Biochimica et Biophysica Acta, 460 (1977) 467–479

BBA 47303

EFFECT OF REDUCTION OF THE REACTION CENTER INTERMEDIATE UPON THE PICOSECOND OXIDATION REACTION OF THE BACTERIOCHLOROPHYLL DIMER IN *CHROMATIUM VINOSUM* AND *RHODOPSEUDOMONAS VIRIDIS*

T. L. NETZEL[a], P. M. RENTZEPIS[a], D. M. TIEDE[b], R. C. PRINCE[b] and P. L. DUTTON[b]

[a]*The Bell Laboratories, Murray Hill, N.J. 07974 and* [b]*The Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pa. 19174 (U.S.A.)*

(Received November 30th, 1976)

SUMMARY

The photo-oxidation of the reaction center bacteriochlorophyll dimer or special pair was monitored at 1235 nm in *Chromatium vinosum* and at 1301 nm in *Rhodopseudomonas viridis.* In both species, the photo-oxidation was apparently complete within 10 ps after light excitation and proceeded unimpeded at low temperatures regardless of the prior state of reduction of the traditional primary electron acceptor, a quinone-iron complex. Thus the requirement for an intermediary electron carrier (I), previously established by picosecond measurements in *Rps. sphaeroides* (see ref. 4), is clearly a more general phenomenon.

The intermediary carrier, which involves bacteriopheophytin, was examined from the standpoint of its role as the direct electron acceptor from the photo-excited reaction center bacteriochlorophyll dimer. To accomplish this, the extent of light induced bacteriochlorophyll dimer oxidation was measured directly by the picosecond response of the infrared bands and indirectly by EPR assay of the triplet/biradical, as a function of the state of reduction of the $I/I^{\dot{-}}$ couple (measured by EPR) prior to activation. Two independent methods of obtaining I in a stably reduced form were used: chemical equilibrium reduction, and photochemical reduction. In both cases, the results demonstrated that the intermediary carrier, which we designate I, alone governs the capability for reaction center bacteriochlorophyll photooxidation, and as such I appears to be the immediate and sole electron acceptor from the light excited reaction center bacteriochlorophyll dimer.

INTRODUCTION

In the bacterial photosynthetic reaction center, the first products of the light-initiated reaction which are stable for milliseconds are the reduced "primary" acceptor, a quinone-iron complex designated $Q^{\dot{-}}Fe$, and the oxidized primary donor, a bacteriochlorophyll dimer $(BChl)_2^{\dot{+}}$ (for a review, see ref. 1). Recent evidence [4] indicates that this reaction actually involves at least two steps with an intermediate electron carrier, I, operating between the chlorophyll dimer and the quinone-iron $[(BChl)_2I]QFe$. In the first step following light excitation, $(BChl)_2$ is oxidized and I is reduced, forming $[(BChl)_2^{\dot{+}}\ I^{\dot{-}}]QFe$ [2, 4] in less than 10 ps. In the second step, the reoxidation of $I^{\dot{-}}$ appears concommitant with the reduction of QFe, yielding $[(BChl)_2^{\dot{+}}\ I]Q^{\dot{-}}Fe$ with a half-time of 100–200 ps [2, 3, 5].

The absorbance changes associated with the transient $[(BChl)_2^{\dot{+}}I^{\dot{-}}]QFe$ state [2, 4] indicate that the I to $I^{\dot{-}}$ transition involves the reduction of a reaction center bacteriopheophytin ($BPh^{\dot{-}}$) [4–11], but may also involve the bacteriochlorophyll absorbing at 800 nm [7–11]. Whether or not any other reaction center components are reduced before those indentified with I at 10 ps is not yet known. At the other extreme of the experimental time scale, I has been trapped in a reduced state for seconds [9–11] or indefinitely [7, 8, 12–14] in reaction center-cytochrome complexes from *Chromatium vinosum* and *C. minutissimum* (both containing bacteriochlorophyll *a* and bacteriopheophytin *a*) and *Rhodopseudomonas viridis* (containing bacteriochlorophyll *b* and bacteriopheophytin *b*). In *C. vinosum* and *C. minutissimum*, the absorbance changes accompanying formation of trapped $I^{\dot{-}}$ display many similarities with those encountered at 10 ps in *Rps. sphaeroides* [2–4] (containing bacteriochlorophyll *a* and bacteriopheophytin *a*). In *Rps. viridis*, I is apparently reducible at equilibrium in the dark, and measurements have yielded an E_m value (pH 10.8) of $\cong -400$ mV ($n \cong 1$) for the $I/I^{\dot{-}}$ couple [15].

In the absence of time-resolved kinetic evidence that I is reduced simultaneously with $(BChl)_2$ oxidation, the major justification for recognizing I as the intermediate and sole electron acceptor from the excited $(BChl)_2$ is based on indirect measurements. The justification rests upon observations of the light-induced spin polarized triplet, or biradical ($BChl^{\dot{+}}-BChl^{\dot{-}}$), which is formed during back-reactions. The EPR signal of ($BChl^{\dot{+}}-BChl^{\dot{-}}$) is readily observed in the light at low temperatures when the normal forward electron transfer from $I^{\dot{-}}$ to QFe is blocked by previous reduction of the QFe [15–19] or Q removal [25]. Under these conditions, illumination still illicits the < 10 ps oxidation of $(BChl)_2$ [4] and reduction of I [2–4], but because the QFe is already reduced, the electron on $I^{\dot{-}}$ is forced to return to the $(BChl)_2^{\dot{+}}$. At low temperatures it is this return, $t_{\frac{1}{2}} \cong 30$ ns, [20, 21] which is thought to be responsible for the formation of the spin-polarized triplet/biradical state ($(BChl)^{\dot{+}}-(BChl^{\dot{-}})I$) [4, 15]. Therefore the triplet EPR signal provides a measure of ($BChl_2$) oxidation when QFe is reduced before illumination, because an electron has to leave $(BChl)_2$ to form the triplet/biradical in the back reaction.

