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Resonance Raman spectroscopy of cytochrome bc_1 complexes from *Rhodospirillum rubrum*: initial characterization and reductive titrations

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Resonance Raman spectra of bc_1 complexes from *Rhodospirillum rubrum* have been obtained. Various resonance conditions and the stoichiometric redox titration of the complex were used to isolate and identify the contributions of the heme c_1 and heme b active sites to the observed spectra. The complex was found to partially photoreduce when exposed to laser excitation.

Introduction

The major pathway for electron flow in purple photosynthetic bacteria involves a multi-subunit protein complex comprised of cytochromes b and c_1 and a high-potential Fe_2S_2 center. This complex catalyzes the net oxidation of ubiquinol by soluble cytochrome c_2 via a series of intracomplex electron transfer reactions. These electron transfer reactions are energetically linked to proton translocation across the membrane and the eventual synthesis of ATP from ADP and inorganic phosphate [1–3]. Thus, the study of electron transport through this cytochrome bc_1 complex is central to an understanding of cellular bioenergetics.

The cytochrome bc_1 complexes isolated from photosynthetic purple non-sulfur bacteria are particularly useful for studying biological mechanisms for electron transfer. They have a relatively simple three or four peptide subunit composition compared with the functionally analogous mitochondrial bc_1 complex, which contains nine to eleven polypeptides [4]. Amino-acid sequences are available for all three chromophore-containing peptides of one bacterial complex, and their electron transfer kinetics have been investigated by transient absorption spectroscopy [7,8].

Recently, a highly stable cytochrome bc_1 complex, containing little or no bacteriochlorophyll, has been purified from *Rhodospirillum rubrum* in concentrations required for resonance Raman studies [9]. This complex contains only three peptide subunits: cytochrome b (35 kDa), cytochrome c_1 (31 kDa) and the Rieske iron-sulfur protein (RISP) (22.4 kDa). The complex catalyzes electron transfer from quinol to cytochrome c (turnover number = 75 s^{-1}) and is inhibited by antimycin A, myxothiazol and quinone analogs. In addition to the stability and simple peptide subunit composition of this complex, it possesses a high-affinity binding site for its electron accepting substrate, cytochrome c_2 [10]. These features offer significant advantages for studying the structures and dynamics of the heme active sites and their protein environments.

In this study, resonance Raman spectroscopy (RRS) has been employed to characterize further the heme electron transfer sites of the complex in various stages of reduction. This is possible due to the differences in midpoint redox potentials among the three hemes present. The measured E_m values (pH 7.4) are: cytochrome c_1, 320 mV, cytochrome b_h −33 mV; and cytochrome b_l, −90 mV [9]. The distinctive redox potentials of the hemes allow for the specific reduction of the higher potential sites by either the addition of stoichiometric amounts of a strong reductant or the use of redox mediators having appropriate reduction potentials. Further, because the Raman intensity of normal modes of vibration is greatly enhanced when the excitation frequency of the laser light source is tuned to a strongly allowed electronic transition [11,12], such as the Soret or Q bands observed in hemes, the differences in the

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absorption bands of the *b*- and *c*-type hemes present may be exploited in differentiating their relative contributions to the Raman spectrum. Here we present preliminary data from redox titrations and resonance enhancement experiments on *R. rubrum* cytochrome bc_1 complex.

Materials and Methods

Wild-type (strain S1) *R. rubrum* cells were grown photosynthetically and chromatophores were prepared as described previously [13]. The *R. rubrum* cytochrome bc_1 complex was solubilized and purified using a modification [9] of the procedure of Ljungdahl et al. [14]. Column fractions containing the purified complex were pooled, glycerol was added to a final concentration of 5% (v/v), and the samples were stored at 77 K until needed.

Raman samples were prepared by concentrating the purified complex using Amicon Centricon 30 microconcentrator to a final concentration of about 100 μM in cytochrome c_1. Samples were then placed in an anaerobic optical cell to which one cytochrome c_1 equivalent of potassium ferricyanide was added. Samples were rigorously degassed, placed under a slightly positive N_2 atmosphere and allowed to incubate for 1 h to obtain the fully oxidized complex. A gas-tight syringe containing a sodium dithionite solution, which had been standardized using an equal volume of 100 μM equine cytochrome *c* (Type VI, Sigma), was then fitted to the optical cell. Single reduction equivalents were added to the sample and the level of reduction monitored by ultraviolet/visible absorption using an HP8452 diode array spectrophotometer interfaced to an Amstrad 512PC computer. Raman spectra corresponding to the fully oxidized and two, three- and four-electron reduced equilibrium species were then obtained using a Molectron UV-24 nitrogen-pumped dye laser (tunable range, 380 to 830 nm) using a 160 degree backscattering collection geometry. Laser energy density was varied from 10–100 mJ/cm^{-1} at the sample using either cylindrical or spherical focusing optics. The scattered light was collected in a Spex 1403 double monochromator with a water-cooled photomultiplier tube (Hamamatsu R928). The spectral band pass was 6–10 cm^{-1} for all spectra. Data were stored on a Spex Industries DM3000 XT system and processed using programs developed in this laboratory. All spectra are the unsmoothed average of three to five scans.

