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ISOLATION AND CHARACTERIZATION OF TWO SOLUBLE HEME *c*-CONTAINING PROTEINS FROM *CHROMATIUM VINOSUM*

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The photosynthetic purple sulfur bacterium *Chromatium vinosum* has been shown to possess two previously undetected heme *c*-containing, soluble proteins. One is an acidic, *c*-type cytochrome with a molecular weight of 12300 and an oxidation-reduction midpoint potential (at pH 8.0) of −82 mV. The other protein is a basic protein with a molecular weight of 11900 and an oxidation-reduction midpoint potential (at pH 8.0) of −110 mV. The basic protein, in both oxidized and reduced forms, has optical spectra similar to those of myoglobin and the oxidized *C. vinosum* protein exhibits a high-spin heme EPR spectrum similar to that of metmyoglobin. Furthermore, the basic *C. vinosum* protein binds CO and O_2. The spectra of the CO and O_2 complexes show significant similarities with the respective myoglobin complexes. Possible functions for an O_2-binding protein in *C. vinosum* are discussed.

Introduction

The photosynthetic purple sulfur bacterium *Chromatium vinosum* is known to possess a considerable number of soluble heme-containing proteins (see Ref. 1 for a recent review). In the course of recent work in our laboratory on the possible role of two soluble *C. vinosum* *c*-type cytochromes in cyclic electron flow [2] and sulfide oxidation [3], previously undetected soluble protein fractions absorbing at 401 nm (in the oxidized form) were detected in *C. vinosum* extracts. The 401 nm-absorbing species have been resolved and characterized. The absorbance at 401 nm in *C. vinosum* extracts can be attributed to two components: (1) An acidic, *c*-type cytochrome with an α-band maximum at 550 nm, a molecular weight of 12300 ± 500 and a midpoint oxidation-reduction potential (E_m) of -82 ± 5 mV at pH 8.0; (2) A basic, high-spin, heme *c*-containing protein with a molecular weight of 11900 ± 500 and $E_m = -110 \pm 15$ mV at pH 8.0. The function of the low-potential *c*-type cytochrome is not known but the high-spin hemoprotein appears to function as an oxygen-binding protein.

Methods

C. vinosum cells were grown and disrupted by sonication in 20 mM Tris-HCl buffer (pH 8.0) containing 300 mM NaCl as described previously [4]. After centrifugation at $255000 \times g$ for 2 h to remove membrane fragments, the bacteriochlorophyll-free supernatant was dialyzed against two changes of 20 mM Tris-HCl buffer (pH 8.0) for 24 h and loaded onto a DEAE-cellulose column (1.5×30 cm) equilibrated with the same buffer. The initial eluent solution was saved and used as the source of the high-spin, heme *c*-containing protein. After washing the DEAE-cellulose column with 20 mM Tris-HCl buffer (pH 8.0)

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containing 40 mM NaCl, the cytochrome was eluted from the column with 20 mM Tris-HCl buffer containing 70 mM NaCl. After dialysis against 20 mM Tris-HCl buffer (pH 8.0), the protein was concentrated by loading on a 1.5 × 10 cm DEAE-cellulose column and eluting with 20 mM Tris-HCl buffer containing 200 mM NaCl. The concentrated protein was chromatographed on a Sephadex G-75 column (2.5 × 90 cm) using 5 mM Tris-HCl buffer (pH 8.0) containing 40 mM KCl. The cytochrome-containing fractions were pooled, diluted 1 : 3 with water, and loaded on a DEAE-cellulose column (1.5 × 20 cm) equilibrated with 20 mM Tris-HCl buffer containing 10 mM KCl. The column was eluted with a linear KCl gradient (10–250 mM) in 20 mM Tris buffer. Cytochrome-containing fractions were pooled, dialyzed and concentrated as described above and the chromatography on Sephadex G-75 repeated. Cytochrome-containing fractions with $A_{401} : A_{280} \geq 1.3$ were pooled and concentrated for use in the studies described below.

The initial eluent solution from the first DEAE-cellulose chromatography was loaded on a CM-Sephadex column (2.6 × 66 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). After washing the column with 20 mM Tris-HCl buffer containing first 40 mM NaCl and then 70 mM NaCl, the hemoprotein was eluted with 20 mM Tris-HCl buffer containing 90 mM NaCl. The protein was dialyzed against 20 mM Tris-HCl buffer, loaded on a 3.4 ml bed volume CM-Sephadex column and concentrated by eluting with 1.0 M NaCl. The concentrated protein was chromatographed on a Sephadex G-100 column (2.5 × 115 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The pure protein was concentrated as described above and passed through a Chelex 100 column (4.2 ml bed volume) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) to remove small amounts of adventitious Cu.