Support for both the kinetic model and the proposition that I is an immediate acceptor from $(BChl)_2$ is provided by experiments which showed that the light-induced triplet/biradical state was markedly diminished when I was reduced before illumination [7, 8, 12]. Indeed this effect was used to measure the E_m of the $I/I^{\dot{-}}$ couple in *Rps. viridis* [15].

However, whether I is the sole electron acceptor from excited $(BChl)_2$ or one of several is open to question. It is possible for example that another electron acceptor from excited $(BChl)_2$ exists which either accepts the electron before I or serves as an alternative acceptor when I is reduced, and that this acceptor fails to generate a detectable triplet/biradical on $(BChl)_2$ in a back reaction. In view of this possibility that the triplet/biradical may not be an obligatory consequence of $(BChl)_2^{\dot{+}}$ generation when QFe is reduced, we have sought further and more direct information about excited $(BChl)_2$ oxidation. To investigate this, we measured $(BChl)_2^{\dot{+}}$ formation by monitoring the picosecond kinetics of reaction center bands in the 1100–1400 nm spectral region.

In the reaction center protein isolated from *Rps. sphaeroides*, an infrared absorbance band centered at 1250 nm appears to be a reliable indicator of the $(BChl)_2^{\dot{+}}$ state (see ref. 4). If this is true, current schemes for the photosynthetic reaction [4, 6, 7, 10, 15] would predict an attenuation of the extent of flash-induced infrared absorption increases, and therefore $(BChl)_2^{\dot{+}}$ formation, following prior reduction of the sole and immediate electron acceptor. The results presented here were obtained from reaction center-cytochrome *c* complexes in which it was possible to generate a stable $I^{\dot{-}}$ state. This was done in two ways: either chemically as in *Rps. viridis* [15] or photochemically as in *Rps. viridis* [12–14] and *C. vinosum* [7, 8, 11, 12]. These results support the current proposal that I is the immediate and sole electron acceptor from light excited $(BChl)_2$; this conclusion is based on the close correlation between the extent of inhibition of both light-induced triplet/biradical formation and infrared absorbance increase, as I is reduced before light activation.

MATERIALS AND METHODS

Preparative procedures

The photosynthetic bacteria, *C. vinosum* and *Rps. viridis* were grown anaerobically in the light as previously described [8, 15]. Chromatophores were prepared by passing the cells through a French press followed by the usual differential centrifugation procedures. The reaction center · cytochrome complex from *C. vinosum* was isolated according to the procedure reported previously [8]; Fig. 1 shows our *Rps. viridis* reaction center complex prepared after the recent improved method of Schaeffer

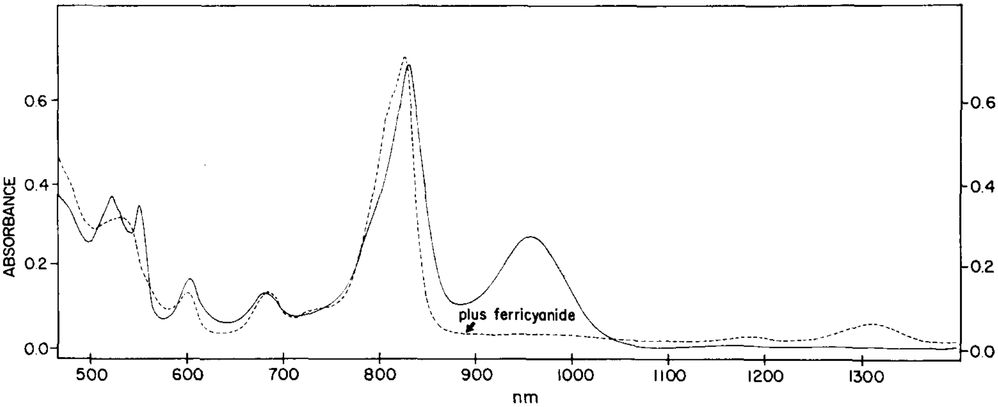


Fig. 1. Absorption spectra of the *Rps. viridis* reaction center · cytochrome complex in the oxidized and reduced forms. The solid line indicates the spectra for the material as isolated in which both the high potential cytochromes c_{558} and low potential cytochromes c_{553} are reduced as a result of the reductant (dithionite) used in the isolation procedure [13, 14]. The suspending medium was 20 mM Tris · HCl, pH 8.0, the temperature 300 K and the light path 1 cm. The addition of ferricyanide to this sample (dashed line), sufficient to raise the redox potential to E_h = 550 mV, results in oxidation of the 960 nm absorbing reaction center $(BChl)_2$ in the reaction center.

et al. [13, 14]. The preparation was fully photochemically active and was capable of supporting normal cytochrome *c* oxidation down to at least 77 K. The dashed line in Fig. 1 shows the chemically oxidized spectrum with the bleaching of the $(BChl)_2$ at 960 nm band and the appearance of the $(BChl)_2^{+\cdot}$ absorption band at 1310 nm; the analogous $(BChl)_2^{+\cdot}$ absorbance band for the *C. vinosum* preparation is at 1235 nm.

The low temperature studies were performed in 50 % ethylene glycol to obtain optically clear glasses. With the *C. vinosum* preparation it was necessary to exchange the preparative cholate detergent for 0.5 % Triton X-100 because the preparation aggregated in the presence of cholate plus ethylene glycol.

