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EPR AND OPTICAL SPECTROSCOPIC PROPERITES OF THE ELECTRON CARRIER INTERMEDIATE BETWEEN THE REACTION CENTER BACTERIOCHLOROPHYLLS AND THE PRIMARY ACCEPTOR IN *CHROMATIUM VINOSUM*

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SUMMARY

1. A reaction center-cytochrome *c* complex has been isolated from *Chromatium vinosum* which is capable of normal photochemistry and light-activated rapid cytochrome c_{553} and c_{555} oxidation, but which has no antenna bacteriochlorophyll. As is found in whole cells, ferrocytochrome c_{553} is oxidized irreversibly in milliseconds by light at 7 K.

2. Room temperature redox potentiometry in combination with EPR analysis at 7 K, of cytochrome c_{553} and the reaction center bacteriochlorophyll dimer $(BChl)_2$ absorbing at 883 nm yields identical results to those previously reported using optical analytical techniques at 77 K. It shows directly that two cytochrome c_{553} hemes are are equivalent with respect to the light induced $(BChl)_2^+$. At 7 K, only one heme can be rapidly oxidized in the light, commensurate with the electron capacity of the primary acceptor (quinone-iron) being unity.

3. Prior chemical reduction of the quinone-iron followed by illumination at 200K, however, leads to the slow ($t_{\frac{1}{2}} \cong 30$ s) oxidation of one cytochrome c_{553} heme, with what appears to be concommitant reduction of one of the two bacteriophytins (BPh) of the reaction center as shown by bleaching of the 760 nm band, a broad absorbance increase at approx. 650 nm and a bleaching at 543 nm. The 800 nm absorbing bacteriochlorophyll is also involved since there is also bleaching at 595 and 800 nm; at the latter wave-length the remaining unbleached band appears to shift significantly to the blue. No redox changes in the 883 absorbing bacteriochlorophyll dimer are seen during or after illumination under these conditions. The reduced part of the state represents what is considered to be the reduced form of the electron carrier (I) which acts as an intermediate between the bacteriochlorophyll dimer and quinone-iron. The state (oxidized c_{553}/reduced I) relaxes in the dark at 200 K in $t_{\frac{1}{2}}$ approx. 20 min but below 77 K it is trapped on a days time scale.

4. EPR analysis of the state trapped as described above reveals that one heme equivalent of cytochrome becomes oxidized for the generation of the state, a result in agreement with the optical data. Two prominent signals are associated with the trapped state in the $g = 2$ region, which can be easily resolved with temperature and

microwave power saturation: one has a line width of 15 g and is centered at $g = 2.003$; the other, which is the major signal, is also a radical centered at $g = 2.003$ but is split by 60 G and behaves as though it were an organic free-radical spin-coupled with another paramagnetic center absorbing at higher magnetic field values; this high field partner could be the iron-quinone of the primary acceptor. The identity of two signals associated with I^- is consistent with the idea that the reduced intermediary carrier is not simply BPh^- but also involves a second radical, perhaps the 800 nm bacteriochlorophylls in the reduced state. As such, the single electron would be shared in some way, and it is probable that one of these centers will be very close to the paramagnetism of the iron-quinone. Alternatively, it is possible that the electron only occupies BPh^- (the optical changes associated with the 800 nm bacteriochlorophyll occurring on a secondary basis) and that some of the BPh^- population of the trapped state is not close enough to interact with the quinone-iron.

5. Light-induced triplet state formation is dramatically diminished in material in which I as well as the quinone-iron is reduced before illumination. This supports the idea that with quinone-iron alone reduced before illumination, triplet formation requires light activated electron transfer from the bacteriochlorophyll dimer to I (not possible if I is already reduced) and that the triplet is formed by the return of the electron from I^- to $(BChl)_2^+$.

6. Results indicate that although the two cytochrome c_{553} hemes may be equivalent at the point of activation, once one has become oxidized the other becomes less competent for oxidation by the $(BChl)_2^+$.

INTRODUCTION

Recent work [1, 2] on primary events in bacterial photosynthesis has shown that in the photosynthetic reaction center protein there exists an electron carrier (designated I), which functions as an intermediate between the reaction center primary electron donor, a bacteriochlorophyll dimer (designated $(BChl)_2$) which in *Chromatium vinosum* absorbs at 883 nm, and the primary electron acceptor, a quinone associated with iron, often designated X, but here less anonymously called QFe (see ref. 3 for a review on reaction center components).

The system can be represented by $[(BChl)_2I]QFe$, and in *C. vinosum* the E_m (pH 8) value for the $(BChl)_2^+/(BChl)_2$ couple is +490 mV [4, 5] and that of the QFe/Q^-Fe couple is −160 mV [6]. The value for I/I^- is very negative; indirect measurements with *Rhodopseudomonas sphaeroides* indicate it is likely to be more negative than −430 mV [1] whereas in *Rhodopseudomonas viridis* it appears to be −400 mV (7); in *C. vinosum* the value is unknown.

Laser induced absorbance changes recorded on a timescale preceding the reduction of the quinone-iron have indicated that the bacteriochlorophyll dimer is oxidized [1] and I is reduced [8, 9] within 10 ps. The I^- of the transient $[(BChl)_2^+ I^-]QFe$ state appears to reoxidise in 100–200 ps, concommitant with the quinone-iron reduction. This leads to the formation of $[(BChl)_2^+I]Q^-Fe$ [8, 9] which is stable for many milliseconds. Chemical reduction of the quinone-iron [10, 11] or physical removal of Q from the reaction center [12] before activation prevents the normal forward electron transfer from I to QFe and this revealed the lifetime of the $[(BChI)_2^+$

I^-] state to be in the nanosecond time range (10 ns at 300 K; 30 ns at 80 K [10, 11]), relaxation occurring with the return of the electron from I^- to the $(BChl)_2^+$.

The nature of the reaction center species which functions as the intermediary carrier is not entirely certain; in accounting for the bacteriochlorophyll dimer oxidation contributions to the light induced [$(BChl)_2^+$ I^-] spectrophotometric changes, Fajer et al. [2] showed the absorbance changes accompanying the I to I^- transition had characteristics of the spectrum generated by bacteriopheophytin (BPh) reduction in vitro. However, other transients are apparent in the subnanosecond time domain which are not accountable for by a simple [$(BChl)_2BPh$] model [2, 12] for the [$(BChl)_2I$] state. This includes transients associated with the BChl abosrbing at 800 nm [9].

In order to overcome some of the problems connected with the measurements of very short laser activated transients in the reaction center, we have looked for a system in which I^- could be trapped in the dark to permit analysis by conventional spectrophotometry and by electron paramagnetic resonance (EPR) techniques. To this end we have exploited the properties of the photosynthetic bacterium *C. vinosum*. This organism possesses two *c*-type cytochromes, cytochrome c_{555}. E_m = 340 mV (pH 8) [4, 13], and cytochrome c_{553}, E_m = 0 mV (pH 8) [4, 5, 13], which are intimately associated with the reaction center and are still firmly associated with the reaction center after its isolation from the membrane [20]. Of the two *c*-cytochromes, cytochrome c_{553} is capable of rapid and irreversible electron donation to the light generated $(BChl)^+$ even down to liquid helium temperatures (at 300 K, $t_{\frac{1}{2}}$ = 1 μs; < 120K, $t_{\frac{1}{2}}$ = 2.5 ms; see refs. 14–17). This irreversible reaction provides a means to trap I in the reduced state in an isolated reaction center-cytochrome complex from *C. vinosum*. This we have done at 200 K using the following rationale: in material with the quinone-iron chemically reduced before activation(i.e., system representable by ferro c_{553} [$(BChl)_2I$]Q^-Fe) steady state illumination continuously regenerates ferro c_{553} [$(BChl)_2^+I^-$]Q^-Fe with a formation halftime of less than 10 ps and decay halftime of approx. 10 ns as described above. The likelihood per photon that cytochrome *c* successfully transfers an electron to $(BChl)_2^+$ ($t_{\frac{1}{2}}$ at 200K is approx. 10 μs; see refs. 16, 19) in competition with the 1000-fold faster I^- back reaction is very small. However, the irreversible nature of the cytochrome electron donation under prolonged illumination at 200 K ultimately leads to the accumulation of the ferri c_{553} [$(BChl)_2$ I^-]Q^-Fe configuration which is stable at low temperature. Thus I^- becomes reduced solely at the expense of cytochrome c_{553} oxidation; net redox changes in the bacteriochlorophyll dimer or the quinone-iron not being expected.

