Primary Processes in Photosynthesis: *In Situ* ESR Studies on the Light Induced Oxidized and Triplet State of Reaction Center Bacteriochlorophyll

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Summary

ESR studies at liquid helium temperatures have been conducted on chromatophore and subchromatophore preparations from *Chromatium* D. If the primary electron acceptor of reaction center bacteriochlorophyll is chemically in a reduced state before illumination, the light activated exited state bacteriochlorophyll is prevented from undergoing oxidation. This is evidenced under these conditions by the absence of the familiar $g \cong 2$ signal. Instead, a new ESR spectrum is generated in the light. This is comprised of both absorption and emission bands. The oxidation-reduction potential dependence and kinetics of the ESR changes, activated by laser pulses, suggest the signals represent bacteriochlorophyll in the triplet state. This state could be a primary intermediate in the early light activated transitions of photosynthesis.

Introduction

The primary events and closely associated transitions of photosynthesis can be effectively isolated at low temperatures. With the photosynthetic bacterium *Chromatium* D spectrophotometric and oxidation-reduction potential studies at liquid nitrogen temperatures (1,2) demonstrate that light induced reactions are limited to the reaction center bacteriochlorophyll (P883) ($E_{m7.4}$ + 475 mV), its primary electron acceptor, "X" ($E_{m7.4}$ - 135 mV) and a primary electron donor, cytochrome c_{553} ($E_{m7.4} \cong$ + 10 mV). The interactions of these components at low temperatures are summarized in Table 1. Three distinct light induced events are evident depending on the state of oxidation-reduction of the components in the dark. Each type of reaction can be observed essentially

Reaction	Conditions Before Freezing	E_m Values +10mV	+475mV	-135mV
I	P883 Red. c_{553} Ox X Ox	c^{III}_{553}	$^{+}P883 \xrightarrow[<50nsec]{h\nu} X^{-}$	
		c^{III}_{553}	$P883 \xleftarrow[25msec]{Dark} X$	
II	P883 Red. c_{553} Red X Ox.	c^{II}_{553}	$^{+}P883 \xrightarrow[<50nsec]{h\nu} X^{-}$	
		$c^{III}_{553} \xrightarrow[Dark]{2msec} P883$ //	//	X^{-}
III	P883, c_{553} and X Red.	c^{II}_{553}	$P883 \xrightarrow{h\nu}$ //	X^{-}

Table 1. Reactions in Chromatium D at low temperatures.

to the exclusion of the other two by suitable adjustment of the oxidation-reduction potential. Thus the reactions may be isolated in the following oxidation-reduction potential regions: I, from +400 mV to +100 mV; II, from 0 mV to -100 mV; and III at potentials less than -200 mV. In regions I and II, P883 can rapidly undergo photooxidation. This reaction is reversed in the dark with a very much slower rate. When cytochrome c_{553} is reduced (region II) this cytochrome can donate an electron to the light generated $P^{+}883$ more than ten times faster than can X (region I). Furthermore, the cytochrome oxidation (and hence $P^{+}883$ re-reduction in this case) is essentially irreversible below liquid nitrogen temperatures; this results in X being permanently reduced after brief illumination at these temperatures. When X is in a reduced state in the dark, whether induced photochemically (II) or chemically (III) the photooxidation of P883 is prevented.

We have studied these oxidation-reduction reactions employing ESR at liquid helium temperatures with emphasis on the photochemistry of P883 when X is in the reduced state. The signals generated by continuous or pulsed light under these conditions are consistent with the formation of the triplet state of the bacteriochlorophyll.

Materials and Methods

Chromatium D were grown heterotrophically, anaerobically in the light. Chromatophores were prepared from 1-2 day old cells by method of Baltscheffsky (3). Subchromatophores (Fraction A) were prepared as described by Thornber (4). Preparations were continuously stirred under strict anaerobic conditions in a vessel (1) which was equipped with electrodes for monitoring oxidation-reduction potential. Redox dyes were employed to promote equilibrium between the platinum electrode and the membrane-bound carriers (1,5). Control experiments showed that the dyes did not contribute in any way to the light induced reactions reported. Samples, taken anaerobically, were rapidly frozen to liquid nitrogen temperatures and then maintained in the dark. ESR was assayed in a Varian E3 ESR spectrometer set at microwave power 1 mW and modulation amplitude 12.5 G. Reactions were activated with saturating 100 mJ, 585 nm pulses from a liquid dye laser (General Laser Corp., GL1000).