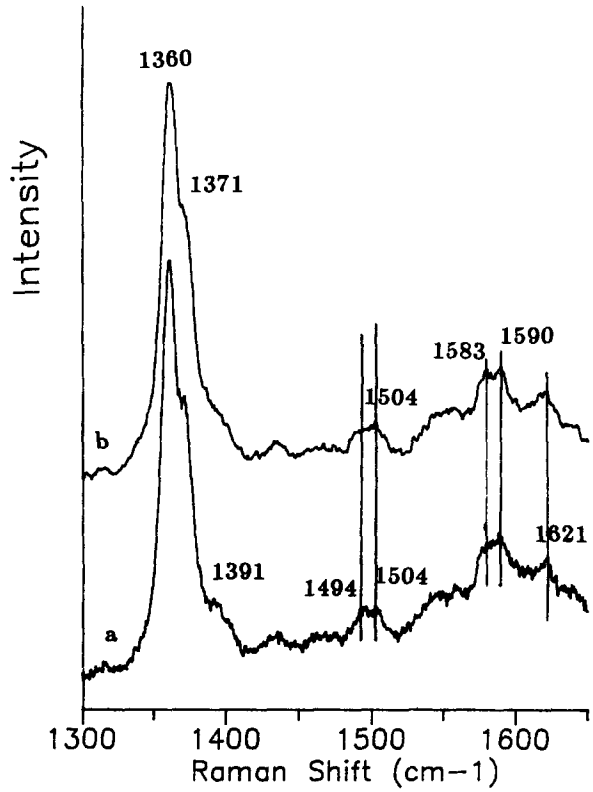

Fig. 1. High-frequency resonance Raman spectra of the cytochrome bc_1 complex from *R. rubrum* (a) as prepared and (b) sodium ascorbate reduced. All samples were approx. 50 μM bc_1 complex in 35 mM Mops buffer (pH 7.4), 1 mM $MgSO_4$, 0.1 mg dodecylmaltoside per ml and 5% (v/v) glycerol. Spectra are the unsmoothed sum of three scans with an average laser power of approx. 5 mW at 15 Hz using 406 nm excitation. The spectral bandpass was 5–8 cm^{-1} for all spectra.

Results

Soret excitation (406–430 nm) preferentially enhances scattering from polarized and depolarized heme bands corresponding primarily to in-plane porphyrin ring vibrations [15]. These skeletal modes are numbered based on calculated and assigned normal-mode frequen-

TABLE I

Raman shift frequencies (in wavenumbers) for cytochrome c, cytochrome b_5, and cytochrome bc_1

	Equine cyt*c*II	Equine cyt*c*III	cytb_5II	cytb_5III	bc_1 c_1III *b*III	bc_1 c_1II *b*III	bc_1 c_1II *b*II
ν_4	1362	1374	1362	1375	1372	1362 1371	1360 1362
ν_2	1590	1580	1586	1581	1581	1590 1583	1590 1582
ν_3	1493	1502	1494	1507	1505	1491 1507	1491 1493
ν_{10}	1622	1636	1617	1619	1639/1621	1621 1621	1621 1617
ν_{19}	1585	1582	1586	1587			1589 1585
ν_{11}	1547						1542 1536

cies for Ni(II) octaethylporphyrin (NiOEP) 16,17]. These bands are most prominent in the high-frequency (1100–1700 cm^{-1}) region of the spectrum and are highly reliable indicators of the heme environment, especially the oxidation and spin-state of the iron ion [18,19].

The locations of these modes for the *R. rubrum* cytochrome bc_1 in various stages of heme reduction are summarized in Table I. Fig. 1 shows the high-frequency spectra obtained with 406 nm excitation of the complex as isolated and the sodium ascorbate reduced cytochrome bc_1 complex. Sodium ascorbate is a relatively mild reductant and should only reduce heme components having E'_m values greater than approx. +50 mV. Raman spectra in Fig. 1 are consistent with reduction of only heme c_1, as evidenced by ν_4 at 1360 cm^{-1}. The presence of oxidized heme *b* is observed by ν_4 intensity at 1371 cm^{-1}. Absorption spectra (not shown) corroborate that ascorbate reduces only the *c*-type heme. The close correspondence between the two spectra indicates