Absorbance spectra were obtained using an Aminco DW-2a spectrophotometer. Oxidation-reduction titrations were performed anaerobically at 4°C, using an electrochemical cell described previously [5]. EPR spectroscopy was performed using a modified JEOL X-band spectrometer equipped with an Air Products cryogenic system for temperature control [6]. SDS-polyacrylamide gel electrophoresis was performed essentially as described by Weber and Osborn [7]. Gels were stained for protein with Coomassie blue and for heme using benzidine [8]. Heme *c* and protoheme analyses were performed as described by Falk [9]. Protein was determined as described by Bradford [10] using cytochrome *c*, hemoglobin or bovine serum albumin as standards.

DEAE-cellulose (DE-52) was obtained from Whatman, Inc.; Sephadex G-75, Sephadex G-100 and CM-Sephadex were obtained from Pharmacia Fine Chemicals. Chelex 100 was obtained from Bio-Rad Laboratories. Protein molecular weight standards, cytochrome *c* (horse heart), hemoglobin, myoglobin and bovine serum albumin were obtained from Sigma Chemical Co.

Results

Fig. 1 shows the spectra of the acidic cytochrome *c*, purified as described in Methods. The oxidized cytochrome has a Soret band maximum at 401 nm and the reduced cytochrome has maxima at 550 (α-band), 521 (β-band) and 419 nm

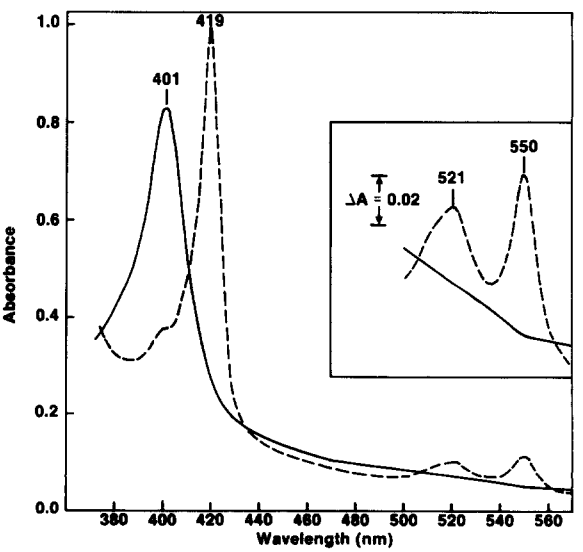

Fig. 1. Absorbance spectra of the oxidized and reduced *C. vinosum* *c*-type cytochrome. The reaction mixture contained the cytochrome (equivalent to 4.38 μM heme *c*) in 40 mM Tris-HCl buffer (pH 8.0) containing 300 mM KCl. The oxidized cytochroem (———) was reduced (— — —) by the addition of small amounts of solid sodium dithionite. The inset showing the α- and β-band region has been expanded 5-fold.

(Soret band). The reduced cytochrome also exhibits a prominent shoulder at 401 nm. The cytochrome exhibits isosbestic points (reduced minus oxidized) at 563, 506, 435 and 410 nm. Alkaline pyridine hemochrome analysis showed a single peak at 551 nm, indicating that heme *c* was the only heme prosthetic group. Heme analysis also allowed determination of the following extinction coefficients (per heme): ε_{401}(oxidized) = 198 $mM^{-1} \cdot cm^{-1}$; ε_{419}(reduced) = 226 $mM^{-1} \cdot cm^{-1}$; ε_{550}(reduced) = 26.0 $mM^{-1} \cdot cm^{-1}$ and $\varepsilon_{550-563}$(reduced minus oxidized) = 13.7 $mM \cdot cm^{-1}$. Using these data we were able to estimate the content of this cytochrome in *C. vinosum* cells to be approx. 3.3 μmol heme *c* per kg wet wt. of cells. The ultraviolet portion of the cytochrome absorbance spectrum is not shown in Fig. 1 because the cytochrome has not yet been purified to homogeneity. Thus, absorbance in the ultraviolet region almost certainly contains significant contribution from proteins other than the cytochrome. At this stage of purification SDS-polyacrylamide gels stained for protein indicated the presence of four to six protein bands in the cytochrome-containing fractions characterized by the highest $A_{401} : A_{280}$ ratios. Staining the gels with benzidine revealed that only a single heme-containing peptide was present in these samples. While reasonable recovery of the cytochrome was observed in the early stages of purification (41% at the stage of the second DEAE-cellulose chromatography), attempts to purify the cytochrome further resulted in large losses. Because of the limited amounts of cytochrome available after the second chromatography on Sephadex G-75 and the fact that the cytochrome was the only heme-containing protein in our samples, it was decided to characterize the cytochrome at this stage of purification.

