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The membrane-bound electron-transfer components of aerobically grown *Chromatium vinosum*

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The electron-transfer chain components of aerobically grown *Chromatium vinosum* have been characterized. Membranes isolated from aerobically grown *C. vinosum* have been shown to contain a Rieske iron-sulfur protein, at least three *c*-type cytochromes and at least one *b*-type cytochrome. Two cytochromes that bind CO appear to be present, one of which may function as a terminal oxidase. Membranes isolated from these cells appear to lack a photochemical reaction center and the high potential (E_m = +340 mV) cytochrome *c*-555 that serves as the immediate donor to the reaction center in photosynthetically grown *C. vinosum*. In addition, the *b*-cytochrome(s) of aerobically grown *C. vinosum* has (have) been shown to be considerably more electronegative (E'_m = −90 mV) than that of photosynthetically grown cells (E'_m = +30 mV).

Introduction

While it has been known for quite some time that the photosynthetic purple non-sulfur bacteria (Rhodospirillaceae) are capable of aerobic growth in the dark, it had been thought that the purple sulfur bacteria (Chromatiaceae) were strict anaerobes [1,2]. Recently, it was demonstrated that several species of purple sulfur bacteria possess the ability to grow under aerobic conditions [3–6]. Kämpf and Pfennig [6] had originally demonstrated growth for Chromatiaceae species only under micro- or semiaerobic conditions. More recently [7], Pfennig and Kämpf have been able to demonstrate growth of one of these species, *Chromatium vinosum*, in the dark under fully aerobic conditions.

In the Rhodospirillaceae, oxygen has been shown to repress the synthesis of bacteriochlorophyll and carotenoids [8]. This also appears to be the case in *C. vinosum*, [7]. In addition, the presence of oxygen induces the synthesis of a terminal oxidase in purple non-sulfur bacteria [9], completing the electron-transfer chain needed for aerobic growth. Other components of the respiratory chain in these bacteria are often identical to and overlap with components of the photosynthetic electron-transport chain [10]. It seemed possible that these phenomena would also be observed in those Chromatiaceae species capable of aerobic growth. The present study concentrates on the electron-transfer chain components of aerobically grown *C. vinosum*, with specific emphasis on the differences between the electron carriers of *C. vinosum* grown aerobically in the dark and anaerobically in the light.

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Abbreviations: E_m, oxidation-reduction midpoint potential; EPR, electron paramagnetic resonance; PMS, phenazine methosulfate; BChl, bacteriochlorophyll.

Materials and Methods

Aerobically grown *C. vinosum* cells were grown in the dark on a thiosulfate-containing medium (at pH 7.4 and 30°C) identical to that used for growing purple sulfur bacteria under microaerobic conditions [6]. Acclimatization of *C. vinosum* to increasing oxygen concentrations was accomplished as follows: (1) low oxygen concentration (3–20 μM oxygen) growth for approx. 4 weeks, followed by (2) intermediate oxygen concentration (30–80 μM oxygen) growth for approx. 4 weeks, and finally (3) high oxygen concentration (100–250 μM oxygen) growth for approx. 6 weeks. Cells were grown at the appropriate oxygen concentration and transferred into fresh growth medium approx. every 3–4 days. Oxygen concentrations under aerobic growth conditions in the dark were monitored with a Clark-type oxygen electrode and a Model 53 polarographic unit from Yellow Springs Instruments. Bacteriochlorophyll *a* (BChl *a*) was determined after each transfer by extraction with 7 : 2 (v/v) acetone/methanol as in Ref. 11. All measurements reported below were conducted on cells grown at high oxygen concentration unless otherwise mentioned. Aerobically grown *C. vinosum* cells were disrupted by sonication in 20 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl as described previously for photosynthetically grown cells [12]. After centrifugation at 255 000 × *g* for 2 h, the sedimented membrane fragments were resuspended in 20 mM Tris-HCl buffer.