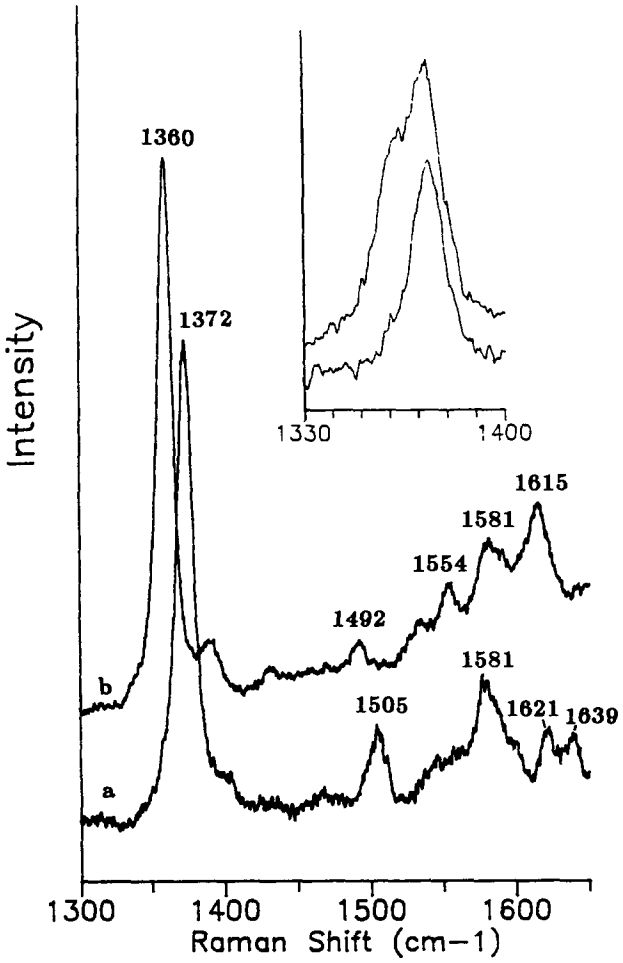

Fig. 2. High-frequency resonance Raman spectra of the cytochrome bc_1 complex (a) potassium ferricyanide oxidized (approx. 10 mM ferricyanide), and (b) sodium hydrosulfite reduced, using 406 nm excitation. All other conditions were the same as in Fig. 1. The inset depicts the behavior of ν_4 for the ferricyanide oxidized sample under high-power (i) and low-power (ii) laser excitation (see text for details).

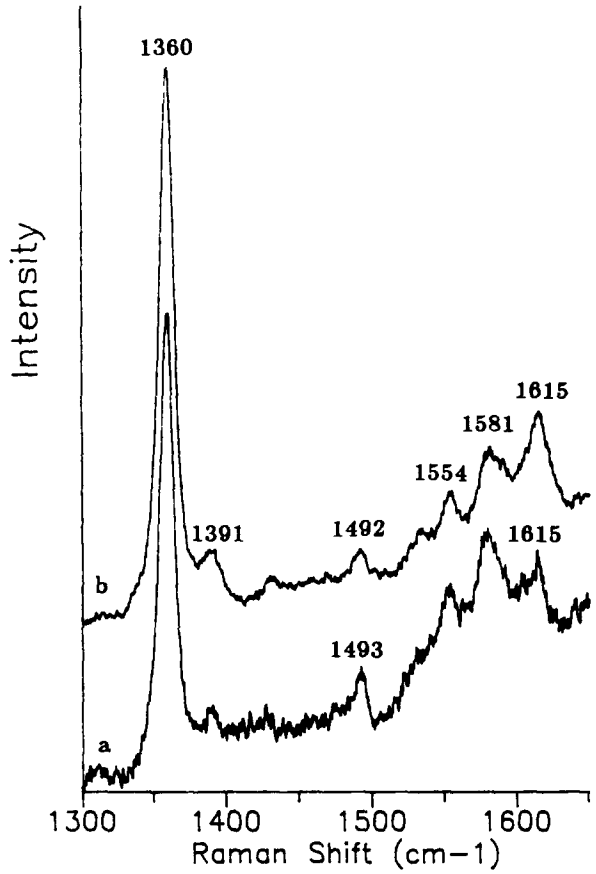

Fig. 3. High-frequency resonance Raman spectra of sodium hydrosulfite fully reduced cytochrome bc_1 complex using (a) 430 nm excitation and (b) 406 nm excitation. All other conditions were the same as in Fig. 1.

that, in the freshly prepared complex, only the heme c_1 is reduced.

The effects of two other redox reagents, dithionite and ferricyanide, upon high-frequency Raman spectrum of the bc_1 complex are shown in Fig. 2. Addition of ferricyanide ($E'_m = +430$ mV) to the solution completely oxidizes all heme sites in the bc_1-complex. Raman bands ν_4 (1372 cm^{-1}), ν_3 (1505 cm^{-1}) and ν_2 (1581 cm^{-1}) appear (Fig. 2a) at frequencies that are characteristic of low-spin ferric hemes. Small additions of sodium dithionite produce changes in the Raman spectrum indicative of formation of low-spin ferrous hemes (Fig. 2b).