Chromatography on a calibrated Sephadex G-75 column, using A_{401} to monitor the location of the oxidized cytochrome, gave a value of 12 700 ± 500 for the molecular weight of the cytochrome. SDS-polyacrylamide gel electrophoresis gave a value of 11 900 ± 500 for the molecular weight of the heme-staining band. Thus, the cytochrome appears to be a monomeric protein with a molecular weight of approx. 12 300. Fig. 2 shows the results of an oxidation-reduction titration of the cytochrome performed at pH 8.0. The cytochrome behaves as a

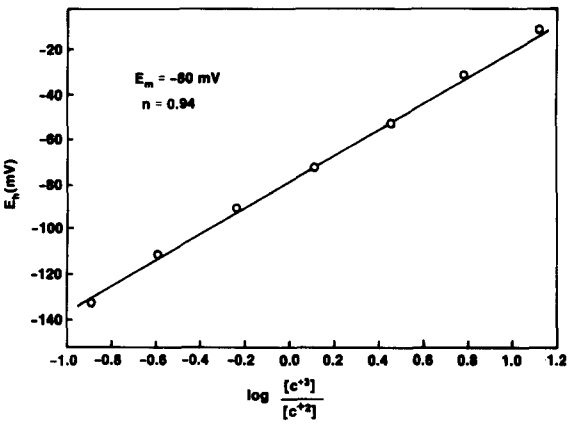

Fig. 2. Oxidation-reduction titration of the *C. vinosum* cytochrome. The reaction mixture contained the cytochrome (equivalent to 13.1 μM heme *c*) in 40 mM Tris-HCl buffer (pH 8.0) containing 300 mM KCl. The following oxidation-reduction mediators were also present: 50 μM indigodisulfonate; 50 μM indigotetrasulfonate and 24 μM ferrous oxalate. The titration was conducted at 4°C and the oxidation state of the cytochrome was monitored by following the absorbance at 419 nm after correction for mediator absorbance.

one-electron carrier ($n = 0.84 \pm 0.08$) with $E_m = -82 \pm 5$ mV (average of three determinations) at pH 8.0. All titrations were fully reversible and the E_m values were independent of mediator concentration. The cytochrome appears to bind CO, as indicated by the change in absorbance of the ferrocytochrome on exposure to CO that is illustrated in Fig. 3.

In contrast to the difficulties experienced in purifying the low-potential cytochrome *c* from *C. vinosum* extracts, the basic, heme *c*-containing protein was readily purified to homogeneity. The protein, purified as described in Methods, showed a constant $A_{401} : A_{280}$ ratio (5.64) in all heme-containing fractions and a single protein-staining band after SDS-polyacrylamide gel electrophoresis. Chromatography on a calibrated Sephadex G-75 column gave a molecular weight of 11 800 ± 500 and SDS-polyacrylamide gel electrophoresis gave a molecular weight of 11 900 ± 500. Thus, the hemoprotein appears to be monomeric with a molecular weight of 11 900.

Fig. 4 shows the absorbance spectra of the oxidized and reduced protein. The spectra are quite different from those expected for a cytochrome and, in fact, bear a striking resemblance to

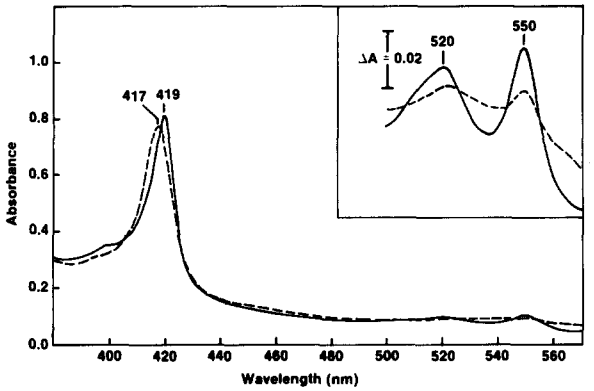

Fig. 3. CO binding by the *C. vinosum* cytochrome. The reaction mixture was as in Fig. 1 except that the reduced cytochrome (———) was present at a concentration equivalent to 3.98 μM. The cytochrome solution was exposed to CO for 10 min and the spectrum of the sample (— — —) measured. The inset showing the α- and β-band region has been expanded 5-fold.

