The Role of High-Potential Iron Protein and Cytochrome c_8 as Alternative Electron Donors to the Reaction Center of *Chromatium vinosum*†

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ABSTRACT: Under anaerobic conditions, intact cells of the purple sulfur bacterium *Chromatium vinosum* exhibit rapid photooxidation of the two low-potential hemes of the *c*-type cytochrome associated with the reaction center, after exposure to two short light flashes separated by a dark interval. Reduction of the photooxidized low-potential hemes is very slow under these conditions. On subsequent flashes, rapid photooxidation of a high-potential reaction center heme occurs and is followed by its rereduction on the millisecond time scale. Cells maintained under aerobic conditions exhibit the millisecond time scale reduction of the photooxidized high-potential heme after each flash. Cells grown autotrophically in the presence of Na_2S and $Na_2S_2O_3$ appear to use the soluble [4Fe-4S]-containing protein, HiPIP, as the only direct electron donor to the reaction center heme under aerobic conditions. In contrast, cells grown in the presence of organic compounds, but in the absence of Na_2S and $Na_2S_2O_3$, appear to use a soluble *c*-type cytochrome (most likely cytochrome c_8) as the only electron donor to the reaction center heme under aerobic conditions. Cells grown autotrophically, in the presence of Na_2S and $Na_2S_2O_3$, have a slightly higher ratio of HiPIP to cytochrome c_8 and a ratio of Rieske iron−sulfur protein to reaction center that is approximately one-half that of cells grown in the absence of Na_2S and $Na_2S_2O_3$ but in the presence of organic compounds.

Light-driven cyclic electron flow in purple photosynthetic bacteria involves two membrane-embedded complexes, the reaction center and the cytochrome bc_1 complex, which are linked by two mobile electron carriers (*1*, *2*). The quinone pool in the membrane serves to carry reducing equivalents from the reaction center to the cytochrome bc_1 complex, and in most cases, a mobile electron-carrying protein located in the periplasmic space conveys reducing equivalents back from the cytochrome bc_1 complex to the reaction center (*1*−*3*). For many years, it was thought that the electron-carrying protein link between the reaction center and the cytochrome bc_1 complex was always a soluble *c*-type cytochrome called cytochrome c_2 (*2*, *3*). However, more recently it has become clear that electron flow from the cytochrome bc_1 complex to the reaction center can be mediated by soluble electron carriers other than cytochrome c_2, such as cytochrome c_8 (*2*, *4*−*9*) and the [4Fe-4S] cluster-containing high-potential iron protein (HiPIP)[1] (*2*, *5*, *8*, *10*−*13*), and by the membrane-associated cytochrome c_y (*3*, *14*−*17*). Recent results (*13*), which implicated HiPIP as the direct reductant for a high-potential heme of the reaction center's cytochrome subunit in intact cells of the photosynthetic purple sulfur bacterium *Chromatium vinosum*, appeared to disagree with earlier experiments with intact cells that implicated a soluble *c*-type cytochrome as the donor to the *C. vinosum* reaction center (*18*). In an attempt to resolve this apparent disagreement, we have investigated the effect of growth conditions for *C. vinosum* on electron donation to the reaction center. (It should be pointed out that this bacterium has recently been reassigned to the genus *Allochromatium*, but we have chosen to refer to it as a *Chromatium* species because it is referred to in this manner in all of the literature references cited herein.)

MATERIALS AND METHODS

C. vinosum (strain DSM 180T), obtained from the DSM culture collection, was grown phototrophically at 30 °C under anaerobic conditions, using white light (25 μmol of photons s^{-1} M^{-2}) supplied by incandescent bulbs. Cells were grown in either Hutner's medium (*19*) or in modified Pfennig's medium (Number 28 in the DSM catalog) for 2 days. The modified Pfennig's medium (hereafter referred to as "plus Na_2S") contains no organic carbon compound and results in autotrophic growth with Na_2S and $Na_2S_2O_3$ supplying the reducing equivalents necessary for carbon assimilation.

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[1] Abbreviations: DAD, 3,6-diaminodurene; EPR, electron paramagnetic resonance; HiPIP, high-potential iron protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PES, phenazine ethosulfate; PMS, phenazine methosulfate; Tris, tris(hydroxymethyl)aminomethane.

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Hutner's medium (hereafter referred to as "minus Na_2S") contains glutamate and acetate but contains neither Na_2S nor $Na_2S_2O_3$ (the source of sulfur for growth is provided by the presence of the sulfate salts of several essential metals).

Cell breakage, the first step in preparing soluble cell extracts used as the starting material for quantitating the amount of HiPIP and of soluble, high-potential *c*-type cytochromes present in *C. vinosum*, was accomplished by passing a suspension of cells which had been washed twice in 20 mM Tris-HCl buffer (pH 8.0) through a French press at 140 MPa three times. The HiPIP and soluble, high-potential *c*-type cytochrome contents were then determined by visible-region absorbance measurements (see below) on supernatants obtained after centrifugation of the cell extracts at 300000*g* for 2 h. These supernatants showed no absorbance features indicative of the presence of either carotenoids or bacteriochlorophyll and thus were judged to be free of membrane fragments. Membrane fragments ("chromatophores") used for measuring the content of Rieske iron−sulfur protein and reaction center hemes were prepared by disrupting cells, suspended in 50 mM MOPS buffer (pH 7.0), with a French press, as described above. The pellet from the high-speed centrifugation, after a preliminary 10 min centrifugation at 10000*g* to remove cell debris, was collected and resuspended in 50 mM MOPS buffer (pH 7.0) and used for obtaining EPR spectra of membrane-bound components, while the supernatant was used for obtaining EPR spectra of soluble components.