Redox potentiometry

The reaction center vessels and other apparatus used for the anaerobic redox poising and transfer of samples for low temperature spectroscopic and EPR analysis have been previously described [22]. When appropirate, all procedures were carried out under minimal lighting conditions to prevent premature photochemistry. The redox dyes used to mediate in the +200 mV, −300 mV and −460 mV region were as follows: 50 μM potassium ferrocyanide, 2 μM 2,3,5,6-tetra methylphenylene diamine (diaminodurol), 5 μM benzyl and methyl violgens and 5 μM 1,1′-trimethylene-2,2′-dipyridylium dibromide ("triquat"). Redox adjustments were made with potassium ferricyanide and sodium dithionite.

Spectrometry

Electron paramagnetic resonance (EPR) spectra were recorded with a Varian E4 EPR spectrometer, equipped with a flowing helium cryostat and temperature controller.

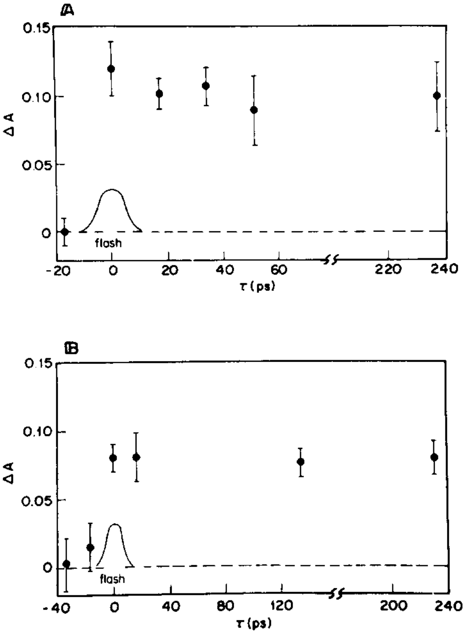
Optical spectra were recorded on Johnson Foundation split beam and dual wavelength spectrophotometers equipped with Dewar flasks for low temperature work.

Picosecond spectroscopy was performed as described previously [23, 24] except that a germanium photodiode detector was used. Excitation light at 530 nm was generated by frequency doubling a single 1060 nm pulse from a Nd/glass, mode locked laser. The infrared light used to interrogate post-excitation kinetics was produced by Raman shifting the 1060 nm laser fundamental in carbon-tetrachloride (20 cm cell length). The coincidence (in time and space) between the infrared observation pulse and the 530 nm excitation pulse was determined by the operation of a picosecond light shutter. The shutter's operation is based on the ability of the 530 nm excitation pulse to produce a picosecond birefringence in a 2-mm CS_2 cell placed between crossed polarizers. The use of this shutter guaranteed that no time measurement errors were made as a result of dispersion. Operation of a mechanical delay stage permitted the measurement of optical density changes in the sample before and after the arrival of the excitation pulse. Low temperatures were maintained in a silver plated, copper cuvette with quartz windows which was attached to a temperature-controlled flowing nitrogen cyrostat.

RESULTS

The kinetics of $(BChl)_2$ photo-oxidation

Fig. 2 shows the picosecond kinetics of the light induced $(BChl)_2^{+\cdot}$ species,



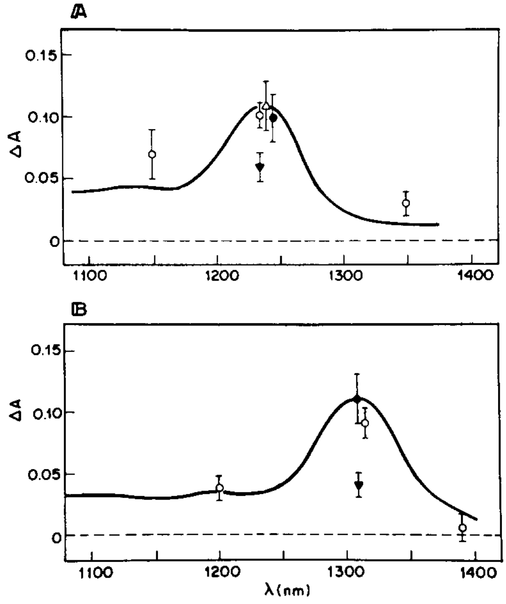


Fig. 2. Picosecond kinetics of the light-induced infrared absorption increases in the *C. vinosum* (A) and *Rps. viridis* (B) reaction center · cytochrome complexes. Reaction center complexes were poised, in a 2 mm optical path length anaerobic redox cuvette, at approx. E_h = 200 mV to insure the full reduction of the $(BChl)_2$ and oxidation of QFe before laser excitation (530 nm) (see Materials and Methods for redox mediating dyes). The extent of absorbance changes observed at times preceding and following excitation was measured at 300 K. Part A shows the kinetics observed at 1235 nm for the *C. vinosum* preparation ($A_{883\,nm}$ = 11 cm^{-1}), suspended in 0.5 % Triton X-100, 20 mM Tris · HCl, pH 8.0; part B, the 1310 nm kinetics for the *Rps. viridis* preparation ($A_{960\,nm}$ = 10 cm^{-1}), suspended in 20 mM Tris · HCl, pH 8.0. The points in this figure, and for the following picosecond data, are the average of at least four determinations, and the error bars represent the standard deviation. The laser flashes were not saturating.

