form was highly stable, and it reacted with carbon monoxide.

Figure 5. Time-course spectra of the "oxygenation" of cytochrome o purified from Azotobacter vinelandii. Curve 1 (———) is the oxidized preparation; curve 2 (-----) the reduced form at one minute after dithionite reduction; curve 3 (-·-·-) the reduced form after ten minutes; curve 4 (·····) the "oxygenated" form.

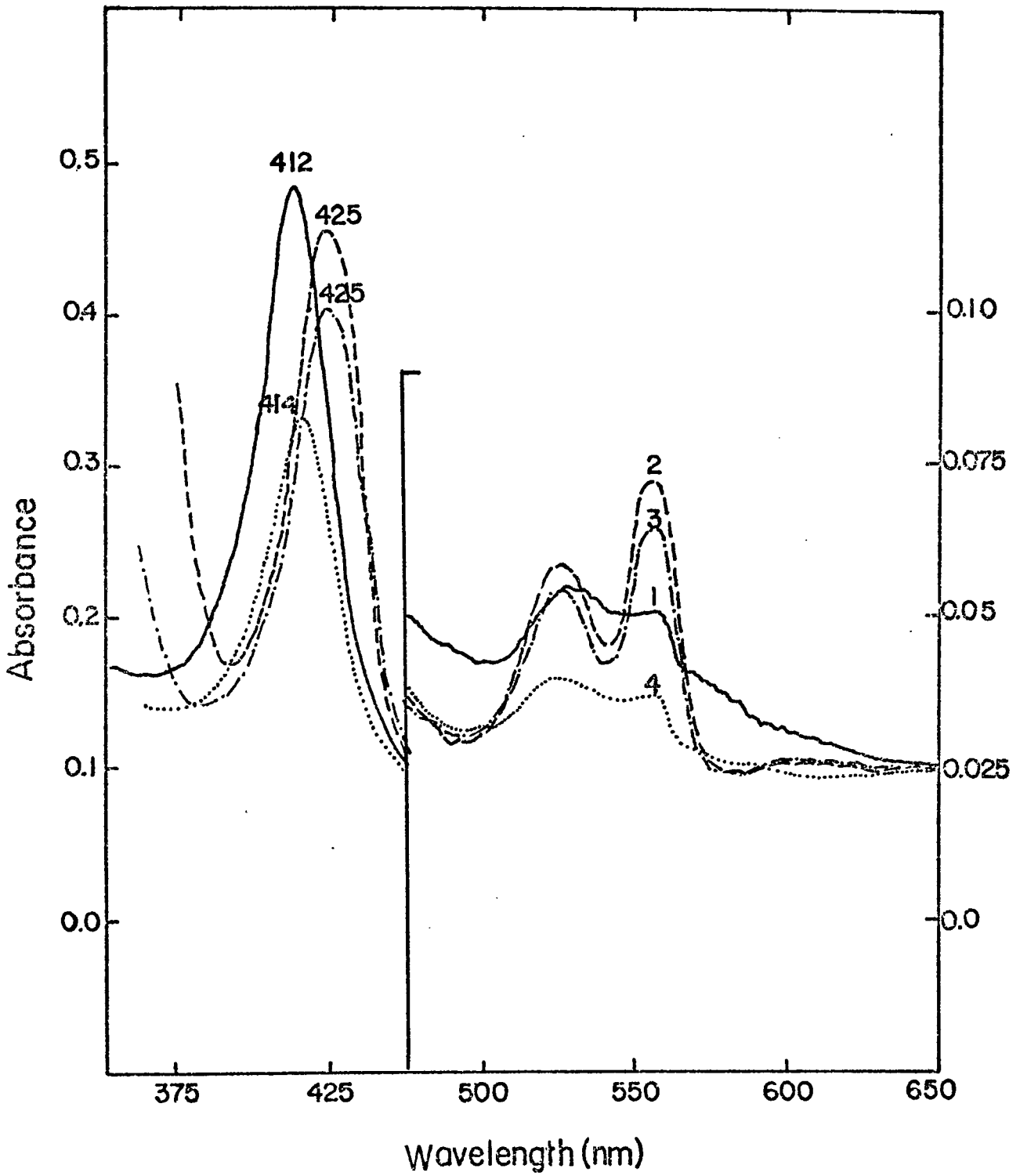

412
425
425
414
2
3
1
4
0.5
0.4
0.3
0.2
0.1
0.0
0.10
0.075
0.05
0.025
0.0
Absorbance
375
425
500
550
600
650
Wavelength (nm)

TABLE 2

Spectral changes during "oxygenation" of the partially purified cytochrome o upon shaking and standing in the air, with reduction accomplished with dithionite. Sample contained 0.5 mg hemoprotein per ml.

	Time elapsed after reduction (minutes)				
	1	10	20	35	untreated
Soret band					
wavelength	423	423	414	414	412
absorbance	0.453	0.402	0.333	0.333	0.481
α band					
absorbance	0.048	0.041	0.016	0.013	0.026

DISCUSSION

The spectral changes that resulted from treating the reduced red oxidase with carbon monoxide have partially confirmed the early work of Castor and Chance on the CO action spectrum studies with Azotobacter oxidase systems (1). The cyanide spectrum studies have also supported previous observations that cytochrome o, in Azotobacter vinelandii, is a cyanide-sensitive oxidase. This is, in part, substantiated by the evidence showing that TMPD oxidase, a complex composed of cytochromes c and o, is very sensitive to the inhibition with cyanide (8,10,11). Unlike the soluble cytochrome o that has been purified from Vitreocilla (12), no NADH-cytochrome o reductase activity could be detected in association with the red oxidase. Throughout the purification process, it was evident that cytochrome o was tightly bound to the membrane and was associated with a c-type cytochrome. No cytochrome a_1 was found to be solubilized together with cytochrome o or with cytochrome c, suggesting that, in the membrane fraction of Azotobacter vinelandii, cytochrome a_1 and cytochrome o may not be associated, as once suggested (4).

An "oxygenated" form of the red oxidase, obtained by aerating the reduced form, was demonstrated to be spectrally different from both the reduced and the oxidized (or untreated) forms. The stability and its reactivity with carbon monoxide suggest a possible functional role in the oxidase reaction mechanism, analogous to that of the mammalian oxidase. Further studies to determine the significance of the "oxygenated" red oxidase are now under way.

ACKNOWLEDGEMENT

We gratefully acknowledge Dr. Graham Palmer of the Biochemistry Department, Rice University, for the assistance provided in the cold-temperature spectral study.

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Title: BIOCHEMICAL CHARACTERIZATION OF CYTOCHROME *O* ISOLATED FROM *AZOTOBACTER VINELANDII*

Running Title: CYTOCHROME *O* OF *AZOTOBACTER VINELANDII*

by

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ABSTRACT

The highly purified cytochrome o, isolated from the electron transport particle of Azotobacter vinelandii, was characterized biochemically. Polyacrylamide gel electrophoresis showed the isolated hemoprotein to be electrophoretically homogeneous. The prosthetic group for cytochrome o was found to be protoheme IV, and the minimum molecular weight, based on protoheme content, was calculated to be 625,000, assuming one mole of heme per mole of protein. The extinction coefficients for cytochrome o were calculated on the basis of the protohemochromogen concentration, and the values obtained are reported. The extinction coefficient for the CO: reduced minus reduced cytochrome o, Δ416 nm (peak) - 431 nm (trough),was 235.4 $mM^{-1}cm^{-1}$. Amino acid analyses revealed the purified cytochrome o to (a) be an acidic protein, (b) be similar to cytochrome p-450 in amino acid composition, and (c) contain high concentrations of hydrophobic residues, indicating a possible structural relationship with membrane phospholipids. Lipid analyses on the cytochrome o revealed the presence of a high concentration of phospholipid; the amound of phospholipid present was 40.5 per cent by weight. Radioautographic analyses, using two-dimensional thin-layer chromatography, revealed the presence of phosphatidylethanolamine and phosphatidylglycerol.

INTRODUCTION

Cytochrome o, a CO-binding hemoprotein, has been found to be a most widely distributed cytochrome component in bacteria, where it is believed to function as a cytochrome oxidase (2,16,20). Chance et al. (4) were the first to observe this unique hemoprotein and to recognize its significance as a new type of CO-reacting cytochrome. Castor and Chance (2) subsequently named this hemoprotein cytochrome o.

Cytochrome o contains protoheme as a prosthetic group, and the spectral characteristics of protoheme is typical of that found for cytochrome b. Cytochrome o can be classified as a CO-reacting b-type cytochrome, whose spectral shifts (as a result of carbon monoxide addition to the reduced hemoprotein) can be specifically defined. It reacts spectrally, not only with carbon monoxide, but with cyanide (29), both of these compounds usually functioning as potent inhibitors of terminal oxidase activity. Cytochrome o is recognized in a bacterium by the following measurements: (i) the absorbance changes of the CO:reduced minus reduced difference spectrum is quite characteristic; and (ii) the nature of the photochemical action spectrum, based on the monochromatic light relief of carbon monoxide inhibition of oxygen uptake, closely resembles that of a b-type cytochrome spectrum (2). For the former determination, one looks for a specific difference spectrum that is characteristic of a CO-reacting o-type cytochrome; this procedure is the one that is most currently used in identifying the presence of cytochrome o in most contemporary studies. Only in a few instances has the latter and more demanding photochemical action spectra measurement been performed to confirm the existence of

cytochrome o as a major terminal oxidase component (20). Chance and his co-workers, as early as 1959, have demonstrated by action spectrum studies that cytochrome o, as well as cytochrome a_1, serves as a terminal oxidase in Azotobacter vinelandii (2). Since then, Jones and Redfearn (14), using action spectra, have verified the presence of cytochrome o, as well as cytochromes a_1 and d, in the electron transport particle of A. vinelandii. Measurements of cytochrome oxidase activity in membrane fractions of A. vinelandii, employing tetramethyl-p-phenylenediamine (TMPD) and dichloroindophenol (DCIP), have shown in one study that partially purified detergent fractions having high DCIP oxidase activity were enriched for both c-type and o cytochrome components (15). In another study, a detergent-treated cytochrome fraction exhibited a twenty-fold increase in TMPD oxidation over that noted for the electron transport particle; the hemoprotein components present in this high TMPD oxidase fraction were identified eventually as cytochromes c_4 + o (17,23).