The fully oxidized complex displayed a propensity for photoreduction even in the presence of ferricyanide. Photoreduction of a fraction of the heme sites was evident in the dependence of ν_4 upon laser intensity (see the inset of Fig. 2). At high laser powers, a lower frequency shoulder (about 1360 cm^{-1}) is clearly evident, suggesting that one or more of the hemes in the complex are photoreducible. In the absence of ferricyanide, high power 440 nm excitation yields a spectrum very similar to that of the ascorbate reduced complex. In Fig. 3, the effects of changes in the excita-

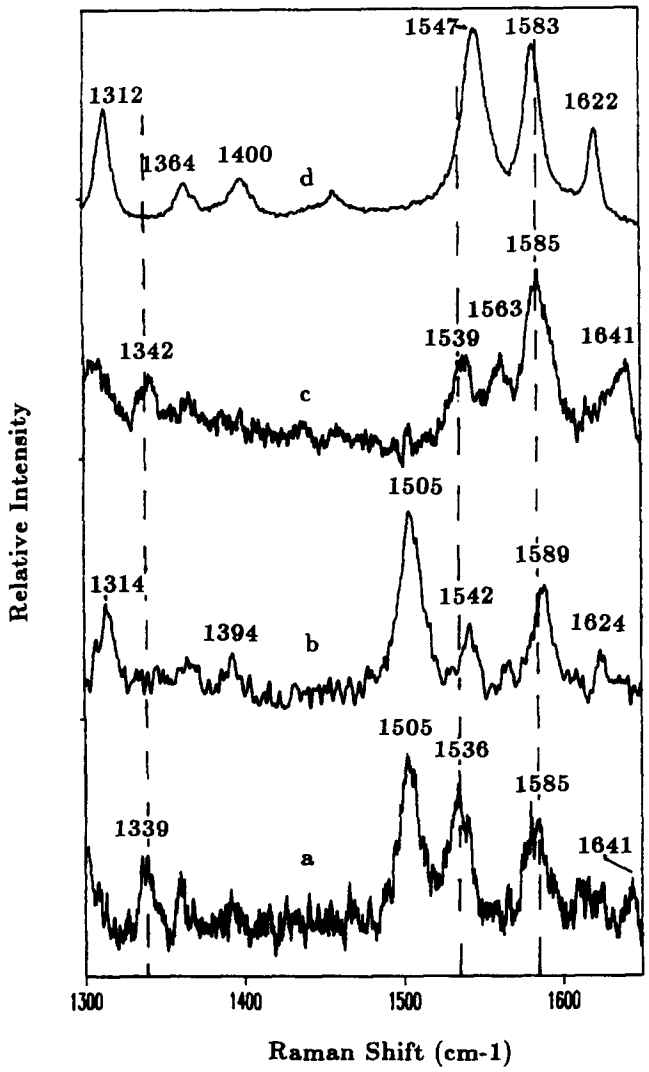

Fig. 4. High-frequency resonance Raman spectra of sodium hydrosulfite fully reduced cytochrome bc_1 complex using (a) 560 nm excitation and (b) 550 nm excitation. All other conditions were the same as in Fig. 1, except the samples were about 200 μM in bc_1 complex. (c) Raman spectrum of reduced mitochondrial cytochrome b_5. (d) Raman spectrum of reduced equine cytochrome *c*. Raman spectra of reduced cytochrome *c* and b_5 were obtained under conditions similar to those used for the cytochrome bc_1 spectra in (a) and (b). In spectra (a) and (b) the baselines have been adjusted to facilitate presentation.

tion frequency within the Soret absorption band of the fully reduced cytochrome bc_1 complex are demonstrated. Excitations at 406 nm (Fig. 3b) preferentially enhance reduced heme c_1, while excitations at 430 nm (Fig. 3a) yield increased scattering from the reduced heme *b* chromophores. Evidence of this may be observed by changes in relative intensity of Raman bands appearing at 1581 cm^{-1} and 1615 cm^{-1}.

Similar effects are observed using Q band (520–560 nm) excitation (Fig. 4). Excitation at 550 nm enhances the reduced heme *c* chromophore, while 560 nm excitation leads to enhancement of the protoheme chromophores of cytochrome *b*. Moreover,the Q band RR spectra are generally dominated by non-totally symmetric vibrational modes, which are responsible for vibronic mixing of the Q_0 and B absorption bands [11]. Thus Q band excitation allows for further separation of the individual heme contributions to the Raman spectrum. For example, using 550 nm excitation, ν_{19} appears at 1589 cm^{-1} (Fig. 4b), but shifts to 1585 cm^{-1} when enhanced at 560 nm (Fig. 4a). Also, ν_{10}, which can be readily identified with 550 nm excitation, is obscured in the Raman spectrum obtained with 560 nm excitation. The band appearing at 1504 cm^{-1} is prominent using either excitation. However, its relative intensity is clearly sensitive to the excitation wavelength. Finally, a band observed at 1542 cm^{-1} (ν_{11}) in Fig. 4b is consistent with a *c*-type cytochrome, while in Fig. 4a this band shifts to 1536 cm^{-1}. Attempts to obtain resonance Raman spectra of the fully oxidized cytochrome bc_1 complex using Q band excitation wavelengths were unsuccessful due to the relatively low optical absorbance of the heme chromophores in this region of the absorption spectrum.