Optical absorbance spectra were measured using a Shimadzu Model UV2100U spectrophotometer at a spectral resolution of 1 nm. Light-induced absorbance changes were measured with an apparatus similar to that described by Joliot et al. (*20*) at a spectral resolution of 1 nm, using intact cells that were suspended in fresh batches of the medium in which they were grown. Excitation was provided by flashes from a xenon lamp (3 μs half-time), filtered through two Kodak Wratten 89B filters. The cell suspensions were made anaerobic by bubbling with nitrogen gas or aerobic by bubbling with air. Under aerobic conditions, the two low-potential *c*-type hemes associated with the reaction center were completely oxidized while the two high-potential *c*-type hemes associated with the reaction center were completely reduced. The rapid absorbance decrease produced at 422 nm by a single saturating flash under aerobic conditions, resulting from the oxidation of one high-potential *c*-type heme per reaction center, was used to estimate the concentration of the reaction center, using a reduced *minus* oxidized extinction coefficient of 75 mM^{-1} cm^{-1} for the cytochrome's Soret band.

Electron paramagnetic resonance (EPR) spectra were recorded using a Bruker ESP 300 X-band spectrometer fitted with an Oxford Instruments liquid helium cryostat and temperature control system. Chromatophores, reduced by the addition of 5 mM sodium ascorbate, were used for the EPR measurements designed to compare the cytochrome bc_1 complex/reaction center ratio for cells grown under different conditions (ascorbate reduces the Rieske iron−sulfur protein subunit of the cytochrome bc_1 complex and the two high-potential reaction center hemes but does not reduce the two low-potential reaction center hemes). The ratios of Rieske iron−sulfur protein to reaction center in cells grown in the two different media were compared using the ratio of the EPR signal amplitude at $g = 1.88$ (*21*) that arises from the [2Fe-2S] cluster of the reduced Rieske iron−sulfur protein (there is one such cluster per cytochrome bc_1 complex; *22*) to the EPR signal amplitude at $g = 2.94$ that arises from the oxidized form of the reaction center's two low-potential hemes, both of which exhibit EPR features at this *g*-value (*23*), in chromatophores prepared from cells grown in the two different media. The EPR spectrum of chromatophores prepared from cells grown on the minus Na_2S medium showed features arising from Mn^{2+}, features which partially obscured those arising from the Rieske iron−sulfur protein. These Mn^{2+} signals were not present in the EPR spectrum of chromatophores prepared from cells grown on the plus Na_2S medium. Thus, to be able to estimate the amount of Rieske protein in chromatophores prepared from cells grown on the minus Na_2S medium, the contribution of Mn^{2+} to the EPR spectrum of this sample was subtracted, after appropriate normalization, using a EPR spectrum obtained for a pure Mn^{2+}-containing salt in solution under the same EPR conditions used to obtain the spectrum of the chromatophores. EPR spectra of HiPIP found in the soluble fraction prepared from these *C. vinosum* cells were obtained as described previously (*10*). Spectra of the EPR feature at $g = 2.98$, arising from the ferric form of the high-potential *c*-type cytochrome (presumably cytochrome c_8) found in the soluble fractions were obtained by subtracting spectra obtained from samples reduced with 5 mM sodium ascorbate from samples oxidized with 1 mM sodium hexachloroiridiate.

The reduced *minus* oxidized difference spectrum for HiPIP is almost featureless in the cytochrome α-band region (*24*), and for this reason, the presence of HiPIP does not interfere with the determination of cytochrome content from redox difference spectra. Thus, no separation of the soluble *C. vinosum* cytochromes from HiPIP needs be carried out prior to measurements of cytochrome difference spectra. The content of high-potential cytochrome *c* was estimated from hydroquinone-reduced *minus* ferricyanide-oxidized difference spectra in the α-band spectral region, using a reduced *minus* oxidized difference extinction coefficient of 14.1 mM^{-1} cm^{-1} at 550 *minus* 540 nm (*7*). As hydroquinone is a relatively weak reductant ($E_m = +290$ mV at pH 7.0), this difference spectrum will reveal only high-potential soluble components.

The relative magnitudes of the cytochrome and HiPIP difference spectra required that HiPIP be completely separated from cytochromes before its content could be quantitated from absorbance spectra. HiPIP was separated from other soluble *C. vinosum* proteins by anion-exchange chromatography using a Poros HQ/M column, at a flow rate of 5 mL/min, and a PerSeptive Biosystems BioCad protein purification system. After the soluble *C. vinosum* fraction was loaded, the column was washed with 20 mM Tris-HCl buffer (pH 8.0) for 1.2 min. Elution was then carried out by addition of NaCl to the buffer so that a NaCl concentration of 210 mM was reached after 6.0 min. The NaCl concentration was then increased to 1.0 M over an additional 8.0 min. The HiPIP eluted from the column was completely reduced and was pure, as judged by the A_{283}/A_{388} absorbance ratio of 2.55 (the ratio reported in the literature for the completely pure protein is 2.52; *24*) of the material collected from the single elution peak. The HiPIP content of the cells was determined, after the addition of sodium ascorbate as a reductant to ensure complete reduction, using an extinction

coefficient of 16.1 mM^{-1} cm^{-1} at 388 nm for the reduced protein (*24*).