We have previously reported the isolation, purification, and spectral characterization of this membrane-bound cytochrome o (28,29). Here, we wish to report on its further biochemical characterization and to show that it is a phospholipid-containing hemoprotein component like the cytochrome a + a_3 oxidase, but has an amino acid composition quite similar to that of cytochrome p-450.

MATERIALS AND METHODS

Preparation of cytochrome o. The membrane-bound cytochrome o was isolated and purified from the electron transport particle, or R_3 fraction, of Azotobacter vinelandii according to the procedure previously described (28,29).

Spectral absorbance measurements. The spectra of the cytochrome o were obtained by using eithr Beckman Model 25 or Cary Model 118 recording spectrophotometers. When necessary, cytochrome o preparations were diluted in 0.05M phosphate buffer, pH 7.5, containing 1% Triton X-100. For anaerobic measurements, 1-cm Thunberg cuvettes, whose contents were evacuated and flushed four times with purified nitrogen gas, were used.

Carbon monoxide treatment. Carbon monoxide was bubbled into all cytochrome o preparations through a capillary tube. The carbon monoxide gas was pretreated by washing with a solution of 10% (w/v) pyrogallol in a 10% (w/v) solution of potassium hydroxide. Usually 15 to 30 seconds of exposure was required for inducing the CO-dependent spectral changes. The heme content of all cytochrome o preparations used varied in concentration from 0.5 to 2.4 μM.

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed in tris-glycine buffer, pH 8.9 (6), with 5% and 7% cross-linked gels, and Coomossie Brilliant Blue was used as the protein stain.

Phospholipid analyses. Total lipid extractions were performed according to the procedure of Folch et al. (11). All lipid extracts were dried under a stream of purified nitrogen gas; the residues were dissolved in a few drops of $CHCH_3$-CH_3OH-H_2O (75:25:2, v/v) and transferred quantitatively to

the thin layer plates coated with silica gel H, 250 μ thick. A two-dimensional separation procedure, similar to that of Getz et al. (12), was used for resolving the individual phospholipid present in the $CHCl_3$-CH_3OH extract. The plate was first developed with $CHCl_3$-CHOH-CH_3-COOH-H_2O (52:20:7:3, v/v), then air-dried, washed with ether, and placed in a vacuum desiccator for twenty minutes. $CHCH_3$-CHOH-40%CH_3NH_2-H_2O (63:31:5:5, v/v) was used for the secondary development step. The controls, consisting of known radioactive phospholipids (9), were co-chromatographically resolved by radioautography using X-ray films which were subjected to 48 to 72 hours of exposure in order to locate the radioactive areas, allowing for the identification of the phospholipids present in the cytochrome o fraction. The phospholipid components on the thin layer plates were stained by exposure to iodine vapor.

Protein determinations. Protein concentrations were determined routinely by a modified Biuret method, as described by Yonetani (30). Protoheme was determined quantitatively by the alkaline extraction method, according to the method of Pappenheimer et al. (24).

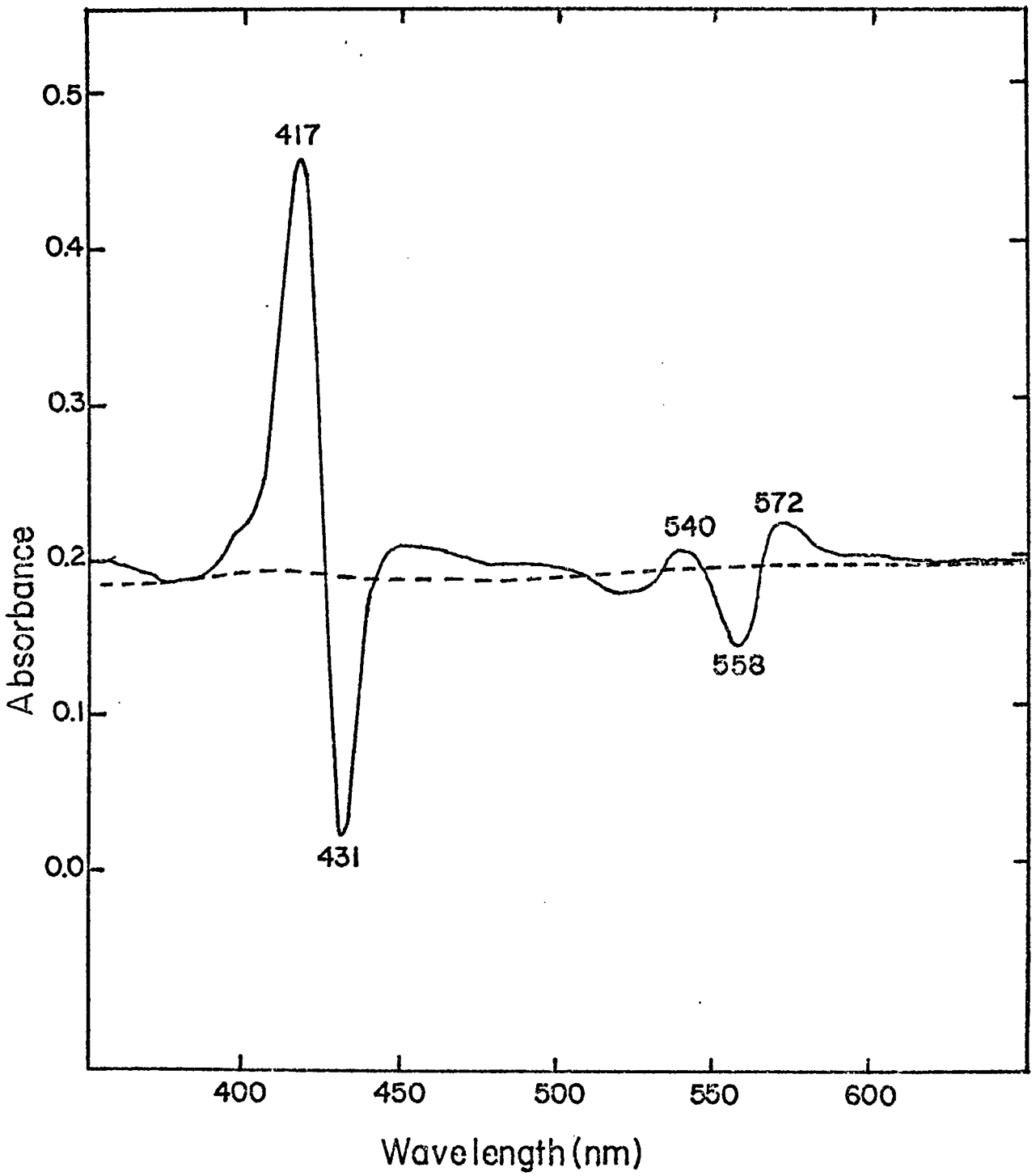
Amino acid analyses. Amino acid analyses were performed according to the method of Spackman et al. (26), using a Beckman Model 120B amino acid analyzer.

RESULTS

Spectral Characteristics and Extinction Coefficients

Figure 1 shows the characteristic CO-binding difference spectrum of the highly purified ferrocytochrome o isolated from Azotobacter vinelandii. One characteristic noted was that, in the Soret region, the absorption spectrum (peak at 417 nm and trough at 431 nm) became sharper as the purity of the cytochrome o preparation increased. This phenomenon was also noted, to a lesser extent, in the visible region for the peaks and trough at 540, 572, and 558 nm. It was also possible to calculate the new extinction coefficients for cytochrome o and to quantitatively compare these values to those previously reported by Chance (3) and Daniel (5). These latter extinction coefficient values are still currently used for estimating cytochrome o concentrations in both bacterial whole cells and in subcellular electron transport-type particles. The two extinction coefficient values previously obtained by Chance and Daniel were derived from measurements of the Soret absorption peak of the CO+ferrocytochrome o difference spectrum, and both values were comparatively lower than that found in this study. The value of Daniel, εmM = 170, was used by us previously (28,29) to estimate the concentration of cytochrome o in both the crude preparations and the highly purified fraction of A. vinelandii. One problem encountered in trying to calculate the extinction coefficient was that the CO+ferrocytochrome o complex exhibited steady-state spectral shifts in the Soret region during the initial exposure to carbon monoxide. This could be demonstrated consistently by saturating a reduced cytochrome o preparation with carbon monoxide and then recording the spectral changes

Figure 1. The CO difference spectrum of a highly purified cytochrome o preparation from Azotobacter vinelandii. The dashed line (-----) shows the reduced minus reduced control; the solid line curve (———) shows the CO:reduced minus reduced spectrum. The concentration of cytochrome o was 2.4 μM; reduction was carried out by the addition of dithionite.


