Coordination and Redox Properties of a Novel Triheme Cytochrome from *Desulfovibrio vulgaris* (Hildenborough)†

Jian Tan and J. A. Cowan*
Evans Laboratory of Chemistry, The Ohio State University, 120 West 18th Avenue, Columbus, Ohio 43210
Received August 15, 1989; Revised Manuscript Received January 10, 1990

ABSTRACT: A high molecular weight multiheme *c*-type cytochrome from the sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) has been spectroscopically characterized and compared with the tetraheme cytochrome c_3. The protein contains a pentacoordinate high-spin heme (g_z 6.0) and two hexacoordinate low-spin hemes (g_z 2.95, g_y 2.27, g_x 1.48). From analysis of the *g* values for the low-spin hemes by the procedure of Blumberg and Peisach (Palmer, 1983) and comparison with the optical spectra from a variety of *c*-type cytochromes, it is likely that these low-spin hemes are bound by two histidine residues. The NO derivative displayed typical rhombic EPR features (g_x 2.07, g_z 2.02, g_y 1.99). Addition of azide does not lead to coupling between heme chromophores, but the ligand is accessible to the high-spin heme. The use of a glassy-carbon electrode to perform direct (no promoter) electrochemistry on the cytochrome is illustrated. Differential pulse polarography of the native protein gave two waves with reduction potentials of −59 (5) and −400 (8) mV (versus NHE). The cyanide adduct gave two waves with reduction potentials of −263 (8) and −401 (8) mV. The cytochrome was found to catalyze the reduction of nitrite and hydroxylamine.

The isolation of cytochrome c_3 (Postgate, 1954) from the sulfate-reducing bacterium *Desulfovibrio vulgaris* marked the initial stages of the isolation and characterization of a large number of multiheme cytochromes from this and related bacterial systems (Ambler et al., 1971; LeGall et al., 1973; Bruschi, 1979; Xavier et al., 1979; Peck et al., 1982; Jones, 1971; Probst, 1977). The four-heme cytochromes c_3 have proved to be archetypal examples of this class of multiheme redox protein and have been the focus of exhaustive spectroscopic and biochemical analysis. Many physical techniques (NMR, ESR, electrochemistry, circular dichroism, and crystallography) have been used to address the importance of heme orientation in intramolecular electron transfer (Guerlesquin et al., 1985; Pierrot et al., 1982), the influence of protein environment in defining redox properties (Niki et al., 1982, 1984; Drucker et al., 1970), and the role of these hemes in electron storage (Yagi et al., 1983), with particular concern toward interactions with other redox enzymes and their catalytic activity (Niki et al., 1982; Dobson et al., 1974). Cytochromes c_3 from a variety of bacterial sources have been found to possess poor sequence homology (DerVartanian et al., 1974), however; all are of low molecular weight ($M_r \sim$ 13000) with extremely negative reduction potentials as a result of bishistidine ligation (DerVartanian et al., 1974). Typically the hemes possess a range of reduction potentials [−220 to −340 mV (NHE)] (Niki et al., 1982). In bacterial cell systems, cytochrome c_3 has been associated with the role of electron carrier to sulfite and thiosulfate reductases and as a biological redox partner to flavodoxin, ferredoxin, and rubredoxin (Postgate, 1984). A variety of related low molecular weight cytochromes possessing negative reduction potentials have been isolated from the *Desulfovibrio* bacteria, including cytochromes cc_3 ($M_r \sim$ 26000) (Guerlesquin et al., 1982) and cytochrome c_{553} ($M_r \sim$ 8000–9000) (Yagi, 1979; Peck et al., 1982), while several larger cytochromes have also been identified. In particular, Liu and Peck have reported the isolation of a novel hexaheme cytochrome ($M_r \sim$ 66000) from *D. desulfuricans* that possesses nitrite reductase activity (Liu et al., 1981). The occurrence of high molecular weight cytochromes ($M_r \sim$ 70000) from the sulfate-reducing bacterium *D. vulgaris*, strains Miyazaki (Yagi, 1969, 1979) and Hildenborough (Higuchi et al., 1987), has also been noted, although neither protein has been characterized in any detail. We also have isolated the large *c*-type cytochrome from *D. vulgaris* (Hildenborough) and in this paper we report the results of detailed studies on the characterization of the coordination states and redox properties of the protein heme units.

A feature of general interest and importance in the study of proteins containing several redox centers is the categorization of those proteins serving an electron-carrier role, from enzymes catalyzing redox chemistry on molecular substrates, i.e., to distinguish electron-transfer from atom-transfer reactivity. An understanding of the structural factors that control the distinction between these two roles would give general insight to a diverse group of metalloredox enzymes and proteins. We have found that the large cytochrome that is the subject of this paper possesses nitrite reductase activity (Tan et al., 1989). In order to better understand the reaction mechanism of this protein and to understand the basis of its functional relationship with other cytochromes, we have studied its coordination and redox chemistry in comparison with the electron-carrier cytochrome c_3 (Meyer et al., 1982) and the novel nitrite reductase isolated by Liu and Peck (Liu et al., 1981).

Materials and Methods

Growth of Organism. D. vulgaris (Hildenborough, NCIB 8303) was grown in an enriched Baars medium [ATCC medium No. 1249] at the Fermentation Laboratory, Department of Biochemistry, University of Wisconsin. The initial stages of the isolation followed the procedure of Peck and co-workers for the isolation of assimilatory sulfite reductase (Huynh et al., 1984). Two hundred fifty grams of wet cells was suspended in sufficient 50 mM Tris-HCl buffer (pH 7.5, 4 °C) to give a 1:1 weight to volume ratio and the cells opened by sonication

† This work was supported by a grant from The Ohio State University.

0006-2960/90/0429-4886$02.50/0

(Fisher Sonic Dismembranator, full power, 3 × 10 min). The temperature was maintained below 15 °C by letting the solution sit in an ice/salt bath. Streptomycin sulfate (2.5 g in 4 mL, 50 mM Tris-HCl) was neutralized and added to the extract, and the solution was stirred for 30 min at 4 °C. Following centrifugation in a Sorvall RC-5B refrigerated centrifuge (13200*g*, 1 h) the supernatant was centrifuged (144000*g*, 90 min) in a Beckman L5-75B ultracentrifuge and filtered through a glass wool plug.