Protein concentration was determined according to the method of Bradford [13] using bovine serum albumin as a standard. Heme *c* and protoheme were determined after extraction of the protoheme by acetone/HCl [14]. Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, was performed on 10–22% gradient slab gels (1.5 mm thickness) prepared according to O'Farrell [15]. Gels were stained for protein with Coomassie brillant blue and for heme with 3,3′,5,5′-tetramethylbenzidine plus hydrogen peroxide [16]. Protein molecular-weight standards were obtained from Pharmacia Fine Chemicals. Absorbance spectra were obtained using Aminco DW-2a and Perkin-Elmer Lambda 5 spectrophotometers. (Reduced + CO)-minus-reduced difference spectra were measured in Thunberg anaerobic cuvettes with a glucose oxidase plus catalase oxygen trap as described in Ref. 17. Following addition of a reductant, under an argon atmosphere, to the membrane suspensions in both the sample and reference cuvettes, the sample cuvette was gased for 15 min with CO in the dark. Gasing for longer times (up to 1 h) produced no additional CO binding. Oxidation-reduction titrations were performed electrochemically in a thin layer cuvette as in Ref. 18 using a minor modification of the procedure described previously [19]. Electron paramagnetic resonance spectra were obtained using a Varian E-109 spectrometer equipped with an Air Products Helitrans flexible helium transfer line and a Varian/Hewlett-Packard E-935 data acquisition system.

Results

Fig. 1 shows the optical spectrum of a membrane preparation from whole cells of *C. vinosum* grown in the dark at high O_2 concentration. This spectrum clearly demonstrates a marked decrease in the contributions from carotenoids and bacteriochlorophyll compared to photosynthetically grown cells [11]. Maxima at 799, 595 and 380 nm (not shown) indicate the presence of some residual BChl *a*. Measurements of BChl *a* content ranged from 0.3 to 1.0 μmol BChl *a*/mg protein for membranes isolated from these cells. Mem-

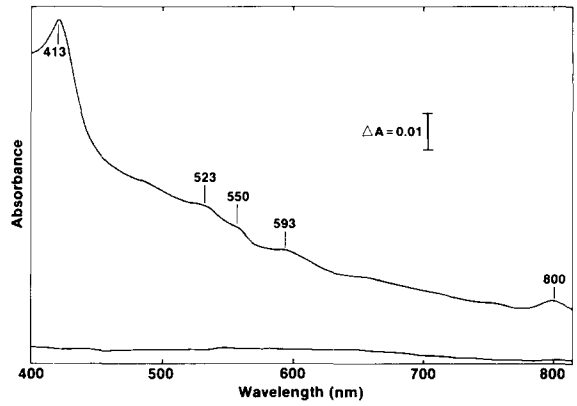

Fig. 1. Absorbance spectrum of aerobically grown *C. vinosum* membranes. Membranes (equivalent to 100 μg protein/ml) were suspended in 20 mM Tris buffer, pH 7.0. Spectral resolution, 1 nm, pathlength, 1 cm. The lower line indicates the instrument baseline.

branes isolated from *C. vinosum* grown at low oxygen concentration showed BChl *a* contents ranging from 4 to 10 μmol BChl *a*/mg protein. The most striking feature of the spectrum of Fig. 1, indicated by large peak at 413 nm arising from the Soret band of oxidized *c*-type cytochromes, is the great enrichment in the cytochrome/BChl *a* ratio as compared to the photosynthetically derived membranes.

To begin characterizing the cytochrome content of these membranes, increasingly stronger reductants were added sequentially to the membranes and difference absorbance spectra recorded in the cytochrome α- and β-band regions. Since hydroquinone is a relatively weak reductant (E'_m = +290 mV), only high-potential cytochromes will be reduced by hydroquinone. Fig. 2 shows the results of adding hydroquinone to the membranes. (Ferricyanide was added to the reference cuvette to insure that the electron carriers in the reference sample were fully oxidized.) The spectrum clearly