0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
417
431
540
558
572
400
450
500
550
600
Wavelength (nm)

that occurred in the Soret region during the first ten-minute interval. Another problem encountered was that the cytochrome o present in the reference cuvette did not remain fully reduced during this ten-minute time interval, because of autooxidation that occurred naturally with the dithionite-reduced enzyme (29). This was circumvented by using either an anaerobic cuvette or by adding an excess amount of dithionite. The steady-state spectral shifts were markedly pronounced in highly purified preparations of cytochrome o; whether or not this same effect occurs with the cytochrome o when bound to the membrane has yet to be established. Although it is unlikely, the exposure of cytochrome o to Triton X-100 might have been a contributing factor which influenced the calculated extinction coefficient values. The εmM value of 235.4 per cm (Table 1) was obtained for the CO-cytochrome o difference spectrum (Δ417 nm minus 432 nm) ten minutes after the addition of carbon monoxide to the ferro-cytochrome o. Accordingly, our previously published purity value for cytochrome o of 1.6 nmoles heme per mg protein, calculated by using the extinction coefficient value of Daniel (28,29), is now correctly 1.2 nmoles of heme per mg protein. The other extinction coefficient values, derived from our highly purified cytochrome o preparations and calculated on the basis of the protohemochromogen content, are reported in Table 1. The extinction coefficient values reported for both the absolute and the difference spectra (in the absence of carbon monoxide treatment) will probably be of limited value if other b-type cytochromes are present in any cytochrome o preparation to be analyzed. To a lesser extent, this would also be true for c-type cytochromes which might have somewhat similar absorption characteristics, as those found for cytochrome o.

TABLE 1

Extinction Coefficients for Cytochrome o of *Azotobacter vinelandii*

Absolute		ε(mM)
α-peak	557-578 nm (reduced)	76.9
Soret	412-455 nm (oxidized)	655.4
Soret	425-455 nm (reduced)	537.0
Difference		
α-peak	557-572 nm (reduced)	60.0
Soret	428-452 nm (reduced)	381.5
CO: Difference		
Soret	417-432 nm (reduced) (peak minus trough)	235.4*

*Calculated from a spectrum taken ten minutes after the initial exposure to carbon monoxide. Steady-state spectral variations occurred upon the addition of carbon monoxide, and the value shown was obtained from a stabilized spectrum.

Characteristics of the Partially Purified Cytochrome o

As mentioned previously, cytochrome o, together with cytochrome c_4, forms an integrated enzyme complex which can be isolated by detergent fractionation and which functions as a TMPD-dependent cytochrome oxidase in A. vinelandii (17,23). A small amount of the c-type cytochrome appeared as a contaminating protein during the purification of cytochrome o, and it was easily detected by its preferential reduction with ascorbate. The spectra shown in Figure 2 demonstrate such a partially purified cytochrome o preparation. The hemoprotein component represented by the broken-line spectrum, absorbing maximally at 552, 522, and 425 nm, represents the residual amount of cytochrome c_4 that can be detected in the partially purified cytochrome o preparation. Cytochrome o is not reduced by ascorbate, and only the subsequent reduction carried out by the addition of dithionite (solid-line spectrum in Figure 2) reveals the presence of cytochrome o, the major hemoprotein component in this fraction.

Component Analyses of Cytochrome o

Table 2 shows the constituents present in the highly purified cytochrome o preparations of A. vinelandii. The protoheme content was calculated from the difference spectrum of the pyridine hemochromogen derivative of cytochrome o, using an extinction coefficient value of 20.7 (10). Phospholipid content was estimated by total phosphorus analyses, and calculations were based on an average molecular weight (for phospholipid) of 775. As shown in Table 2, the phospholipid content of cytochrome o was high, 1.32 μmoles of phospholipid per 1.5 mg protein; this concentration on a protein basis was 40.5%.

Figure 2. Difference spectra of a partially purified cytochrome *o* preparation (2.0 mg protein per ml) from *Azotobacter vinelandii*. Cytochrome c_4 was detected as a residual contaminant using a differential reduction method. The broken line curve (-·-·-) indicates the reduction carried out by 1 mM ascorbate, revealing the presence of cytochrome c_4. The solid line curve (———) represents the reduction of cytochrome *o* achieved by the addition of dithionite.

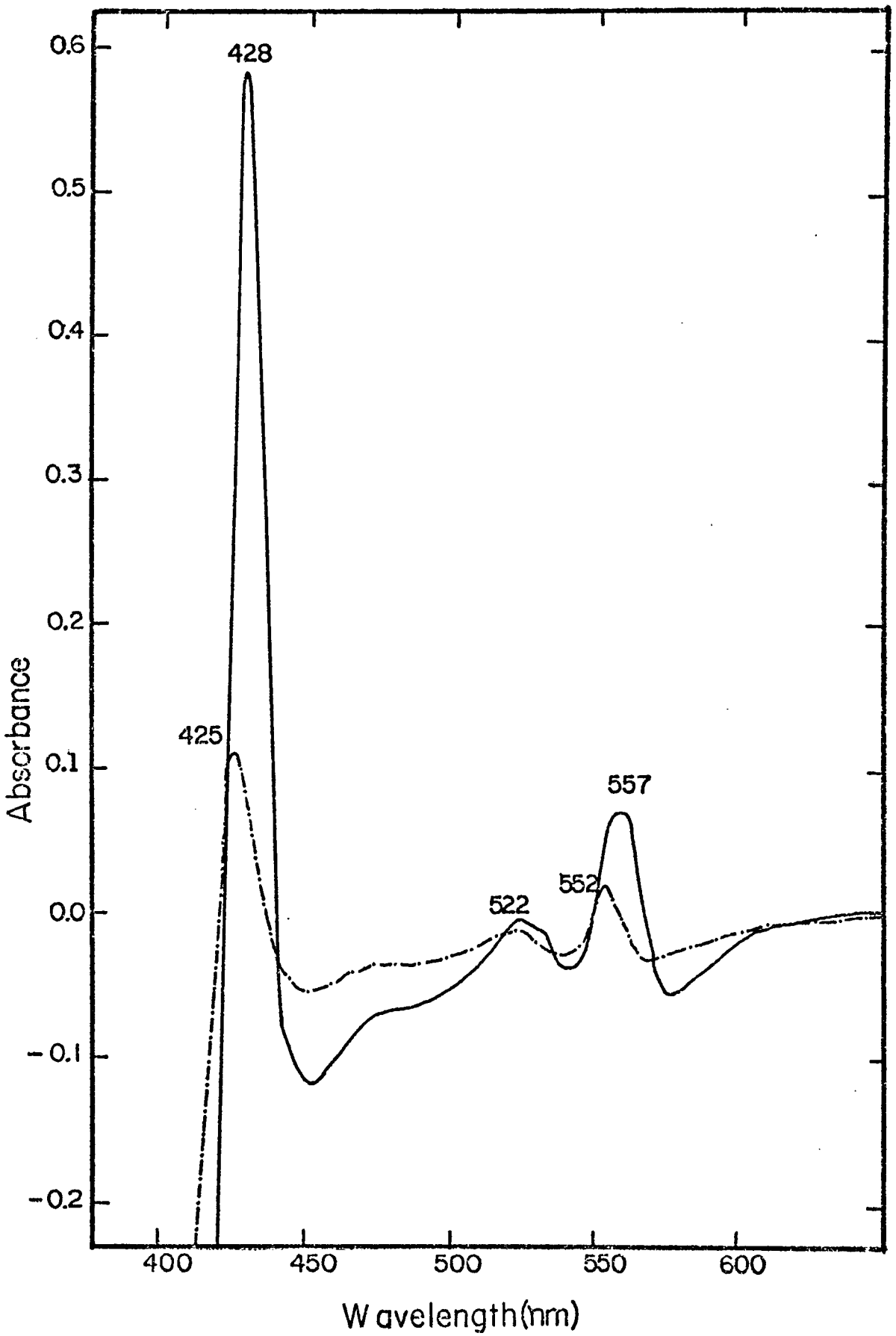

0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
Absorbance
428
425
557
552
522
400
450
500
550
600
Wavelength(nm)

TABLE 2

Composition of the Purified Cytochrome o of *Azotobacter vinelandii*

Component	Concentration	Per Cent (w/w)
Heme (protoheme IV)	2.4 nmoles*	—
Protein	1.5 mg	59.4
Phospholipid	1.32 μmoles	40.5**

*The minimum molecular weight of cytochrome o was estimated to be 625,000, based on the chemical stoichiometry of one molecule of protoheme per molecule of protein.

**This value excludes the content of the detergent, Triton X-100, present in the cytochrome o.

Phospholipid Composition of Cytochrome o

The phospholipids present in the A. vinelandii electron transport particle have previously been analyzed and have been found to contain phosphotidylethanolamine(62%), phosphotidylglycerol(28%), and cardiolipin (8%) (16,18). On a protein basis, the electron transport particle has a phospholipid content analogous to that found in mitochondria (24-26%). Therefore, it is evident that the purified cytochrome o contains almost twice the concentration of phospholipid found in the original electron transport fraction. Two of these phospholipids, namely phosphotidylethanolamine and phosphatidylglycerol, were readily identified as major components present in highly purified cytochrome o preparations. Figure 3 shows a two-dimentational thin-layer chromatogram of the lipid extract obtained from a purified cytochrome o preparation. The presence of phosphatidylethanolamine and phosphatidylglycerol was readily established, using an autoradiographic technique. Trace amounts of known radioactive phospholipids, like the phosphatidylinositol shown in Figure 3, were used as radioactive markers for the identification of the phospholipids present in the cytochrome o fraction. Cardiolipin, which represents only a small percentage of the total phospholipids found in the membranes of the electron transport fraction (16,18), could not be detected, but its presence may have been masked by the residual amount of Triton X-100 that was also detected on the thin layer plate.