Isolation of Cytochrome. All purification procedures were performed at 4 °C and all buffers, unless otherwise stated, were adjusted to pH 7.6 (4 °C). The crude extract was loaded onto a hydroxylapatite column (5 × 15 cm) that had previously been equilibrated with 100 mM Tris-HCl. After running a descending Tris gradient (100 mM to 20 mM, 1000-mL total volume) the cytochrome fraction was obtained by applying a potassium phosphate gradient (0.01–0.5 M, total volume 1000 L). Desulfoviridin was eluted first, followed by an orange cytochrome fraction at 0.25 M phosphate. The cytochrome eluent was concentrated by ultrafiltration [Amicon, YM-8 membrane (Pharmacia/LKB)] and diluted with 5 mM potassium phosphate and the process repeated twice more. After loading onto a DE-52 (Whatman) column (4 × 15 cm) equilibrated in 5 mM potassium phosphate, the cytochrome fraction was eluted with the equilibration buffer. The solution was then loaded onto a CM-52 (Whatman) column (5 × 15 cm) equilibrated with 5 mM potassium phosphate. A stepwise phosphate gradient [10 mM (300 mL), 15 mM (400 mL), 30 mM (1 L), and 50 mM (400 mL)] was run. The large cytochrome was eluted at 15 mM phosphate.

Analysis for Heme Content. The total number of heme units was determined by using the pyridine hemochrome test. A known mass of the protein (determined by hydrolysis of an aliquot of protein solution followed by quantitative amino acid analysis) was added to an alkaline [0.075 M NaOH(aq)] pyridine (25%) solution and reduced by adding a few crystals of sodium dithionite. The heme content was calculated by using $\epsilon_{550nm} = 29.1 \times 10^3\ M^{-1}\ cm^{-1}$ (Falk, 1964). The ratio of high- and low-spin hemes also was determined by relative integration of the high- and low-spin EPR features in the native enzyme by using standard Bruker software. The number of high-spin (HS) hemes was estimated from integration of the ESR signatures of the HS signal relative to a sample of metmyoglobin fluoride (Mb.F) of known concentration.

Amino Acid Analyses. Quantitative amino acid analyses were performed at the campus Biochemical Instrument Center. Amino acid compositions were determined after hydrolysis of purified protein for 24 h in hydrochloric acid vapors at 110 °C in an evacuated vial. Cysteine was determined after oxidation of the protein in performic acid for 24 h at −20 °C followed by acid hydrolysis for 24 h as above. Amino acid analyses were performed after precolumn derivatization with phenyl isothiocyanate on a Waters Associates Pico Tag amino acid analysis system according to standard manufacturer protocols.

Gel Electrophoresis. SDS–PAGE was run on a Phast electrophoresis system (Pharmacia/LKB) at 15 °C using premade 20% homogeneous gels (Pharmacia/LKB). A sample of protein (4 μL of a 2.5 mM solution) was treated with an SDS gel loading buffer solution (4 μL, prepared according to the manufacturer's protocols), warmed at 100 °C for 2 min, and a 0.5-μL sample was applied to the gel by using an automated gel loading applicator (Pharmacia/LKB) and run under recommended preprogrammed operating conditions. Molecular weight determinations were referenced to commercially available standards (BRL, large range). The cytochrome ran as a single band (developed using the Pharmacia silver staining procedure) with a MW ~68000, in line with previous determinations (Higuchi et al., 1987). Analytical IEF gels were run on a LKB Multiphor instrument using a premade PAG plate (pH 3.5–9.5) at 10 °C and pI's were referenced to oxidized/reduced Mb [pI 8.1 and 7.4, respectively (Cowan et al., 1989)] and high-potential iron protein (*C. vinosum*) [pI 3.6 (Bartsch, 1971, 1978)]. Typically 10 μL of a 2.5 mM protein solution was applied to each lane. Running [1500 V (final); 30 W (initial); constant current] and staining (Coomassie) conditions followed recommended operating procedures. A single isolated band with a pI of 8.9 ± 0.1 was observed.

Spectroscopic and Electrochemical Methods. Electronic absorption spectra were obtained on a Perkin-Elmer Lambda 6 spectrophotometer. Typically a ca. 0.7 μM solution in 50 mM phosphate buffer [pH 7.0, 298 (2) K] was used. Reduced cytochromes were obtained by adding a few crystals of sodium dithionite to the solution. Ligand anions were introduced by the addition of 5 mg of the sodium salt containing the anion of interest. In the case of hydroxylamine, the hydrochloride was used and the pH adjusted to 7.0 with a concentrated stock solution of 10 M NaOH(aq) prior to the addition of protein or anion.

ESR spectra were measured on a Bruker ESP-300 instrument equipped with an Oxford helium cryostat operating at 8 (3) K and an integration program in the internal Bruker software package. Solutions contained ca. 0.5 mM protein in 50 mM phosphate buffer (pH 7.0). Typically, a modulation amplitude of 20 G and a microwave power of 1 mW were used. Azide derivatives were formed by dissolving sodium azide (5 mg) in the solutions prior to freezing. NO adducts were formed by adding 50 μL of a NO-saturated solution of buffer to a degassed sample (purged for 1 h with oxygen-free nitrogen) of the dithionite-reduced protein. These latter spectra were obtained at 150 K by using a modulation amplitude of 2.5 G. Electrochemical potentials were determined by differential-pulse polarography using a Princeton Applied Research (PAR) Model 174A polarographic analyzer at a scan rate of 2 mV s^{-1}, modulation amplitude of 25, and a 0.5-s drop time. Results were recorded on a Houston Instrument Omnigraphics 2000 recorder. A homemade electrochemical cell was used. A 2.5 × 4 cm glass vial was fitted with a plastic cap containing holes for access by electrodes and an inlet for a needle connected to a nitrogen supply. A glassy-carbon working electrode [Bioanalytical Systems (BAS)], platinum-wire counter electrode, and SSCE reference (corrected later to NHE) were routinely used. Potentials were calibrated against a MeV^{2+}/MeV^{+} couple [$E^{\circ\prime}$ −446 mV (Kuwana, 1977)]. Electrochemical experiments were carried out in 50 mM sodium phosphate buffer (1 mL, pH 7.0, 298 K). Protein concentrations were typically ca. 5 mM and no redox promoter was employed. Solutions were degassed by flushing the surface with nitrogen for 15 min with occasional stirring. The glassy-carbon electrode was polished with fine alumina in a water suspension prior to use. The results reported were reproducible within the error limits quoted (generally within ±10 mV). Experiments were performed on samples from at least two different batches of protein from distinct cell preparations.

