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THE TRIPLET STATE OF REACTION CENTER BACTERIOCHLOROPHYLL: DETERMINATION OF A RELATIVE QUANTUM YIELD

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SUMMARY

The low temperature EPR signal of the excited triplet state of bacteriochlorophyll has been quantitatively studied in reaction centers from *Rhodopseudomonas spheroides* (carotenoid free R 26 mutant). Using laser flash excitation the light saturation curve of the triplet signal has been compared with that of the free-radical formation due to photooxidation of P870 under identical optical conditions. This comparison shows that the quantum yield of triplet formation is nearly the same as that of the photochemical bleaching of bacteriochlorophyll.

INTRODUCTION

Characterization of the chemical components of the electron transport reactions of bacterial photosynthesis has proven very rewarding in recent years and even the difficult problem of identification of the primary electron acceptor has met with some success [1–4]. However, there is still a glaring omission in our knowledge of the primary photoacts, there is no data pertaining directly to the nature of the photochemically active excited state of the reactive chlorophyll.

Fluorescence from the bulk (antenna) chlorophyll of both bacteria and higher organisms exhibits a variable yield which seems to show approximately inverse relationships to photochemistry [5, 6]. When isolated reaction centers from the purple bacterium *Rhodopseudomonas spheroides* were found also to exhibit a fluorescence yield which varied inversely with the photochemical activity [7], it seemed not unreasonable to take this as evidence for a minimum hypothesis that the photochemically active state was the excited singlet level of the reactive bacteriochlorophyll, P870, and that fluorescence and photochemistry were competing pathways for deexcitation of this state.

In solution photochemistry of chlorophylls it has been shown that the first excited triplet state can be formed with high yield [8, 9] and most characterized

Abbreviations: LDAO, lauryldimethylamine-*N*-oxide; MOPS, 3-(*N*-morpholino)-propanesulfonic acid.

photochemical reactions of purified chlorophylls are believed to proceed via the triplet level [10]. Until very recently there was no evidence for triplet states of chlorophylls in photosynthetic systems except in highly damaged tissues [11]. The apparent correlation between fluorescence and photochemical activity has been enough to endorse the general belief that the primary photochemical act occurs through the excited singlet level. Recently, however, a light-induced EPR signal identified with a triplet state of reaction center bacteriochlorophyll has been observed in a wide variety of preparations from several species of photosynthetic bacteria [12].

The triplet signal is apparent when the reaction center primary electron acceptor is chemically reduced, (i.e. closed traps). The light-induced signal cannot be saturated by continuous illumination at normal intensities due to its very rapid decay; laser flash excitation shows it to decay with a half-time of about 5 μs [16]. We have measured the flash induced light saturation of the triplet EPR signal and have obtained a relative quantum yield by comparison with the light saturation of $P870^+$ photooxidation (as detected by the EPR signal at g 2.00 [14, 15]) under identical optical conditions.

METHODS AND MATERIALS

Reaction centers from the blue-green mutant of *Rps. spheroides* (strain R26) were prepared by disruption of chromatophores with the zwitterionic detergent lauryldimethylamine-*N*-oxide (LDAO, a gift from the Onyx Chemical Company). The reaction center fraction was recovered and purified either by repeated $(NH_4)_2SO_4$ precipitations [1, 17] or by chromatography on DEAE-cellulose. The LDAO was fully exchanged for Triton X-100 in both cases by DEAE column chromatography because of the apparent instability of LDAO at redox potentials below approx. -50 mV. The reaction centers were eluted in 0.05% Triton X-100, 10 mM Tris buffer, pH 8.0, but were then adjusted to pH 7.0 with concentrated MOPS (3-(*N*-morpholino)-propanesulfonic acid) because at the time the redox characteristics of the primary acceptor in reaction centers had only been determined with certainty for this pH ($E_{m7} = -50$ mV, refs 18, 19).

The EPR studies were performed with a Varian E3 EPR spectrometer. Flash excitation (flash half-width, 0.4 μs) was provided by a liquid dye laser (rhodamine 6G) tunable in the 600-nm region appropriate for the red peak of bacteriochlorophyll. The wavelength was chosen by scanning the effect of a non-saturating flash and setting at the peak of this crude action spectrum; there was no noticeable difference between using the g 2.00 or triplet signal for this procedure. Actinic intensities were varied by using neutral density filters of various types: calibrated wire mesh, Kodak Wratten gelatin filters and Baird-Atomic thin metal film neutral density filters.