Electro-phoretic Analyses of Cytochrome o

Electrophoretic analyses of the purified cytochrome o were performed using 5% acrylamide gel in tris-glycin buffer at pH 8.9. As shown in

Figure 3. Two-dimensional thin-layer chromatogram used for identifying the phospholipids present in cytochrome o of Azotobacter vinelandii. Known radioactive phospholipids (9) were co-chromatographed with the lipid extract obtained from cytochrome o. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI) were used as the radioautographic markers for the identification of the unknown phospholipids. The phospholipid components of cytochrome o were readily stained by iodine vapor.

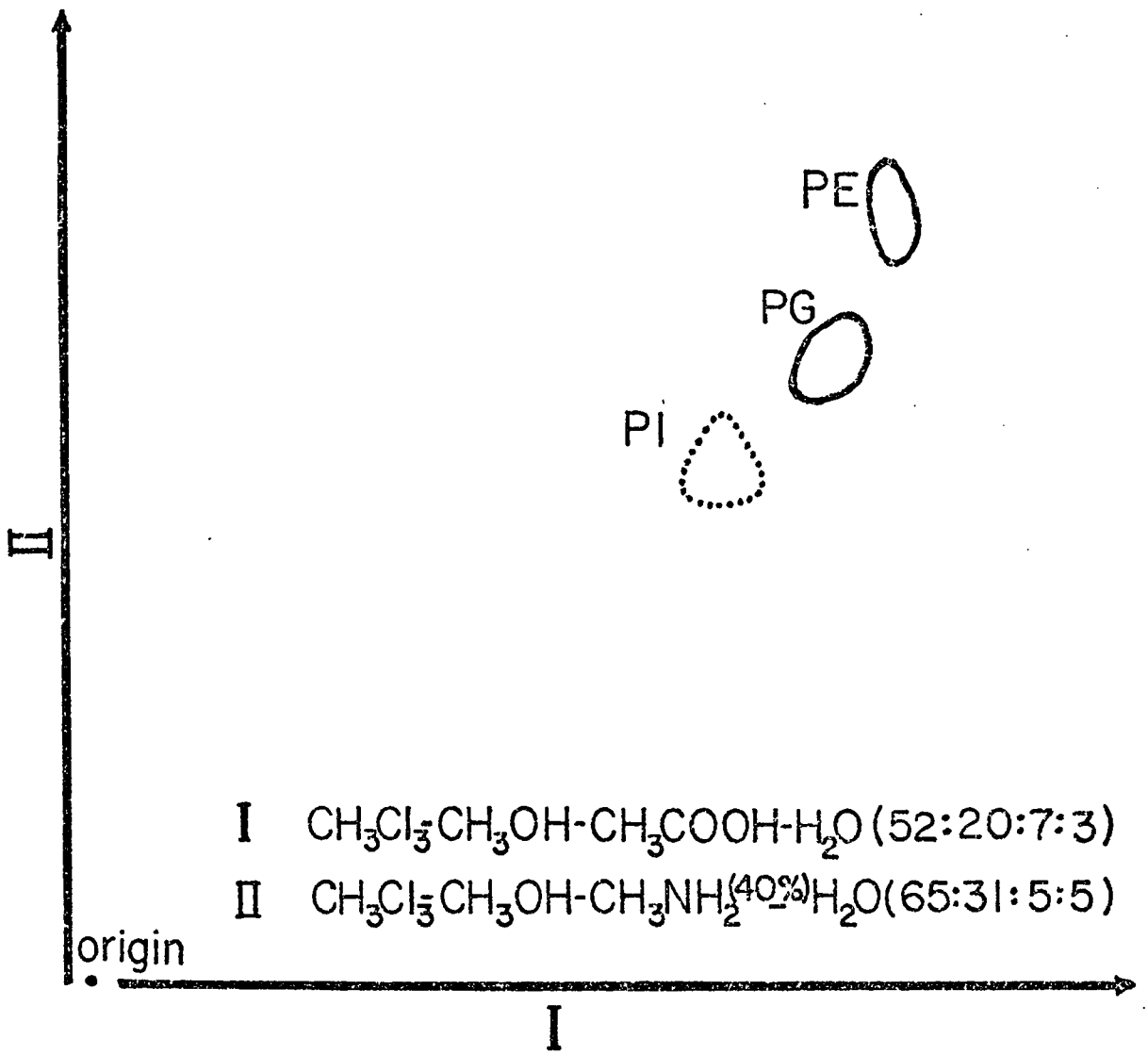
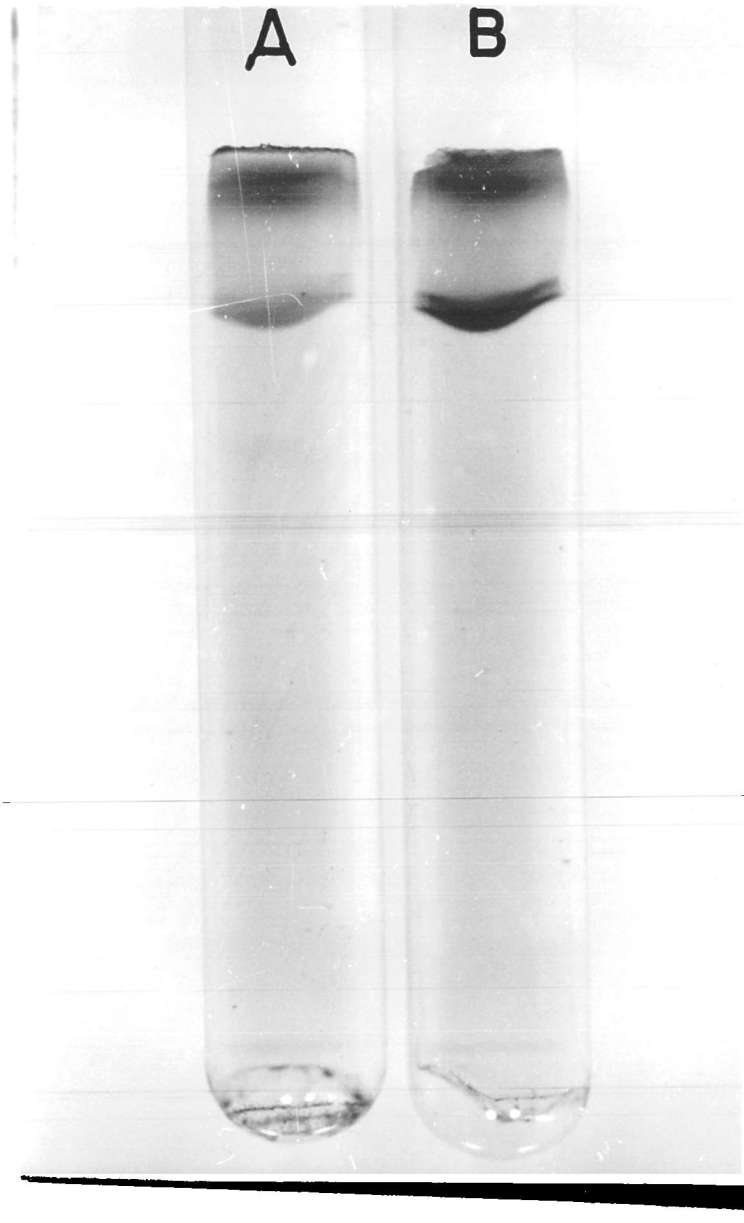

PE
PG
PI
II
I
origin
I
I $CH_3Cl_3CH_3OH$-CH_3COOH-H_2O (52:20:7:3)
II $CH_3Cl_3CH_3OH$-CH_3NH_2(40%)H_2O(65:31:5:5)

Figure 4, gel A represents a partially purified cytochrome o preparation, while gel B shows a purified cytochrome o. In both cases, the cytochrome o preparations migrated as broad diffused bands, with some minor contaminant shown in gel A. Part of the hemoprotein was retained at the edge of the cathode terminal, which was probably due to the initial fouling of the gel by the lipid and the Triton X-100 that were present in the sample. An attempt was made to circumvent this problem by delipidating the cytochrome o sample prior to electrophoresing; however, this treatment resulted in the aggregation of the hemoprotein. Attempts at removing some of the residual Triton X-100 present in the purified cytochrome o preparations, via gel filtration chromatography, also led to the same type of unsatisfactory results. We conclude that the presence of phospholipid and Triton X-100 are essential for maintaining the cytochrome o preparation in a "solubilized" state, and the presence of these two components might have affected its electrophoretic mobility. The initial mild detergent treatments of the A. vinelandii electron transport fraction, with Triton X-100 and deoxycholate, did not "solubilize" the cytochrome o. The cytochrome o remained tightly bound to the electron transport particle, and its solubilization could only be successfully accomplished by using large amounts of Triton X-100 in the presence of a 1M KCl solution (28,29). Any attempt made at altering the "hydrophobic milieu" of the cytochrome o preparation by the removal of phospholipids or the residual Triton X-100 resulted in the direct aggregation of this hemoprotein.