Fig. 3. Spectra of the infrared absorbance changes measured 17 ps after laser activation. The open circles (○) represent changes measured at 300 K and E_h = 200 mV as described in Fig. 2. The other symbols represent data obtained at low temperature: the reaction center · cytochrome complexes were suspended in 50 % ethylene glycol, 20 mM Tris · HCl, pH 8, 0.5 % Triton X-100, poised anaerobically at −300 mV (QFe reduced), transferred to 2 mm cuvettes, and frozen to 120 K. The absorbance increases were measured at (A) 1235 nm for *C. vinosum* ($A_{883\,nm}$ = 11 cm^{-1}) and (B) 1310 nm for *Rps. viridis* ($A_{960\,nm}$ = 12 cm^{-1}) before (●) and after (▼) low temperature (110–150 K) illumination. The lamp used for continuous illumination in this and other experiments was a focused Unitron lamp (8V, 5A). The illumination period for *C. vinosum* preparation was 30 min, and for the *Rps. viridis* preparation was 50 min. In the case of *C. vinosum* the sample treated by prolonged illumination in the 110–150 K range was warmed to 300 K for 20 s and recooled to 120 K in the dark, and the absorbance change remeasured (△).

measured by the appearance of the absorption increases at 1235 and 1310 nm for the *C. vinosum* and *Rps. viridis* reaction center-cytochrome complexes, respectively. Fig. 3 shows light-induced spectra of these absorption increases, measured 17 ps after excitation, superimposed upon spectra of chemically generated $(BChl)_2^{+}$. These data establish that the photo-oxidation of the $(BChl)_2$ occurs within 10 ps of excitation in both *C. vinosum* and *Rps. viridis*. Similar results have been presented earlier for *Rps. sphaeroides* [4]. Fig. 2 also shows that there is no significant decay of the oxidized dimer within 240 ps after excitation, which is again in agreement with data for *Rps. sphaeroides* [4]. Under these conditions, the oxidized dimer is expected to be present until it is reduced after several microseconds by one of the high potential *c*-type cytochromes: cytochrome c_{555} in *C. vinosum* [26] or cytochrome c_{558} in *Rps. viridis* [26].

The effect of I reduction on $(BChl)_2$ *photo-oxidation*

a) Chemical reduction of I. If the model and propositions which are described in the Introduction are correct, the reduction of the $I/I^{\overline{\cdot}}$ couple before activation should prevent the photo-oxidation of the $(BChl)_2$ and hence the appearance of the 1235 or 1310 nm bands. I can be chemically reduced in chromatophores from *Rps. viridis* where the E_m is −400 mV at pH 10.8 [15]. Fig. 4 shows that anaerobically poising *Rps. viridis* reaction centers at an E_h of approximately −470 mV (pH 9.8) diminishes the extent of light induced 1310 nm absorption increase by approximately 65 % relative to its original E_h = 200 mV (i.e., I oxidized) value. Unfortunately we were unable to maintain the ambient redox potential below −470 mV for the long periods of time required for the picosecond experiments. However, one minute of illumination at room temperature of the E_h = −470 mV sample [13, 14] further inhibited the laser-induced 1310 nm change yielding an 82 % inhibition compared to the original value. Restoring the ambient redox potential to +200 mV to reoxidize $I^{\overline{\cdot}}$, promptly returned the 1310 nm change to almost the original level. The fact that the return was not entirely complete is probably not significant, but rather a reflection of some reaction center attrition resulting from the long exposure to low potential/high pH environments. Typically, approx. 6 h were required for data accumulation to complete an experimental series.

As a control, the light-induced triplet/biradical EPR signal was measured. Reaction centers were treated as described above at room temperature, and then

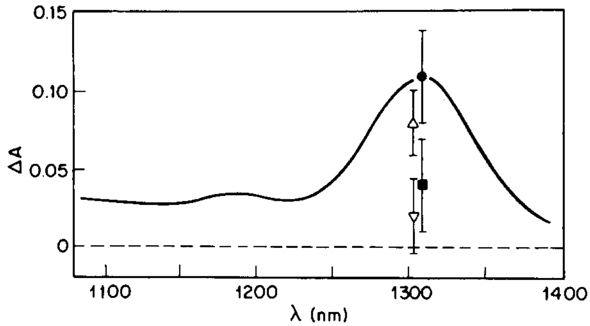


Fig. 4. Redox potential dependence of the picosecond flash induced absorption increases for the *Rps. viridis* reaction center complex. The 1310 nm absorption increases were measured 34 ps after laser excitation (530 nm) at 300 K, with the reaction center cytochrome complex ($A_{960\,nm}$ 12 cm^{-1}) suspended in 100 mM glycine 100 mM Tris · HCl pH 9.8 and contained in a 2 mm light path anaerobic cuvette. The 1310 nm absorption increases were assayed sequentially for the following conditions: E_h = +200 mV (●); E_h = −470 mV (■); E_h = −470 mV after 1 min of continuous illumination at room temperature (▽); following the return of the redox potential to E_h = +200 mV (△).