The g 2.00 signal was monitored at the positive peak of the derivative spectrum using a microwave power of 2 mW. The triplet signal was taken directly from the preamplifier of the EPR spectrometer in order to circumvent the limitation of the response time of the main amplifier. No field modulation was used and AFC modulation was adjusted to minimum. The microwave power was also 2 mW. In the microsecond time range applicable to the triplet measurement there was a small, light intensity dependent but field-independent laser artifact. To overcome the interference of this artifact on the measurements, the triplet was measured at two positions in the

spectrum for each light intensity — at the microwave absorption peak at $g = 2.11$, and at an equivalent emission peak at $g = 1.91$. Spin polarization of the triplet EPR spectrum has been previously described (see preceding paper [21]). The difference between these two measurements was taken to eliminate the laser artifact.

The laser flash induced signals were recorded on a high speed storage oscilloscope (Tektronix 5103 N) triggered from the laser. The g 2.00 signal was recorded at sweep rates of 10 or 20 ms/cm and the triplet was recorded at 2 or 5 μs/cm.

Treatment of the reaction centers with sodium dodecylsulfate was done according to a modification of the initial method of Feher [1, 21]. The reaction centers were dialysed in the dark for 21 h at room temperature against 0.1% sodium dodecylsulfate, 0.02% LDAO and 1 mM EDTA in 50 mM Tris–HCl, pH 8.0. The LDAO was then removed by further dialysis for 15 h against 0.05% Triton, 50 mM Tris–HCl, pH 8.0. The control sample was dialysed without sodium dodecylsulfate for 36 h against 0.05% Triton, 50 mM Tris–HCl, pH 8.0.

For redox potential work the following mediators were used: phenazine methosulfate (Sigma Chemical Co.), phenazine ethosulfate and pyocyanine (K and K Laboratories, Plainview, N.Y.) and 2-hydroxy-1,4-naphthoquinone (Eastman Organic Chemicals, Rochester, N.Y.). The technique of preparing EPR samples at a known redox potential has been previously described [12].

RESULTS

A preliminary experiment was performed in which the triplet and $g2$ signals were measured in different samples; in one, for observation of the triplet, the primary electron acceptor was reduced by addition of excess dithionite (closed traps). The other had no additions and was in the photochemically competent state with the acceptor oxidized (open traps). Although this experiment (No. I in Table 1) was reported in the Abstracts of the 17th Annual Meeting of the Biophysical Society at Columbus, Ohio, in March 1973, it was soon found to be anomalous when the experimental procedure was redesigned to make the triplet and g 2.00 data completely comparable. It was decided to perform subsequent experiments on samples poised at a redox potential in the region of the mid-point potential of the primary electron acceptor ($E_{m7} = -50$ mV). Thus each sample would have a mixed population of reaction centers, some fully reduced and therefore capable of giving the triplet signal, and some with oxidized acceptor being capable of producing the g 2.00 signal. In this way the optical equivalence of the two measurements was assured. A few additional samples were prepared in the absence of redox dyes in either the fully reduced (plus excess dithionite) or oxidized (no additions) states; in these cases care was taken to choose sample tubes of equivalent dimensions — a possible source of the anomalous result of the preliminary experiment.

The actual oscilloscope tracings of one sample are shown in Fig. 1. Relative quantum yields were determined by comparison of the half-saturation intensities obtained from double reciprocal plots as shown in Fig. 2. In all cases the data of both signals conformed well to a linear regression.

The results of all experiments are shown in Table I, some of which require comment. It had been observed previously that prolonged exposure of reaction centers to a strongly reducing environment at room temperature causes a loss of the

TABLE I

RELATIVE QUANTUM YIELDS OF FORMATION OF TRIPLET AND $g2$ EPR SIGNALS IN R 26 REACTION CENTRES

80 μM reaction centers in 50 mM MOPS, 0.05 % Triton, pH 7.0. Flash illumination at 600 nm from liquid dye laser. Liquid helium temperature. Redox dyes as in Methods and Materials.