Amino Acid Composition of Cytochrome o

The amino acid composition of the highly purified cytochrome o preparation is shown in Table 3. The data shows that cytochrome o contains approximately 38% apolar amino acid residues and 25% charged

Figure 4. Electrophoretic polyacrylamide gels of cytochrome o fractions of Azotobacter vinelandii. In (A), 50 µg of a partially purified fraction was electrophoresed, while (B) shows a 100 µg sample of a highly purified cytochrome o preparation.


A
B

residues. The other 36% of the amino acid residues were of intermediate polarity, the latter parameter having little effect on the solubility of this hemoprotein in either the aqueous or organic phases. The amino acid analyses show cytochrome *o* to be an acidic protein; it is also more "hydrophobic" in nature than a typical water-soluble protein, or even the protein isolated from the membrane of the red blood cell (27). Furthermore, the extent of the hydrophobicity noted for cytochrome *o* is similar to that reported for some "intrinsic" membrane proteins, such as those associated with the microsomal cytochrome b_5 (21), and the mammalian mitochondrial cytochrome *a* + a_3 oxidase (1).

Table 3 also compares the amino acid composition of cytochrome *o* to that of two types of cytochrome p-450 (p-450_{cam} and p-450_{LM}), whose amino acid contents have been analyzed previously (8). As shown, the amino acid composition of cytochrome *o* and the two p-450 cytochromes are very similar, and this relationship even holds true for the amino acid composition data presented for the purified p-450 cytochrome of *Rhizobium japonicum* (7). No such overall similarity in amino acid content was noted in comparisons made to the mammalian cytochrome *a* + a_3 oxidase (1).

The *A. vinelandii* cytochrome *o*, like all cytochrome p-450 analyzed to date, possesses a protoheme prosthetic group capable of reacting with carbon monoxide, and one is tempted to point out that, if cytochrome *o* were in any way "functionally" similar to cytochrome p-450, it might also possess oxygenase activity, in addition to oxidase function.

TABLE 3

Amino Acid Composition of the Highly Purified Cytochrome o of Azotobacter vinelandii Compared to Two Types of Cytochrome p-450[a]

Amino acid	Cytochrome o (A) Residues[b]	Cytochrome o (B) ½ Residues	$p\text{-}450_{cam}$	$p\text{-}450_{LM}$
Asp	45	22	27	21
Thr	37	19	19	23
Ser	35	17	21	26
Glu	51	26	42	24
Pro	26	13	27	24
$CysO_3H$	ND[c]	ND	6	6
Gly	63	31	26	30
Ala	64	32	34	23
Val	40	20	24	27
Met	8	4	9	8
Ile	40	20	24	19
Leu	87	43	40	46
Tyr	20	10	9	11
Phe	48	24	17	28
His	12	6	12	11
Lys	25	12	13	19
Arg	30	15	24	29
Trp	ND	ND	1	1

[a]Cytochrome p-450 values are taken from reference 8 and represent those of the camphor-oxidizing Pseudomonas putida ($p\text{-}450_{cam}$) and the membrane-bound p-450 of liver microsomes ($p\text{-}450_{LM}$).

[b]HCl hydrolysis carried out for 24 hours.

[c]ND - not determined.

DISCUSSION

There is now evidence in the literature which suggests that cytochrome *o* is perhaps the most important terminal oxidase found in bacterial respiratory systems. Cytochrome *o* is found in bacteria with greater frequency than cytochrome *a* + a_3; for examples, see references 13 and 16. There are now some reports indicating that cytochrome *o* may be found in some yeast (22) and in *Tetrahymena* (25). However, the presence of cytochrome *o* in various organisms can be effectively questioned, as pointed out earlier by Lemberg, since the rigorous criterion of demonstrating this hemoprotein by the photochemical action spectrum technique is seldom used (20).

There has been some doubt cast over the role of cytochrome *o* serving as a terminal oxidase in *A. vinelandii* (19), because of its low redox potential, in measurements which employ whole cells. However, certain data presented here might alter this interpretation. Our studies demonstrate that the α peak of cytochrome *o* absorbs maximally at 557 nm rather than at 560 nm, which was the wavelength used in determining the redox potential of this hemoprotein. The absorbance measurements at 560 nm would predominantly reflect absorbance changes attributed to cytochrome b_1.

The data presented herein represents the first successful attempt at biochemically characterizing a membrane-bound cytochrome *o*. Studies on the spectral characterization of this highly purified cytochrome *o* have already been published (28,29). It was possible to isolate and purify the *A. vinelandii* cytochrome *o* because it was one of two cytochrome components having high TMPD oxidase activity which were concentrated in a detergent

fraction. TMPD oxidation (i) is dependent on the presence of a c-type cytochrome functioning in an integrated manner with a terminal oxidase, like cytochrome o or a + a_3; and (ii) is commonly used as an assay for measuring cytochrome oxidase activity (17).

Like other terminal oxidases, cytochrome o in A. vinelandii is tightly bound to the membrane, and, together with cytochrome c_4, it functions as an enzyme complex which carries out carbon monoxide- and cyanide-sensitive TMPD, DCIP, and phenazinemethosulfate (PMS) oxidase reactions that are associated with the electron transport particle of this organism (17). Of these two hemoproteins, only cytochrome o is capable of reacting with carbon monoxide and cyanide readily, producing recognizable spectral shifts (28,29). Cytochrome o is also highly autooxidizable, and the ferrous form reacts with oxygen to form an oxygenated species (29). In many respects, its biochemical properties resemble the classical cytochrome a + a_3 oxidase found in mammalian mitochondria. The highly purified cytochrome o preparation contained two of the three phospholipids found in the electron transport particle of A. vinelandii. It is possible that these phospholipids may be essential for oxidase activity, as is the case for the mammalian cytochrome oxidase. Cytochrome o also aggregates when the purified preparation is delipidated, another property that is associated with the mitochondrial cytochrome oxidase. The phospholipid (and detergent) may well play a role essential for maintaining the native or "solubilized" state of the cytochrome o Amino acid analyses on cytochrome o and mitochondrial cytochrome a + a_3 oxidase show that both contain high concentrations of amino acids rich in hydrophobic residues. This same finding was true for the amino acid composition of two forms of cytochrome p-450 (see Table 3), but even more

striking was the similarity in amino acid content found for cytochrome o and these forms of cytochrome p-450. The fact that cytochrome o contains a CO-reacting protoheme as a prosthetic group, as does cytochrome p-450, and since the amino acid content of cytochrome o is similar to that of cytochrome p-450, suggests that cytochrome o might function both as an oxidase and as an oxygenating hemoprotein.

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DISCUSSION

Azotobacter vinelandii is a free living, nitrogen-fixing obligate aerobe, which uses molecular oxygen as terminal acceptor for respiration. It is an unusual organism because it possesses the highest respiratory rate of any known living cell. It is because of this extremely high respiratory activity (and its nitrogen-fixing capability) that a great deal of work has been done in attemtping to elucidate the nature of its electron transport system. Unfortunately, A. vinelandii also possesses a very complex electron transport chain. For instance, membrane associated dehydrogenases have been demonstrated for L-malate (15,21), D-lactate (15,20), succinate (15,22), L-glutamate (23), NADH (35), and NADPH (2,8). In addition to these flavoprotein-type membrane-bound dehydrogenases, of the four cytochrome oxidases that are known to exist in bacterial electron transport systems three have been found in A. vinelandii (6,16,25). The three terminal oxidases that can react with molecular 0_2, are cytochromes a_1, a_2 (or d) and o, and cytochrome oxidase activity has been demonstrated using assay systems employing ascorbate-TMPD (18), ascorbate-DCIP (16), reduced Azotobacter cytochrome c_4+c_5 (16,19), as well as reduced mammalian cytochrome c (19). In addition to coenzyme Q-8 and noheme iron proteins, both multiple c- and b-type cytochroms have also been implicated as functional components in the electron transport system of this organism (16,40). All these cytochrome components and related electron carriers are membrane-bound, and intergrated functionally. The isolation and purification of each of these individual components, for reconstituting

enzyme activity, has yet to be accomplished for the A. vinelandii respiratory chain system. To date, only the c-type cytochromes have ever been isolated and purified from the membrane fraction of A. vinelandii (40). However, due to the "harsh" extraction procedures employed, the purified cytochromes c_4 and c_5 no longer possessed any cytochrome oxidase activity; hence the functional role of these c-type cytochromes are still essentially unknown. Consequently, only chemical and spectral information was obtained from such purification studies (40).