We have previously reported a preliminary EPR characterization of I^- generated in this manner [18]; here we present spectrophotometric and further EPR properties of I^-, together with details of the factors governing its generation in the trapped state; we also describe how I in the trapped reduced state affects low-temperature photochemical events in the reaction center.

MATERIALS AND METHODS

Chromatophores

The photosynthetic bacterium *Chromatium vinosum* was grown anaerobically in the light on a mineral medium with succinate as sole carbon source as previously

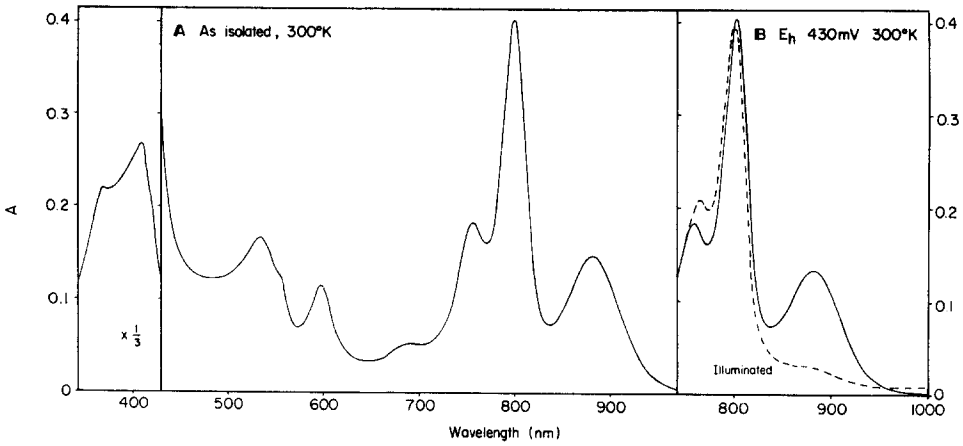


Fig. 1. The reaction center-cytochrome *c* complex of *C. vinosum*. On the left, is shown the absorption spectrum of the preparation as isolated, where the high potential cytochrome c_{555} complement is normally in a reduced state and the low potential cytochrome c_{553} is oxidized. The suspending medium was 1 % cholate in 50 mM Tris · HCl, pH 8.0; the temperature was 300 K and the measuring light path was 1 cm. On the right is the near infra-red spectrum of the same material (solid line) poised at a redox potential of 430 mV which is sufficient to oxidize essentially all of the cytochrome c_{555}, but no more than 10 % of the 883 nm absorbing reaction center $(BChl)_2$; under these conditions, illumination (dashed line) causes the familiar bleaching of the 883 nm band associated with $(BChl)_2$ oxidation, and the apparent blue shift of the 800 nm absorbing bacteriochlorophylls.

described [6]. Chromatophores were then prepared by passing the cells through a French press, followed by the usual differential centrifugation procedures.

Reaction center-cytochrome complex

The reaction center containing subchromatophore preparation from *C. vinosum* was prepared with the detergent Triton X-100 [20], according to procedures adapted from Thornber [20]. Antenna bacteriochlorophyll and a large proportion of the carotenoid pigments were then removed from the subchromatophore particles by a low-temperature acetone extraction as follows: the subchromatophore particles were stirred into acetone previously chilled by a dry ice/acetone bath. The volume of acetone was selected to result in a final 9 : 1 (v/v) acetone to water ratio. The mixture was stirred gently in the dry ice/acetone bath for about 10 min, and the precipitate then collected and dried on a Whatman No. 42 filter. The precipitate was then extracted with 2 % cholate and 0.5 M NaCl in 50 mM Tris · HCl, pH 8.0, for 24 h at cold room temperatures, followed by centrifugation to remove material not solubilized by the cholate. A spectrum of the material at this point very closely resembles that of a reaction center; the preparation could be further improved by passage through a hydroxyapatite column [20] equilibrated with the cholate extraction buffer. The reaction center-cytochrome complex eluated with the wash, while particles enriched with 800 nm absorbing chlorophyll and carotenoids were retained by the column. The reaction center-cytochrome complex was then precipitated with ammonium sulfate at 16 % (w/v), and resuspended in 1 % cholate, 50 mM Tris · HCl pH 8.0. This medium was used in all experiments.

The spectrum of the preparation is shown in Fig. 1; it is spectrophotometrically similar to that of Lin and Thornber [28] prepared in a different way. The near infra red region clearly shows the usual absorption characteristics of reaction center bacteriochlorophylls (800 and 883 nm) and bacteriopheophytin (760 nm), and the 800/883 nm absorbance ratio was 2.6. The absorbance in the 500–550 nm region arises in part from carotenoids and cytochromes which partially obscure the 535 nm bacteriopheophytin band. The preparation was stable for weeks stored on ice, and no deterioration was seen in months when stored at −10 °C. The functional integrity of the reaction center preparation seemed no different from that observed in the parent whole cell. The preparation was capable of supporting normal photochemistry and at room temperature displayed the microsecond oxidation rates of cytochromes c_{555} and c_{553} that are encountered in chromatophores or whole cells; furthermore, cytochrome c_{553} was oxidizable rapidly and irreversibly at 10 K.

Redox potentiometry

Reaction vessels and apparatus used for the anaerobic redox poising and transfer of samples for low temperature analysis by optical and EPR techniques have previously been described [5, 21]. All procedures were carried out in near darkness to prevent premature photochemistry from occurring. The redox mediators, with their approximate E_m values at pH 7.0, used in the various potential regions were: (i) redox potentials about 400 mV: 300 μM potassium ferri/ferrocyanide (E_m = 450 mV); (ii) redox potentials about 220 mV: 50 μM diaminodurol (E_m = 250 mV) 50 μM *N*-methyl-phenazonium methosulfate (E_m = 80 mV); (iii) redox potentials from 100 mV to −100 mV: 50 μM *N*-ethyl phenazonium ethosulfate (E_m = +55 mV); 50 μM *N*-ethyl phenazonium ethosulphate (E_m = 50 mV); 50 μM pyocyanine (E_m = −40 mV); 50 μM 2-hydroxy-1,4 napthoquinone (E_m = −125 mV); (iv) redox potentials from −100 mV to −400 mV: 100 μM, 2-hydroxy-1,4 napthoquinone; 50 μM benzyl viologen (E_m = −310 mV); 50 μM methyl viologen (E_m = −440 mV).

Spectrometry

Optical spectra and light-induced absorbance changes were recorded on Johnson Foundation split beam and dual wavelength spectrophotometers respectively, both equipped with Dewar flasks for low temperature work. Actinic illumination originated from a tungsten source (Unitron lamp, 8 V; 5A), filtered through 4 cm of water and a Wratten 88A filter. A Corning blue-green filter (9788) protected the photomultiplier from near infrared cross illumination.

Electron spin resonance spectra were recorded with a Varian E4 EPR spectrometer, equipped with a flowing helium cryostat and temperature control. A Nicolet C-1024 computer was used to obtain difference spectra.