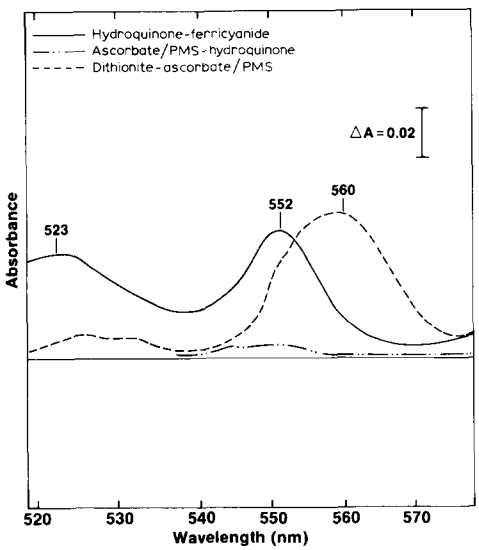

Fig. 2. α- and β-band difference spectra of membrane-bound cytochromes from aerobically grown *C. vinosum*. Membranes (equivalent to 50 μg protein/ml) were suspended in 20 mM Tris buffer, pH 7.0. The corresponding difference spectra were recorded after addition of the indicated reductant. Spectral resolution, 1 nm; pathlength, 1 cm. Hydroquinone, potassium ferricyanide, sodium ascorbate and sodium dithionite were added as small solid aliquots until no further absorbance changes were detected. PMS was present at a final concentration of 5 μM.

indicates the presence of a hydroquinone-reducible cytochrome with an α-band maximum at 552 nm and a β-band maximum at 523 nm. This hydroquinone minus ferricyanide spectrum differs significantly from that observed with membranes derived from photosynthetically grown cells, which shows a prominent α-band maximum at 555–556 nm due to cytochrome *c*-555 (E'_m = +340 mV, Refs. 12 and 20–24). Fig. 2 also shows the ascorbate/phenazine methosulfate (PMS) minus hydroquinone spectrum of the membranes. Ascorbate plus PMS is a stronger reductant than hydroquinone (E_h values near +100 mV were typically observed in membrane samples to which ascorbate plus PMS were added) and components not reducible by hydroquinone are often reduced by ascorbate plus PMS. As can be seen, there is only a very small increment in absorbance produced by ascorbate/PMS compared to that produced by hydroquinone. However, this difference spectrum suggests that a small additional reduction of a *c*-type cytochrome with α- and β-band absorbance maxima near 552 and 523 nm, respectively, may occur. Fig. 2 also shows the dithionite minus ascorbate/PMS difference spectrum of these membranes. Dithionite is a sufficiently strong reductant that its addition to the sample cuvette should result in reduction of all cytochromes present in the membranes. This difference spectrum shows a broad absorbance band, probably arising from the presence of multiple components. Along with a shoulder at 552 nm caused by the reduction of an additional *c*-type cytochrome, a peak near 560 nm is present, probably arising from the reduction of one or more *b*-type cytochromes. The dithionite minus ascorbate/PMS absorbance spectrum also appears to be somewhat asymmetric on the long wavelength side of the 560 nm maximum, suggesting that a small shoulder at 565–566 nm may also be present. Should such a shoulder be present one could not conclude that more than one *b* cytochrome is present, as some *b* cytochromes have split α-band maxima [25]. Difference spectra similar to these of Fig. 2 were obtained with membranes derived from cells grown at low oxygen concentration.

Fig. 3 shows the results of analysis of the membranes for protoheme and hemes *a*, *c* and *d*. After preliminary extraction of the membranes with

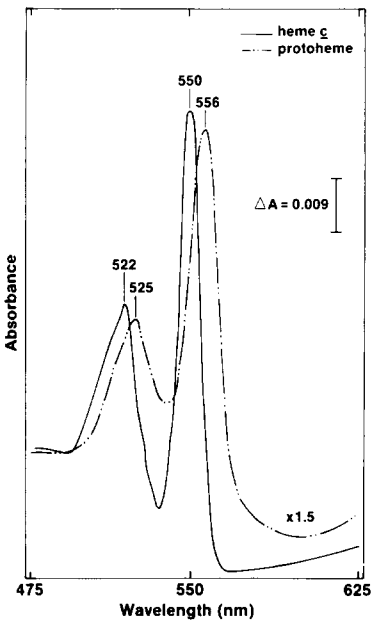

Fig. 3. Absorbance spectra of the pyridine hemochromogen derivatives of membranes from aerobically grown *C. vinosum* cells. The acid-acetone extract of membranes (equivalent to 30 μg/ml protein) were prepared as described in Materials and Methods. The resulting mixture was then placed in both sample and reference cuvettes (1 cm path length), dithionite was added to the sample cuvette, and the spectrum was recorded at a spectral resolution of 1 nm; pathlength, 1 cm.