Figs. 5 and 6 summarize the results of studies in which reductant was added stoichiometrically to fully oxidized samples. Electrochemical redox titrations of the cytochrome bc_1 complex indicate that the cytochrome c_1 component has a midpoint redox potential of

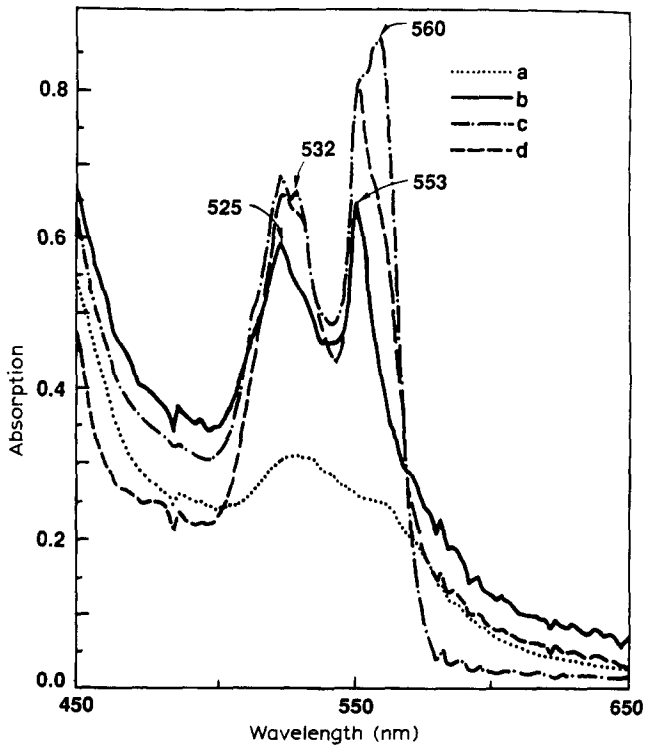

Fig. 5. Ultraviolet/visible absorption spectra of the cytochrome bc_1 complex which has been stoichiometrically reduced with sodium hydrosulfite. (a) Zero electron equivalent (fully oxidized). (b) One electron equivalent (only heme c_1 reduced). (c) Three electron equivalents (heme c_1 and heme *b* reduced). (d) Four electron equivalents (fully reduced).

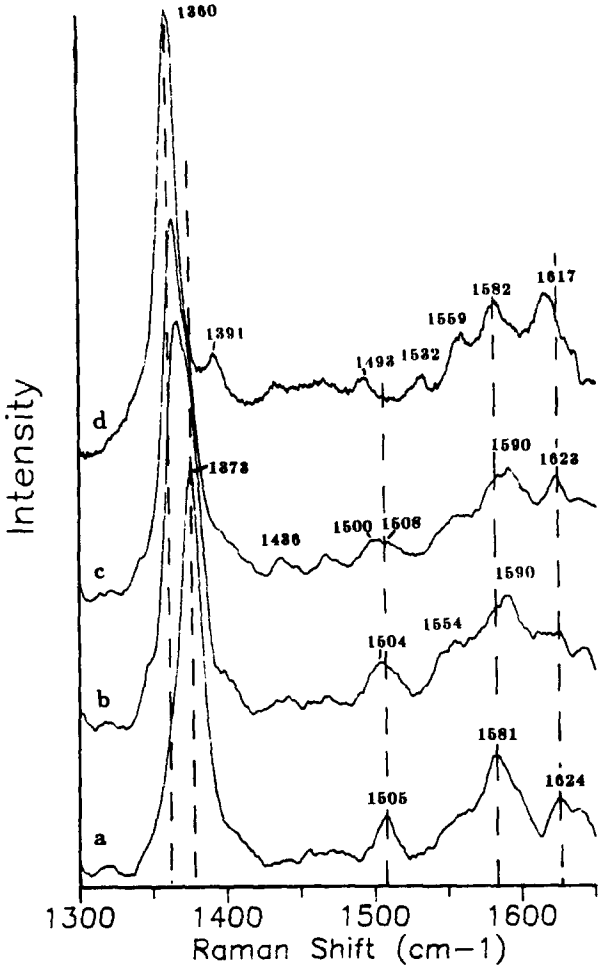

Fig. 6. Sodium hydrosulfite redox titration of the cytochrome bc_1 complex from *R. rubrum*, which had been completely oxidized with a stoichiometric amount of potassium ferricyanide. (a) Zero electron equivalents. (b) One-electron equivalent. (c) Three-electron equivalents. (d) Four-electron equivalents. All spectra taken using 410 nm laser excitation. Sample and spectral parameters were the same as in Fig. 1.

320 mV [9], slightly greater than that observed in redox titrations of the complex in situ using *R. rubrum* chromatophore membranes at pH 7.0 [20]. Preliminary potentiometric titrations (Robertson, D.E., Güner, S. and Knaff, D.B., unpublished data) indicate that the Rieske iron-sulfur protein ($E'_m = 310$ mV) is approximately isopotential to cytochrome c_1 in the isolated *R. rubrum* complex. However, the behavior of the Q-band absorption of bc_1 complexes during stoichiometric titrations with sodium dithionite indicates that the initial electron added to the complex resides on the c_1 heme. Furthermore, addition of the second electron initially reduces the *b*-type heme sites. This is followed by a kinetically slow ($\tau_{1/2} \approx 2$ h) oxidation of the cytochrome *b*.