RESULTS

Spectrophotometric properties of I⁻

Fig. 2A shows the absorption spectrum (solid line) of the *C. vinosum* reaction center-cytochrome *c* complex taken at a redox potential of −440 mV established before freezing to 200 K. At this potential, all relevant components except I are chemically

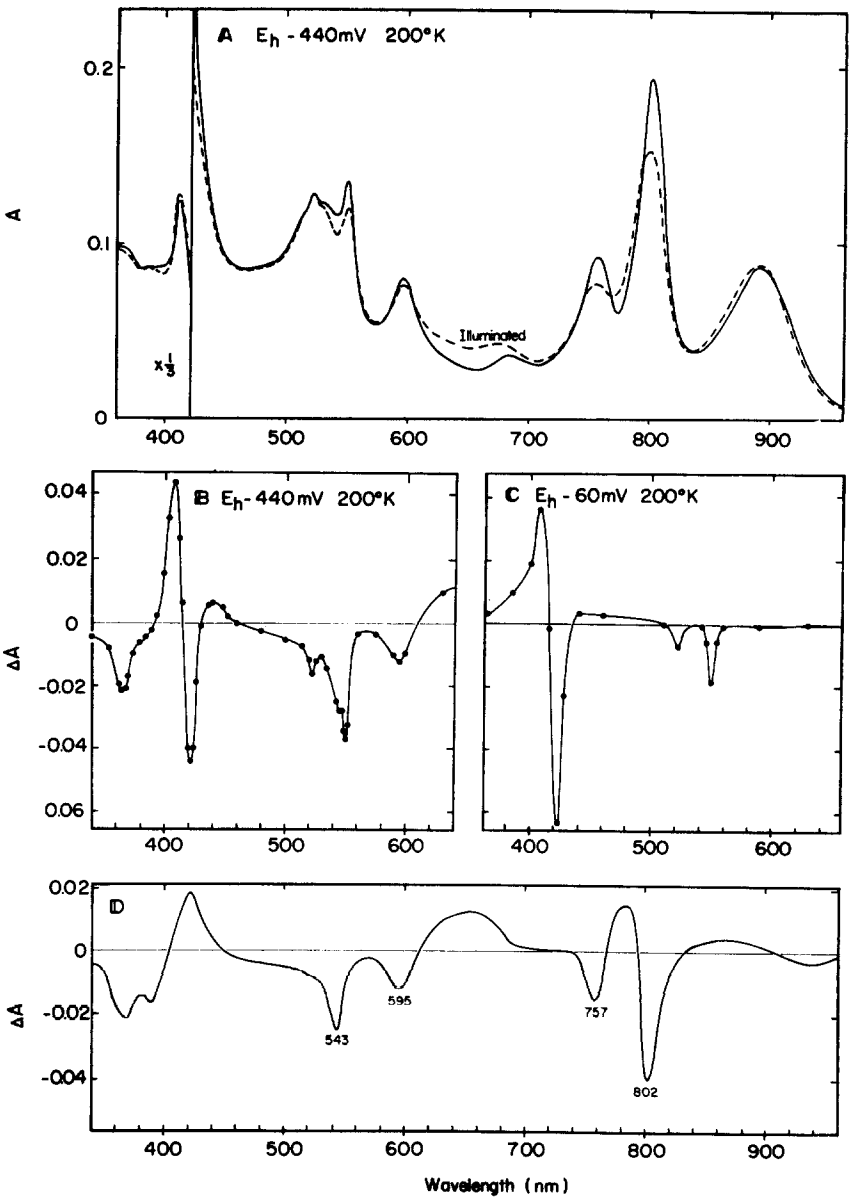


Fig. 2. The reaction center-cytochrome complex and the trapping of the reduced intermediate I^-. Part A. The solid line shows the spectrum measured at 200 K of the preparation in 1 % cholate, 50 mM Tris · HCl buffer, pH 8.0, poised at a redox potential of −440 mV established at room temperature; the measuring light path was 1 mm. The dotted line is a spectrum of the same sample which had been illuminated for 3 min, at 200 K. Part B shows, under the same conditions, a treated (3 min illumination at 200 K) minus an untreated difference spectrum of the preparation obtained using a dual wavelength spectrophotometer. Each point represents a fresh sample, the absorbance change at each wavelength being referred to a 460-nm reference wavelength (see Fig. 3 for more details). Part C shows the difference spectrum of material poised at a redox potential of −60 mV and illuminated for a few seconds at 200 K minus similar material in the dark (see Fig. 4 for more details). Part D, from 360 to 660 nm, is the difference between spectra B and C; beyond 660 nm, the difference is taken directly from A.

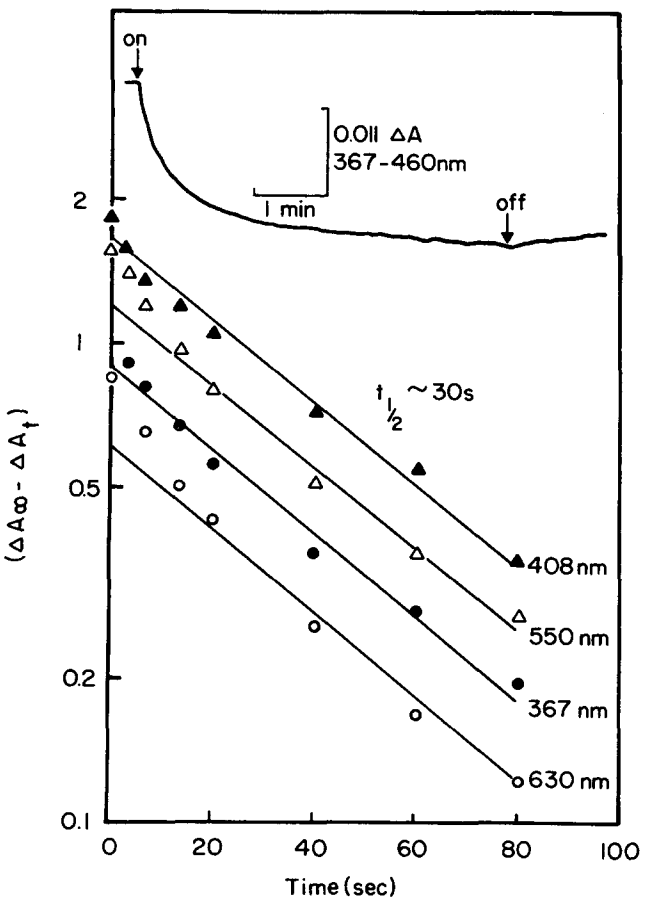


Fig. 3. The time-course of the absorbance changes induced by light at 200 K in the reaction center-cytochrome complex poised at −440 mV. At the top is shown a typical dual wavelength trace of the light induced reaction (see also Fig. 4A). Below are several semi-log plots of traces taken at various points in the spectrum of Fig. 2B. Other experimental conditions are as described in Fig. 2.

reduced (i.e., the system can be represented by ferro c_{553} $[(BChl)_2I]Q^-Fe$. A 3-min period of illumination of the sample at this temperature generates the spectrum shown by the dashed line in Fig. 2A. The new spectrum is unaltered whether measured during illumination or in the dark. Its relaxation in the dark back to the original one is quite slow at 200 K, displaying a halftime of about 20 min. The relaxation is undetectable on an hours basis if after the 200 K illumination the temperature of the sample is lowered to 80K or further; this provides the means to trap the state for long time periods.

The generation of the new spectrum at 200 K follows a roughly exponential course displaying, under the prevailing condition of light intensity, a halftime of approx. 30 s; there is an indication of a more rapid phase, but this has not been investigated yet. Measurement of the course of the generation of the spectrum at various

wavelengths yields similar kinetics (Fig. 3) implying that all the absorbance changes are associated with the same events.

Comparing the two spectra of Fig. 2A in the near-infra red region it is clear that the bacteriochlorophyll dimer of the 883 nm band does not undergo any detectable redox changes (cf. Fig. 1) but the absorbance of the bacteriopheophytin band at 760 nm is significantly diminished. There is also a partial absorbance decrease of the bacteriochlorophyll band at 800 nm and an apparent shift to the blue. A broad absorbance increase is evident from 620 to 700 nm.

No effort was made to examine the details of the visible region either directly as discussed above in Fig. 2A, or by split-beam difference spectrophotometry, since it was clear that a higher degree of resolution was needed than is given by these methods. Fig. 2B shows the difference spectrum in the visible region generated at 200K by 3-min illumination of material poised at −440 mV as described for Fig. 2A but measured in a dual wavelength spectrophotometer as shown in Fig. 3. Points taken at various wavelengths each represent a different sample. Sharp absorbance decreases at about 550, 520 and 420 nm show that cytochrome c_{553} undergoes oxidation during the 200K illumination. The prominent absorbance decrease shoulder at 543 nm is consistent with a bacteriopheophytin redox change companion to that observed at 760 nm; that at 595 nm is consistent with a bacteriochlorophyll absorbance change companion to those at 800 nm. The absence of change at 605 nm is in accord with the absence of changes seen in the main band at 883 nm.