Results

Isolation and Characterization. The enzyme was isolated in a simple three-step procedure from the crude extract by passage over a hydroxylapatite affinity column, followed by DE-52 and CM-52 ion-exchange chromatography. The elution

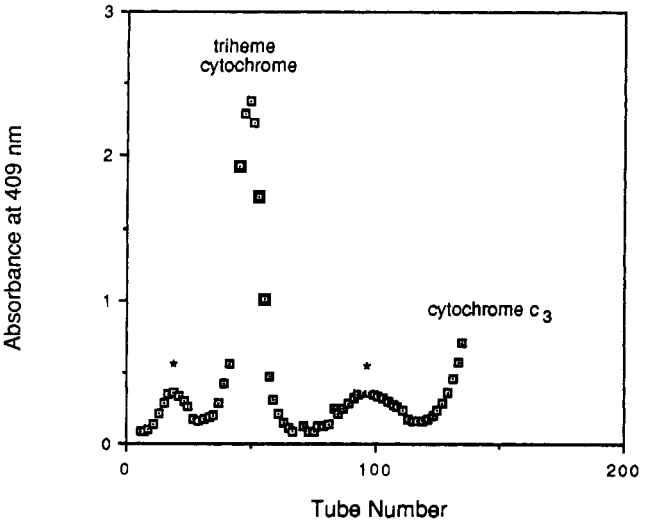

FIGURE 1: Elution profile from a CM-52 chromatography column run at 4 °C. Each tube held 7 mL of solution. A stepwise gradient was employed, using sodium phosphate, pH 7.0, as eluent. At tubes 1, 25, 60, and 110, the concentration of the elution buffer was changed to 10, 15, 30, and 50 mM, respectively. Bands marked * indicate additional uncharacterized cytochromes.

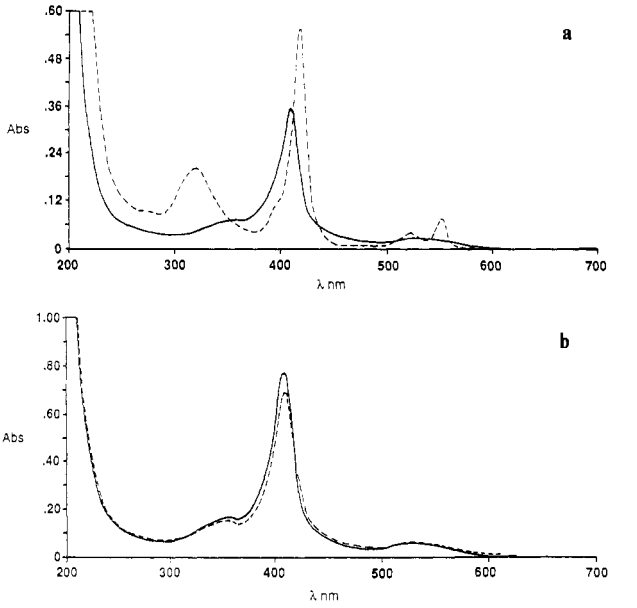

FIGURE 2: Electronic absorption spectra comparing (a) oxidized (—) and reduced (---) cytochrome and (b) oxidized cytochrome (—) and the cyanide derivative (---). Spectra were obtained using a ca. 0.7 μM protein solution at 298 K, in 50 mM sodium phosphate buffer, pH 7.5. Cyanide spectra were obtained following the addition of 5 mg of NaCN.

profile in Figure 1 illustrates how the large *c*-type cytochrome can be cleanly separated from other contaminating cytochromes on the final CM-52 column. A single isolated band was observed in both SDS and IEF gels, while the pI of 8.9 ± 0.1 and the purity index $A_{ox}(280)/A_{red}(553) = 3.40 \pm 0.05$ were both in agreement with the values reported elsewhere (Higuchi et al., 1987). The similarity of the electronic absorption spectrum of the large cytochrome to that of cytochrome c_3 (Meyer et al., 1982) leads us to classify this enzyme as a high molecular weight *c*-type cytochrome. In keeping with the results of Higuchi (Higuchi et al., 1987) the molecular weight of the polypeptide backbone was determined to be 68 (2) × 10^3 by comparison with standard molecular weight markers following SDS–PAGE electrophoresis. We have independently determined the number of hemes present and in addition have detailed their coordination and spin states and