the spectra of metmyoglobin and myoglobin, respectively [11]. Despite the spectral resemblances between the *C. vinosum* protein and myoglobin, pyridine hemochrome analysis indicated that the *C. vinosum* protein contained covalently bound heme *c* rather than noncovalently bound protoheme. A single absorbance peak at 550 nm was observed upon addition of pyridine-NaOH and dithionite to the aqueous phase and no heme was found in the organic phase after treatment of an aliquot of the protein with acidified methyl ethyl ketone [9]. Heme analysis on samples of known absorbance allowed calculation of extinction coefficients (per heme) of 149 $mM^{-1} \cdot cm^{-1}$ at 401.5 nm for the oxidized protein and 99.3 $mM^{-1} \cdot cm^{-1}$ at 428 nm for the reduced protein. Using these extinction coefficients, it was possible to estimate the final yield of the pure heme *c*-containing protein in *C. vinosum* to be 3.7 μmol per kg (wet wt.) of cells. Spectra of the oxidized protein at higher concentration and higher sensitivity than those of Fig. 4 showed no absorbance feature at 695 nm, suggesting that the protein does not contain methionine as an axial heme ligand [12].

Fig. 5 shows the EPR spectrum of the oxidized *C. vinosum* protein. The EPR spectrum, which is quite similar to that of metmyoglobin, indicated that the oxidized protein contains high-spin ferric heme [13]. Fig. 6 shows the results of an oxidation-reduction titration of the protein, which behaves as a one-electron carrier ($n = 1.1 \pm 0.13$) with $E_m = -110 \pm 15$ mV (average of three determinations) at pH 8.0. The higher uncertainty in these values compared to those presented above for the acidic *C. vinosum* cytochrome may be due to the fact that the high-spin hemoprotein equilibrated very slowly with the electrode via the oxidation-reduction mediators available. This

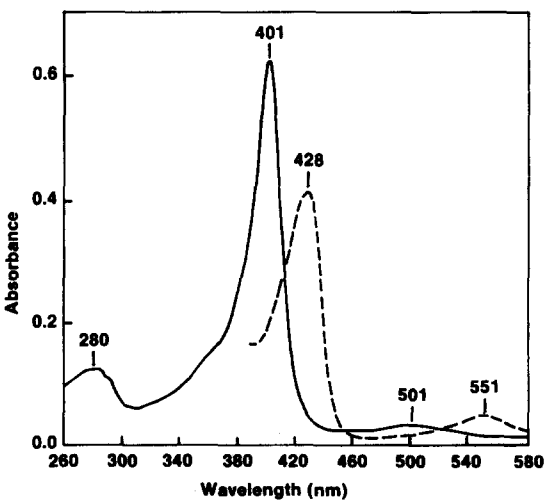

Fig. 4. Absorbance spectra of the oxidized and reduced *C. vinosum* high-spin hemoprotein. The reaction mixture containing the *C. vinosum* high-spin hemoprotein (equivalent to 0.84 μM heme *c*) in 0.8 mM Tris-HCl buffer (pH 8.0) containing 38.5 mM NaCl. The oxidized protein (———) was reduced (— — —) by the addition of small amounts of solid sodium dithionite.

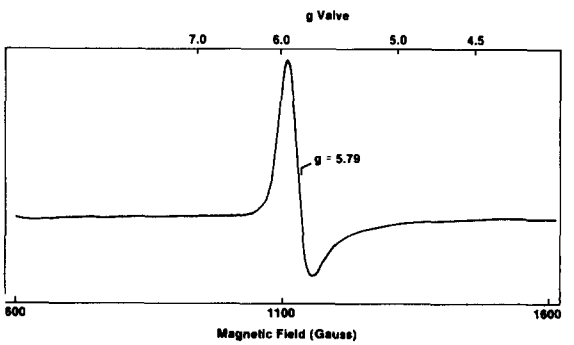

Fig. 5. EPR spectrum of the oxidized *C. vinosum* high-spin hemoprotein. The reaction mixture contained the *C. vinosum* hemoprotein (equivalent to 23.9 μM heme *c*) in 20 mM Tris-HCl buffer (pH 8.0). EPR conditions: temperature, 12 K; frequency, 9.2116 GHz; microwave power, 3 mW; modulation amplitude, 10 G.