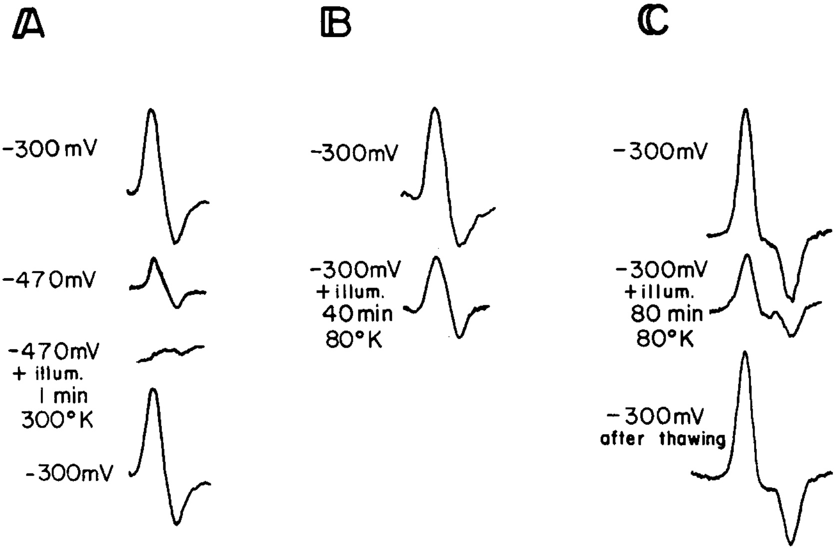


Fig. 5. Light induced triplet EPR signals in the *Rps. viridis* (A and B) and *C. vinosum* (C) reaction center complexes. The extent of triplet formation was assayed by the amplitude of the low field portion of the triplet signal ($g \cong 2.1$) observed at 5 K. Part A shows the results observed for samples treated under identical conditions to those listed in Fig. 4, but transferred anaerobically to EPR tubes and rapidly cooled to 80 K before triplet assay. Parts B and C follow the protocol listed for the low temperature experiments in Fig. 3, except that continuous illumination treatment was done in 3 mm internal diameter EPR tubes submerged in liquid nitrogen. The instrument settings for the EPR assay were 1 mW microwave power, 12.5 G modulation amplitude, 100 Hz modulation frequency (see ref. 15).

cooled to liquid helium temperatures for assay. As shown in Fig. 5, there was a good correlation between the amount of triplet formation and the extent of the 1310 nm absorption increase under similar conditions. Although after illumination there was a 95% diminution of triplet formation compared with 82% of flash-induced 1310 nm increase, this difference is within the resolution of the experiments, and may be of doubtful significance.

b) Photochemical reduction of I

Because chemical reduction is a somewhat non-specific treatment, particularly under strongly reducing conditions (i.e., other electron acceptors alternative to I may have similar E_m values and could be reduced together with I), we have taken advantage of the ability to trap photo-chemically $I^{\bar{\cdot}}$ in the *C. vinosum* [7, 8, 12] and *Rps. viridis* [12–14] reaction center-cytochrome complexes. In this way, $I^{\bar{\cdot}}$ is generated in the relatively mild and well-studied redox environment just sufficient to reduce QFe. This procedure has the additional advantage of forming the $I^{\bar{\cdot}}$ state as a product of the light-induced $(BChl)_2$ oxidation.

The photo-chemical trapping technique, which we have discussed at length [7, 8], starts with the system

ferrocytochrome $c[(BChl)_2\, I]Q^{\bar{\cdot}}Fe$

and prolonged illumination at low temperatures is proposed to yield:

ferricytochrome c $[(\mathrm{BChl})_2\,\mathrm{I}^{\overline{\cdot}}]\mathrm{Q}^{\overline{\cdot}}\mathrm{Fe}$

The rate of the forward reaction is dependent on the incident light intensity, while the rates of both forward and reverse reactions depend on the temperature. At 200 K the reverse reaction has a halftime of approx. 20 min, but at 80 K it is so slow that no decay has been detected even after several days; thus $\mathrm{I}^{\overline{\cdot}}$ is trapped indefinitely at low temperatures and is produced at the expense of the one-electron oxidation of one cytochrome c heme.

The study of the picosecond kinetics of $(\mathrm{BChl})_2$ photo-oxidation in reaction centers with $\mathrm{I}^{\overline{\cdot}}$ photochemically trapped at low temperatures presented several technical restrictions. Because these studies required optically clear samples, ethylene glycol was added to the reaction center preparation to produce clear glasses at cryogenic temperatures. However, the properties of the reaction center · cytochrome complex suspensions in ethylene glycol constrained the available temperature range because the frozen suspension caracked as the temperature went below 100 K, and became opaque (unless thawed) as the temperature approached 160 K. A consequence of having to work in this temperature range was that the halftime of the generation of $\mathrm{I}^{\overline{\cdot}}$ was expected to be much longer than the 30 s halftime which was previously established at 200 K.

Because $\mathrm{I}^{\overline{\cdot}}$ is photochemically trapped only when ferrocytochrome c successfully and irreversibly reduces $(\mathrm{BChl})_2^{\dot{+}}$ before $\mathrm{I}^{\overline{\cdot}}$ does so reversibly in the back reaction, the trapping of $\mathrm{I}^{\overline{\cdot}}$ is very sensitive to the rates of these competing reactions. While the back reaction is not very temperature sensitive, at least in *Rps. sphaeroides* [20, 21], the rate of cytochrome c oxidation decreases markedly as the temperature is lowered. In all three species where this has been fully described (*C. vinosum* [27, 28], *Rps. gelatinosa* [29] and *Rps. sp. N.W.* [29]), cytochrome c oxidation displays an activation energy of 2–4 kcal/mol as the temperature is lowered from room temperature to between 120 and 150 K, and then below this point the reaction approaches temperature insensitivity ($E_a = 0$ kcal/mol). Thus below the 120 to 150 K transition we might expect the generation time of $\mathrm{I}^{\overline{\cdot}}$ to become essentially temperature insensitive.

Fig. 6 shows the time of formation for $\mathrm{I}^{\overline{\cdot}}$, as determined by EPR spectroscopy, under conditions similar to those mandated by the picosecond experiments. In these EPR experiments, as in picosecond experiments, reaction center · cytochrome complexes in 50 % ethylene glycol were anaerobically poised at -300 mV and then frozen. The extent of the light-induced triplet signal was first determined at 5 K; then the samples were illuminated continuously at liquid nitrogen temperatures. At various points during the illumination treatment the extent of light-induced triplet signal, and the amount of $\mathrm{I}^{\overline{\cdot}}$ signal generated were assayed at 5 K. In both *Rps. viridis* and *C. vinosum*, the halftime of $\mathrm{I}^{\overline{\cdot}}$ trapping and decrease in the light-induced triplet/radical is about 60 min, although no attempt was made to pursue the reaction to completion. The importance of Fig. 6 is that it establishes a similar time course for the amount of I reduction and the extent of light-induced triplet inhibition. This result complements previous inverse correlations between the two signals and the redox potential of the reaction center [8].