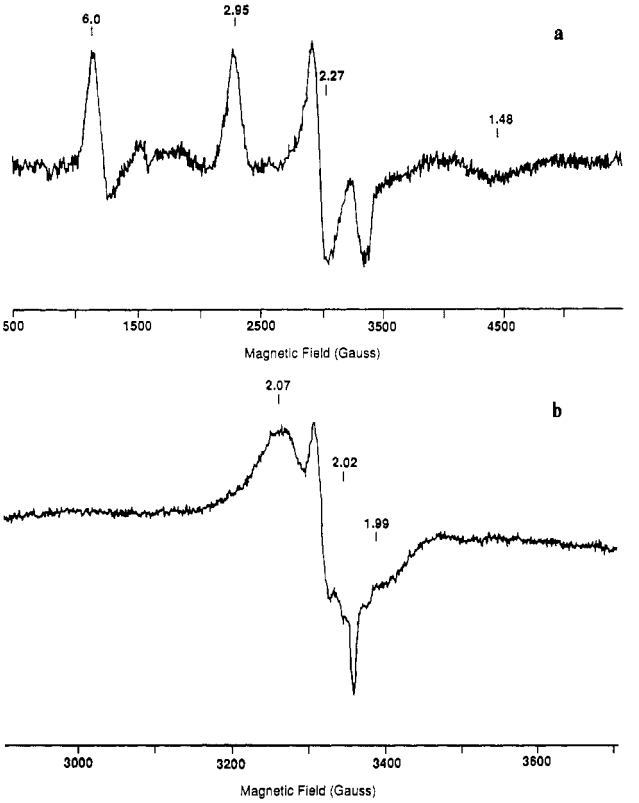

FIGURE 3: (a) EPR spectrum of native triheme cytochrome, obtained at ca. 8 (3) K. Scan width 5000 G, modulation amplitude 20 G, frequency 9.44 GHz, microwave power 1 mW. (b) EPR spectrum of the NO adduct of native triheme cytochrome, obtained at ca. 150 K, pH 7.0, with 50 mM potassium phosphate buffer. Scan width 800 G, modulation amplitude 2.5 G, frequency 9.44 GHz, microwave power 1 mW. Solutions contained ca. 0.5 mM protein in 50 mM phosphate buffer, pH 7.0.

redox properties. Contrary to previous reports (Higuchi et al., 1987), a total of three hemes per cytochrome was determined by the pyridine hemochrome test. The quantity of protein used was accurately measured by quantitative amino acid analysis; the dry weight method of protein determination is very prone to error (Meyer et al., 1982). The results of two independent runs with batches from distinct cell preparations gave a total of 2.94 and 3.09 hemes. In keeping with the similarity of the electronic spectra to those *c*-type cytochromes known to possess histidine-coordinated hemes and by an analysis of the EPR spectra and electrochemical data described below, we consider the coordination spheres of the hemes in this larger cytochrome to be dominated by histidine ligation. Both low-spin hexacoordinate (bishistidine) and high-spin pentacoordinate (monohistidine) heme units have been identified. For the purpose of simplifying the discussion, we shall consistently refer to the high-spin component with one coordinating histidine as a pentacoordinate species. It is possible that a water molecule may be bound to the distal site; however, we currently have no conclusive evidence for this. The relative number of high- and low-spin hemes was estimated by integration of the EPR spectrum described below.

EPR. In comparison with cytochrome c_3 (DerVartanian et al., 1971; LeGall et al., 1971), both high- and low-spin features were observed (Figure 3a) with the following *g* values: g_z 6.0 [the weaker high-field signal was lost under the manganese impurity at $g = 2.02$ (LeGall et al., 1971)] and g_z 2.95, g_y 2.27, g_x 1.48, respectively. Relative spin integration of two independently prepared samples from two distinct cell preparations yielded ratios of 0.52 and 0.55 ± 0.1 for the number of high- to low-spin ferric centers. Since the protein contains

Table I: Comparison of Optical and Physical Properties of Triheme Cytochrome and Cytochrome c_3

	triheme cytochrome[a]				cytochrome c_3			
native	$\epsilon_{ox}(409)$	401.0	$\epsilon_{red}(419)$	612.4	$\epsilon_{ox}(410)^b$	460	$\epsilon_{red}(419)^b$	688
	$\epsilon_{ox}(528)$	35.6	$\epsilon_{red}(523)$	52.3	$\epsilon_{ox}(530)^b$	37	$\epsilon_{red}(524)^b$	57
			$\epsilon_{red}(553)$	90.0			$\epsilon_{red}(552)^b$	110
+cyanide	$\epsilon_{ox}(410)$	363.0	$\epsilon_{red}(420)$	407.0	$\epsilon_{ox}(410)$	460.2	$\epsilon_{red}(421)$	447.2
	$\epsilon_{ox}(530)$	33.3	$\epsilon_{red}(525)$	46.4	$\epsilon_{ox}(530)$	37.5	$\epsilon_{red}(525)$	58.9
			$\epsilon_{red}(554)$	57.5			$\epsilon_{red}(553)$	57.8
pI	8.9 ± 0.1^c				10.5^d			
M_r	$68\,000^c$				$13\,000^d$			
heme content	2 low-spin ferric, 1 high-spin ferric				4 low-spin ferric			

[a] Extinction coefficients (ϵ_{ox} and ϵ_{red}) are in mM^{-1} cm^{-1}. Measurements were made under aerobic conditions. [b] Yagi et al., 1983. [c] Higuchi et al., 1987; and this work. [d] Postgate, 1984.

only three heme units we determine one high- and two low-spin hemes. Addition of azide led to the disappearance of the high-spin bands due to the formation of a hexacoordinate ligand environment and the resulting low-spin g components were similar to those noted for native enzyme (g_z 2.95, g_y 2.27, g_x 1.45). Relative to Mb.F, the cytochrome contained a total of 1.1 ± 0.2 high-spin hemes, in support of the above results. We have analyzed the anisotropic EPR signals of the low-spin hemes using the methodology of Taylor (Taylor, 1977) to determine values for the rhombicity V/Δ and tetragonal field splitting Δ/λ. These parameters have been correlated (Blumberg et al., 1971) to give a measure of the strength and asymmetry of the two apical ligands bound to the heme. In particular, comparison can be made with the many results that are available for cytochromes, myoglobulins, hemoglobins, and model systems (Palmer, 1983).

The relevant equations (after Taylor, 1977) are noted below and values of 0.6 and 3.1 were obtained for V/Δ and Δ/λ, respectively. These values are in keeping with a bishistidine

$$V/\lambda = g_x/(g_z + g_y) + g_y/(g_z - g_x)$$

$$\Delta/\lambda = g_x/2(g_z + g_y) + g_z/(g_y - g_x) - g_y/2(g_z - g_x)$$

ligand set (Palmer, 1983). The g values are also similar to those for low-spin cytochrome c_3 (DerVartanian et al., 1971), where a bishistidine environment has been demonstrated crystallographically (Haser et al., 1979; Higuchi et al., 1981).