The change contributed by cytochrome c_{553} oxidation to the overall absorbance change of Fig. 2B was accounted for as follows: material of the same concentration was poised at a redox potential of −60 mV before freezing. Under these conditions, the primary acceptor (E_m = −160 mV at pH 8) is essentially oxidized but cytochrome c_{553} reduced (E_m = 0 mV at pH 8) and the system before freezing can be represented by ferro c_{553} [$(BChl)_2I$]QFe. Illumination generates ferri c_{553} [$(BChl)_2I$]Q^-Fe in an essentially irreversible manner in microseconds; the light induced absorbance difference is almost uniquely that of oxidation of the cytochrome c_{553} as shown in Fig. 2C (no obvious absorbance change at 450 nm (X450) associated with the accompanying quinone-iron reduction is evident; it has a low difference extinction coefficient relative to that of cytochrome oxidation [22]). The time-course of these changes is shown in Fig. 4 and contrasted with the much slower changes seen under the much more negative redox conditions with quinone-iron already reduced before illumination. The extent of the low temperature change seen at −60 mV should represent the oxidation of only one cytochrome c_{553} heme (see refs. 5, 6, 16, 21). Subtraction of this cytochrome c_{553} one-heme equivalent absorption from the composite spectrum in Fig. 2B quantitatively eliminates cytochrome c_{553} from that spectrum and reveals the difference spectrum in Fig. 2D (with the longer wavelength difference taken directly from Fig. 2A). This absorbance difference, as interpreted from the rationale outlined in the Introduction represents changes as I becomes reduced.

The difference spectrum accompanying the I to I^- reaction displays several features which are qualitatively similar to the absorbance difference encountered in vitro for bacteriopheophytin reduction [2], particularly the double-peaked absorbance decrease between 360 and 390 nm, the absorbance increase at about 425 nm, the broad peak from 600–680 nm and the bleaching at about 760 nm. The absorbance

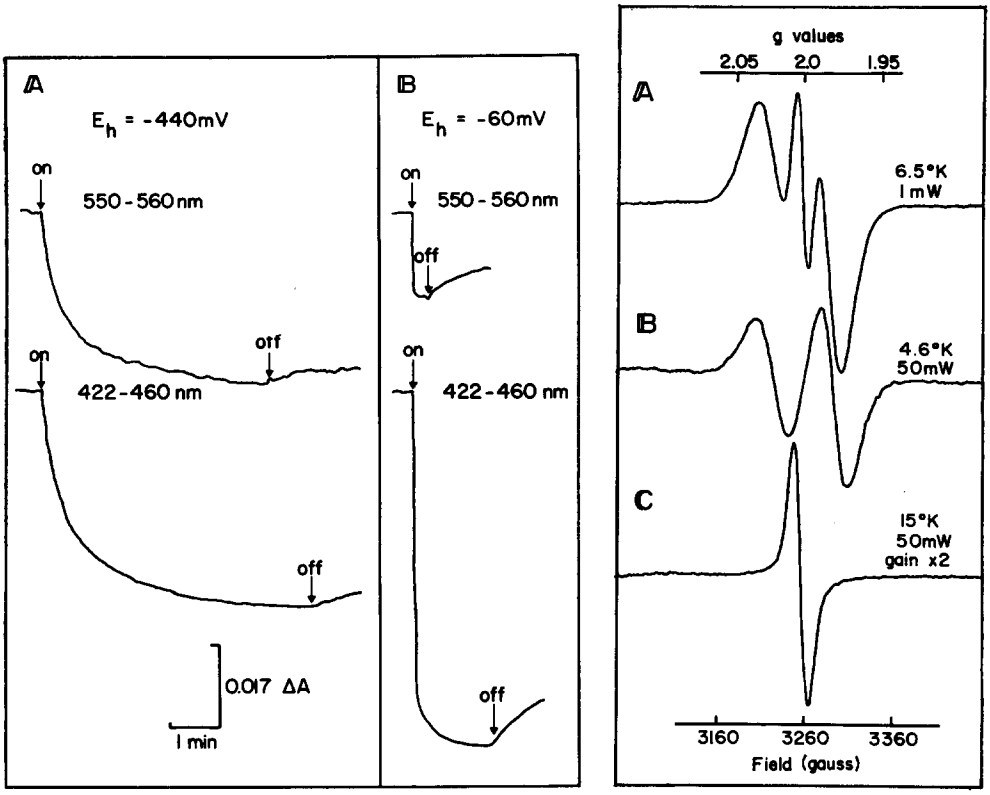


Fig. 4. Light induced cytochrome c_{553} oxidation at 200 K in the reaction center-cytochrome complex poised at redox potentials of −440 and −60 mV. Part A shows the absorbance changes similar to those described at −440 mV in Fig. 3 for the spectrum described in Fig. 2B. The absorbance changes recorded include contributions from those occurring as the intermediate is reduced. Part B shows absorbance changes occurring at −60 mV which are exclusively those associated with cytochrome c_{553} oxidation, as shown in Fig. 2C. Other conditions are as described in Fig. 2.

Fig. 5. EPR spectrum associated with the reduced intermediate I^-. Reaction center-cytochrome complex ($A_{883\,nm}$, 3.0 cm^{-1}) was poised in 1 % cholate, 50 mM Tris · HCl buffer, pH 8.0, at −440 mV before freezing to 200 K. The EPR signals are the difference between signals taken after 3 min illumination at 200 K and the untreated material, which had a small (<10 %) free radical signal due to the redox mediators. The EPR spectrometer modulation amplitude was 5.0 G; other settings including temperature are as indicated on the figure.

decrease in vivo at 543 nm seems to be a red-shifted equivalent of the 520 nm bleaching seen in vitro, and is similar in wavelength to that identified as I^- seen in *R. sphaeroides* reaction centers in the nanosecond [9–11] and picosecond [12] time domains. In this organism, the 542–543 nm bleaching has been identified [23] with the longer wavelength member of the two bacteriopheophytins which are spectrophotometrically resolvable in the 535 nm region. The presence of carotenoids in the *C. vinosum* preparation does not permit a direct assessment of the extent of bleaching at 543 nm with respect to the absolute absorbance of bacteriopheophytin in this region. However, the extent of the bleaching at 543 nm in Fig. 2D is that expected for the complete

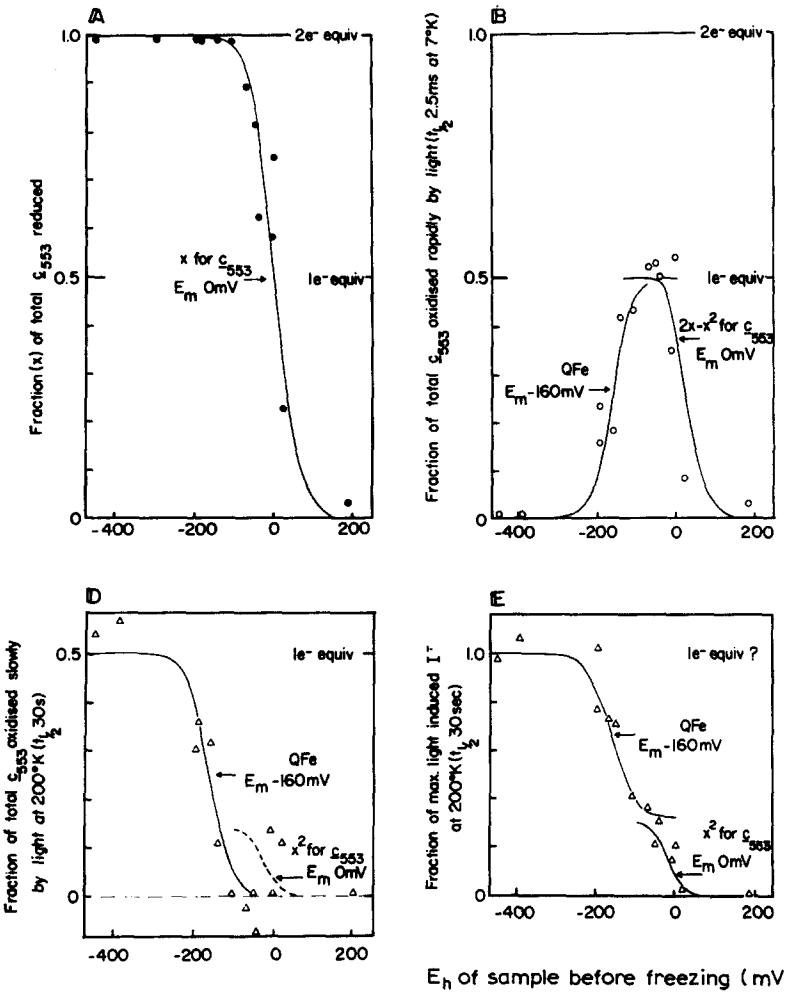
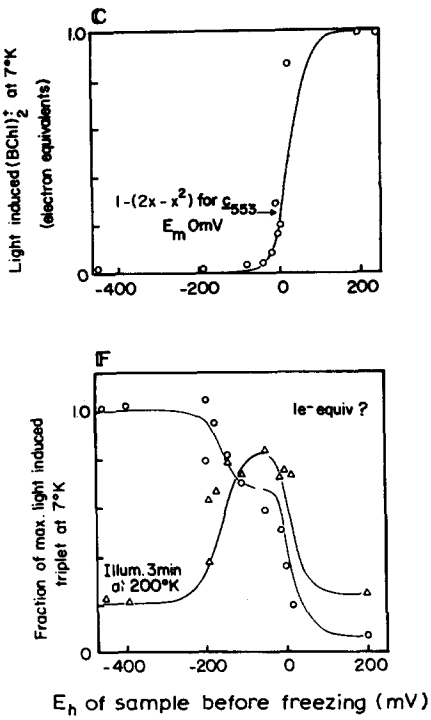