Fig. 6 consists of resonance Raman spectra from the partially reduced bc_1 complexes. The absorption spectra of these species (shown in Fig. 5) are consistent with a fully oxidized, heme *c* reduced, partial heme *b* reduction, and the fully reduced complex, respectively. For these initial Raman experiments, a single laser excitation wavelength at 410 nm was employed. The behavior of ν_4 (Fig. 6) is indicative of the progressive reduction of the hemes. The addition of one electron equivalent of dithionite to the complex (Fig. 6b) produces a mode at 1590 cm^{-1} consistent with ν_2 observed for most ferrous *c*-type cytochromes. Further additions of dithionite shift ν_2 to 1582 cm^{-1}, with a shoulder at 1590 cm^{-1} (Fig. 6d). A similar shift occurs in the position of ν_{10} for the complex. In Fig. 6b, ν_{10} at 1623 cm^{-1} is consistent with that found for other *c*-type cytochromes; while in Fig. 6d, ν_{10} is observed at 1617 cm^{-1} (with a shoulder at 1623 cm^{-1}), a position similar to that observed for cytochrome b_5.

Discussion

Recent studies have provided substantial molecular details for some of the protein complexes involved in the initial phases of light transduction in photosynthetic bacterial membranes. For instance, high-resolution crystallographic data are now available for the reaction centers (RC) from *Rhodopseudomonas viridis* [21] and *Rhodobacter sphaeroides* [22]. These important structural studies have revealed the molecular framework responsible for the initial electron transfer reaction in bacterial photosynthesis. No crystal structures exist, however, for the cytochrome bc_1 complexes of photosynthetic bacteria. These complexes are responsible for secondary electron and proton transfers that lead to the reduction of the photooxidized reaction centers via cytochrome c_2 [1–3,8]. Thus, resonance Raman data for the cytochrome bc_1 complexes are germane to the determination of the roles played by the structure and dynamics of their heme active sites in photosynthetic electron transfer.

Resonance Raman spectra for the cytochrome bc_1 complex isolated from the photosynthetic bacterium, *R. rubrum* have been obtained in successive states of reduction for the heme *b* and heme *c* components, and resonance Raman enhancement techniques have been employed to further separate the relative contributions of the different heme chromophores to the spectra.

Fully oxidized and fully reduced complexes

Spectra of the fully oxidized or reduced heme complex were obtained by addition of excess ferricyanide or dithionite, respectively. Excess ferricyanide was required to maintain complete oxidation of the hemes due to photoreduction of at least one of the hemes within the complex during the course of the scattering experiments. Heme photoreduction is quite evident in spectra of the fully oxidized bc_1 complexes obtained at moderate to high laser powers. Similar phenomena have

been observed in other membrane bound protein complexes, such as mitochondrial cytochrome oxidase [28,29] and chloroplast b_6f complexes (Hobbs and Ondrias, unpublished results). Photoreduction of bc_1 complexes is evidently a very efficient process, competing effectively with the rapid reoxidation of the complex by ferricyanide. Neither cytochrome b_5 nor cytochrome c exhibit any photoreduction under similar conditions (Hobbs and Ondrias, unpublished results). This clearly implies that the heme photoactivity in bc_1 complexes is a direct consequence of active site environment(s) within the complex.

Preliminary studies in our laboratory indicate that both B and Q-band excitation are effective in producing reduced heme c_1 within the approx. 10 ns laser pulsewidth. This strongly suggests that the heme c_1 itself is the locus of the photoactivity and that heme excited states accessible from both B and Q band optical transitions act as electron acceptors. The fact that photoreduction is apparent within 10 ns of excitation further suggests that the unidentified immediate electron donor must be close to the heme c_1 site.

Spectra of the fully oxidized complex (Fig. 2A) obtained using low power Soret excitations (406–430 nm) exhibit the polarized and Jahn-Teller activated depolarized modes typically enhanced via Franck-Condon scattering from hemes. The positions and relative intensities of these modes are consistent with all of the heme sites possessing a low-spin six-coordinate configuration. The oxidation state marker, ν_4, was observed at 1372 cm^{-1} and has a linewidth at half maximum approximately equal to 11 cm^{-1}, a value similar to the homogeneous linewidths obtained from proteins containing a single heme. Thus, the ν_4 bands for the ferric c_1 and ferric b hemes in the complex apparently have coincident positions and linewidths despite the large differences in their redox potentials and porphyrin peripheral substituents. This is somewhat surprising in view of the dependence of ν_4 upon metal → porphyrin ($d_\pi \rightarrow e_g(\pi^*)$) backbonding [12]. Another Raman band, ν_3, which has been identified as being sensitive to the iron spin-state and number of the axial ligands [25], is also relatively narrow and thus is consistent with similar ligand field strengths among the (six-coordinate, low-spin) oxidized hemes present. Raman bands appearing in the 1500–1700 cm^{-1} region of the spectrum are diagnostic of the iron spin state as well as the porphyrin core size [18]. In our results, these bands were broad as compared to ν_4 and thus represent more than one distinct heme configuration. These were not directly assignable to the b or c_1 hemes using Soret excitation wavelengths.