The 150 K EPR spectrum of the NO derivative (Figure 3b) displays the rhombicity frequently found in heme proteins (g_x 2.07, g_z 2.02, g_y 1.99) (Kon et al., 1969). The NO derivative of cytochrome c_3 produced a more axial spectrum ($g_{x,y}$ 2.03 and g_z 2.01) with no resolved coupling to the nitrogen in g_z. These differences in the EPR spectra are not necessarily indicative of major differences in the environments of the hemes in these two proteins. The tensor components are particularly sensitive to minor differences in the relative energies of d_{xz} and d_{yz} that can arise due to a slight asymmetry along the x and y axes (Palmer, 1979; Kon et al., 1969). The magnitude of the hyperfine coupling from ^{14}NO (12 ± 1 G) observed in the z component in the former case is typical for coordination to heme (Hille et al., 1979), while a reduction in the size of this coupling has previously been noted in the case of axial symmetry (Kon et al., 1968).

Electronic Absorption. Oxidized State. From the spectrum of a known mass of the protein (43.6 μg in 1 mL of 50 mM sodium phosphate, pH 7), determined by hydrolysis of an aliquot of protein solution followed by quantitative amino acid analysis, we have determined the extinction coefficients listed in Table I. The ratio $A(409)/A(530)$ was found to be 11.3 for the triheme cytochrome in comparison with 10.9 for c_3 (Yagi et al., 1983). The absorption spectrum for the large cytochrome is qualitatively similar to that of cytochrome c_3; the absorption bands occur at similar wavelengths, although the extinction coefficients (Table I) are larger (14–22% per heme) in the case of the triheme cytochrome. This is in keeping with the generally larger extinction coefficients for pentacoordinate high-spin hemes (Antonini et al., 1971). The absorption spectra for the native and cyanide derivatives of the triheme cytochrome are compared in Figure 2. The wavelengths of the Soret and Q bands were similar to those of the corresponding bands in c_3, although the absorption intensities were reduced (Table I). The reduction in intensity is consistent with other observations on the coordination of cyanide to pentacoordinate hemes (Antonini et al., 1971). In the case of cytochrome c_3, however, we have found the spectrum of the cyanide derivative to be similar to that of the native protein with no obvious change in band position or absorbance. Interestingly, the extinction coefficients at the absorbance wavelengths (per heme) for the cyanide adduct of the larger cytochrome were in line with those determined for c_3, reflecting the change from penta- to hexacoordination (high to low spin) at one of the heme centers. The addition of F^-, N_3^-, NH_2OH, SO_3^{2-}, or NO_2^- to the oxidized state of the protein produced no significant change in the spectrum.

Reduced State. Again, the spectrum for the larger cytochrome is qualitatively similar to that of the c_3 protein, with $A(419)/A(553)$ of 6.8 for the former and 6.3 for the latter (Yagi et al., 1983). The extinction coefficients determined for the reduced protein, and the cyanide adducts, are listed in Table I and the absorption spectra of reduced and oxidized forms are compared in Figure 2. Although the absorbance wavelengths are similar, the extinction coefficients of the c_3 were 8–18% smaller per heme in comparison with the larger cytochrome. The protein demonstrated diverse behavior in the presence of a variety of anionic and neutral ligands. The addition of N_3^-, F^-, and SO_3^{2-} produced little change in the spectrum; however, the addition of either NO_2^- or NH_2OH resulted in reoxidation to the ferric form.

Electrochemistry. Good results were obtained by using a glassy-carbon electrode (gce) and are shown in Figure 4. Such an electrode possesses a large overpotential for production of hydrogen and dissolved oxygen and can be usefully employed in aqueous solutions. More significantly, the electrochemistry was performed in the absence of added redox promoter. Under the conditions employed, an average reduction potential was determined for the four hemes in cytochrome c_3 (Table II) that was in good agreement with that previously noted by Niki et al. (1982, 1984). The heme potentials in the tetraheme cytochrome c_3 are known to vary from −220 to −340 mV (NHE); however, only a single wave (ca. −324 mV) is generally observed in direct electrochemical experiments, which can be studied further by deconvolution analysis (Niki et al., 1982, 1984). The good agreement with the published data on c_3 provided confidence in the use of this electrode system. The

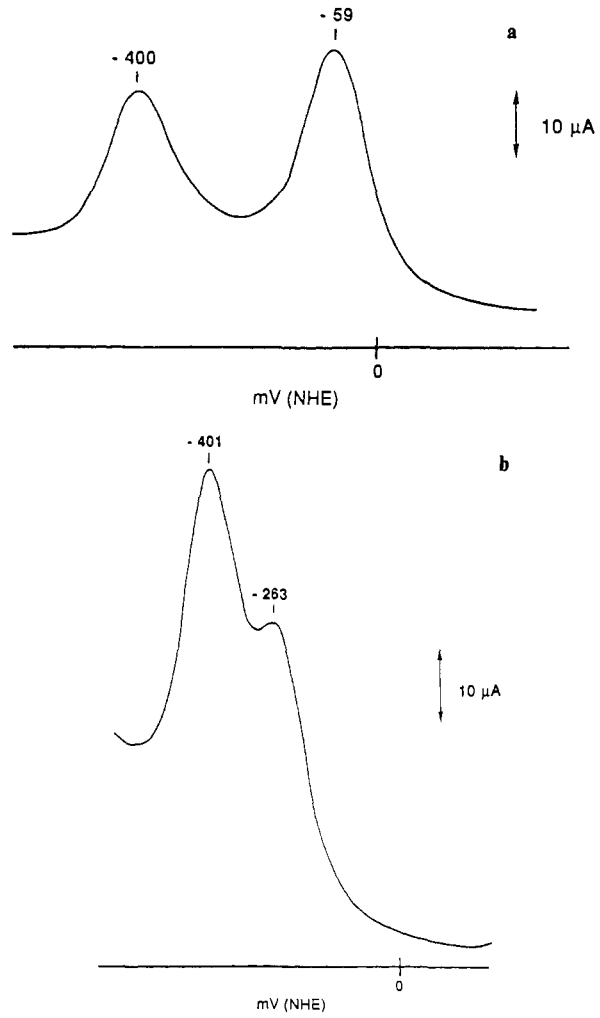

FIGURE 4: Differential pulse polarograms for (a) native triheme cytochrome and (b) the cyanide derivative. Results were obtained with a protein concentration of ca. 5 mM using a glassy-carbon electrode in 50 mM sodium phosphate buffer, pH 7.0, 298 K. The quoted values refer to cathodic scans. In general, anodic scans were 5–10 mV less negative.