In the companion studies of the picosecond kinetics of the infrared bands, re-

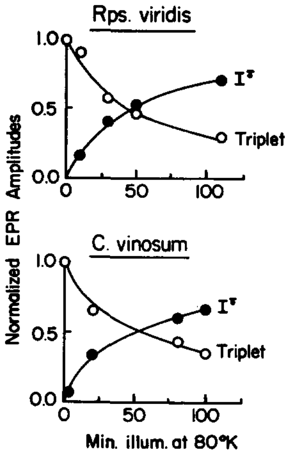


Fig. 6. The approximate kinetics for the appearance of I⁻ and triplet EPR signals following illumination at 80 K. *Rps. viridis* (top) and *C. vinosum* (bottom) samples were poised at $E_h = -300$ mV at room temperature, as described in Fig. 3 and Fig. 5 (B and C), then cooled and illuminated in liquid nitrogen. Samples tubes were withdrawn at various times and assayed at 5 K for triplet and I⁻ EPR signals. The triplet assays are described in Fig. 5. The extent of I⁻ accumulation was determined by the amplitude of the low field signal at $g = 2.034$ in *C. vinosum*, and by the amplitude of the $g = 2.00$ signal in *Rps. viridis*. EPR spectrometer settings were the same as listed in Fig. 5.

action center · cytochrome complexes in 50 % ethylene gycol were poised anaerobically at an ambient redox potential of —300 mV in the dark, transferred anaerobically to the cuvette, and then cooled rapidly into the 110–150 K range in order to form an optically clear glass. Before proceeding, we checked that the light induced infrared absorbance increase measured at 110–150 K was comparable to that measured at room temperature. Fig. 3 (a and b) show that this was the case in both *C. vinosum* and *Rps. viridis*.

In order to trap I⁻, we illuminated the *C. vinosum* reaction center · cytochrome complex for 30 min near 120 K (using the same lamp system was as used for the EPR experiments). This resulted in approximately 40 % inhibition of the flash-induced 1235 nm absorption increase (Fig. 3a). A similar treatment of the *Rps. viridis* sample at 120 K (Fig. 3b) caused a 60 % inhibition of the 1310 nm absorption increase. Relaxation of the trapped I⁻ state by thawing the samples, followed by immediate recooling to the 110–150 K region, restored the light induced infrared absorption band increase to its original level.

Although the EPR (Fig. 6) and picosecond cryogenic experiments (Fig. 3) were not done under precisely the same conditions (namely, the geometry of the cuvettes and the temperature of the continuous illumination treatments were different), they are qualitatively comparable. Together they suggest that the low temperature illumination for the picosecond experiments (Fig. 3) would trap approximately half the reaction centers in an I⁻ state. The experiments show that in both *C. vinosum* and *Rps. viridis* this level of I reduction prior to activation is reflected not only in the diminished generation of the light-induced triplet/biradical signal (Figs. 5 and 6), but also in the diminished generation of the infrared absorbancy increase in the 1100–1400 nm region (Fig. 3).

The optical spectrum of the changes accompanying I reduction in Rps. viridis

In an earlier paper [7, 8] we presented the optical spectrum accompanying the trapping of $I^{\cdot-}$ in *C. vinosum*. Fig. 7 illustrates a similar experiment performed on the *Rps. viridis* reaction center · cytochrome complex. Part A shows the absolute absorption spectrum of reaction centers poised at $E_h = -300$ mV, both before and after illumination for 50 min at 80 K. Part B shows the difference spectrum taken from Part A. This represents the combined absorbance changes for the reduction of I and the oxidation of cytochrome c_{553}.