Oxidation−reduction titrations were carried out as described by Dutton (*25*). Concentrated soluble fractions from *C. vinosum* cells, prepared as described above, were diluted to a total volume of 9.0 mL in buffer that contained the following redox mediators, all at a concentration of 25 μM: 1,1′-dimethylferrocene, 1,4-benzoquinone, DAD (3,6-diaminodurene), 2,5-dimethyl-1,4-benzoquinone, 1,2-naphthoquinone, PES (phenazine ethosulfate), and PMS (phenazine methosulfate). Absorbance changes in the cytochrome α-band region were used to monitor the redox state of the cytochrome, and the absorbance vs E_h data were fitted to the Nernst equation for a single, $n = 1$ component using Microsoft Excel.

RESULTS

Figure 1A shows the response of *C. vinosum* reaction center hemes to excitation by a series of single-turnover flashes under either aerobic (closed circles, upper trace) or anaerobic (closed squares, lower trace) conditions. In both cases, a *c*-type heme associated with the reaction center is rapidly photooxidized (as indicated by the absorbance decreases in the Soret band region of the spectrum). However, while the photooxidized heme is rapidly rereduced after every flash in cells kept under aerobic conditions, reduction of the heme that is photooxidized after the first two flashes is much slower under anaerobic conditions. Under anaerobic conditions, rapid reduction of photooxidized reaction center heme occurs only on the third and subsequent flashes. Figure 1B, which shows the light *minus* dark difference spectra, in the α-band region, obtained under the two conditions, indicates that a heme with an α-band maximum at 556 nm is photooxidized by the first flash under aerobic conditions, while a heme with an α-band maximum at 552 nm is photooxidized by the first flash under anaerobic conditions. Difference spectra (not shown) indicate that the heme which is photooxidized on the second flash under anaerobic conditions also has an α-band maximum at 552 nm, while the heme oxidized on the third and subsequent flashes under anaerobic conditions has an α-band maximum at 556 nm. These difference spectra, when compared to reduced *minus* oxidized spectra obtained using chemical oxidants and reductants (*26*−*32*), indicate that each flash excitation under aerobic conditions (and excitation by the third and subsequent flashes under anaerobic conditions) results in oxidation of a high-potential reaction center heme (*c*-556, with $E_m = +330$ mV), while excitation by the first two flashes under anaerobic conditions results in oxidation of first one and then the other of the two low-potential reaction center *c*-552 hemes (hemes with very similar α-band spectra and E_m values of +30 mV and −10 mV, respectively).

The results of Figure 1 were obtained with cells grown on plus Na_2S medium, but qualitatively similar results were obtained with cells grown on minus Na_2S medium. Thus, when both the low-potential and high-potential reaction center-associated hemes are reduced prior to the flash and available for oxidation in intact *C. vinosum* cells, a low-potential heme is preferentially photooxidized, regardless of whether the cells have been grown in the presence or absence

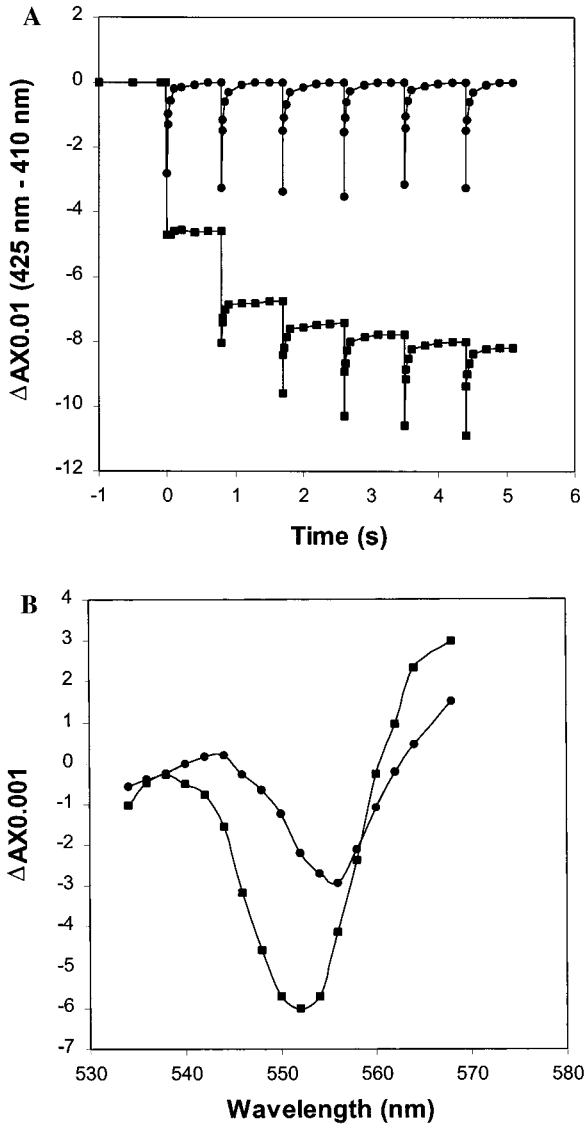

FIGURE 1: Flash excitation of intact cells of *C. vinosum* under aerobic and anaerobic conditions. (A) *C. vinosum* cells, kept under either aerobic (closed circles) or anaerobic (closed squares) conditions, were exposed to a series of six consecutive saturating flashes, spaced 800 ms apart. The cells were grown on plus Na_2S medium. (B) Difference spectra for *C. vinosum* cells, kept under either aerobic (closed circles) or anaerobic (closed squares) conditions, represent the absorbance 50 μs after the first flash *minus* the absorbance before the flash. The cells were grown on plus Na_2S medium.