Raman spectra of the fully reduced complex obtained with Soret excitation (Fig. 2B) also give evidence for similar electronic ground states of the heme b and c_1 of the complex. Here again, the linewidths of ν_4 (1360 cm^{-1}) and ν_3 (1494 cm^{-1}) composite bands are relatively narrow, indicating that the ferrous hemes in the complex have similar metal-porphyrin and ligand field interactions. Varying excitation wavelengths within the Soret band produced some changes in the relative intensity of several core-size-sensitive bands (Fig. 3), but no obvious shifts were observed in vibrational modes that were specifically useful in assigning heme c_1 and heme b bands.

Resonance Raman scattering for excitations in resonance with heme Q-bands occurs via the Hertzberg–Teller mechanism. This preferentially enhanced heme modes of depolarized or anomalously polarized character [11]. Spectral isolation of the different hemes within the fully reduced complex proved more effective in the Q band spectral region of the chemically reduced species for several reasons. The reduced hemes have larger scattering cross-sections due to their greater extinction coefficients for both Q_{00} and Q_{01} transitions. Moreover, relative absorption differences between the two heme types are greater in the α band. Excitation at 550 nm preferentially enhances scattering from ferrous heme c_1 sites, while slightly redder excitation (560 nm) was used to maximally enhance the contributions of the ferrous b hemes to the composite spectrum. As points of comparison, spectra of equine cytochrome c and mitochondrial b_5 were obtained using similar excitations and sample conditions (Figs. 4d and 4c, respectively).

The sensitivity of the RRS spectra (Fig. 4) to excitation wavelength can be used to assign bands at 1589 cm^{-1} and 1542 cm^{-1} to ν_{19} and ν_{11} of heme c_1, respectively. With 560 nm excitation, these bands shift to 1585 cm^{-1} and 1536 cm^{-1}, respectively, and are assigned to be predominantly ν_{19} and ν_{11} of the ferrous heme b sites. Two porphyrin ring modes at 1304 cm^{-1} and 1339 cm^{-1} are also preferentially enhanced with excitation into the heme b α-band (560 nm). These modes are diagnostic for the presence of vinyl substituents.

Both ν_{11} and ν_{19} are quite sensitive to axial ligation and/or geometric distortions of the heme macrocycle [15,24]. Their positions in the bc_1 spectra are generally consistent with low-spin six-coordinate heme c_1 and heme b in the complex. The near coincidence of the positions of these modes for cytochrome b_5 and the heme b sites of the bc_1 complex strongly suggests that they possess identical axial ligation (His-His) and heme geometries (Ref. 3 and references therein). On the other hand, comparison of ν_{11} and ν_{19} for heme c_1 and equine cytochrome c reveals significantly lower frequencies for the heme c_1 site, even though the axial ligands of these species are presumed to be equivalent [5,6,30]. This indicates that the putative His-Met heme c_1 configuration may be perturbed from the rhombic symmetry observed for most cytochromes c [26].

The intense mode appearing at 1504 cm^{-1} (see Fig. 4) in the Q-band spectra is anomalous, but is probably not due to contaminants such as bacteriochlorphylls or carotenoids. These adventitious contaminants are not observed in the ultraviolet absorption spectrum or as background fluorescence in the Raman spectra. This band appears more intensely in the spectra obtained at 550 nm and thus may be associated with the c_1 heme component of the complex. No such band, however, is observed in the Raman spectra of either equine cytochrome *c* or mammalian cytochrome b_5. Further studies will address the assignment of this band in an isolated heme c_1 subunit.

Reductive titrations

Sequential reduction of the cytochrome bc_1 complexes proved to be an effective means of separating and identifying contributions from the *b* and c_1 heme sites to the Raman spectra using Soret band excitation. Since the redox potentials of the three heme sites are well separated, the stoichiometric addition of strong reductants ($E'_m > -300$ mV) allows for the preparation of equilibrium $C_1^{3+}b_h^{3+}b_L^{3+}$, $C_1^{2+}b_h^{3+}b_L^{3+}$, $C_1^{2+}b_h^{2+}b_L^{3+}$, and $C_1^{2+}b_h^{2+}b_L^{2+}$ complexes.

This, in turn, permits the direct assignment of several important Raman shift frequencies for reduced c_1 and the still oxidized *b* type hemes (see Table I). In general, these assignments are consistent with those observed for reduced equine cytochrome *c* and oxidized cytochrome b_5 (Table I). Further mode assignments were made following this general method while stoichiometrically reducing the complex.

As in the ascorbate reduced samples, addition of one cytochrome c_1 electron equivalent to the fully oxidized complex reduced only the higher potential cytochrome c_1 heme site. Several vibrational mode assignments for the heme *c*(FeII) and the heme *b*(FeIII) can be made from spectra of this species. In the spectra of the one-electron reduced complex, the contributions of the reduced heme c_1 dominate that of the oxidized *b* type hemes. This is clearly observed in the greater intensity of the 1360 cm^{-1} oxidation state band for the single heme c_1 versus that at 1373 cm^{-1}, which represents the two oxidized *b*-type hemes. By and large, the Raman modes assignable to cytochrome c_1 (ν_4, 1362; ν_2, 1590; ν_3, 1491 and ν_{10}, 1622 cm^{-1}) resemble those determined for equine cytochrome *c* (ν_4, 1362; ν_2, 1590; ν_3, 1493; and ν_{10}, 1622 cm^{-1}).