This past decade, a major attempt has been made toward understanding the role of branched electron transport systems and their concomitant terminal oxidases in the respiration of bacteria (4,12,16,24,25,29, 39,44). This has been particularly true for Azotobacter vinelandii (12,24). Although, a tentative branched scheme of the A. vinelandii electron transport chain was first proposed in 1967 by Jones and Redfearn (16), the exact sequence and nature of the terminal branched chain remained uncertain even today (24,47). The branching point site (s) and its relationship to the substrate oxidation site as well as to the multiple oxidase pathways, through which the electrons are sequentially transferred have been subjected to several revisions (12). Even today the current scheme (s) proposed are grossly oversimplified; and there is evidence which suggests that the actual sequences of the electron transport carrier in A. vinelandii may be more complicated than it were originally thought. Most of the information obtained in constructing the original scheme were from the studies on (a) substrate-oxidation and reduction kinetics, (b) inhibition patterns, and (c) photo-

chemical action spectra (16). From such studies, which employ either the whole cells or the membrane particles isolated from the electron transport chain, some ideas of a possible sequence of electron transfer as well as the association of substrate with the terminal oxidase system can be obtained. But, the data available for evaluating each individual redox potential and consequently for assigning the sequence of each electron carriers are not complete. This can be exemplified by the studies of Jones and Redfearn who examined the substrate reduction of the cytochrome components in A. vinelandii, and found that cytochrome a_1 and o could not be subjected to such reduction analysis (16). Therefore, the lack of the understanding of these components function during oxidation and reduction, plus the heterogenerous nature of the electron carriers as revealed for b-type cytochrome (s), have complicated the interpretation of the sequence of the electron carriers to some extent (16). Consequently, the functional roles and the significance of each components to the overall scheme remain in doubt. Unlike the mitochondrial electron transport system, which has been physically and functionally fractionated into three segments, and then reconstituted together to demonstrate the overall sequences of electron flow, no such major studies has been undertaken in any microbial systems (24,29,44). Only recently has it been possible to isolate functional enzyme complexes that have been "solubilized" from the Azotobacter electron transport particle by detergent fractionation procedures (25, 32). Furthermore, the limited knowledge that has accumulated, in this past decade, which indicates the high degree of complexcity of the

electron transport systems in microorganisms when compares to that of mitochondria (11,29).

The work in elucidating the overall scheme of electron transfer systems in bacterial membrane, using isolated enzyme complexes, has just begun. This has been due to the recent development of practical techniques of membrane fractionation which utilized certain types of detergents as well as some lipolytic enzymes (11,24,29). Thus, it is now possible to obtain membrane-bound cytochrome components and enzymes that can be isolated (or released) from membrane fragments into a "solubilized state". These solubilized components can then be further purified by concentional biochemical techniques. The isolation and purification of a segment (or a complex) of the electron transfer chain, such as the flavoprotein dehydrogenases, or a terminal oxidase, is a mandatory requirement so that it may be studied as a simplified model system. Such simplified models can then be used as a base for reconstructing or reconstituting by enzyme activity the entire respiratory chain system, as exemplified by the work of Hatefi with the mitochondrial system (13). This approach was most effectively used by David Green's Laboratory at the Enzyme Institute in examining the function of membrane-bound enzyme complexes.

The work presented in this dissertation represents a similar attempt for reconstructing such a simplified model, which begins with the terminal oxidase cytochrome o, which could be isolated and purified from the electron transport chain of A. vinelandii. These studies are the first to report on the successful isolation and purification

of a "membrane-bound" cytochrome o from any bacterial electron transport system. Because of the difficulties encountered in assaying oxidases and isolating membrane-bound cytochromes, very little has been done in isolating and purifying bacterial cytochrome oxidases. Consequently, little is known about any oxidase components in bacteria, except cytochrome aa_3 which has been extensively studied because of its being the major oxidase in mammalian and eucaryotic organisms.

Cytochrome oxidases of A. vinelandii

As stated before, A. vinelandii contains three terminal cytochrome oxidases; these cytochrome components are cytochromes a_1, a_2 and o. Cytochromes a_1 and o were first detected in 1959 and reported to be the functional oxidases in A. vinelandii by Castor and Chance (6). Although the action spectra analyses of these investigators did not reveal the presence of cytochrome a_2 in A. vinelandii, it was concluded that cytochrome a_2 (or d) could not be excluded as being a significant oxidase of this organism (6). Smith had demonstrated spectrally that cytochrome a_2 was present in A. vinelandii membrane fraction (6). Jones and Redfearn (16), and more recently Frikson and Diehl (10) have shown, by use of action spectra, that cytochrome a_2 is unquestionably a functional oxidase in A. vinelandii.

It has been pointed out in earlier studies, that photochemical action spectra cannot discriminate at a given wavelength between the "relief" of CO inhibition of a single or multiple species of a CO-reacting hemoprotein (s), and, as such the data may reflect the presence of more than one pigment (6). Also, no simple relationship exists be-

tween the degree of oxidase activity of the CO-hemoprotein pigment and the height of the "relief" bands of the photoaction spectrum (6). The fact, that both earlier studies of Castor and Chance (6) and of Jones and Redfearn (16) are uncomparable in regards to the nature of the action spectra obtained, could be due to several reasons. First, the strain of A. vinelandii, used by these two groups of investigators may not have been the same, and this could have accounted for the difference in the cytochrome constituents, as has been found in the case of Acetobacter suboxydans (29). Secondly, the difference might have occurred in the growth (and aeration) conditions, as well as possible differences in the stages of the growth cycle when the cells were harvested. It was demonstrated earlier by Castor and Chance (6) that the cytochrome components of stationary and log phases cells contained different terminal cytochrome oxidases in organisms like E. coli and A. aerogenes.

Recently, Erikson and Diehl (10), reinvestigated the respiratory electron transport chain of A. vinelandii, again using action spectra studies, confirmed that cytochromes $\underline{a}_1$, $\underline{a}_2$ and $\underline{o}$ were the terminal oxidases for the physiological substrates such as NADH and L-malate. Using the TMPD ascorbate electron donor system, coupled phosphorylation could not be obtained; mass action in spectra indicated the involvement of cytochromes $\underline{a}_1$ and possibly and $\underline{o}$ type. The electrons generated by the ascorbate-DCIP system gave coupled phosphorylation using neither cytochromes $\underline{a}_1$ nor $\underline{a}_2$. They reported that all three terminal oxidases ($\underline{a}_1$, $\underline{a}_2$ and $\underline{o}$) were involved in NADH-dependent

respiration; and these investigators confirmed the previous finding of Jones and Redfearn in that the TMPD-ascorbate electron donor system did not use cytochrome $\underline{a}_2$ as an electron acceptor. Their data also indicated as yet an unidentified o-type cytochrome, different from the one involved in the NADH oxidation pathway, was possibly present as an active oxidase. From these studies, it seems undoubtly that cytochromes $\underline{a}_1$, $\underline{a}_2$, and o are all capable of serving as terminal cytochrome oxidases in Azotobacter vinelandii.

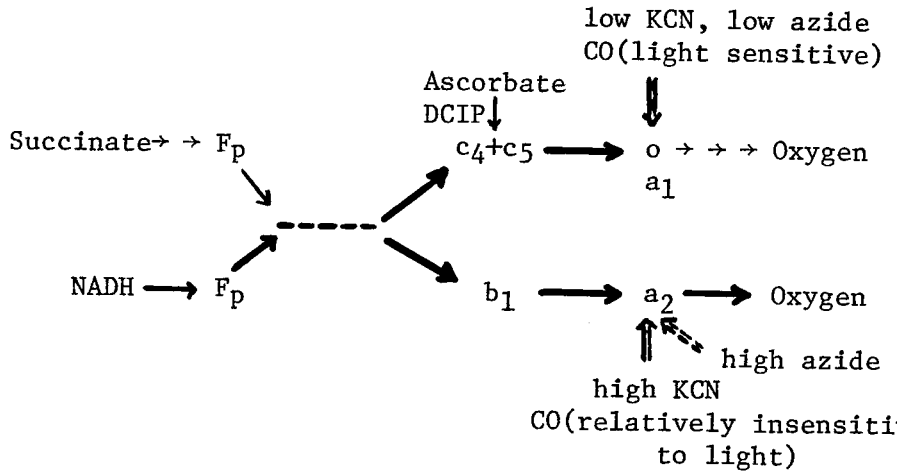
Electron transport chain of A. vinelandii

Jones and Redfearn (16), examining the A. vinelandii electron transport particle, found that at low concentrations cyanide (50μM) ascorbate-DCIP oxidase was maximally inhibited whereas NADH oxidase activity was not significantly affected by this concentration of cyanide. These results, together with other inhibitor data, showed that cyanide and azide could increase or alter the aerobic steady-state reduction of cytochrome $\underline{c}_4+\underline{c}_5$, but not of $\underline{b}_1$, suggested the presence of a branched cytochrome system, and each branch linked to a functionally separate oxidase system. This idea was supported by the findings of Jurtshuk et al., who examined the cyanide sensitivity of all the substrate linked oxidases in the electron transport particle of A. vinelandii (24). They showed that all the oxidases activities were cyanide sensitive, however, the variation and degree of the cyanide sensitivity exhibited was very great. Of all the flavoprotein-dependent electron transport oxidases examined the most sensitive one was the succinate oxidase, which showed 50% inhibition

at a cyanide concentration of $6X10^{-6}$M, while the least sensitive activity was noted for the D-lcatate oxidase which required $9X10^{-4}$M or almost a 1 mM concentration of cyanide to obtain the same degree of inhibition. It was concluded from such studies, that the Azotobacter vinelandii succinate oxidase was 150 times more sensitive to cyanide than the D-lactate oxidase, which suggested that there were probably at least two (or more) different oxidases functioning at the terminal end of the electron transport chain.