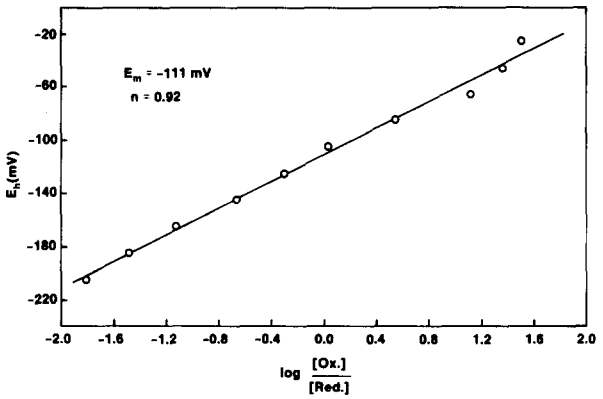

Fig. 6. Oxidation-reduction titration of the *C. vinosum* high-spin hemoprotein. The reaction mixture contained the *C. vinosum* hemoprotein (equivalent to 80 μM heme *c*) in 25 mM Tris-HCl buffer (pH 8.0) containing 120 mM NaCl. The following oxidation-reduction mediators were also present: 10 μM benzyl viologen, 10 μM safranine O; 10 μM anthraquinone-1,5-disulfonate; 10 μM anthraquinone-2-sulfonate and 10 μM indigodisulfonate. The titration was conducted at 4°C and the oxidation state of the protein was monitored by following the absorbance at 401 nm after correction for mediator absorbance.

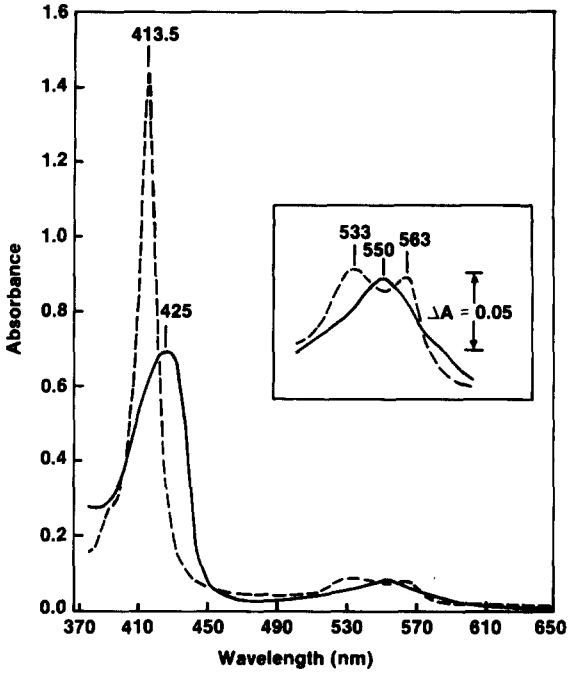

Fig. 7. CO binding by the *C. vinosum* high-spin hemoprotein. The reaction mixture contained the *C. vinosum* hemoprotein (equivalent to 8.8 μM heme *c*) in 20 mM Tris-HCl buffer (pH 8.0) containing 360 mM NaCl. The protein was reduced (———) under an N_2 atmosphere with sodium dithionite and then exposed to CO for 1.75 m (— — —). The inset, showing the spectra from 500 to 600 nm, has been expanded 4-fold.

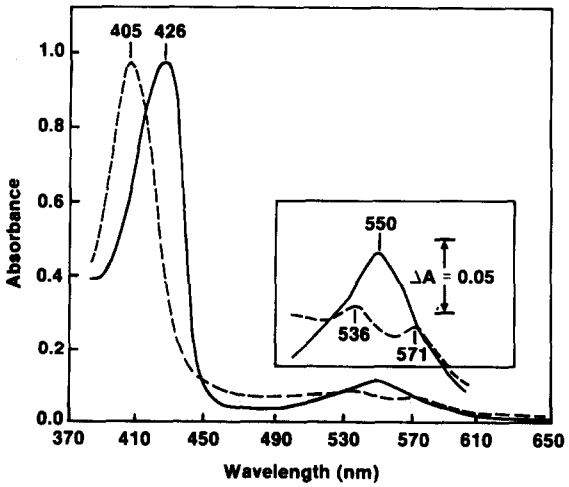

Fig. 8. O_2 binding by the *C. vinosum* high-spin hemoprotein. The reaction mixture contained the *C. vinosum* hemoprotein (equivalent to 12.0 μM heme *c*) in 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl. The protein was reduced (———) with sodium dithionite under an N_2 atmosphere and then exposed to O_2 for 1.75 m (— — —). The inset, showing the spectra from 500 to 600 nm, has been expanded 4-fold.

necessitated the use of high mediator concentrations which, in turn, resulted in substantial mediator absorbance corrections. However, the titrations were reversible and the E_m and *n* values obtained were independent of mediator concentration.