Fig. 6. Redox potential dependence of light-induced reactions of cytochrome c_{553}, bacteriochlorophyll dimer, I and the spin-polarized triplet state in the reaction center-cytochrome complex. The reaction center-cytochrome preparation ($A_{883\,nm}$ 3.0 cm^{-1}) in 1% cholate, 50 mM Tris · HCl, pH 8.0, was poised in the dark at various redox potentials before transfer to EPR tubes and subsequent freezing. The experimental protocol is given in detail in the text. The EPR settings for the assays were as follows: ferri-cytochrome c_{553} was measured at 7 K by the amplitude of the g_z band at 2.95 (microwave power 1 mW; modulation amplitude 12.5 G); $(BChl)_2^+$ was measured at 7 K by the high to low field amplitude of the $g = 2.003$ signal (microwave power, 1 mW; modulation amplitude, 5.0 G); I^- was measured at 7 K by the amplitude of the low field signal at $g = 2.034$ (microwave power 1 mW; modulation amplitude 5.0 G); and the triplet or biradical signal was assayed at 7 K from the low field absorption signal at $g = 2.1$ (microwave power, 1 mW; modulation amplitude, 12.5 G). In Part A the line drawn through the points is an $n = 1$ Nernst curve $E_m = 0$ mV plotted as "x", the fraction of total reduced cytochrome c_{553}, with respect to the redox potential established before freezing. In Part B, the theoretical line drawn through the points represents a $(2x-x^2)$ dependency on cytochrome c_{553} reduced which is expected if there are two cytochrome c_{553} which are functionally identical in electron transfer to the light generated $(BChl)_2^+$. At more negative potentials the line drawn through the course of attenuation of the light induced cytochrome c_{553} oxidation reaction is related to the $n = 1$ Nernst curve [$E_m = -160$ mV (pH 8.0)] for the



reduction of the primary acceptor QFe. In Part C, the line drawn through the points for the extent of detected $(BChl)_2^+$ is $1-(2x-x^2)$, x being the fraction of cytochrome c_{553} reduced before illumination, and reflects the presence of two cytochrome c_{553} hemes per bacteriochlorophyll dimer as discussed for Part B. Part D presents the extent of cytochrome c_{553} oxidized during the 3-min illumination treatment at 200 K. The solid line drawn through the points reflects the $n = 1$ Nernst curve for the primary acceptor; the dotted line is a "best-fit" theoretical line for the x^2 dependency of the "second" of the two cytochrome c_{553} hemes being partially available for oxidation during the treatment (see Discussion). Part E is the extent of I^- generated during the 3 min illumination at 200 K. The lines drawn through the points are best-fits for the $n = 1$ Nernst curve of QFe and the x^2 dependency for the "second" of the two cytochrome c_{553} hemes (see Discussion). Part F presents the extent of triplet signal generated in the light before (○) and after (△) the 3 min illumination at 200 K. In the former case, the lines drawn through the points represent theoretical best fits for the $2x-x^2$ dependency on the cytochrome c_{553} complement and the normal $n = 1$ Nernst curve for QFe $E_m = -160$ mV (pH 8.0).

reduction of the 543 nm bacteriopheophytin species, assuming that the spectrophotometric properties for the bacteriopheophytin complement of *C. vinosum* are similar to those of *R. sphaeroides*. The bleaching seen at 760 nm, where both bacteriopheophytin species have superimposed absorption maxima, is consistent with the reduction of one bacteriopheophytin in the I^- state, although the extent of the bleaching is less than the 50 % expected. However, this is probably due to the apparent blue shift of the 800 nm bacteriochlorophyll band which leads to an absorbance increase in the 760 nm region and thus opposes the 760 nm absorbance decrease arising from bacteriopheophytin reduction. The broad absorbance increase from 600–700 nm is also characteristic of the BPh to BPh^- difference spectrum. However, the BChl to $BChl^-$ difference spectrum is also similar in this respect [2].

The partial bleaching and blue shift of the 800 nm band and the similar behavior of the companion 595 nm band (although whether there is also a blue shift here is uncertain) brings the bacteriochlorophylls responsible for these absorptions into consideration as being involved in the I^- state. Whether the involvement is secondary (e.g., electrochromic effects) as may be indicated, in part at least from the apparent 800 nm blue shift, cannot be decided from the available data. However in spite of the possibility that the absorbance decreases at 595 and 800 nm could also be secondary hypochromic effects, the 800 nm absorbing bacteriochlorophylls cannot be ruled out as being a possible part of I^- and capable of undergoing reduction.

Electron paramagnetic resonance of I^-.

A preliminary note on the paramagnetic properties of what is probably I^- in *C. vinosum* has been presented [18], in which EPR analysis of the reaction center-cytochrome complex treated to generate I^-. in the way described in the previous section revealed prominent signals about $g = 2.0$. The spectrum shown in Fig. 5 (top) was found to be composed of a radical signal centered at $g = 2.003$ but split by 60 G to form a doublet (Fig. 5, center) and also a free radical signal also centered at $g = 2.003$ but only 15 G in width (Fig. 5, bottom). Here, we present a detailed account of the factors which control the generation of the I^- signals with respect to the state of reduction of cytochrome c_{553} and quinone-iron before freezing, and also examine the effects on light activated reactions in the reaction center at 7K when I is in the reduced state before illumination.

Samples of the reaction center-cytochrome complex were poised at known redox potentials over the +200 to −450 mV range, transferred anaerobically to EPR tubes and stored in darkness at 80K; each sample was dealt with (see the legend of Fig. 6 for full details) as follows:

(A) Ferri-cytochrome c_{553} was assayed by EPR at $g = 2.95$ [21] in the dark to determine the equilibrium redox state of the cytochrome as a function of redox potential. This was to provide a measure of the total cytochrome c_{553} ($E_m = 0$ mV, pH 8, $n = 1$) content of the reaction center and to verify its electrochemical homogeneity in the preparation.

(B) The portion of cytochrome c_{553} rapidly (i.e., milliseconds) oxidizable by light at 7K was assayed to determine what fraction of the total cytochrome c_{553} could be oxidized at 7K by the light generated reaction center $(BChl)_2^+$.

(C) The extent of $(BChl)_2^+$ generated by light at 7 K was measured at $g = 2.0$ to provide a calibration for oxidizing equivalents (one per reaction center) for cytochrome c_{553} oxidation. This assay, together with (B), was in essence the same experiment done previously with spectrophotometry at 80 K [5].

Each sample was then illuminated for 3 min at 200 K and then cooled back to 80 K or below, to trap the state which included I^-.