Jurtshuk et al. (22) also noted earlier that a substantial reduction of cytochromes c_4+c_5 occurred by the sole addition of ascorbate to the A. vinelandii electron transport particle. The concomitant reduction of cytochromes a_1 and a_2 did not occur by the addition of ascorbate. This suggests that electrons from ascorbate are capable of reducing cytochrome c_4+c_5 but they cannot be transferred to cytochromes a_1 and a_2 until TMPD is added. It also implies that A. vinelandii c-type cytochromes are similar in function to the mammalian cytochrome c, in that it can be reduced by ascorbate, but its reoxidation requires TMPD, as well as a terminal oxidase like cytochromes a+a_3 (36) or possibly cytochrome o in Azotobacter vinelandii. It is possible cytochromes a_1 and a_2 may also serve in this capacity as does cytochrome o.

The branched chain, cytochrome-dependent electron transport system in A. vinelandii was first postulated by Jones and Redfearn (16) and its original form can be diagramatically shown as:



The aerobic steady-state reduction values indicate that ascorbate-DCIP donates electrons at the level of cytochromes $\underline{c}_4+\underline{c}_5$. The relatively high aerobic steady-state reduction of the cytochrome $\underline{c}_4+\underline{c}_5$, that occurs after the addition of natural substrates such as succinate and NADH, together with the observation that an increase in this reduction occurs upon the addition of low concentrations of KCN or azide, supports the concept of a $\underline{c}_4$ $\underline{c}_5 \rightarrow o/\underline{a}_1 \rightarrow O_2$ terminal oxidases pathway. Jurtshuk et al. (25,32) have also demonstrated by isolating and purifying an A. vinelandii cytochrome oxidase, which actively oxidized reduced TMPD, that this terminal oxidase contained c-type cytochromes integrated with cytochrome o. More recent studies, Yang and Jurtshuk (45) have provided evidence which indicates that the purified cytochrome oxidase of A. vinelandii, is actually a enzyme complex of cytochrome $\underline{c}_4+o$. No cytochrome $\underline{c}_5$ could be detected in highly purified preparations which suggest that cytochromes $\underline{c}_4$ and $\underline{c}_5$ may not be closely associated components in the electron transfer pathway of A. vinelandii. This is supported by the finding that purified cytochromes $\underline{c}_4$ and $\underline{c}_5$ from A.

vinelandii yielded significantly different P/O ratios when used as electron mediators between ascorbate and the respiratory electron transport chain (1). These two cytochromes may actually operate in parallel pathways rather than in series in a single pathway. In the original formulation of the branched-chain cytochrome respiratory system of A. vinelandii, the splitting of the chain was postulated to occur at the quinone level to yield two terminal oxidase pathways the major ($b_1 \rightarrow a_2$) and a minor pathway ($c_4+c_5 \rightarrow a_1/o$).

The evidence for cytochrome a_2 serving as the functional oxidase for the major pathway, was obtained from studies on the electron transport particle using an electron donors such as NADH, as well as in A. vinelandii whole cells using photochemical action spectra. The pertinent findings were (a) CO-inhibition NADH oxidase was relatively insensitive to white light, (b) only high intensity red-light (but not blue light) could "relieve" substantially the CO inhibition; (c) low concentrations of KCN could not abolish this red light relief of CO-inhibition and (d) NADH oxidase system was relatively insensitive to both cyanide and azide.

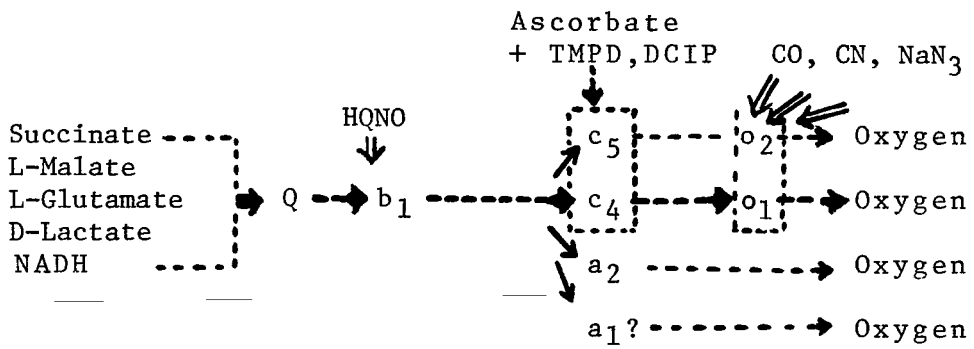
Kauffman and van Gelder, in studying the binding of cyanide to cytochrome a_2, had reported a decrease in the intensity of absorption of cytochrome a_2 after the addition of cyanide (27). This decrease corresponded with a gradual increase in the inhibition of NADH oxidation. This result confirmed that cytochrome a_2 was involved in the oxidation of NADH, supporting the conclusion previously reported by Jones and Redfearn (16,26).

The existence of a major cytochrome $b_1 \rightarrow a_2$ pathway was partially supported by other data, namely on the partial purification of an NADH

oxidase in the earlier studies of Repaske and Josten (35). The content of cytochromes $\underline{b}_1$ and $\underline{a}_2$ of the NADH oxidase preparation was on a relative basis higher than the concentration of cytochromes $\underline{c}_4+\underline{c}_5$. However, cytochrome $\underline{a}_1$ was also present in this partially purified oxidase preparation, which suggested that cytochrome $\underline{a}_1$ as well as $\underline{a}_2$ might be responsible for carrying out NADH oxidation. This early work also indicated that cytochrome $\underline{c}_4$ $\underline{c}_5$ was present in NADH oxidase preparation whether or not all of the cytochromes found, ($\underline{a}_1$, $\underline{a}_2$, $\underline{b}_1$ and $\underline{c}_4+\underline{c}_5$) were involved in electron transport reaction involving NADH oxidation to oxygen could not be definitely established. However, Repaske and Josten (35) demonstrated that all cytochromes associated with the NADH oxidizing particle did undergo rapid oxidation and reduction, and that cytochrome $\underline{c}_4+\underline{c}_5$ was enzymatically more active than the added cytochrome $\underline{c}_4+\underline{c}_5$ isolated and partially purified from this Azotobacter species. This suggested that cytochrome $\underline{c}_4+\underline{c}_5$ could also have been involved in the oxidation of NADH. Since the concentration of cytochrome $\underline{a}_1$ was obviously low as judged from the spectra shown (although not quantitatively estimated), no comment was made concerning the role of cytochrome $\underline{a}_1$ in NADH oxidation. However, Jones and Redfearn had interpreted these findings to indicate that cytochromes $\underline{a}_1$ along with $\underline{c}_4+\underline{c}_5$, had decreased during the purification of the NADH oxidase preparation and therefore suggested that only a "casual relationship" existed between these two cytochrome components to NADH oxidase activity.

The excluding of cytochrome $\underline{b}_1$ as being in the minor pathway was deduced from the apparent inability of 2-n-alkyl-4-hydroxy-qunioline-N-oxide (HQNO) to inhibit NADH oxidation completely. However, Jurtshuk

et al. (24) subsequently showed complete inhibition by HQNO of most substrate oxidations associated with the respiratory chain of A. vinelandii, and further analysis of the mutual depletion kinetics of this inhibition suggested that HQNO combined tightly with the reduced form of cytochrome b_1. The modified scheme is illustrated below which represents the A. vinelandii electron transport chain based on all current information available to date:



Unlike past schemes which were orginally proposed by Jones and Redfearn, the above pathway is modified so that the branches of the chain passing through the c-type cytochromes carries the major share of electrons from NADH and succinate.

In considering (a) the respiration in the membranes of A. vinelandii all oxidases are readily inhibited by cyanide, HQNO and most probably carbon monoxide, (b) the CN and CO readily inhibite ascorbate-TMPD and ascorbate-DCIP oxidase system, (c) the cytochrome c_4+o complex isolated using ascorbate-TMPD assay was highly reactive with CO, cyanide and oxygen, and (d) TMPD oxidase activity quantitatively reflects the actual respiration rate in whole cells of A. vinelandii. It is therefore,

concluded that cytochrome $\underline{c}_4$ → $\underline{o}$ → oxygen pathway represents the major electron transport pathway in A. vinelandii and it appears to be responsible for coupled site III phosphorylation (1).

Jones (47), in his latest revised scheme, has also indicated separated cytochrome $\underline{c}_4$ and $\underline{c}_5$ pathways, which channel into the terminal oxidases cytochromes $\underline{a}_1$ and $\underline{o}$ respectively. However, his $\underline{c}_5$ → $\underline{o}$ and $\underline{c}_4$ → $\underline{a}_1$ pathways are not accurate, and there is no new evidence to support the $\underline{c}_4$ → $\underline{a}_1$ pathway. Our recent studies of the purified cytochrome $\underline{c}_4$+$\underline{o}$ complex weighes heavily against the proposed cytochrome $\underline{c}_5$ → $\underline{o}$ pathway in the respiratory chain of A. vinelandii (45).

Jones and Redfearn also fractionated the electron particle of A. vinelandii, using sodium deoxylate, in the presence of high KCl concentration, followed by sucrose density gradient centrifugation and obtained a "classical" red-green split (17). The color difference observed between these two types of solubilized electron transport particles reflected their cytochrome contents, as determined by spectral analyses. The red particle supernatant contained enriched amounts of cytochromes $\underline{c}_4$+$\underline{c}_5$, $\underline{b}_1$ and $\underline{o}$, and the concentration levels of cytochromes $\underline{a}_1$ and $\underline{a}_2$ were relatively low. On the other hand, the green residue particle were enriched for cytochromes $\underline{a}_1$ and $\underline{a}_2$ (17). Jones and Redfearn analyzed the distribution of cytochrome $\underline{a}_1$ in the red supernatant and green residue fractions and suggested that this component was functionally more closely linked to cytochrome $\underline{a}_2$ than to the c-type cytochrome. Both a-type cytochromes were found in higher concentrations in the green residue pellet, which essentially exhibited no cytochrome

oxidase activity. The red supernatant particles, which also contained high levels of flavoprotein coenzyme Q, non-heme iron and cytochromes c_4+ c_5, b and o, also catalyzed the oxidation of succinate, ascorbate-DCIP, ascorbate-TMPD or reduced by cytochrome c_4+c_5. The latter finding again suggests that cytochrome oxidase activity in A. vinelandii is directly related to the presence of c-type cytochromes and cytochrome o.