Results

Figure 1 shows the kinetics of the laser-induced g 2 signal which is generally accepted (see refs. 6-8) to be the paramagnetic oxidized bacteriochlorophyll ($P^{+}883$). The chromatophores were poised before freezing at an oxidation-reduction potential of +115 mV; this is in the potential region for reaction I. The laser induced P883 oxidation (as given by the g 2 signal) at liquid helium temperatures occurs faster than the response time of the instrument (half-time 40 μsec) and becomes re-reduced following the laser flash with a half-time of 25 msec (cf. ref. 7). The reaction is repeatable. However, at -80 mV (potential region for reaction II) the rate of re-reduction of $P^{+}883$ is more than ten times more rapid (half-time 2 msec). This is concomitant with the irreversible oxidation of cytochrome c_{553} (2,9). X now cannot react with P883 which is already reduced. Conversely the second and subsequent laser pulses elicit no further g 2 signal

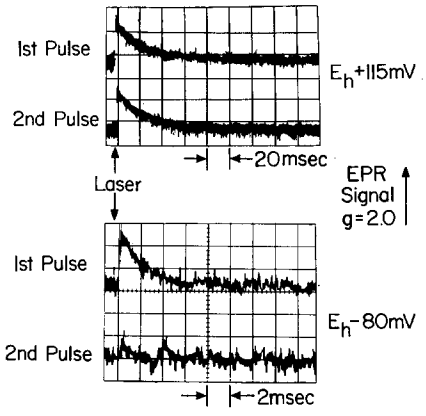

Figure 1. Laser activated g 2 signal (P^+883) at liquid helium temperature in chromatophores from Chromatium D in oxidation-reduction potential regions I (+115 mV) and II (-80 mV). The bacteriochlorophyll concentration was 1.8 mM. The following redox mediators were present: diaminodurol 20μM, phenazine methosulphate 20 μM, pyocyanine 5μM, duroquinone 40 μM, 2-hydroxy-1,4-naphthaquinone 20 μM, anthraquinone-2-sulphanate 20 μM and methylviologen 10 μM.

since P883 photooxidation is prevented by the prior reduced state of X. The behavior of the g 2 signal is entirely consistent with P883 photooxidation and dark reduction assayed spectrophotometrically at liquid nitrogen temperatures (2).

Under any conditions when both X and P883 are in reduced states at liquid helium temperatures (region II after brief illumination; region III without treatment) the light induced g 2 signal is absent and a new light induced ESR signal is clearly apparent. Figure 2 shows the liquid helium ESR spectra of dark chromatophores poised at -260 mV (region III). The top spectrum is taken in the dark; the middle spectrum is of the same material when continuously illuminated with infrared light. The light minus dark difference is shown at the bottom. The light induced ESR changes are rapidly and completely reversible. Figure 3 shows the kinetics of the changes at various oxidation-reduction potentials. At +110 mV (region I) when P883 photooxidation (g 2) is apparent (Figure 1), little laser induced change is observed. At -80 mV (region II), when P883 photooxidation and cytochrome c_{553} oxidation occur after the first laser pulse, there is

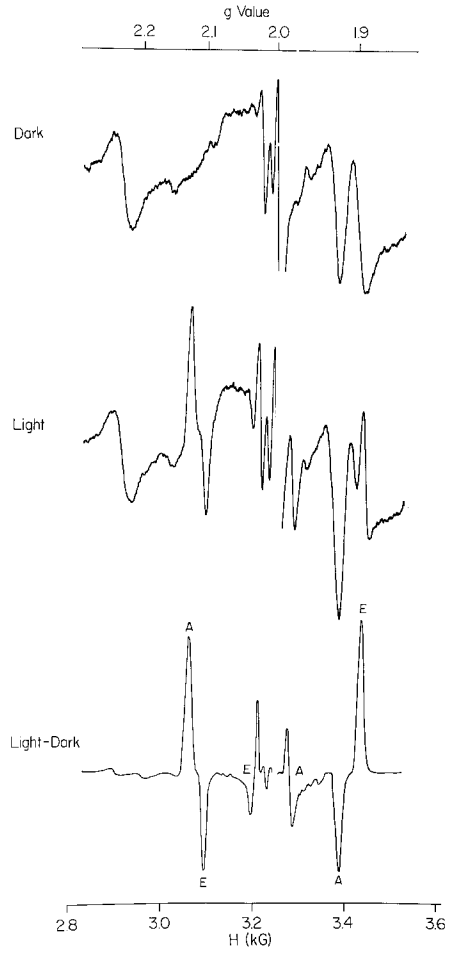

Figure 2. ESR spectra at liquid helium temperature of chromatophores from Chromatium D. The bacteriochlorophyll concentration was 2.0 mM. The oxidation-reduction potential was -260 mV. The following mediators were present: phenazine ethosulphate 20 μM, pyocyanine 5 μM, 2-hydroxy-1,4-naphthaquinone 20 μM and anthraquinone-2-sulphonate, 20 μM. The top spectrum is of chromatophore maintained in the dark; the center spectrum is the same sample but illuminated with a Unitron tungsten lamp (8v;5A) passed through a Wratten 88A filter and 2 cm of water. The lower spectrum is the light minus dark difference. Peaks labeled A and E result from microwave absorption and emission, respectively.