(D) Any further cytochrome c_{553} oxidized at 200 K beyond that oxidized at 7 K in (B) was assayed at 7 K. This was to provide a measure of how much cytochrome c_{553} was oxidized for the generation of the I^-, and would be relatable to the spectrophotometric assays done in Fig. 2B and 2C.

(E) The extent of the I^- signals generated during the 200 K treatment were assayed at 7 K.

(F) The preparation was also examined for the light-inducible spin polarized

triplet or biradical EPR signal both before and after the 3 min illumination at 200 K. This parameter will be discussed later.

Fig. 6A shows the dark redox titration of cytochrome c_{553} in the reaction center-cytochrome complex. The points fit well on an $n = 1$ Nernst curve with a mid-point potential of 0 mV, as would be expected for the electrochemically homogeneous presence of cytochrome c_{553}. Fig. 6B shows the amount of cytochrome c_{553} which is oxidized rapidly and irreversibly by light at 7 K. The maximum extent encountered is half the total cytochrome c_{553} content. The attenuation of the light-induced cytochrome oxidation as the redox potential is lowered follows the expected Nernst curve for the reduction of quinone-iron (E_m −160 mV, pH 8; $n = 1$); this is expected since the reduction of quinone-iron before illumination prevents long lived (i.e., only 30 ns instead of 20 ms halftime) light-induced $(BChl)_2^+$ formation, which in turn prevents cytochrome c_{553} from undergoing prompt (i.e., a 2.5 ms $t_{\frac{1}{2}}$ capability) oxidation.

Fig. 6C shows the extent of the light induced $(BChl)_2^+$ measurable as the potential is lowered; this attenuates to zero as the cytochrome c_{553} is increasingly reduced before freezing and thus becomes available to rapidly reduce light-induced $(BChl)_2^+$ in 2.5 ms. (In the −100 to 0 mV range with quinone-iron oxidized and cytochrome c_{553} reduced, there would in fact be a 2.5 ms halftime $(BChl)_2^+$ transient which would not be detected without a rapidly responding instrument, see refs. 17 and 24). The fact that no $(BChl)_2^+$ is detectable when cytochrome c_{553} is reduced, implies that all the bacteriochlorophyll dimers in our reaction center-cytochrome complex are associated with at least one functional cytochrome c_{553} heme.

The results in Fig. 6B and C are replicas of the measurements made previously by spectrophotometric techniques [5]. As was found with those measurements, the redox potential dependent course of the attenuation of the light-induced $(BChl)_2^+$ does not follow the normal Nernst curve for the reduction of cytochrome c_{553} as is encountered in Fig. 6A. In fact, if in Fig. 6A "x" is the fraction of the cytochrome reduced at any redox potential, the amount of $(BChl)_2^+$ (Fig. 6C) is proportional to the probability of both cytochromes being oxidized before illumination, namely $1-(2x-x^2)$. Appropriately, the redox potential course of the extent of cytochrome c_{553} oxidized at 7 K follows the $2x-x^2$ curve. These data (see refs. 5 and 13) imply that there are two cytochrome c_{553} hemes which are identical with respect to their electron donating capabilities to the light induced $(BChl)_2^+$, but that only one can be oxidized under the low temperature conditions. The maximum extent of light induced cytochrome c_{553} oxidized at low temperatures thus represents one cytochrome c_{553} heme of two present. This work therefore confirms the tentative conclusion made before [5] that there are two cytochrome c_{553} hemes or molecules which are functionally identical with respect to the single oxidizing equivalent of the $(BChl)_2^+$ per reaction center. Here, however, the direct measurement of the total amount of cytochrome c_{553} as being a factor of two greater than that oxidized in the light at 7 K makes this relationship much more certain.

Fig. 6D shows the amount of cytochrome c_{553} oxidized during the 3 min illumination at 200 K as a function of redox potential. The amount of cytochrome c_{553} oxidized follows the Nernst curve for the reduction of the quinone-iron (although there is some uncertainty in the 0 to −100 mV region), and maximises at half the total cytochrome c_{553} content. Thus, one electron equivalent of cytochrome c_{553} is

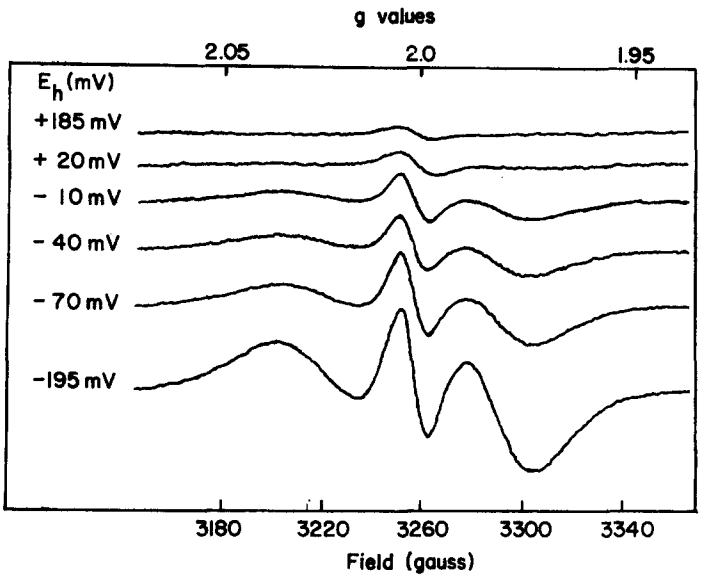


Fig. 7. Redox potential dependence of the generation of I^- in the reaction center cytochrome complex. The preparation ($A_{883\,nm}$, 3.0 cm^{-1}) in 1 % cholate Tris · HCl buffer, pH 8, was poised at the redox potentials indicated, cooled to 200 K and illuminated for 3 min, and then cooled to 7 K and assayed. EPR spectrometer settings were microwave power 1.0 mW; modulation amplitude, 5.0 G.

oxidized during the 200 K illumination treatment. This would suggest that one electron equivalent should appear on I during this treatment, and that the formation of I^- should follow a similar redox potential dependency.

Fig. 7 shows the EPR signal measured at 7 K of I^- generated during 3 min in the light at 200 K in samples at redox potentials over the +200 to −200 mV range. Some is generated in the 0 to −100 mV range, but most is generated following the Nernst curve of the quinone-iron reduction, as seen graphically in Fig. 6E.

The redox potential dependency of the 7 K light induced bacteriochlorophyll dimer triplet or biradical signal before and after the 3 min 200 K illumination treatment shown in Fig. 7F will be described in the Discussion.

DISCUSSION

Summary of electron transfer reactions at low temperatures in photosynthetic bacteria. Fig. 8 summarizes the components associated with the photosynthetic reaction center which function efficiently down to liquid helium temperatures, the three sections representing the electron transfer reactions which can occur at various states of reduction of the system before illumination. The scheme, based on the working model [1] derived from studies with the *R. sphaeroides* reaction center, which lacks *c*-type cytochromes, has been expanded to include data from *C. vinosum*. As such, it serves as a digest of the early photosynthetic reactions and their decay processes. Reference sources are listed in the figure legend.