of organic compounds. However, the extremely slow rate of rereduction of the photooxidized low-potential hemes observed under anaerobic conditions, in contrast to the more rapid reduction of the photooxidized high-potential heme, is not easily reconciled with a role for the low-potential hemes in cyclic electron flow. Results similar to those described above after the first flash have previously been reported for intact cells and membrane fragments ("chromatophores") of *C. vinosum* (*27*, *29*, *33*) exposed to a single flash, but the response to a series of flashes has not previously been reported for this bacterium. Furthermore, there have been no previous reports of the effect of growth medium on the response of intact cells to a train of flashes.

Figure 2 shows the kinetics of reduction of the photooxidized, high-potential reaction center *c*-556 heme, under aerobic conditions, obtained with intact cells of *C. vinosum*

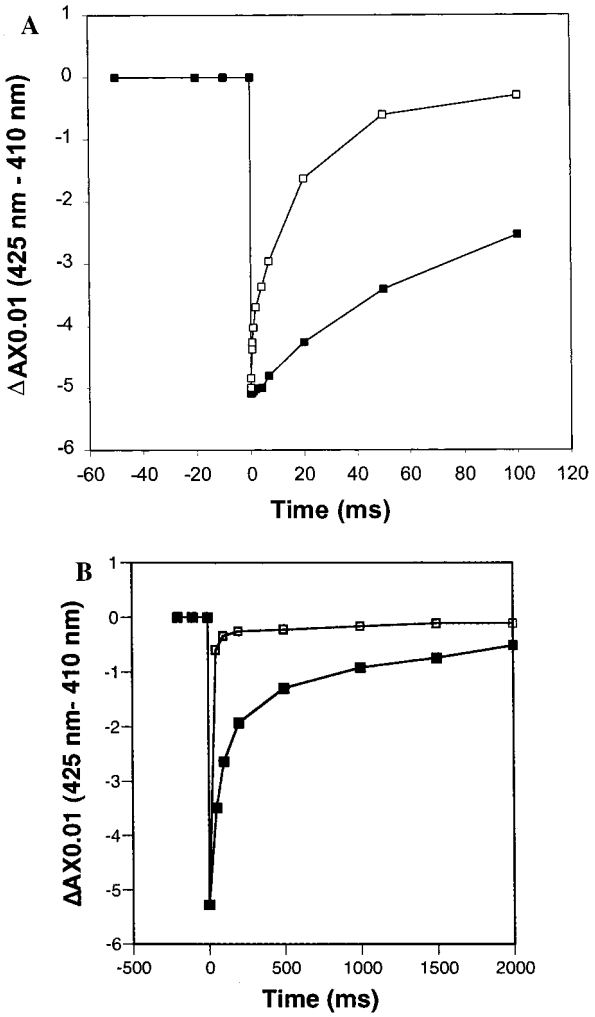

FIGURE 2: Kinetics of *C. vinosum* reaction center cytochrome *c*-556 reduction after photooxidation under aerobic conditions. Cells were grown on either plus Na_2S (open squares) or minus Na_2S (closed squares) medium. Panel A represents the fast phase, and panel B shows the complete time course.