nonpolar organic solvents to remove pigments and lipids, the membranes were treated with acidic acetone to extract specifically protoheme, heme *a* and heme *d*. The spectrum of this extract, after resuspension in an alkaline pyridine solution and reduction with dithionite, shows an absorbance maximum at 556 nm characteristic of protoheme [14]. The results of several such analyses indicate an average protoheme content of 3.3 μmol per mg protein. No contributions from heme *a* or heme *d* were detectable at 587 nm or 616 nm, respectively. Fig. 3 also shows the alkaline pyridine hemochromogen spectrum of the residue remaining after extraction with acidic acetone. The absorbance maximum at 550 nm is consistent with the presence of heme *c* [14]. Several analyses for heme *c* indicated a protoheme/heme *c* ratio of 2 : 5.

Typical results from an oxidation-reduction titration, monitoring the oxidation state of the cytochromes from α-band region spectra, are shown in Fig. 4. Measurements were made at two wavelength pairs, 550–540 nm and 560–575 nm, to maximize contribution from *c* and *b* type cytochromes, respectively. The data in Fig. 4 show three distinct features in the titration curve measured at 550–540 nm. The three features correspond reasonably well to $n = 1$ titrations of components with E'_m values of +340 mV ($n = 0.90$), +180 mV ($n = 0.94$) and +40 mV ($n = 1.3$). The E_m values of the three components were reproducible to within ±5 mV. Complete α-band spectra of each of these components showed wavelength maxima at 551 nm for each component (data not shown), similar to those observed in the difference spectra of Fig. 2. Since these wavelength maxima are in the region where *c*-type cytochromes absorb, we attribute these features to the presence of at least three *c*-type cytochromes in the membranes. Based on the magnitudes of the absorbance changes associated with reduction of the +340, +180 and +40 mV components, it would appear that the three cytochromes are present in approximately equimolar amounts. In addition to these *c*-type cytochromes, Fig. 4 also shows the contribution from a lower potential,

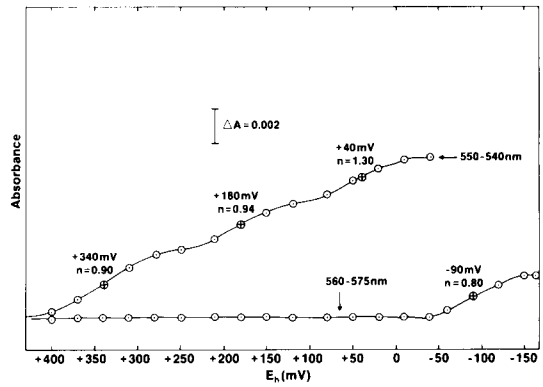

Fig. 4. Oxidation-reduction titration of membranes from aerobically grown *C. vinosum* cells. The titration was conducted as described in Materials and Methods using a reaction mixture of membranes (equivalent to 500 μg protein/ml) in 20 mM Tris buffer, pH 7.0. The following oxidation-reduction mediators were also present: 8 μM pyocyanin/25 μm ferricyanide/50 μM hydroquinone/50 μM diaminodurene/50 μM 1,2-naphthoquinone/80 μM duroquinone/25 μM PMS/30 μM phenazine ethosulfate/25 μM anthroquinone disulfonate. The titration was conducted at 4°C with 1 nm spectra resolution. Optical pathlength, 0.3 mm.

approximately $n = 1$ component which exhibits absorbance changes at 560 nm, but does not contribute to the absorbance at 550 nm. The E'_m value of this component was -90 mV ($n = 0.80$). The spectrum of the $E'_m = -90$ mV component showed a maximum at 560 nm, consistent with this feature in the titration resulting from the reduction of one or more *b*-type cytochromes in the membranes. Difference spectra in the -50 to -150 mV E_h region (not shown) also indicated the possibility of a shoulder at 565–566 nm in addition to the maximum at 560 nm. The titrations were fully reversible and the E_m and n values obtained for all four components were independent of mediator concentrations over a 10-fold range. Similar electrochemical titrations were performed on membranes isolated from cells grown under low oxygen concentrations with essentially the same results.