Comparison of the spectra of the equilibrium two-electron reduced complex with that of the four-electron, fully reduced complex allows assignment of vibrational frequencies associated with ν_2, ν_3 and ν_{10} of the ferrous *b*-type hemes. The positions of these porphyrin modes compare well with those reported for ferrocytochrome b_5, corroborating the behavior of ν_{19} and ν_{11} observed with Q-band excitation. In particular, ν_3 (1493 cm^{-1}), ν_{10} (1617 cm^{-1}) and ν_{19} (1585 cm^{-1}) determined in this study for the *b*-type hemes of the bc_1 complex are virtually identical with those found for mitochondrial cytochrome b_5 and thus reflect strong similarities in porphyrin core-size and ligand field strength for these species.

Local environments of hemes

It is possible to exploit the spectral differences among the hemes in bc_1 complexes to obtain insight into their structures and environments. Selective resonance enhancement and sequential stoichiometric reduction of the complex are particularly useful for isolating the spectra of the *c* and *b* type hemes. Using these methods, it is possible to assign most of the high-frequency Raman bands for these redox sites in the *R. rubrum* cytochrome bc_1 complex. In general, Raman modes observed for the hemes *b* and c_1 of the complex are similar to those reported for isolated mammalian cytochrome b_5 and cytochrome *c*. In particular, ν_4, the Raman band most associated with electron density of the porphyrin π^* molecular orbital, is narrow for both the fully oxidized and fully reduced complex. This finding is consistent with similar interaction between the d_π orbital of the heme iron and the porphyrin $e_g(\pi^*)$ orbitals in the ferric *b*- and *c*-type as well as the ferrous *b*- and *c*-type hemes.

The core-size sensitive Raman bands (1500–1700 cm^{-1}), however, are broad (> 20 cm^{-1}) and suggest that different heme environments exist among the active sites. This result is plausible in view of the differences in the distal axial ligation state and peripheral substituents of the two heme types. These variances in core size and porphyrin bond strengths are consistent with those observed between equine cytochrome *c* (which has a His/Met axial ligand configuration and covalent linkages to the apoprotein at two adjacent pyrrole rings) and mammalian cytochrome b_5 (which has a bis-histidine axial ligand configuration and two free pyrrole vinyls). Thus, Raman spectra using B band excitation alone would imply that there are few significant differences in the low-spin *b* and *c* type hemes of the bc_1 complex as compared to other low-spin *b* and *c* type hemes. It is clear, however, from Raman excitations within the Q_{00} absorption bands for the heme *b* (560 nm) and heme *c* (550 nm) chromophores that the heme sites of the complex differ from those of structurally related *b*- and *c*-type heme proteins. Moreover, since Q band Raman excitations enhance primarily antisymmetric A_{2g}, B_{1g} and B_{2g} vibrational modes in D_{4h} point group symmetry, it is possible that perturbations to the heme c_1 environment are of similar symmetry. For instance, ν_{11} (B_{1g}) and ν_{19} (A_{2g}) observed, using 550 nm laser excitations, at 1542 and 1589 cm^{-1}, respectively, for cytochrome c_1 and differ from the values obtained for equine cytochrome *c* (ν_{11}, 1547 cm^{-1} and

ν_{19}, 1585 cm^{-1}). Although these modes have been shown to be influenced by heme ligation state and core size, it is clear from the absence of shifts in the core-size sensitive A_{1g} vibrational modes, ν_2 and ν_3 (see Table I), that core-size is not the determinative factor in these differences. More likely, ν_{11} and ν_{19} are affected by peripheral heme–protein interactions. In particular, ν_{11} has been shown to be sensitive to the conformation of vinyl pyrrole substituents, and their planarity [26], relative to the heme plane, could easily be directed through Van der Waal's contacts with individual peptides of the protein. In the cytochrome bc_1 complex of *R. rubrum*, we observe shifts in ν_{11} to lower frequency for both the *b*- and *c*-type of the complex. These shifts to lower vibrational energy may be due to out-of-plane distortion of the heme substituents and thus loss of π-bonding with the tetrapyrrole ring.

Conclusions

In this study, it is evident that stoichiometric redox titrations allow preparation of partially reduced forms of cytochrome bc_1 complexes. These stages compare well with those obtained using redox mediators and thus there appear to be no obvious reductant dependent effects. The isolated cytochrome bc_1 complex is quite stable during the course of Raman experiments. Resonance Raman spectra of these species obtained with a variety of excitation wavelengths allow for the deconvolution of heme *c* and heme *b* contributions to the spectra. The vibrational properties of the heme chromophores provide some initial insights into their local environments within the complex. Future studies will be directed at separating the heme *b* contributions to the Raman spectra as well as inhibitor binding effects.

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