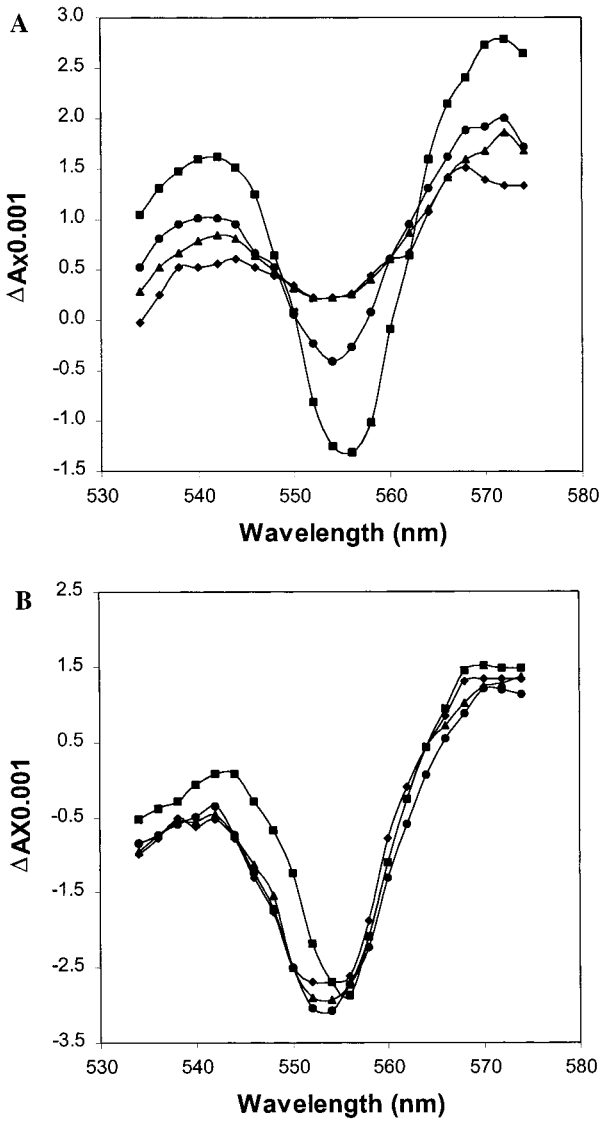

FIGURE 3: Time-dependent difference spectra associated with reduction of *C. vinosum* reaction center cytochrome *c*-556 under aerobic conditions. *C. vinosum* cells were grown on either plus Na_2S (panel A) or minus Na_2S (panel B) medium. The filled circles represent points taken 50 μs after the flash, the filled squares represent points taken 6 ms after the flash, the filled triangles represent points taken 20 ms after the flash, and the filled diamonds represent points taken 50 ms after the flash.

grown on either minus Na_2S (closed squares) or plus Na_2S (open squares) media. The rereduction of the photooxidized cytochrome is considerably more rapid in cells grown on the plus Na_2S medium than it is with cells grown on the minus Na_2S medium. In typical experiments, ca. 80% of the photooxidized heme is reduced with a half-time of 100 ms in cells grown on the minus Na_2S medium, with the remaining 20% reduced in a slow phase with a half-time between 1 and 2 s. The more rapid reduction seen with cells grown on the plus Na_2S medium is characterized by a fast phase, accounting for between 30% and 50% of the decay, with a half-time of 300 μs and a slower phase with a half-time between 20 and 30 ms (the fast and slow phases of the kinetics are shown in panels A and B of Figure 2, respectively).

In addition to the different reduction kinetics observed for reaction center reduction under aerobic conditions in the cells grown in the two different media, striking differences were observed in the difference spectra (shown in Figure 3) obtained at different times after the flash with cells grown on the two different media. For cells grown on the plus Na_2S medium (Figure 3A), the time period after the flash is characterized by a significant increase in absorbance in the α-band region with little significant change in the location of the absorbance minimum. This pattern has been previously interpreted as indicative of the reduction of the reaction center *c*-type heme by HiPIP (*8*, *10*−*13*). As the oxidized *minus* reduced difference spectrum of HiPIP is relatively featureless in the α-band spectral region, the absorbance changes are dominated by the reappearance of the reaction center heme's α-band during its rereduction (*8*, *10*−*13*). In contrast, for cells grown on the minus Na_2S medium (Figure 3B), the time after the flash is characterized by an absorbance increase that is small in magnitude and is accompanied by a shift in the absorbance minimum from 556 to 553 nm. This pattern is likely to result from the reduction of the reaction center heme by another *c*-type cytochrome, where the simultaneous oxidation and reduction of two cytochromes with similar, but not identical, α-band spectra produce little net absorbance change but a shift in the absorbance minimum as one cytochrome reduces another (*7*, *8*, *18*). Previous studies,

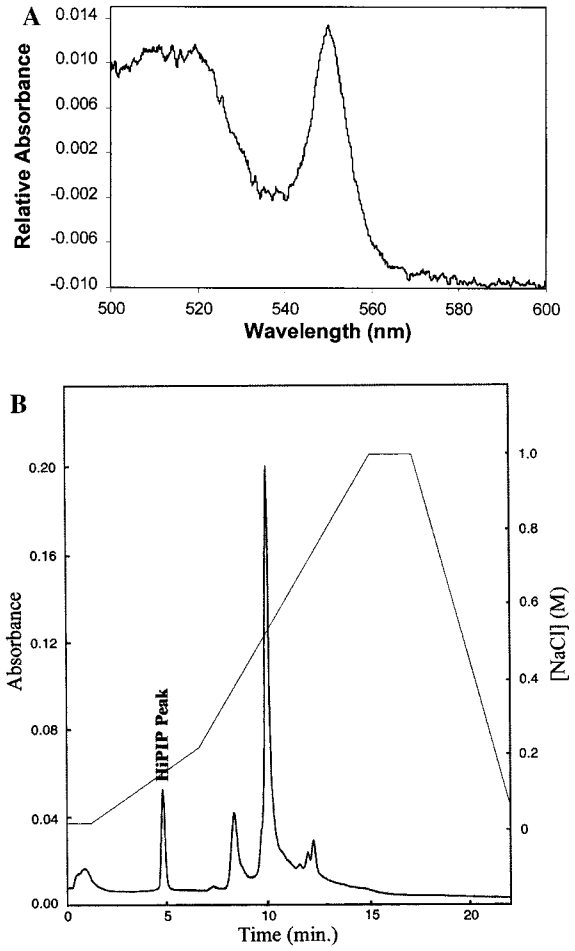

FIGURE 4: High-potential cytochrome *c* and HiPIP contents of *C. vinosum* cells grown on plus Na_2S medium. (A) Hydroquinone-reduced *minus* ferricyanide-oxidized difference spectrum. Identical portions of the concentrated soluble fraction were placed in the sample and reference cuvettes to establish a baseline, and then small crystals of hydroquinone and potassium ferricyanide were added to the sample and reference cuvettes, respectively. (B) Elution profile from the Poros HQ/M column (see Materials and Methods for details of the chromatographic protocol). Absorbance was monitored at 388 nm. The profile of the NaCl concentration in the eluting buffer is also shown.

comparing results obtained with intact cells and membrane fragments from *C. vinosum*, suggest that the *c*-type cytochrome that reduces the reaction center heme is a soluble component and is not membrane-bound (*18*, *34*).