SDS-polyacrylamide gel electrophoresis was performed on membranes denatured by heating in the presence of SDS and 2-mercaptoethanol. The gels were subsequently stained for heme and, as can be seen in Fig. 5, such staining reveals the presence of two prominent heme-containing peptides with apparent molecular weights of 24 and 20 kDa. Two additional minor heme-staining bands, with apparent molecular weights of 28 and 12 kDa, could be observed in gels which were loaded with higher amounts of membrane protein (data not shown). The 44 kDa heme-staining band which is present in membranes isolated from photosynthetically grown *C. vinosum* cells, due to the presence of cytochrome *c*-555 [26–28], was not observed in this experiment, nor was any 44 kDa heme-staining band observed in experiments utilizing higher amounts of membrane protein. However, the 44 kDa heme-containing peptide could be readily detected in control experiments with membranes isolated from photosynthetically grown cells. These data, as well as the difference spectrum of Fig. 2, suggest that the cytochrome *c*-555 is absent from the membranes of aerobically grown *C. vinosum*.

Fig. 6 shows the CO difference spectra [(reductant + CO)-minus-(reductant, CO absent)] of the membranes in the presence of ascorbate/PMS or dithionite, as reductants. Fig. 6 shows that CO binding to a heme component does occur in membranes reduced with ascorbate/PMS (E_h, approx. +100 mV). This difference spectrum, with maxima at 416, 543 and 573 nm and minima at 430, 516 and 561 nm, resembles the CO difference spectrum for an *o*-type oxidase [29,30]. Fig. 6 shows the CO difference spectrum of the same sample, following dithionite reduction and subsequent gasing with CO as described in Methods. This spectrum, with wavelength maxima at 416, 539 and 572 nm and minima at 431, 521 and 557 nm, also resembles the CO difference spectrum reported for an *o*-type oxidase [29,30]. An additional feature at approx. 610 nm can be observed

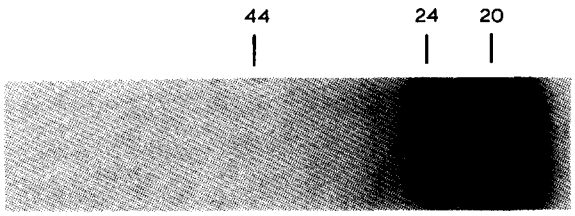

Fig. 5. The heme containing peptides of aerobically grown *C. vinosum* membranes. Membranes equivalent to 100 μg protein in 100 μl volume were applied to the gels and stained for heme as described in Materials and Methods. Molecular weight standards used: bovine serum albumin, ovalbumin, chrymotrypsinogen A, ribonuclease A and cytochrome *c*. Figures are molecular masses in kDa.

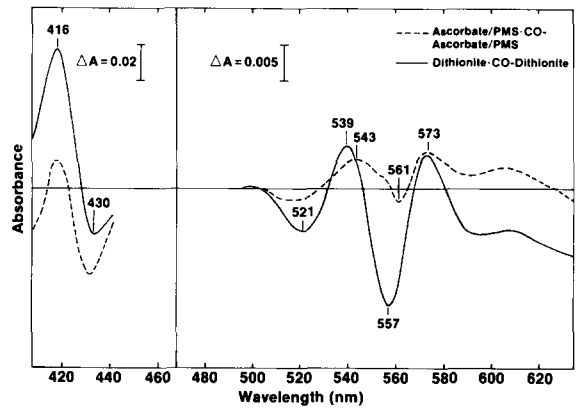

Fig. 6. CO difference spectra of membranes from aerobically grown *C. vinosum* cells. Membranes (equivalent to 25 μg protein/ml) were suspended in 20 mM Tris buffer, pH 7.0. The difference spectra were recorded following exposure to CO for 10 min. Spectral resolution, 1 nm, pathlength, 1 cm. Ascorbate, dithionite and PMS were added as in Fig. 2.

in both spectra, but we have not yet been able to assign this feature. The two difference spectra of Fig. 6 differ slightly in the positions of absorbance features but more strikingly in the relative intensities of the absorbance bands, suggesting the possible presence of two CO-binding heme components in the membranes. No CO binding was observed in the presence of hydroquinone as the reductant.