The spectral similarities between the *C. vinosum* high-spin hemoprotein and myoglobin suggested that the *C. vinosum* protein might bind CO and/or O_2. Fig. 7 shows the spectra of the reduced *C. vinosum* protein before and after exposure to CO. CO binding clearly occurs and is associated with spectral changes similar to those accompanying carboxymyoglobin formation [11]. Fig. 8 shows that the *C. vinosum* protein also binds O_2. Exposure of the reduced *C. vinosum* protein to O_2 for 1.75 min produces a complex with a spectrum that bears some similarities to that of oxymyoglobin [11] and that differs from those of both the oxidized and reduced protein.

Discussion

In addition to the large number of soluble heme-containing proteins previously reported to be present in the photosynthetic purple sulfur bacterium *C. vinosum*, the results described above indicate that this bacterium contains at least two additional soluble hemoproteins. Elucidation of a possible function for the acidic, *c*-type cytochrome ($E_m = -82$ mV) described in Figs. 1–3 will require additional experimentation. Such experiments are currently underway in our laboratory.

The most striking result of our recent investigation of soluble *C. vinosum* hemoproteins is the presence in this bacterium of a protein resembling myoglobin in spectral characteristics and its capacity for binding O_2. Based on a brief description of optical spectra, it appears likely that similar proteins have been detected in two species of photosynthetic purple nonsulfur bacteria, *Rhodopseudomonas sphaeroides* and *Rps. capsulata*, as 'minor components' [1]. However, the proteins detected in these *Rhodopseudomonas* species were not characterized further. No mention was made in this brief report [1] of a protein with similar spectral characteristics in any photosynthetic purple sulfur bacterium. Thus, the results presented above represent the first report of such a protein in any purple sulfur bacterium and of such a protein as a major constituent in any photosynthetic bacterium. Most significantly, the data presented above provide the first evidence for an O_2-binding protein in any photosynthetic bacterium.

One problem that arises from our preliminary characterization of the *C. vinosum* protein is that of how to classify it. Although the *C. vinosum* protein resembles myoglobin insofar as its optical and EPR spectra and the optical spectra of its CO and O_2 complexes are concerned, it clearly cannot be classified as a myoglobin. It contains protein-bound heme *c* rather than protoheme as its prosthetic group and also differs considerably from myoglobin in its molecular weight (11 900 for the *C. vinosum* protein vs. 16 900 for myoglobin). The two proteins also differ substantially in their E_m values, with the *C. vinosum* protein exhibiting a considerably more electronegative value ($E_m = -110$ mV) than the metmyoglobin/myoglobin couple ($E_m = +46$ mV at pH 7.0 [14]).

An obvious question that arises about the *C. vinosum* protein concerns the possible function of an O_2-binding protein in a photosynthetic bacterium. The data of Fig. 8 clearly demonstrate that the *C. vinosum* high-spin hemoprotein can bind O_2. However, at this stage it is perhaps too early to conclude that the function of the *C. vinosum* protein in vivo actually involves O_2 binding. The argument for O_2 binding as the in vivo function is strengthened by the recent work of Kämpf and Pfennig [15] demonstrating that several species of photosynthetic purple sulfur bacteria, previously thought to be obligate anaerobes, are capable of chemotrophic growth under semi-aerobic or microaerobic conditions. *C. vinosum* was one of the species that showed the capacity for growth and respiration in the presence of low oxygen concentrations [15]. Furthermore, Takamiya and co-workers [16] have demonstrated that cell-free, membrane preparations from *C. vinosum* exhibit NADH- or succinate-dependent O_2 uptakes that are sensitive to a number of classical respiratory inhibitors. These results support that idea that *C. vinosum* can respire [15,16] but can only grow if the O_2 concentration is low. Thus, the bacterium may require O_2-binding protein(s) which serve to regulate its internal p_{O_2}. Investigations are currently underway in our laboratory to explore the possibility that the *C. vinosum* high-spin, heme *c*-containing protein functions in this manner.

Acknowledgments

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