Figure 4A shows the hydroquinone-reduced *minus* ferricyanide-oxidized spectrum of the soluble fraction of *C. vinosum* grown on plus Na_2S medium. The difference spectrum obtained from the extract prepared from cells grown on minus Na_2S medium (not shown) was virtually identical insofar as the location of the absorbance maximum and the bandwidth are concerned. The location of the α-band maximum at 550 nm indicates that the component is likely to be a *c*-type cytochrome. While the location of this maximum does not allow one to discriminate between cytochromes c_8 and c_2, recent data suggest that cytochrome c_8 is the predominant (and perhaps the only) high-potential, soluble *c*-type cytochrome present in *C. vinosum* (*2*, *4*). Figure 4B shows a typical elution profile for anion-exchange chromatography of the soluble fraction of *C. vinosum* grown on plus Na_2S medium. A very similar profile was obtained with extracts from cells grown on minus Na_2S medium (not shown). The peak labeled HiPIP contains essentially pure HiPIP, as judged by its absorbance spectrum in the visible and near-ultraviolet regions (not shown). In particular, the peak labeled as HiPIP showed no evidence for the presence of any cytochromes. The other peaks, which were not characterized in detail, appear to contain more than one component when examined by polyacrylamide gel electrophoresis under denaturing conditions.

Assuming that all of the cytochrome detected is cytochrome c_8, the ratio of HiPIP to cytochrome c_8 present in *C. vinosum* cells grown on plus Na_2S medium was calculated to be 2.5. Similar experiments performed on extracts of *C. vinosum* cells grown on the minus Na_2S medium indicated a HiPIP/cytochrome c_8 ratio of 1.7 for these cells. Cells grown on the plus Na_2S medium contained, on an equal reaction center basis, more HiPIP (4.0-fold) and cytochrome c_8 (2.8-fold) than did cells grown on the minus Na_2S medium. Our attempts to obtain a HiPIP-containing fraction that was completely free of cytochromes could, in principle, have resulted in some loss of HiPIP during the separation procedure and thus may have produced an underestimate of the HiPIP content. The use of hydroquinone as a reductant for assaying the presence of high-potential, soluble *c*-type cytochromes produced ambient potentials (E_h) that were typically near +190 mV, making it very likely that low-potential cytochromes did not contribute significantly to the difference spectra and cause an overestimate in the amount of cytochrome c_8. For example, the hemes of the soluble, abundant flavocytochrome *c*-552 ($E_m = +30$ mV; *35*) would only be ca. 0.1% reduced at this ambient potential. It is also possible that soluble, high-potential *c*-type cytochromes other than cytochrome c_8 could have contributed to hydroquinone *minus* ferricyanide difference spectra, resulting in an overestimate of the cytochrome c_8 content and an underestimate of the values presented for the HiPIP/cytochrome c_8 ratio. It appears unlikely that cytochrome c_4 (*36*) could make such a contribution, as this high-potential cytochrome is typically membrane-bound (*36*) and only the soluble fraction of *C. vinosum* was used to obtain these difference spectra. Furthermore, all of the hydroquinone *minus* ferricyanide difference spectra measured in this study exhibited symmetrical α-bands with maxima at 550 nm (like purified cytochrome c_8), whereas cytochrome c_4 has an asymmetric α-band with a maximum at 553 nm (*36*). It is important to note that exactly the same procedure was used for estimating HiPIP and cytochrome c_8 in extracts from cells grown on the two different media. Thus, it appears unlikely that significant errors have been made in these relative measurements. It can therefore be concluded that, on a comparative basis, the HiPIP/cytochrome c_8 ratio appears to be altered by only a relatively small extent by a change in growing conditions from one of the growth media used in these experiments to the other.

Previous reports, from three different laboratories, describing the properties of a soluble, high-potential *c*-type cytochrome from *C. vinosum* with an α-band maximum at 550−551 nm included redox titrations which showed an E_m value for the cytochrome at pH 7.0 between +240 and +260 mV (*18*, *37*, *38*). Figure 5 shows the results of a redox titration, carried out at pH 7.0 over the E_h range from +350 to +100 mV, of the soluble fraction isolated from *C. vinosum* cells grown on the plus Na_2S medium. (The lower concentration