Fig. 7 shows the electron paramagnetic resonance (EPR) spectrum (measured at 12.6 K) of *C. vinosum* membranes reduced with hydroquinone. The spectrum reveals an EPR feature at $g = 1.90$, a value diagnostic for the presence of a reduced Rieske iron-sulfur protein [31–33]. No increase in the amplitude of the $g = 1.90$ feature was observed if dithionite was substituted for hydroquinone as a reductant, suggesting that this Rieske iron-sulfur protein has an $E'_m \geq +280$ mV, similar to the E_m values reported for other Rieske iron-sulfur proteins in many other organisms [33]. In addition to the EPR signal arising from the Rieske protein, a signal at $g = 2.0$, presumably arising from one or more free radicals, is present. The identity of the species responsible for this signal has not yet been determined. Also present are signals at $g = 2.02$ and $g = 1.96$ which resemble [34,35] those due to the semiquinone dimer, interacting with iron-sulfur center S-3 [36], of mitochondrial succinate dehydrogenase. It thus seems likely that membranes of

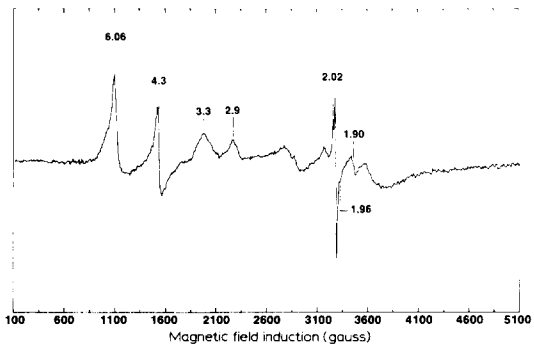

Fig. 7. EPR spectrum of hydroquinone reduced, membranes from aerobically grown *C. vinosum* cells. Membranes (equivalent to 2 mg protein/ml) were suspended in 10 mM Tris buffer, pH 7.0. Hydroquinone was added as a solid. The conditions of EPR spectroscopy were: microwave frequency, 9.150 GHz; microwave power, 3 mW; modulation amplitude, 8 gauss; modulation frequency, 100 kHz and temperature, 12.6 K.

aerobiclly grown *C. vinosum*, like those of photosynthetically grown cells [37], contain an enzyme similar to mitochondrial succinate dehydrogenase. In addition to the above-mentioned signals, several unidentified signals at $g = 3.3$, $g = 2.9$ and $g = 2.2$ were detected and are almost certainly due to low spin, oxidized cytochromes. No EPR signals characteristic of Cu^{2+} were observed. In addition to the signal at $g = 4.3$, probably arising from non-specifically bound iron, one additional EPR signal at $g = 6.06$ can be observed in the membranes, presumably arising from an oxidized high spin heme. Photosynthetically grown *C. vinosum* contains a cytochrome *c'*, that exhibits a high-spin EPR signal near $g = 6.0$ and, although largely present as a soluble species, can associate with the membrane [34]. Although the EPR signal at $g = 6.06$ in membranes from aerobically grown cells differs somewhat from that of cytochrome *c'*, the signal may in fact arise from cytochrome *c'*, since our preliminary investigation of the soluble protein of aerobically grown *C. vinosum* indicates the presence of cytochrome *c'*. This high spin signal also closely resembles a signal at $g = 6.0$ measured for the *o*-type oxidase isolated from *Pseudomonas aeruginosa* by Yang [29]. Additional EPR experiments were performed in an attempt to detect the presence of any residual reaction center in the membranes. No signal at $g = 1.82$ from the primary acceptor [24,38,39] could be detected at 10 K, either during illumination or after addition of dithionite. Similarly, no appearance of an additional $g = 2.0$ EPR signal, due to P-870$^+$, was observed during prolonged illumination of the membranes at 18 K. It thus appears that, within the limits of our detection, no reaction center is present in the membranes isolated from *C. vinosum* cells grown at high O_2 concentration.