similarly little change. However, the second and subsequent pulses (when X is now already photoreduced) elicit the ESR changes observed in Figure 2. When X is chemically reduced (region III) before freezing the signal is observed following the first flash. The kinetics of the rise and decay of the signal are instrument limited. At all potentials assayed the signal forms in less than half-time 40 μsec and; once formed, decays in less than

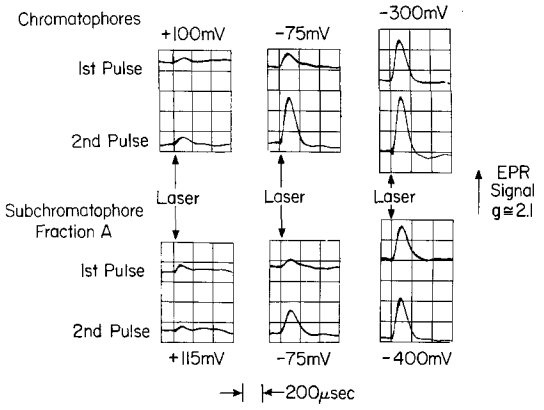

Figure 3. Laser activated ESR measured at g 2·12 (labeled A in Figure 2) at liquid helium temperatures. The details are as described in Figure 1.

half-time 40 μsec. The subchromatophore preparation, fraction A of Thornber (4) displays the same light induced ESR changes as the parent chromatophore.

Discussion

The kinetics of the laser induced g 2 ESR signal are essentially the same as the spectrophotometrically assayed P883 photooxidation (2). These data provide firm support for previous evidence that g 2 signal is the oxidized form of the bacteriochlorophyll reaction center (see refs. 6-8). It may be further stated that the reduced primary electron acceptor (X) of P883 does not display a similar narrow free radical-type signal at g 2. This seems clear from the lower trace (first pulse) in Figure 1 where the g 2 signal returns to the baseline leaving no net g 2 signal as $P^{+}883$ becomes reduced by cytochrome c_{553}. Since these processes (II) leave X permanently reduced at liquid helium temperatures, it is apparent that the reduced primary electron acceptor makes no contribution to the g 2 signal under these conditions.

Under conditions when P883 cannot undergo photooxidation to form the characteristic g 2 signal, because of the prior reduced state of X, other

light induced signals are clearly apparent. The overall properties of these signals suggest they represent bacteriochlorophyll in the triplet state.

In a strong magnetic field, triplet states have three distinct energy levels, which may be labeled by m_S values (+1, 0 and -1). The $\Delta m_S = 1$ transitions which appear in the g 2 region occur between the $+1 \leftrightarrow 0$, and the $0 \leftrightarrow -1$ levels. Theoretical ESR lineshapes for randomly oriented triplets have been described (10,11). Each transition generates three spectral peaks. Triplet ESR spectra are characterized by zero field splitting parameters designated D and E, which are a measure of the dipolar coupling between two electrons. Analysis of the triplet ESR signals observed for bacteriochlorophyll in Figure 2 yields values of 0.0154 cm^{-1} and 0.0031 cm^{-1} for D and E respectively. One of the transitions corresponds to microwave absorption (peaks labeled A in Figure 2); the other is "inverted" (peaks labeled E) which corresponds to microwave emission. This situation may occur when the $m_S = \pm 1$ levels are roughly equally populated and the $m_S = 0$ level contains either a surplus or deficit number of spins. As a result no triplet g 4 ($\Delta m_S = 2$) signals are observed. Selective population (and/or depopulation) of the triplet levels implies that spin lattice relaxation of the electron spins is slow at these temperatures.

A proposed role of the triplet state P883 (P^3883) in early photochemical events is summarized in Figure 4. Important questions still to be answered pertain to the position of the triplet state in energy transfer from the act of light absorption to charge separation. It seems reasonable to consider that the triplet state is a primary intermediate in these processes. The reduced state of X, in preventing charge separation would enhance the level of P^3883. The rates of formation of P^3883 presumably from the exited singlet P^*883 would expected to be significantly faster than the inherent fluorescence rate of $\sim 10^8$ sec^{-1}.

The generality of the phenomenon in photosynthetic bacteria seems

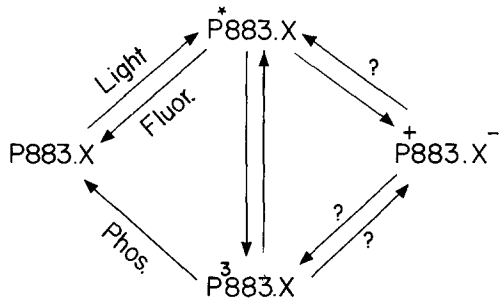

Figure 4. Working scheme for the early photochemical events in bacterial photosynthesis.

probable. Very similar light induced triplet ESR spectra are readily observable in the carotenoid-free mutant of R. rubrum (G 9) and Rps. spheroides (J.S. Leigh and P.L. Dutton, unpublished results).

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