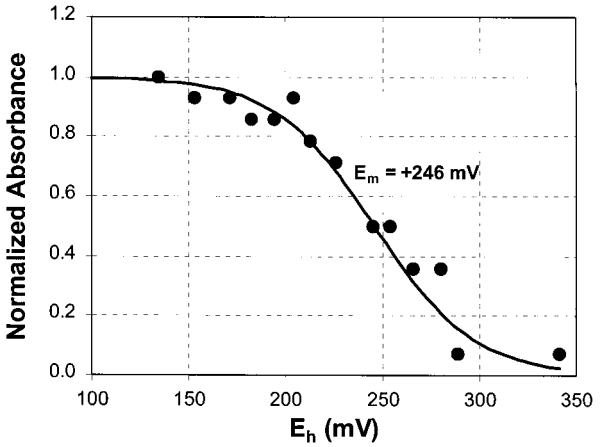

FIGURE 5: Oxidation−reduction titration of the high-potential soluble cytochrome *c* from *C. vinosum*. The soluble fraction from *C. vinosum* cells grown on plus Na_2S medium was diluted in 50 mM MOPS buffer (pH 7.0) containing 100 mM KCl and the redox mediators listed in Materials and Methods. The titration was carried out in the reductive direction, using small aliquots of sodium dithionite to lower E_h. The absorbance at 550 nm *minus* 570 nm was used to monitor the oxidation state of the cytochrome.

of hydroquinone-reducible, cytochrome *c* present in the soluble fraction isolated from cells grown on the minus Na_2S medium made it difficult to obtain reliable titration data from extracts of those cells.) The data obtained in the titration shown in Figure 5 give a good fit to the Nernst equation for a one-electron redox couple with an E_m value of +246 mV. Difference spectra, taken over this E_h range, all showed a maximum at 550 nm. The 550 nm location of the cytochrome's α-band, combined with its +246 mV E_m value, makes it appear likely that the soluble cytochrome detected in the current experiments is identical to the cytochrome designated as cytochrome *c*-551 in the earlier studies, and which we now refer to as cytochrome c_8.

As the oxidized form of low-spin *c*-type cytochromes (*39*) and the oxidized form of HiPIP (*40*) exhibit characteristic EPR signals, an attempt was made to check the HiPIP/cytochrome c_8 ratios determined from absorbance measurements (see above) by quantitating the EPR signals, attributable to HiPIP and high-potential *c*-type cytochrome(s), detected in the soluble fractions prepared from *C. vinosum* cells grown under the two conditions. Unfortunately, although the HiPIP EPR signals observed during these measurements were of sufficiently large amplitudes to give quantifiable results, the cytochrome quantitation was much less reliable, due to the considerably lower magnitude of the EPR signals that could be attributed to a high-potential, low-spin *c*-type cytochrome. Nevertheless, despite the absence of EPR data accurate enough to give exact HiPIP/cytochrome c_8 ratios, the data did show that these ratios did not differ greatly in *C. vinosum* cells grown on the two different media.

EPR spectroscopy can also be a valuable tool for comparing the amount of Rieske iron−sulfur protein present in different preparations, utilizing the characteristic feature found near $g = 1.9$ in the EPR spectra of the reduced protein (*21*) as a measure of the amount of iron−sulfur protein present. Figure 6 shows EPR spectra of chromatophores isolated from *C. vinosum* cells grown on either plus Na_2S (heavy line) or minus Na_2S (light line) medium. Both of the chromatophore samples were treated with sodium ascorbate

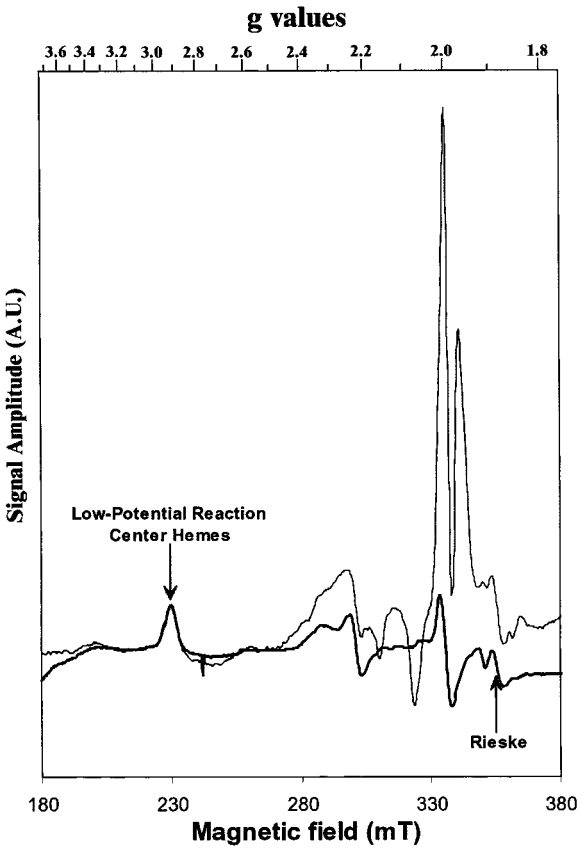

FIGURE 6: EPR spectra of chromatophore membranes isolated from *C. vinosum* cells. Chromatophores isolated, grown on either plus Na_2S medium (heavy line) or minus Na_2S medium (light line), were incubated with sodium ascorbate. EPR measurement conditions: temperature, 15 K; microwave power, 6.3 mW; microwave frequency, 9.42 GHz; modulation amplitude, 3.2 mT.