Part A outlines the reactions which occur when only the bacteriochlorophyll

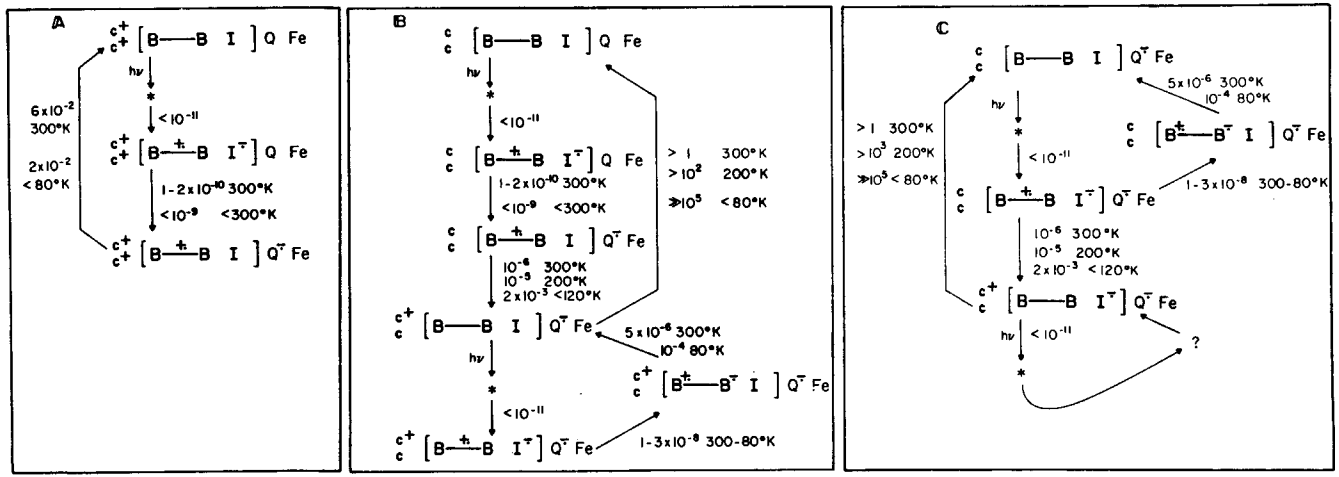


Fig. 8. Summary of light activated reaction center and cytochrome electron transfer reactions which can operate at low temperatures. In the figure, the bacteriochlorphyll dimer is represented by B-B, the *c* represents ferrocytochrome c_{553} and c^+ represents ferricytochrome c_{553}. The star represents the singlet excited state of the reaction center complement. The numbers associated with the reactions are the approximate reaction half times in seconds given with the temperature of measurement. Parts A, B and C are the reactions expected starting from different reduction states at equilibrium before light activation. All the times and details for the reactions occurring within the bacteriochlorophyll dimer, I and quinone-iron components are from work on *R. sphaeroides* reaction centers; (the forward reactions, refs. 1, 8, 9; decay reactions, refs. 1, 8, 9). The return of the electron from QFe to $(BChl)_2^+$ is for both *C. vinosum* and *R. sphaeroides*; [17, 21–26]. The cytochrome c_{553} oxidation reactions are for *C. vinosum* (refs. 5, 13–17, 21 and this work).

dimer is reduced before activation. This scheme is identical to that for *R. sphaeroides* and all the reactions are reversible. The temperature dependence of the I^- to QFe reaction has not been investigated, but large temperature effects are not expected for two reasons: firstly, because the quantum efficiency of the light reaction does not change greatly as the temperature is lowered [23] so the I^- to QFe reaction must always be considerably faster than the I^- to $(BChl)_2^+$ back reaction [$t_{\frac{1}{2}}$ = 30 ns at 80K and below [10, 11]]. Secondly, as discussed below, any back reaction from I^- to $(BChl)_2^+$ would be expected to generate the triplet/biradical signal on the bacteriochlorophyll dimer (see ref. 1), and since very little is detected under these conditions (see refs. 24, 25, 27 and Fig. 6F), it might be supposed that the I^- to QFe rate never exceeds a nanosecond halftime; for instance, the 4-ns possibility discussed in the section on structural aspects would cause only a 10–20 % drop in quantum efficiency from 300 K to 8 K, but would yield a level of light-induced triplet at 8 K significantly higher than is evident in Fig. 6F (one point taken at 200 mV). It may now be appropriate to examine in more detail the light-induced triplet levels under these conditions. As for the return of the electron from Q^-Fe to $(BChl)_2^+$, the oxidation ($t_{\frac{1}{2}}$ = 20 ms) of a single-turnover flash induced Q^-Fe [21, 25] and the concomitant re-reduction of the flash induced $(BChl)_2^+$ as seen by EPR [21, 25, 26] or spectrophotometry [17, 26] is well documented in both *R. sphaeroides* and *C. vinosum*.

Part B shows the situation when the cytochromes c_{553} are also reduced before activation. The first turnover generated $(BChl)_2^+$ is reduced by ferrocytochrome c_{553}, which easily competes with the back reaction from Q^-Fe which is evident in Part A; even at 4 K, it is faster by a factor of 10. The resulting state represented by ferricytochrome c_{553} $[(BChl)_2I]Q^-Fe$ is formed in an essentially irreversible manner; at 300 K, the forward vs. decay halftime ratio is 10^6, and at 80 K no detectable back reaction occurs even after several weeks. With an electron stranded on Q^-Fe, subsequent turnovers do not elicit a long-lived $(BChl)_2^+$, or further cytochrome c_{553} oxidation [17, 24], but they do generate $[(BChl)_2^+I^-]$ within the state [8–12], and because Q^-Fe is already reduced, the electron on I^- is forced to return to the $(BChl)_2^+$ ($t_{\frac{1}{2}}$ = 10 ns at 300 K; 30 ns at 15 K and 80 K, [10, 11]). Under these conditions the triplet or biradical state of the bacteriochlorophyll dimer (depicted in the figure as B^+–B^-). is generated [24, 25, 27]. If this triplet state is the same as that observed by spectrophotometry and designated P^R [10], then its decay back to the ground state is 6 μs at 300 K and 120 μs below 120 K [10]. The decay of the EPR detectable signal measured at 8 K is much faster ($t_{\frac{1}{2}}$ = 6 μs [27]), but this is probably due to the spin-lattice relaxation time (T_1) of the polarization rather than the decay of the state itself. At low temperatures (80 K for P^R [10]; 10 K for the EPR measurements [29]) the triplet is generated with a quantum efficiency approaching 100 %, relative to $(BChl)_2^+$ formation under the conditions of Part A, but this drops to only 10–20 % (P^R measurements) as the temperature is raised to 300 K [10] indicating that other decay processes become involved at warmer temperatures.

Previous single turnover flash experiments [27] showed that the triplet was not significantly formed on the first flash when normal forward progress was unhindered, but that it was formed on subsequent flashes when the quinone-iron was reduced to block the I^- to QFe reaction.

The redox potential dependence of the steady state (multiple turnover) light-

induced triplet state on the prior state of reduction of cytochrome c_{553} (Fig. 6F) is consistent with these data, and the model (but see final section of Discussion).

In Part C, the quinone-iron is already reduced, and thus triplet formation occurs on all single turnovers of the reaction center, including the first [27]. The highly unfavored competition of cytochrome c_{553} to donate an electron to $(BChl)_2^+$ ($t_{\frac{1}{2}}$ = 10 μs at 200 K, [16, 17]) rather than the back reaction from I^- ($t_{\frac{1}{2}}$ = 10 ns, [10]) means that on a single turnover basis hardly any cytochrome would be oxidized (10^{-1}% at 200 K, 10^{-4}% under 80 K). However the irreversibility of the cytochrome oxidation at 200 K allows prolonged illumination to generate the state where I is trapped in its reduced form. The stability of this state, once formed, has a high temperature sensitivity, decaying promptly at room temperature, but lasting apparently indefinitely below 80 K.

Since both I^- and the bacteriochlorophyll dimer are reduced in this state, further illumination should not generate the triplet signal, and Fig. 6F shows that this is indeed the case. As I^- is generated (dependent predominantly on the prior state of reduction of the quinone-iron, Fig. 6E) the amplitude of the light-induced EPR triplet signal diminishes by 5-fold. How the absorbed light energy is dissipated under these conditions is a question of considerable interest.

The nature of I/I⁻. The expected behavior of the triplet state lends major support to the notion that the spectra and EPR signals of the trapped state indeed represent I^-, and not the product of a side reaction. Both the optical and EPR results (Figs. 2C and 6D) indicate that I^- is generated by a one-electron reduction since only one heme of cytochrome c_{553} is oxidized during the 200 K illumination, but the spectra associated with I^- reduction (Figs. 2D and 5) pose a problem to a simplistic view of the identity of I as solely one of the bacteriopheophytin complement of the reaction center. If BPh^- fully accommodates one electron, then the other optical changes associated with the bacteriochlorophyll of the 800 nm band must either be secondary, possibly electrochromic, effects, or the results of reduction from an agent other than cytochrome c_{553}. The possibility that the reductant is ferrocytochrome c_{555} (in addition to ferrocytochrome c_{553}) seems unlikely since there is no evidence for oxidized cytochrome c_{555} (as seen from its g = 3.1 signal or from optical data) after the 200K illumination treatment, even under higher potential conditions when this cytochrome is the only one reduced. The alternative consideration is that in the single electron proposal, the electron is shared between bacteriopheophytin and the 800 nm bacteriochlorophyll(s) with effects (the apparent blue shifts) acting on the remaining population of unreduced bacteriochlorophyll. In this respect it may be significant that published P^F (ie., $[(BChl)_2^+I^-]$) spectra [9–11] indicate that absorbance decreases at 595 nm (part of I^- formation?) may also be accompanying those of $(BChl)_2^+$ formation at 605 nm. Taken together with the flash-induced alterations at 800 nm [9–11], this suggests a more than passive role for the 800 nm bacteriochlorophyll(s) in the electron transfer reaction. A close spectrophotometric examination of the flash-induced intermediary state is now warranted.