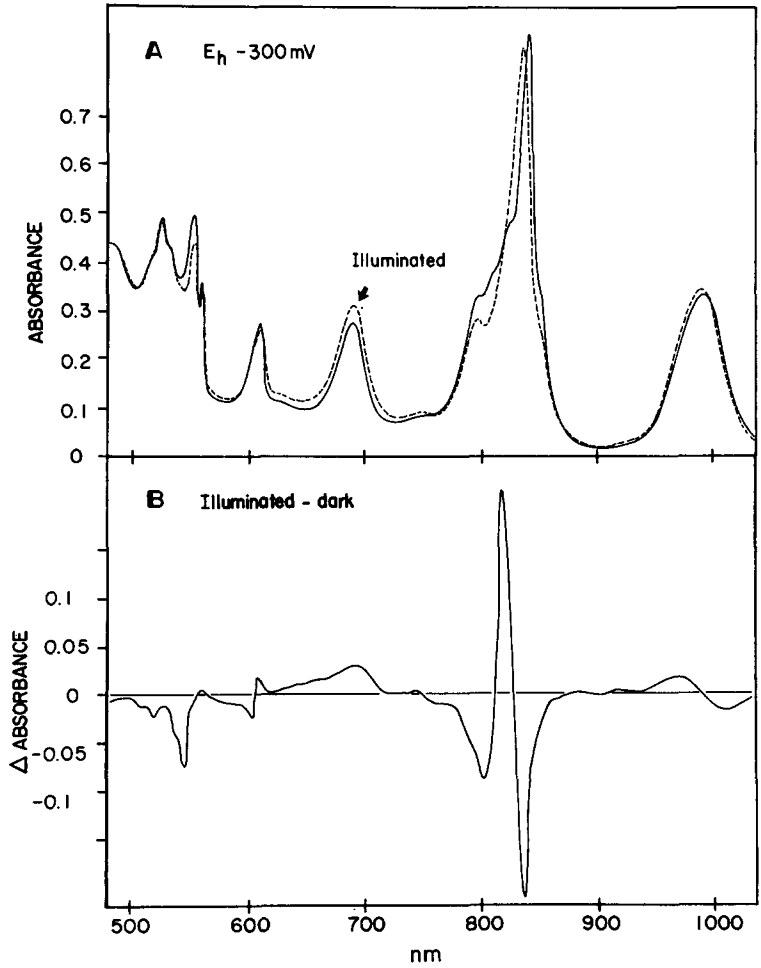


Fig. 7. Optical spectra for the *Rps. viridis* reaction center · cytochrome complex and the partially accumulated $I^{\cdot-}$ state. The solid line, Part A, shows the absorbance spectrum measured at 80 K for the *Eps. viridis* preparation suspended in 50 % ethylene glycol, 50 mM Tris · HCl, pH 8.0. As described in Fig. 3, the material was first posed at $E_h = -300$ mV at room temperature, then anaerobically transferred to a 2 mm low temperature cuvette and cooled to $\cong$ 80 K in liquid nitrogen. The dashed line shows the spectrum after 50 min of continuous illumination of the cuvette submerged in liquid nitrogen. Part B shows the illuminated minus dark difference spectrum, taken from the spectrum in Part A.

DISCUSSION

The kinetics of $(BChl)_2$ photo-oxidation

An essential part of these studies was the extension to *C. vinosum* (which possesses bacteriochlorophyll and bacteriopheophytin *a*) and *Rps. viridis* (which possesses bacteriochlorophyll *b* and bacteriopheophytin *b*) of the measurement of the rate of formation of the infrared absorption band characteristic of the oxidized reaction center bacteriochlorophyll dimer, $(BChl)_2^{+\cdot}$. The rate of $(BCHl)_2^{+\cdot}$ formation had been measured previously in *Rps. sphaeroides* reaction centers [4]. It now appears that in all three species the photo-excited $(BChl)_2$ becomes oxidized in less than 10 ps and, as expected for $(BChl)_2$ oxidation, the reaction is unaffected by temperature decrease to at least 120 K. Furthermore it is established now in all three species that the reaction proceeds whether or not the traditional "primary" acceptor, QFe is reduced. These results expand into a more general phenomenon, the demonstration of an obligatory requirement for an intermediary electron carrier reacting directly with the $(BChl)_2$.

The role of the component designated I

The main part of this work was designed to acquire more information about the intermediary electron carrier recognized by optical and EPR spectroscopy and designated $I/I^{-\cdot}$. In particular, we sought to determine whether its prior reduction would impede $(BChl)_2$ photo-oxidation, because such an effect is an essential pre-requisite of its postulated role as sole electron acceptor from the photo-excited $(BChl)_2$.

The room-temperature optical experiments on *Rps. viridis* showed that poising the reaction center · cytochrome complex at an ambient redox potential of −470 mV caused a 65 % inhibition in the ability of the $(BChl)_2$ to undergo photo-oxidation (compared to the extent of photo-oxidation encountered at −300 mV or higher). A similar inhibition in the extent of triplet/biradical formation was found by EPR experiments. If we assume that the reduction of I in the reaction center · cytochrome complex follows a Nernst curve similar to the one for I reduction in chromatophores [$n \cong 1$ when $(BChl)_2$ photooxidation was measured indirectly by monitoring light-induced triplet signal], the E_m of the $I/I^{-\cdot}$ couple in the reaction center · cytochrome complex would be −450 mV compared with the −400 mV measured in chromatophores [15]. This is neither a large nor an unprecedented difference, since the E_m of the $(BChl)_2/(BChl)_2^{+\cdot}$ couple of *Rps. viridis* has also been reported to exhibit a 50 mV negative shift upon isolation (cf. refs. 15, 30). Differences for the E_m of QFe in *Rps. sphaeroides* between chromatophores and reaction centers have also been observed [31].

The photochemical trapping of $I^{-\cdot}$ is a more strigent test of the function of the $I/I^{-\cdot}$ couple, since the chemical reduction might have reduced alternative acceptors with similar or higher E_m values than the $I/I^{-\cdot}$ couple. These photo-trapping experiments established a good correspondence between the $I^{-\cdot}$ formation and the inhibition of $(BChl)_2^{+\cdot}$ formation as indicated by the light-induced triplet/biradical (Fig. 6); this augmented the same relationship acquired on a redox potentiometric basis [7, 8]. Also, the extent of flash induced $(BChl)_2^{+\cdot}$ formation indicated by the absorbancy increase of the 1310 nm or 1235 nm bands (Fig. 3) was related to the expected amount of $(BChl)_2^{+\cdot}$ formation as indicated by the triplet/biradical experiments

(Figs. 5 and 6).

Using the above indicators of $(BChl)_2^{+\cdot}$, it seems clear that the state of reduction of the I/$I^{-\cdot}$ couple governs the ability of $(BChl)_2$ to undergo photo-oxidation. Thus I satisfies this criterion for the role of the immediate electron acceptor from the excited $(BChl)_2$. Furthermore, since the $(BChl)_2$ photo-oxidation measured by the infrared absorption and by the triplet EPR signal give the same results, it would appear that I is the sole electron acceptor from the excited $(BChl)_2$. There seems to be little or no room left for an alternate acceptor which does not generate triplet/biradical signals in re-reducing $(BChl)_2^{+\cdot}$.