Discussion

From the results described above, the membranes isolated from aerobically grown *C. vinosum* cells have been shown to contain at least three *c*-type cytochromes, each possessing an α-band wavelength maximum at 551–552 nm. No component with an absorbance maximum at 555–556 nm was detected. These results and the absence of any heme-staining peptide with $M_r = 44$ kDa in the

SDS-polyacrylamide gel electrophoresis separation (Fig. 5) suggest that cytochrome *c*-555 is not present in membranes of aerobically grown *C. vinosum*. In membranes from photosynthetically grown cells, this cytochrome functions in cyclic electron flow as the immediate donor to P-870$^+$ [20,21,23]. As cyclic electron flow probably cannot function in membranes isolated from aerobically grown cells because of their apparent absence of reaction centers, the lack of cytochrome *c*-555 is not surprising. The high-potential *c*-type cytochrome ($E'_m = +340$ mV), observable in our oxidation-reduction titrations, has a similar potential to cytochrome *c*-555 ($E'_m = +340$ mV), but apparently represents a different *c*-type cytochrome, not present in photosynthetically grown cells. The function of the $E'_m = +340$ mV cytochrome (α-band maximum, 551–552 nm) is presently under investigation in our laboratory.

The *c*-type cytochrome with $E'_m = +180$ mV may possibly be related to the cytochrome c_1, recently discovered in photosynthetically grown cells [28], from *C. vinosum*. While the E'_m of this component is approx. 50 mV lower than that estimated for cytochrome c_1 in a detergent-solubilized fraction from photosynthetically grown *C. vinosum*, the α-band spectrum of this component and the presence of a heme-staining peptide with $M_r = 28$ kDa [28,33] are at least consistent with the possible presence of cytochrome c_1.

The low potential ($E'_m = +40$ mV) *c*-type cytochrome component closely resembles the cytochrome *c*-552 from photosynthetically grown *C. vinosum* in both E'_m (+10 mV, Ref. 22–24) and α-band spectrum. The SDS-polyacrylamide gel electrophoresis data are also consistent with the present of cytochrome *c*-552 ($M_r = 23$ kDa, Ref. 26). However, as cytochrome *c*-552 appears to be closely associated with the photosynthetic reaction center [23,40–42], it was somewhat surprising to find what appears to be cytochrome *c*-552 in membranes apparently free of reaction center. While the above experimental results point to the presence of a *c*-type cytochrome in membranes isolated from aerobically grown *C. vinosum* cells similar to cytochrome *c*-552, we cannot conclude with certainty that the two cytochromes are, in fact, identical.

One difficulty with the assignment of the $E'_m = +180$ mV component to a putative cytochrome c_1 with $M_r = 28$ kDa and the +40 mV component to a cytochrome *c*-552 with $M_r = 20$ or 24 kDa is that, while the optical titrations (see Fig. 4) suggest that the two *c*-type cytochromes are present in approximately equimolar amounts, SDS-polyacrylamide gel electrophoresis (Fig. 5) suggest that the 28 kDa heme-containing peptide is present in relatively small amounts compared to the 20 kDa and particularly compared to the 24 kDa components. It has recently been shown [43,33] that some *c*-type cytochromes may lose considerable iron under the conditions we have used for preparing our samples for SDS-polyacrylamide gel electrophoresis, and thus may show considerably lower staining intensity using the H_2O_2 plus 3,3′,5,5′-tetramethylbenzidine assay for heme. This phenomenon may possibly have influenced the relative heme-staining intensities we have observed.

Protoheme analysis (Fig. 3), optical spectra (Fig. 2) and electrochemical titrations (Fig. 4) support the presence of at least one *b*-type cytochrome in membranes of aerobically grown *C. vinosum*. The presence of a Rieske iron-sulfur protein (see fig. 7) suggests that some *b* cytochromes are likely to be components of a cytochrome bc_1 complex, known to contain this iron-sulfur protein in a variety of organisms [33]. Evidence for such a complex has recently been obtained in photosynthetically grown *C. vinosum* [28,45]. However, the *b*-cytochromes of aerobically grown cells are considerably more electronegative ($E'_m = -90$ mV) than those in photosynthetic cells ($E'_m = +30$ mV; Refs. 12 and 46). In photosynthetically grown *C. vinosum*, it appears likely that a soluble, high-potential ($E_m = +240$ mV) cytochrome *c*-550 [20,47,48], related to mitochondrial cytochrome *c* [49], acts as the electron acceptor for the cytochrome bc_1 complex [45]. Preliminary investigation of the soluble heme proteins of aerobically grown *C. vinosum* in our laboratory indicates that cytochrome *c*-550 is also present in aerobically grown *C. vinosum*. The observation of the known electron acceptor for the cytochrome bc_1 complex makes it appear likely that the aerobically grown cells of this purple sulfur bacterium also contain such a complex.