Table II: Electrochemical Reduction Potentials for Triheme Cytochrome Relative to Cytochrome c_3[a]

protein	$E^{\circ\prime}$ (mV) (NHE)
triheme cytochrome	−400 (8), −59 (5)
triheme cytochrome + CN^-	−401 (8), −263 (8)
cytochrome c_3	−346 (8)
cytochrome c_3 + CN^-	−409 (8)

[a] Measured with a glassy-carbon electrode in 50 mM sodium phosphate buffer, pH 7.0, 298 (2) K. Protein concentration, ca. 5 mM. The quoted values refer to cathodic scans. In general, anodic scans were 5–10 mV less negative.

results of experiments on the triheme cytochrome are noted in Table II and, in line with results from optical spectra and EPR, suggest the presence of two distinct types of heme environment in the larger cytochrome. The results were reproducible within the limits listed beside each potential and the quoted values refer to cathodic scans. In general, anodic scans were 5–10 mV less negative. The observed wave at ca. −59 mV was found to be reproducible during cathodic scans using a freshly polished electrode surface; however, no signal was observed either on the return scan or during repetitive cathodic scans. Previous studies on cyt c_3 using a hanging mercury-drop electrode had indicated the disappearance of signal following the initial observation of a cathodic wave. No such disappearance was noted with the wave at −400 mV. The forward and reverse waves peaked at similar potentials. The reason for this behavior is not clear; presumably the heme giving rise to the former signal undergoes an ill-defined irreversible change in coordination state or local environment that prohibits subsequent electron transfer. The value of −59 mV determined for the higher potential wave can be compared to that found for sperm-whale myoglobin ($E^{\circ\prime}$ 47 mV) using a platinum electrode (Brunori et al., 1971). In the latter case, the oxidized heme is coordinated by a histidine and a water molecule that is lost on reduction (Crutchley et al., 1985). The relative similarity in potential leads us to assign this wave to the high-spin heme. The wave at −400 mV compares well with that determined for cytochrome c_3 (−346 mV) by using similar experimental conditions, the latter possessing bishistidine-ligated hemes.

Addition of cyanide to the triheme cytochrome resulted in a shift of the higher potential wave to −263 mV, whereas the low potential wave was relatively unchanged ($E^{\circ\prime}$ −401 mV), and in this case both waves were reproducible in repeated scans. We attribute the former shift to cyanide binding to the high-spin pentacoordinate heme site. The average potentials found for the larger enzyme are therefore in line with the average from the c_3 protein (representing bishistidine coordinated hemes) and sperm whale myoglobin (representing monohistidine coordinated hemes).

Discussion

In this paper we have characterized both the coordination and redox chemistry of a large *c*-type cytochrome from *Desulfovibrio vulgaris* (Hildenborough). The similarities in pI, M_r, and $A_{ox}(280)/A_{red}(553)$ are in line with those determined for the cytochrome isolated previously by Higuchi et al. (1987). The only discrepancy between our characterization and prior work lies with the calculation of heme content, and it is likely that previous estimates were higher than expected as a result of a mistaken dry-weight measurement of protein mass. Such errors are not uncommon when using this method (Meyer et al., 1982), while in all other respects our data agree with those from the previous characterization of the protein. We therefore refer to this molecule as a triheme cytochrome. Triheme cytochromes have previously been identified in *Desulfuromonas acetoxidans* (Probst et al., 1977) and the mixed culture *Chloropseudomonas ethylica* (Gray et al., 1973; Gray 1977). Both possessed a low $E^{\circ\prime}$ that was similar to the potential for cytochromes c_3, suggesting hexacoordinate heme units with histidine ligation. Although these proteins have been termed cytochromes c_7, it has recently been suggested that the generic title of cytochromes c_3 might best describe both this and other c_3 and cc_3-type cytochromes (Pettigrew et al., 1987). A preliminary crystal structure of the low molecular weight *Desulfuromonas* cytochrome c_7 has been reported (Haser et al., 1979). The identification of distinct types of heme units in the triheme protein reported in this paper has been conclusively demonstrated by both EPR (high- and low-spin hemes) and electrochemistry, suggesting that the function of the protein in bacterial metabolism is distinct from that of the c_3 and c_7 group of proteins. The observation of both high- and low-spin hemes and the reactivity of the reduced protein with nitrite and hydroxylamine suggest comparison with the hexaheme cytochrome isolated from *D. desulfuricans* (Liu et al., 1981). The latter was recently described as a new type of nitrite reductase that was isolated following growth of the bacterium in a nitrate-enriched medium. There are, however, notable differences in reactivity and these shall be discussed in a later paper. The nature of the EPR spectra is also quite distinct. The *g* values determined for the triheme cytochrome

were typical of rhombic low-spin and high-spin heme environments (g_z 2.95, g_y 2.27, g_x 1.48 and g_z 6.0, respectively), whereas the data reported for the hexaheme cytochrome [low-spin heme, g_z 3.02, g_y 2.29, g_x 1.52; high-spin heme, g_z 9.26, g_{xy} 3.81; heme in an unidentified spin state, g 4.84] are unusual (Liu et al., 1981). In particular the high-spin features in the latter were attributed to a severe rhombic distortion of the heme environment.

The redox potentials of the heme units for the triheme cytochrome were in line with those expected for penta- and hexacoordination by comparison with hemes in c_3 and Mb. The similarity in $E^{\circ\prime}$ for the low potential waves, in comparing the native proteins and cyanide adducts, suggests that these centers might not readily bind exogenous ligand. This would implicate the pentacoordinate site as the origin of reduction chemistry following addition of substrate. Recently the hexaheme nitrite reductase isolated by Liu and Peck (Costa et al., 1989) has also been shown to possess a unique high-spin site.