to ensure that the Rieske protein was fully reduced and the $g = 1.88$ feature in its EPR spectrum was thus at its maximal amplitude (*22*). As ascorbate is not a sufficiently strong reductant to reduce the two low-potential hemes of the reaction center (see above), the EPR feature at $g = 2.94$ that arises from the ferric form of these two low-spin hemes (*23*) remains at maximal amplitude, even in the presence of ascorbate, and provides an internal standard that can be used for estimating the relative cytochrome bc_1 complex/reaction center ratios in the membranes isolated from cells grown under the two different conditions. The EPR spectrum of membranes isolated from cells grown on the plus Na_2S medium (Figure 6) allows direct observation of both the $g = 1.88$ Rieske protein signal and the $g = 2.94$ reaction center heme signal. In the case of the EPR spectrum of membranes isolated from cells grown on the minus Na_2S medium, features arising from an unidentified Mn^{2+}-containing species overlap with those arising from the [2Fe-2S] cluster of the Rieske protein. However, after correcting the EPR spectrum of membranes isolated from cells grown on the minus Na_2S medium for the presence of this Mn^{2+}-containing compound (see Materials and Methods for details of the correction procedure) and then comparing the EPR signals arising from the Rieske protein and the low-potential reaction center hemes, the data of Figure 6 indicate that the cytochrome bc_1 complex/reaction center ratio is approximately twice as large for cells grown on the minus Na_2S medium as it is for cells grown on the plus Na_2S medium.

DISCUSSION

The results presented above should help to reconcile the apparently conflicting earlier reports that either HiPIP (*13*) or a soluble *c*-type cytochrome (*18*, *34*), but not both, serves as the exclusive mobile reductant for the high-potential reaction center heme of *C. vinosum*. It has now been demonstrated that both soluble proteins are capable of reducing the high-potential reaction center heme of *C. vinosum*. Furthermore, the results of this study show that the growth conditions can change the identity of the electron donor to the reaction center in this purple sulfur bacterium. It should be pointed out that it is not possible to compare our results with the earlier results of van Grondelle et al. (*18*) on the reduction of the reaction center by a soluble *c*-type cytochrome in every detail because of differences in the growth conditions (and, as the strain used by van Grondelle et al. was not specified, possibly also in the strains of *C. vinosum*) used. In our study, autotrophic growth was accomplished using a medium that contained both Na_2S and $Na_2S_2O_3$, and the cells used by van Grondelle et al. were grown on a medium that contained $Na_2S_2O_3$ but not Na_2S (*18*). It should be pointed out that van Grondelle et al. (*18*) noted that ca. 40% of the total absorbance decrease arising from photooxidation of the reaction center heme was recovered over the time period between 0.15 and 3 ms after the flash (i.e., during the 300 μs phase). As the reaction center heme and cytochrome *c*-551 (as van Grondelle et al. designated what is probably cytochrome c_8) have very similar reduced *minus* oxidized extinction coefficients (*18*), it is likely that a significant amount of the photooxidized reaction center heme had been reduced by a component that does not have a substantial reduced *minus* oxidized extinction coefficient in the Soret band region in these earlier experiments. From these considerations, van Grondelle et al. estimated that cytochrome *c*-551 reduces only 60−70% of the photooxidized reaction center heme (*18*). It is thus possible that the remaining 30−40% portion of reaction center reduction could have occurred with HiPIP serving as the donor, in a reaction undetected in this earlier investigation.

While the exact nature of the "switch" in electron donor to the *C. vinosum* reaction center that we have observed is not known, it may be possible that the ambient redox potential has some effect. If one speculates that the growth conditions used by van Grondelle et al. (i.e., autotrophic growth in the presence of $Na_2S_2O_3$ but the absence of Na_2S) are intermediate, in terms of ambient potential, between the more oxidizing conditions of heterotrophic growth and the more reducing conditions of autotrophic growth in the presence of both $Na_2S_2O_3$ and Na_2S used in our study, then it can be hypothesized that a more reducing environment may favor HiPIP as the reaction center donor and a more oxidizing environment may favor cytochrome c_8. However, any control of the electron donor by ambient potential is unlikely to occur through the preoxidation of one of the possible donors, given the E_m value of +350 mV for HiPIP (*24*) and the likely E_m value near +250 mV for the cytochrome (see Figure 5). Thus, the situation in *C. vinosum* differs from that in *Rhodocyclus tenuis*, where cytochrome c_8 has a more positive E_m than does HiPIP and so is the only donor available for reaction center reduction at high ambient potentials (*8*).

We have demonstrated, perhaps not surprisingly, that altering the growth medium produces changes in the amounts of HiPIP, cytochrome c_8, and the cytochrome bc_1 complex, compared to the amount of reaction center present. However, the data presented above clearly show that the change in growth conditions for *C. vinosum* does not simply eliminate the presence of one soluble electron donor and replace it with another. The relatively modest difference in HiPIP/cytochrome c_8 ratios observed for cells grown under the two different conditions used in this study do not seem likely to explain the observation that HiPIP functions as the exclusive donor under one condition and cytochrome c_8 is the exclusive donor when the growth conditions are altered. Thus the mechanism for the *C. vinosum* switch must differ from that occurring in some algae and cyanobacteria, where plastocyanin is replaced by cytochrome c_6 as the soluble electron donor to photosystem I when cells are grown in Cu-deficient media (*41*).

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