The identification that the 800 nm bacteriochlorophylls are involved with the I^- state trapped as a stable entity in the dark removes the possibility that the 800/595 nm changes observed in the flash-induced kinetic studies in *R. sphaeroides* are the result of direct light or multiple photon excitation, or arise from other unrelated "side-reactions" (This conclusion is relevant to all the other changes seen in the visible

region and associated with I^-). It also indicates that the source of the 800-nm changes cannot be a secondary reaction with the $(BChl)_2^+$ alone of the $[(BChl)_2^+I^-]$ state, which unavoidably accompanies the flash induced kinetic work, since this species is absent in the trapped I^- state; in this case it would have to be a similar response to the accompanying ferricytochrome c_{553} which seems unlikely. The 800 nm changes might be a response to the electric dipole between I^- and its oxidized partner, but some evidence that this may not be the case comes from a recent report [30] with *R. viridis* reaction center-cytochrome complex (functionally similar to the *C. vinosum* preparation used here), where illumination at room temperature in the presence of dithionite caused an apparent blue shift of the 830 nm bacteriochlorophyll band (analagous to the 800 nm bacteriochlorophyll of *C. vinosum*) in addition to net bacteriopheophytin reduction. Under these conditions the ferricytochrome would be reduced by the dithionite in the external medium, so that if the bacteriochlorophyll shifts are due to some form of electrostatic interaction, this must arise from I^- (i.e., BPh^-) alone.

The EPR spectrum associated with I^- also has two components: the doublet accounts for the majority of the spins in the state, but the narrow free radical signal cannot be ignored. The proportions of the two signals appears to be constant under any given assay conditions, regardless of whether or not redox mediator dyes are present before the 200 K illumination, or whether the electron in the I^- state comes from the first cytochrome heme oxidized (i.e., quinone-iron reduced before illumination) or the second (i.e., quinone-iron oxidized before illumination), although in the latter case both the signals are much smaller. Both signals are evident in whole cells and chromatophores; indeed, the signals were first revealed in chromatophores from *C. vinosum* illuminated while freezing in the presence of dithionite [31]. They are also present in whole cells of *R. viridis*, although in this organism the splitting exhibited by the doublet is greater (approx. 100 G) than in *C. vinosum*. These data contribute to suggest that the narrow radical signal is a bona fide part of the I^- state.

With the above considerations, we tentatively conclude that the optical and EPR data represent a single electron reduction of I, and that I certainly involves bacteriopheophytin, but may also involve an electron sharing with bacteriochlorophyll (800 nm) and/or a perhaps secondary electrochromic effect on this bacteriochlorophyll(s).

Some structural aspects. The split nature of the major part of the I^- radical to form a doublet implies that the unpaired spin on I^- is spin-coupled to another paramagnetic center [18]. Analagous, but not entirely similar lineshapes are recognized in other biological systems [32–35], such as reactions catalyzed by enzymes requiring vitamin B_{12}, where Schepler et al. [32] have proposed an exchange interaction between organic radicals at about $g = 2$ and the divalent low-spin cobalt at lower magnetic fields. By analogy, in the photosynthetic reaction center I^- may be the low field partner of the higher field signals of Q^-Fe ($g_y = 1.82$; $g_x = 1.68$ in *R. sphaeroides* [25] and *R. viridis* [7], and 1.62 in *C. vinosum* [21]) or perhaps only the iron of QFe (see ref. 36). Encouraging evidence for the interaction of I with quinone-iron comes from current preliminary work that shows the two centers to have similar relaxation times, as indicated by the similar and rather unusual power/temperature dependencies, and the significant modification of the Q^-Fe signal at $g = 1.82$ when I^- is present. An exchange coupling as proposed by Schepler et al.

[32] would require that the ratio of the integrated intensities of the I^- doublet be 1.5 : 1 for the high to low field lines. In this respect, the experimentally observed ratio seems to be greater than unity. Indeed, taking the ratio from the unsaturated derivative amplitudes a value of 1.6 is obtained, but further quantitative work will be required to establish this firmly. Nevertheless, regardless of whether the two signals of I^- reflect BPh^- in two populations or BPh^- and $BChl^-$, there are strong indications that there will be a significant overlap of the wave functions of the interacting radicals. If this proves to be between I^- and Q^-Fe, then this may be important to the mechanism by which electron transfer from I to QFe occurs. Considering this further, and calculating the exchange rate between I^- and Q^-Fe from the I^- splitting (60 G), a value of $2 \cdot 10^8\ s^{-1}$ ($t_{\frac{1}{2}} \cong 4$ ns) is obtained. Assuming that this exchange rate has any relevance to electron transfer rates (i.e., from I^- to QFe), than this would indicate that in *C. vinosum* the I to QFe electron transfer halftime is approx. 4 ns at 8 K.

Further considerations and anomalies. In part B of Fig. 8, where both cytochromes are reduced but QFe oxidized before activation, it is not clear why once one of the cytochrome c_{553} hemes has been oxidized to irreversibly reduce Q^-Fe, the other is not fully available to generate I^-. If this were the case, then the cytochrome oxidized during the 200K illumination would have followed an x^2 dependency (i.e., where x is the fraction of the total cytochrome reduced at equilibrium as a function of redox potential, as in Fig. 6A) and would have represented those reaction centers with both cytochrome c_{553} hemes reduced before activation. The extent of 200 K light-induced cytochrome oxidation would have achieved a maximum one electron equivalent following this curve without any reliance on the prior state of reduction of quinone-iron, and in the final state with I^- trapped, both cytochromes would be oxidized, one rapidly to cause the irreversible reduction of QFe, the other slowly to cause the reduction of I. Experimentally, there was only a partial oxidation of the "second" cytochrome c_{553} heme (Fig. 6D). Although the results from EPR were variable because these points are the result of two subtractions from three separate measurements of the $g = 2.95$ signal, very often (and further experiments support this) there were 0.2–0.3 electron equivalents of cytochrome c_{553} oxidized during the 200K treatment. Spectrophotometric analysis of material poised at −60 mV (i.e., Fig. 8, Part. B conditions) and illuminated at 200 K also indicated a small amount of slowly oxidized cytochrome (approx 30 %) in addition to the rapidly oxidized cytochrome which follows the reduction of QFe. Whatever the reason for the apparent incompetence, the amount of "second" cytochrome oxidized is commensurate on an electron equivalent basis with the amplitude of I^- generation in this range, and the points fit quite well (although there is scatter in the data points) on the expected x^2 curve for the "second" cytochrome c_{553}.

One possible explanation for this unexpected behavior could be that, although both cytochromes c_{553} hemes are originally thermodynamically and kinetically equivalent with respect to the light generated $(BChl)_2^+$, once one is oxidized most of the remaining heme becomes functionally incompetent. This phenomenon may be related to that seen with cytochrome c_{555} and many other cytochrome reactions which rapidly lose competency as temperatures are lowered below 270 K.

A second result which cannot readily be understood is why (Fig. 6F) maximum triplet formation was not observed following the expected $(2x-x^2)$ curve for the cytochrome c_{553} but only as the quinone-iron was also reduced. The absence of any

reversible light-induced $(BChl)_2^+$ at -60 mV implies that all the QFe population was reduced after the first turnover, apparently ruling out the possibility that this result is due to a portion of the reaction centers being devoid of cytochrome c_{553}. Nor do we understand why there is still some triplet signal after the 200 K treatment; all the extents in Fig. 6F appear to be 0.2 equivalents above what would have ideally been expected; all the triplet signal did disappear as 1^- was reduced in *R. viridis* [7]. Hopefully further experiments will clarify this point.

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