Of additional interest is the observation of relatively clean electrochemical response with a glassy-carbon electrode in the absence of added redox promoter. Normally such direct measurements are extremely difficult owing to poor electron exchange between the protein and the electrode surface (Albery et al., 1981). Hill and co-workers have documented an array of pyridine- and thiol-based redox promoters that facilitate rapid electron transfer between electrodes and metalloproteins (Albery et al., 1981). Initial study was directed toward gold electrodes; however, later work described the direct electrochemistry of redox proteins at pyrolytic graphite electrodes (Armstrong et al., 1984, 1985). Such electrodes were home built. The commercial availability of glassy-carbon electrodes suggests that this electrode system may be of value for routine potential measurements on a variety of metalloproteins.

In this paper we have focused on the characterization of the redox and coordination chemistry of the triheme cytochrome, leaving the reactivity of the reduced protein with nitrogenous substrates to be discussed in detail elsewhere. As with any newly characterized cytochrome, and unfortunately with many well-characterized proteins, the precise physiological function is not clear. The mixture of penta- and hexacoordinate hemes is suggestive of a role involving enzymatic chemistry on a substrate molecule, the hexacoordinate hemes serving an electron-storage function (cf. cytochrome c_3) with substrate binding at the pentacoordinate site. Such considerations are necessarily speculative at this time.

Acknowledgments

We thank Russ Hille for running low-temperature ESR spectra and Jane Tolley in the Biochemical Instrument Center for carrying out the amino acid analyses.

Registry No. Fe, 7439-89-6; Heme, 14875-96-8; nitrite reductase, 9080-03-9; hydroxylamine, 7803-49-8; cytochrome *c*, 9007-43-6.

References

Albery, W. J., Eddowes, M. J., Hill, H. A. O., & Hillman, A. R. (1981) *J. Am. Chem. Soc. 103*, 3904–3910.

Ambler, R. P., Bruschi, M., & LeGall, J. (1971) in *Recent Advances in Microbiology*, 10th Int. Congr. Microbiol., Mexico City, pp 25–34.

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland Publishing Company, London.

Armstrong, F. A., Hill, H. A. O., Oliver, B. N., Walton, N. J. (1984) *J. Am. Chem. Soc. 106*, 921–923.

Armstrong, F. A., Hill, H. A. O., Oliver, B. N., & Whitford, D. (1985) *J. Am. Chem. Soc. 107*, 1473–1476.

Bartsch, R. G. (1971) *Methods Enzymol. 23*, 644–649.

Bartsch, R. G. (1978) *Methods Enzymol. 53*, 329–340.

Blumberg, W. E., & Peisach, J. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, B., Yonetoni, T., Mildvan, A. S., Eds.) Vol. 2, pp 215–229, Academic Press, New York.

Brunori, M., Saggese, U., Rotilio, G. C., Antonini, E., & Wyman, J. (1971) *Biochemistry 10*, 1604–1609.

Bruschi, M. (1979) *Biochem. Biophys. Res. Commun. 91*, 623–8.

Costa, C., Moura, J. G., Wang, Y., Liu, M. Y., LeGall, J., Huynh, B. H., Moura, I. (1989) *J. Inorg. Biochem.* (Abstracts from the Fourth International Conference in Bioinorganic Chemistry) *36*, 176 (Abstract No. B027).

Crutchley, R. J., Ellis, W. R., & Gray, H. B. (1985) *J. Am. Chem. Soc. 107*, 5002.

DerVartanian, D. V., & LeGall, J. (1971) *Biochim. Biophys. Acta 243*, 53–65.

DerVartanian, D. V., & LeGall, J. (1974) *Biochim. Biophys. Acta 346*, 79–99.

Dobson, C. M., Hoyle, N. J., Geraldes, C. F., Bruschi, M., LeGall, J., Wright, P. E., & Williams, R. J. P. (1974) *Nature 249*, 425–429.

Drucker, H., Campbell, L. L., & Woody, R. W. (1970) *Biochemistry 9*, 1519–1527.

Falk, J. E. (1964) in *Porphyrins and Metalloporphyrins*, p 240, Elsevier, Amsterdam.

Gray, B. H. (1977) *Int. J. Syst. Bacteriol. 27*, 168.

Gray, B. H., Fowler, C. F., Nugent, N. A., Rigopoulos, N., & Fuller, R. C. (1973) *Int. J. Syst. Bacteriol. 23*, 256–264.

Guerlesquin, F., Bovier-Lapierre, G., & Bruschi, M. (1982) *Biochem. Biophys. Res. Commun. 105*, 530–8.

Guerlesquin, F., Bruschi, M., & Wuthrich, K. (1985) *Biochim. Biophys. Acta 830*, 296–303.

Haser, R., Payan, F., Bache, R., Brusci, M., & LeGall, J. (1979a) *J. Mol. Biol. 130*, 97–98.

Haser, R., Pierrot, M., Frey, M., Payan, F., Astier, J. P., Bruschi, M., & LeGall, J. (1979b) *Nature (London) 282*, 806.

Higuchi, Y., Bando, S., Kusunoki, M., Matsuura, Y., Yasuoka, N., Kakudo, M., Yamanaka, T., Yagi, T., & Inokuchi, H. (1981) *J. Biochem. (Tokyo) 89*, 1659.

Higuchi, Y., Inaka, K., Yasuoka, N., Yagi, T. (1987) *Biochim. Biophys. Acta 911*, 341–348.

Hille, R., Olson, J. S., & Palmer, G. (1979) *J. Biol. Chem. 254*, 12110–12120.

Huynh, B. H., Kang, L., DerVartanian, D. V., Peck, H. D., & LeGall, J. (1984) *J. Biol. Chem. 259*, 15373–15376.

Jones, H. E. (1971) *Arch. Mikrobiol. 80*, 78–86.

Kon, H. (1968) *J. Biol. Chem. 243*, 4350–4357.

Kon, H., Kataoka, N. (1969) *Biochemistry 8*, 4757–4762.

Kuwana, T. (1977) in *Electrochemical Studies of Biological Systems* (Sawyer, D. T., Ed.) ACS Symposium Series, No. 38, American Chemical Society, Washington, DC.

LeGall, J., & Postgate, J. R. (1973) *Adv. Microb. Physiol. 10*, 81–133.

LeGall, J., Bruschi-Heriand, M., & DerVartanian, D. V. (1971) *Biochim. Biophys. Acta 234*, 499–512.