Expt No.	Sample and description	$\Phi_{\frac{1}{2}}$ (trip)	$\Phi_{\frac{1}{2}}$ ($g2$)	$\Phi_{\frac{1}{2}}$ (trip)/ $\Phi_{\frac{1}{2}}$ ($g2$)
1	1. no additions		7.6	
	2. plus dithionite	2.6		0.34
2	1. −85 mV	4.0	3.9	1.03
	2. −85 mV	3.1	2.7	1.15
3	1. −55 mV	3.3	3.0	1.10
4	1. no additions		2.9	
	6. −140 mV	2.6		0.90
	9. plus dithionite	2.9		1.00
	2. +105 mV	1.9	2.3	0.83
	4. −50 mV	3.3	3.8	0.87
	5. −25 mV	2.0	3.6	0.55
	8. +100 mV no X^- EPR signal	1.9	2.3	0.83
5	11. dialysed control, plus dithionite	1.8		1.30
	12. dialysed control, no additions		1.4	
	13. dialysed control, −35 mV	2.6	3.1	0.84
	14. sodium dodecylsulfate-dialysed, −35 mV no X^- EPR signal	1.2	1.7	0.71
Average over Expts 2–5				0.93±0.19

photoredoxin signal at $g = 1.82$. Dispersion in phospholipid (egg yolk lecithin) instead of detergent seems to protect against this effect (Dutton, P. L. and Leigh, J. S., unpublished). Despite the loss of the g 1.82 signal the bacteriochlorophyll photochemistry is not appreciably disturbed in that the light saturation curve of the g 2.00 signal is similar and the low temperature dark decay rate of the g 2.00 signal is essentially unchanged ($t_{\frac{1}{2}} = 18$ ms). In sample 8 of Expt 4, the reaction centers had been exposed to potentials as low as −200 mV for some time before returning to the potential of +100 mV; the g 1.82 signal was not observable. However, the relative quantum yield of triplet formation was not significantly different from the other samples.

Mild sodium dodecylsulfate treatment (0.1%) has been reported to cause partial dissociation of one of the protein subunits of reaction centers [1]. It seems probable that the mild treatment followed by dialysis against Triton as used here does not cause detachment of a protein component since the absorption spectrum is unaltered unlike that of the partially dissociated particles [3]. Sample 14 (Expt 5) shows the light saturation data for triplet and g 2 signal formation of such a preparation. The relative quantum yields are not appreciably affected; however, the photoredoxin EPR signal is effectively lost. The apparent loss is most likely caused by protein structural modifications which greatly broaden the g 1.82 signal. A very broad EPR signal of this type has been described previously by Feher [13].

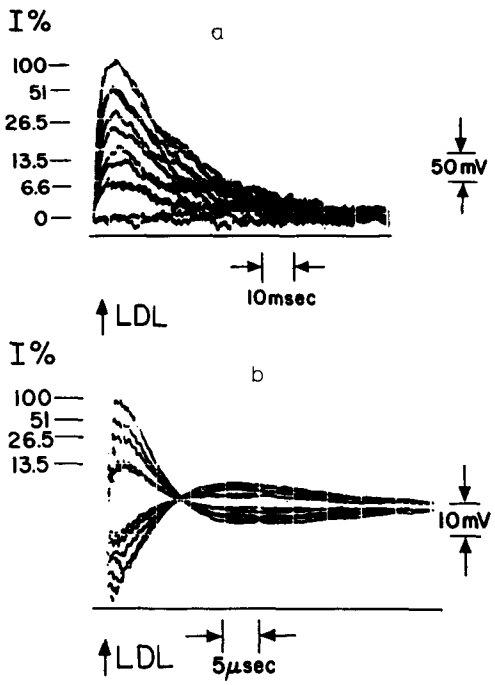

Fig. 1. Kinetics of low temperature laser flash induced EPR signals in reaction centers. Reaction centers (80 μM) in 0.05 % Triton, 50 mM Tris adjusted to pH 7.0 with 1 M MOPS; redox potential set at -35 mV in the presence of 120 μM pyocyanine, 40 μM 2-hydroxy-napthoquinone. Excitation with liquid dye laser (LDL) attenuated with neutral density filters to give intensities as indicated. Liquid helium temperature. (a) '$g2$' signal of photooxidized bacteriochlorophyll. (b) triplet signal; the positive and negative signals were taken at each light intensity and the difference was used for further processing (see Methods and Materials).