The question of the identity of the terminal oxidase in aerobically grown *C. vinosum* was first raised when Kämpf and Pfennig [6] demonstrated

that this phototrophic purple sulfur bacterium, previously thought to be an obligate anaerobe, could grow and respire in the dark under semi- or microaerobic conditions. Furthermore, Takamiya and co-workers [50] have demonstrated that cell-free membrane preparations from photosynthetically grown *C. vinosum* exhibit NADH- or succinate-dependent O_2 uptake that is sensitive to a number of classical respiratory inhibitors. More recently, Kämpf has demonstrated that respiration by *C. vinosum* cells is markedly inhibited by the cytochrome oxidase inhibitors azide and cyanide [7]. In each of the above cases, the data are suggestive of a 'cytochrome oxidase' functioning as a terminal electron acceptor. Since in general [51] such oxidases bind CO in the reduced form, it seemed reasonable to use CO difference spectra in an attempt to identify the *C. vinosum* terminal oxidase. The difference spectra of Fig. 6 suggest the presence of at least two CO-binding heme components in aerobically grown *C. vinosum*. Both spectra show considerable similarities to those reported for *o*-type oxidases [29]. One difficulty present in the interpretation of our data is that CO-binding but no *b*-type cytochrome reduction is observed in the presence of ascorbate plus PMS (see Figs. 2 and 6). As *o*-type oxidases resemble *b*-type cytochromes spectrally because of their common protoheme prosthetic group [51], it could be argued that the CO-binding component reduced in the presence of ascorbate plus PMS cannot be an *o*-type oxidase. A more likely explanation may be that there are two protoheme-containing components with E'_m values too similar to be resolved and that one of these components has its E'_m value shifted to a considerably more positive value by CO binding. As CO binds only to ferrous hemes, thermodynamics would predict such an E_m shift in the presence of CO.

As no CO binding was observed in the presence of hydroquinone, any putative terminal oxidase(s) that bind CO will be relatively electronegative ($E'_m \lesssim +200$ mV). While examples of low potential *o*-type oxidases are known [51–54], it is difficult to reconcile a low potential oxidase with the presence of a 'normal' high potential (i.e., hydroquinone reducible) Rieske iron-sulfur protein and the soluble cytochrome *c*-550 ($E_m = +240$ mV at pH 8.0, Refs. 47 and 48), since the Rieske iron-sulfur protein and cytochrome *c*-550 would likely form a portion of the electron-transfer chain donating electrons to the terminal oxidase. The only component present in the membranes of aerobically grown *C. vinosum* with a significantly higher E_m than that of the Rieske iron-sulfur protein ($E'_m = +300$ mV in photosynthetically grown *C. vinosum*; D. Knaff, unpublished observation) and cytochrome *c*-550 is the +340 mV *c*-type cytochrome which appears to be unique to aerobically grown *C. vinosum*. There is, in fact, some precedent for bacterial oxidases containing heme *c* [55,56], but, unlike the *C. vinosum* +340 mV component, these appear to bind CO [55,56]. Additional experiments are clearly required to identify the terminal oxidase(s) of aerobically grown *C. vinosum*. However, since the membranes of aerobically grown *C. vinosum* contain neither heme *a* nor heme *d*, it can be concluded that the oxidase is neither a cytochrome aa_3 type nor a cytochrome *d* type. The absence of an EPR detectable Cu^{2+} signal characteristic of copper-containing aa_3 oxidases [57,58] is consistent with the absence of such an oxidase in aerobically grown cells.

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