Liu, M.-C., & Peck, H. D. (1981) *J. Biol. Chem. 256*, 13159–13164.

Liu, M.-C., DerVartanian, D. V., & Peck, H. D. (1980) *Biochem. Biophys. Res. Commun. 96*, 278–285.

Meyer, T. E., & Kamen, M. D. (1982) *Adv. Protein Chem. 35*, 105-212.
Niki, K., Yagi, T., & Inokuchi, H. (1982) *Adv. Chem. Ser. 201*, 199-218.
Niki, K., Kawasaki, Y., Nishimura, N., Higuchi, Y., Yasuoka, N., & Kakudo, M. (1984) *J. Electroanal. Chem. 168*, 275-86.
Palmer, G. (1979) *The Porphyrins* (Dolphin, D., Ed.) Vol IV, pp 313-353, Academic Press, New York.
Palmer, G. (1983) in *Iron Porphyrins* (Lever, A. B. P., Gray, H. B., Eds.) Physical Bioinorganic Chemistry Series, Vol. 2, Chapter 2, pp 43-88, Benjamin Cummings, Menlo Park, CA.
Peck, H. D., & LeGall, J. (1982) *Philos. Trans. R. Soc. London, B 298*, 443-66.
Pettigrew, G. W., & Moore, G. R. (1987) *Cytochromes c (Biological Aspects)* p 26, Springer-Verlag, New York.
Pierrot, M., Haser, R., Frey, M., Payan, F., & Astier, J.-P. (1982) *J. Biol. Chem. 257*, 14341-14348.
Postgate, J. R. (1954) *Biochem. J. 56*, xi.
Postgate, J. R. (1984) *The Sulphate-Reducing Bacteria*, Chapter 5, Cambridge University Press, New York.
Probst, I., Bruschi, M., Pfennig, N., & LeGall, J. (1977) *Biochim. Biophys. Acta 460*, 58-64.
Tan, J., & Cowan, J. A. (1989) (unpublished results).
Xavier, A. V., Moura, J. J. A., LeGall, J., & DerVartanian, D. V. (1979) *Biochimie 61*, 689-95.
Yagi, T. (1969) *J. Biochem. (Tokyo) 66*, 473-8.
Yagi, T. (1979) *Biochim. Biophys. Acta 548*, 96-105.
Yagi, T., Inokuchi, H., & Kimura, K. (1983) *Acc. Chem. Res. 16*, 2-7.
Yonetani, T., Hamamoto, H., Erman, J. E., Leigh, J. S., & Reed, G. H. (1972) *J. Biol. Chem. 247*, 2447-2455.

Temperature-Dependent Conformational Changes in the Bacteriopheophytins of *Rhodobacter sphaeroides* Reaction Centers†

Jeffrey M. Peloquin,‡ Carol A. Violette,§ Harry A. Frank,§ and David F. Bocian*,‡
Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, and Department of Chemistry, University of Connecticut, Storrs, Connecticut 06269
Received October 10, 1989; Revised Manuscript Received January 2, 1990

ABSTRACT: Resonance Raman (RR) spectra are reported for the photosynthetic reaction center (RC) protein from *Rhodobacter sphaeroides* 2.4.1. The spectra were obtained with a variety of excitation wavelengths, spanning the UV, violet, and yellow-green regions of the absorption spectrum, and at a number of temperatures ranging from 30 to 270 K. The RR data indicate that the frequencies of certain vibrational modes of the bacteriochlorin pigments in the RC shift with temperature. These shifts are reversible and do not depend on external factors such as solvent or detergent. The acetyl carbonyl bands exhibit the largest shifts with temperature. These shifts are attributed to thermal effects involving the torsional vibrations of the acetyl groups of several (or all) of the bacteriochlorins rather than to specific pigment-protein interactions. The frequency of the structure-sensitive skeletal mode near 1610 cm^{-1} of one of the two bacteriopheophytins (BPhs) in the RC is also sensitive to temperature. In contrast, no temperature sensitivity is observed for the analogous modes of the bacteriochlorophylls or other BPhs. Over the range 160-100 K, the skeletal mode of the BPh upshifts by $\sim$4 cm^{-1}. This upshift is attributed to a flattening of the macrocycle at low temperatures. It is suggested that the BPh active in the electron-transfer process is the pigment whose structure is temperature dependent. It is further suggested that such structural changes could be responsible in part for the temperature dependence of the electron-transfer rates in photosynthetic RCs.

The X-ray crystal structures recently reported for the reaction center (RC)[1] proteins from *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* elegantly elucidate the macromolecular arrangement of the pigments in the protein matrix (Deisenhofer et al., 1984, 1985; Michel et al., 1986; Chang et al., 1986; Allen et al., 1987, 1988). These data delineate the distances between and relative orientations of the bacteriochlorophyll (BChl) and bacteriopheophytin (BPh) pigments which constitute the active sites for the initial steps of light-induced charge separation (Okamura et al., 1982). They also provide insight into the structure of the individual chromophores and the nature of the interactions between these species and the amino acid residues of the protein. Spectroscopic techniques provide other means for probing the structure of RCs [for reviews, see Parson (1982), Hoff (1984), Kirmaier and Holten (1987), and Friesner and Won (1989)]. In addition, these techniques provide information regarding the time scales of the electron-transfer events. Along these lines, optical absorption studies have shown that the initial light-induced transfer of an electron from the special pair donor $BChl_2$ to the primary acceptor BPh_L occurs in $\sim$3 ps at room tem-

† This work was supported by Grants GM-39781 (D.F.B) and GM-30353 (H.A.F.) from the National Institute of General Medical Sciences and by Grant 88-37130-3938 (H.A.F.) from the Competitive Research Office of the U.S. Department of Agriculture.
‡ Carnegie Mellon University.
§ University of Connecticut.

[1] Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; C_aC_m and C_bC_b, stretching modes of the carbon-carbon bonds in the methine bridges and β positions of the pyrrole rings, respectively, of the bacteriochlorin macrocycle; LDAO, lauryldimethylamine *N*-oxide; L and M, light and medium polypeptides, respectively, of the reaction center; Q_A, quinone; RR, resonance Raman; RC, reaction center; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/90/0429-4892$02.50/0