The problem of cytochrome a_1 in bacterial electron transport systems

Cytochrome a_1 has been isolated and partially purified from Nitosomonas europaea (9). Unfortunately, this partially purified preparation contained only rather weak cytochrome c oxidase activity (9), which could not account for the oxygen uptake exhibited by the whole cell oxidation rate. The cytochrome c oxidase activity was inhibited by cyanide (10^{-5}M); CO inhibition could not be demonstrated. Cytochrome a_1 was also noted in semianaerobic Hemophilus parainfluenzae cells (43). The concentration of cytochrome a_1 was found to be greater in cells grown anaerobically, in the presence of nitrate (38,42), and in such cells cytochrome a_1 served as a nitrate reductase. Other observations on cytochrome a_1 in particle preparations have been made with Nitrobacter (3), Acromobacter (31). Acetobacter (6), P. vulgaris (6), E. coli (4), Ferrobacillus ferrooxidans (5), Holobacterium cutirubrum (30), H. halobium (7), and A. vinelandii (6).

From early studies of Jones and Redfearn (17), and Jurtshuk et al. (22), it was evident that the concentration of cytochrome a_1 in A. vinelandii apparently was low in the cells harvested at log or late log phases of growth. Kauffman and van Gelder also reported that in some of their preparations cytochrome a_1 could not be detected (28), and by moni-

toring NADH or ascorbate-DCIP oxidations in some of these preparations the activity was found to be as high as in other preparations which contained a substantial amount of cytochrome a_1 (28). This observation indicates that there is a questionable role that cytochrome a_1 plays in the integral part of the A. vinelandii respiratory chain. Other data gives additional, yet conflicting functional roles of this oxidase in A. vinelandii. For example, Erickson and Diehl (10) showed that in an action spectrum, obtained using ascorbate-TMPD as substrate, the presence of cytochrome a_1 and possibly another o type cytochrome, which was different from the cytochrome o found when NADH was used as the reductant. This finding suggests that cytochrome a_1 functionally participates in the ascorbate-TMPD oxidation pathway, as was originally proposed by Jones and Redfearn (16).

However, in studying the respiratory activity of the A. vinelandii electron transport system, in the presence of cyanide, Kauffman and van Gelder found that spectrally cyanide had no effect on this cytochrome, and they concluded that cytochrome a_1 was unable to function as a cyanide-sensitive oxidase (28), which is in direct contrast to the findings reported by Jones and Redfearn. Jurtshuk et al (25,32) further demonstrated that on purification of a cyanide sensitive cytochrome oxidase which carrying active ascorbate-TMPD oxidation, no cytochrome a_1 was found to be associated with such an oxidase. This suggested that cytochrome a_1, while insensitive to cyanide inhibition, may not be associated with cytochrome c_4 and o in functioning in the same pathway. Yang and Jurtshuk (unpublished observation) found that when ascorbate-TMPD was used as the reductant, the spectral changes in the electron transport

particle revealed a major band at 551 nm and no absorption change was noted in the red region (580-600 nm), further indicating the lack of participation of cytochrome of a-type in reduction involving ascorbate-TMPD.

While there is sufficient evidence for linking cytochrome c_4 and o in the electron transport chain of A. vinelandii, there is no evidence to indicate the functional association of cytochromes c_5 and a_1 in any Azotobacter electron transfer reactions. To date, only limited work has been done in attempting to establish the significance of cytochrome a_1 in serving as a functional bacterial oxidase. There is also conflicting evidence as to the actual electron transferring pathway in which this cytochrome serves as a terminal electron acceptor, and its true role remains uncertain.

Multiplicity of the cytochrome (s) o component

As mentioned previously, Jones and Redfearn (16) carried out substrate reduction and examined spectrally the changes in the individual cytochrome components in the electron transport particle of A. vinelandii. Although the reduction of cytochromes c_4+c_5, b_1 and a_2 could be followed with accuracy, cytochromes a_1 and o could not be examined in this manner. Therefore, no such reduction data were reported for these two cytochrome components. Since the absorption spectrum of cytochrome b_1 obviously overlapped with that of cytochrome o, the consequent complication in the reduction kinetics observed for cytochrome b_1 was not surprising. More than one b-type cytochrome component was implicated in A. vinelandii based on the above reduction analyses. This finding is compatible with

the observations reported by other workers. Shipp (37), studying the spectral characteristics of bacterial cytochrome α-bands that could be resolved by fourth-order finite difference analyses of the low-temperature (77°K) absorption spectra, found that in A. vinelandii at least two types of b- cytochromes were present. At least one of these b-type cytochrome components is undoubtly cytochrome o.

Erikson and Diehl (10) showed that photochemical action spectra obtained using NADH with phosphorylating particles from A. vinelandii, was very similar in the visible region to that obtained for whole cells which oxidized endogenous substrate. All three terminal oxidases, i.e., cytochromes a_1, a_2 and o, were involved in the NADH-dependent respiration. Cytochrome o was identified with characteristic light relief maxima at 570,537 and 417 nm. This was remarkablely similar to the results reported much earlier by Castor and Chance (6). However, when ascorbate-TMPD was used as reducing substrate, the action spectrum obtained showed light maximum at 560 nm, indicating yet another identified o-type cytochrome which was obviously different from the cytochrome o_{570} found when NADH was used as the reducing substrate. There is also other evidence suggesting the presence of multiple cytochrome o in different bacterial electron transport systems, as mentioned in the Introduction discussing individual microorganism. Furthermore, these multiple cytochrome o components can undoubtedly be found in the same species of certain organisms; as indicated from the studies on Vitreoscilla spp.and A. suboxydans (29,41). The preliminary studies of Yang and Jurtshuk, in fractionating the entire membrane particle of A. vinelandii, also seemed to indicate the possible presence of two multiple cytochrome o components, as orginally

suggested by Erikson and Diehl (10). It was previously reported that cytochrome o was tightly bound to the membrane fraction of A. vinelandii (46), yet during fractionation studies, Yang and Jurtshuk have noticed that some "less tightly" bound cytochrome o fractions could be obtained separate from the cytochrome o component associated with the TMPD-dependent cytochrome oxidase activity. Spectral analysis revealed that these two different cytochrome o preparations reacted with cyanide in different ways, judged from the distinct spectral products that are derived from CN-cytochrome o complexes. The question to be asked is that what, if any, functional difference does exist between these two distinct types of cytochromes o's? Are both cytochromes o serving as terminal oxidases, as indicated in the electron transport pathway presented previously. If this is the case, it would be expected that both cytochromes o (designated as cytochrome o_1 and o_2) may serve in different pathways. However, it is also possible that one of these two cytochromes o's components is not terminal oxidase, but an oxygenating-type cytochrome like P-450. When the amino acid composition of our purified cytochrome o was compared to the amino acid content of cytochrome P-450 (cam) and P-450 (lm), the similarity noted was quite striking. The fact that both cytochromes o and P-450, have CO-reacting protoheme as prosthetic groups, and almost similar amino acid content leads one to believe that there may be a functional similarity between these two hemoproteins. If so, then one cytochrome o may well serve both as a terminal oxidase, the other as an oxygenase and among the biological oxidations that cytochrome o may carry out is a mixed function oxidase reaction.

Cytochrome o as a possible oxygenating component for mixed function oxidases

There is evidence already which suggests that cytochrome o may function as a mixed-function oxidase. Cytochrome o content in Pseudomonas oleovorans, grown in n-hexane as the sole carbon source, was found to be very high, cytochrome P-450, the functional group of the methylene hydroxylase, was not detected in this organism, either in the presence or absence of the mixed function oxidase system. Since the content of cytochrome o was markedly high in cells grown on n-hexane and no P-450 cytochromes were present, the possibility exists that cytochrome o needs to oxygenate n-hexane in order to utilize it as a growth substrate (34). Even though the ω-hydroxylation of n-hexane is not inhibited by carbon monoxide (33), this observation may not be a significant deterrent to the above possibility. There is evidence indicated that some cytochrome o's have a low affinity for CO, and is possible that such a low affinity would account for the uninhibited hydroxylation required for bacterial growth.

Limitation of the studies of cytochrome o

Although that cytochromes o in this study has been found to be a phospholipid-containing hemoprotein; the quantitative studies of these phospholipids are limited here. Hence, the amount of phospholipids, that are associated with the cytochrome o, shall not be considered as a defined quantity. As shown by the lipid analyses that both phosphotidylglycerol and phosphotidylethanolamine are undoubtly present in the cytochrome o preparation, however, the presence of cardiolipin is uncertain. A further quantitative analysis is required to establish the absence (or presence) of this phospholipid. Finally the homogeneity of this cytochrome o preparation, although can not be definitelyjudged by electrophoretic pattern, can be best shown by cold temperature analyses.

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