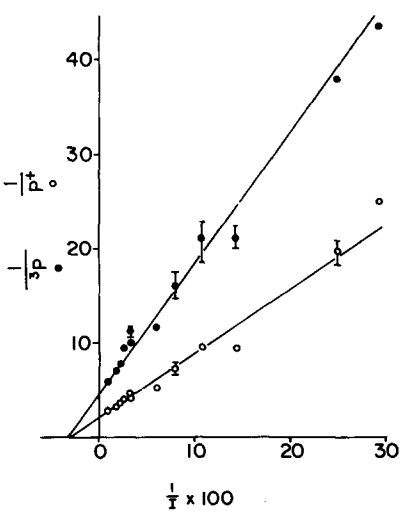

Fig. 2. Double reciprocal plots of the light saturation curves of triplet and $g2$ EPR signals. Conditions as for Fig. 1, except 50 μM pyocyanine, 150 μM 2-hydroxynaphthoquinone, $E_h = -55$ mV. Open circles, $g2$ (P^+); closed circles, triplet (^3P).

The mean value of the relative quantum yield of triplet formation taken over all samples in Expts 2–5 was 0.93 ± 0.19 of the $g2$ yield.

DISCUSSION

In the calculation of the average triplet yield all samples were included and treated alike; any plausible selection of data such as omission of those samples lacking the photoredoxin EPR signal, or omission of the two extreme values, only results in higher average yield. It has recently been shown that the absolute quantum yield of P870 bleaching at room temperature is very close to one (1.02 ± 0.04) [25] and, if we apply this same value to the low temperature oxidation generating the g 2 signal, then the yield of triplet formation is at least 90%. The close equivalence of the triplet and g 2 light saturation curves strongly indicates that only one triplet can be generated per reaction center.

In some samples and preparations there is a small amount of observable triplet signal even with the acceptor in the fully oxidized state, however, this is not usually the case and it seems probable that the occasional presence of a triplet signal under oxidizing conditions is attributable to a small population of damaged reaction centers in the preparation. The sensitivity of detection is such that the yield of microsecond triplet in samples with undamaged, oxidized acceptors must be less than about 5% of that in samples with reduced acceptors.

These results, and the recent confirmation of the absolute quantum yield of P870 photooxidation as very close to unity, render untenable the current, simple picture of photochemistry and fluorescence competing for the excited singlet state. The experimental observation that the reaction center fluorescence yield rises 3-fold as the primary electron acceptor is reduced has been taken to indicate a photochemical yield of only 70% [7, 24]. In order to accommodate the data presented here it is necessary to assume that the rates of non-radiative decay processes do not stay constant as the acceptor is reduced, for example the triplet yield would appear to increase from near zero to approximately one. Alternatively, it is appropriate to seriously consider that the photoreactive state may be the excited triplet; this would account very simply for the high triplet yield observed with reduced acceptors and the unobservability of the triplet EPR signal in samples with oxidized acceptors, since with oxidized acceptor the triplet state would be utilized at a much faster rate than our available time resolution. The three fold rise in fluorescence yield must be accounted for by an adventitious decrease in the intersystem crossing rate, from singlet to triplet, as the acceptor is reduced.

At the same time as this work was in progress Parson and Clayton [27] were reinvestigating a laser flash-induced absorbance change at about 430 nm first described in dithionite-reduced chromatophores of *Rhodospirillum rubrum* [26]. This rapidly decaying absorbance increase had been tentatively identified as triplet–triplet absorption in bacteriochlorophyll; in support of this identification was a corresponding decrease in the normal (singlet–singlet) bacteriochlorophyll absorbance peaks (P 870). Pulsed light saturation studies of the 430-nm absorbance change at room temperature indicated a relative yield only 1/3 that of P 870 photooxidation. If these optical changes in the presence of dithionite are due to the triplet state of P 870, then this value for the quantum yield of formation is in conflict with our low tem-

perature work*.

Notwithstanding this uncertainty it is clear from this work and that of Wraight and Clayton [25] that the total reaction centre fluorescence is not the quantitative assay of the photochemical yield that the simple reactive singlet model predicts. Since fluorescence data are the mainstay of this model, and in view of the present work, the possibility of triplet-state mechanisms for the primary photochemistry should be seriously reconsidered.

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* Work in progress by Dr W. W. Parson indicates that there is a very rapidly decaying component of the 430-nm absorbance change which was not detected in earlier work. It is hoped that this development will soon clarify the apparent discrepancy between the EPR and optical studies.

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