Further spectroscopic information on the nature of $I^{-\cdot}$

Fig. 7 presents the optical spectrum accompanying I reduction in the *Rps. viridis* reaction center · cytochrome complex. In essence, it is the low temperature analog of the spectrum presented by Schaeffer et al. [13, 14], and as such includes a contribution from oxidized cytochrome c_{553}. This spectrum exhibits some major differences when compared to the $I^{-\cdot}$ spectrum of *C. vinosum* [7, 8, 11] and *C. minutissimum* [9, 10]. In both *Chromatium* species there was not only a blue shift, but also a partial bleaching, of the 800 nm bacteriochorophyll. In *Rps. viridis* where the analogous bacteriochorophyll *b* band is at 830 nm, there is only the blue shift of the BChl with no concommitant bleaching. There are also other minor absorbance changes, such as the apparent band shifts observed in the 960 and 600 nm regions. The significance of these perhaps secondary effects is uncertain at present.

In the *C. vinosum* case, we considered the possibility that the bleaching of the 800 nm absorbing bacteriochlorophyll indicated the generation of $BChl^{-\cdot}$, which might perhaps combine with $BPh^{-\cdot}$ to produce $I^{-\cdot}$. The absence of such bleaching in *Rps. viridis* renders this less likely, at least as a general phenomenon, and leaves $BPh^{-\cdot}$ as the major contender for a role in $I^{-\cdot}$. This of course leaves unanswered questions concerning the role of the 800 nm bacteriochlorophylls and the remaining bacteriopheophytin.

ACKNOWLEDGEMENTS

Part of this work was supported by an NSF grant PCM 76-14209 to P.L.D. P.L.D. is the recipient of a PHS RCDA award no. 1-K4-GM 70771. This support is gratefully acknowledged.

REFERENCES

1 Parson, W. W. and Cogdell, R. J. (1975) Biochim. Biophys. Acta 416, 105–149
2 Kaufmann, K. J., Dutton, P. L., Leigh, J. S., Netzel, T. L. and Rentzepis, P. M. (1975) Science 188, 1301–1304
3 Rockley, M. G., Windsor, M. N., Cogdell, R. J. and Parson, W. W. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2251–2255
4 Dutton, P. L., Kaufmann, K. J., Chance, B. and Rentzepis, P. M. (1975) FEBS Lett. 60, 275–280
5 Kaufmann, K. J., Petty, K. M., Dutton, P. L. and Rentzepis, P. M. (1976) Biochem. Biophys. Res. Commun. 70, 839–845
6 Fajer, J., Brune, D. C., Davis, M. S., Forman, A. and Spaulding, L. D. (1975) Proc. Natl. Acad. Sci. U.S. 72, 4956–4960

7 Dutton, P. L., Prince, R. C., Tiede, D. M., Petty, K. M., Cobley, U. T., Kaufmann, K. J., Netzel, T. L. and Rentzepis, P. M. (1976) Brookhaven Symp. Biol. 28, in the press

8 Tiede, D. M., Prince, R. C. and Dutton, P. L. (1976) Biochim. Biophys. Acta, in the press

9 Shuvalov, V. A., Klimov, V. V., Krahmaleva, I. N., Moskalerke, A. A. and Krasnovskij, A. A. (1976) Doklady. Acad. Sci. U.S.S.R. 227, 984–987

10 Shuvalov, V. A. and Klimov, V. V. (1976) Biochim. Biophys. Acta 440, 587–599

11 Van Grondelle, R., Duysens, L. N. M. and van der Wal, H. N. (1976) FEBS Lett., in the press

12 Tiede, D. M., Prince, R. C., Reed, G. H. and Dutton, P. L. (1976) FEBS Lett., 65, 301–304

13 Trosper, T. T., Benson, D. L. and Thornber, J. P. (1976) Abstract of the 4th Annual Meeting of the American Society for Photobiology, p. 63

14 Schaeffer, T. T., Benson, D. L. and Thornber, J. P. (1976) Photochem. Photobiol., in the press

15 Prince, R. C., Leigh, J. S. and Dutton, P. L. (1976) Biochim. Biophys. Acta 440, 622–636

16 Dutton, P. L., Leigh, J. S. and Seibert, M. (1973) Biochem. Biophys. Res. Commun. 46, 406–413

17 Duttton, P. L., Leigh, J. S. and Reed, D. W. (1973) Biochim. Biophys. Acta 292, 654–664

18 Leigh, J. S. and Dutton, P. L. (1974) Biochim. Biophys. Acta 357, 67–77

19 Thurnauer, M. C., Katz, J. J. and Norris, J. R. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3270–3274

20 Parson, W. W., Clayton, R. K. and Cogdell, R. J. (1975) Biochim. Biophys. Acta 387, 365–278

21 Cogdell, R. J., Monger, T. G. and Parson, W. W. (1975) Biochim. Biophys. Acta 408, 189–199

22 Dutton, P. L. (1971) Biochim. Biophys. Acta 226, 63–80

23 Rentzepis, P. M. (1973) Adv. Chem. Phys. 28, 189

24 Busch, G. E., Jones, R. P. and Rentzepis, P. M. (1973) Chem. Phys. Lett. 18, 178–185

25 Okamura, M. Y., Isaacson, R. A. and Feher, G. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3491–3495

26 Case, G. D., Parson, W. W. and Thornber, J. P. (1970) Biochim. Biophys. Acta 223, 122–128

27 DeVault, D. C. and Chance, B. (1966) Biophys. J. 6, 825–847

28 Dutton, P. L., Kihara, T., McCray, J. A. and Thornber, J. P. (1971) Biochim. Biophys. Acta 226, 81–87

29 Kihara, T. and McCray, J. A. (1973) Biochim. Biophys. Acta 292, 297–309

30 Thornber, J. P. and Olson, J. M. (1971) Photochem. Photobiol. 14, 329–341

31 Dutton, P. L., Leigh, J. S. and Wraight, C. A. (1973) FEBS Lett. 36, 169–173