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THE EFFECT OF AMBIENT REDOX POTENTIAL ON THE TRANSIENT ELECTRON SPIN ECHO SIGNALS OBSERVED IN CHLOROPLASTS AND PHOTOSYNTHETIC ALGAE *

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Time-resolved electron spin echo (ESE) studies were carried out at room temperature on chloroplast preparations and whole cells of photosynthetic algae. The signals observed exhibit the unexpected special ESE signal which we have proposed to be the result of transient interactions between P^+-700 and an early electron acceptor of Photosystem I (Thurnauer, M.C. and Norris, J.R. (1980) Chem. Phys. Lett. 76, 557–561). The intensity of the special ESE signal decreases with the chemical reduction of the Center A-Center B complex. The results suggest that in the untreated photosynthetic systems we are initially observing P^+-700 as it interacts with the reduced acceptor which precedes the Center A-Center B complex. Then the decay of the special ESE signal (approx. 170 ns) gives the lifetime of this reduced acceptor as it participates in forward electron transport.

Introduction

In the last few years there have been substantial advances in the understanding of the primary reactions of bacterial photosynthesis [1,2]. It now appears that there are several similarities between these reactions and the primary reactions of green plant Photosystem II (PS II) so that some direct comparisons can be made. Except for the fact that we have developed ideas about the necessary conditions for forward electron transport, fewer direct analogies can be made between the bacterial primary events and those of green plant Photosystem I (PS I). Present evidence suggests that following the photoexcitation of the PS I primary electron donor P-700 to its first excited singlet state, there is rapid electron transfer according to the sequence P-700 → A_1 → X → (Center A-Center B). It has been suggested that A_1 [3] is a chlorophyll dimer [4–7] or a chlorophyll or pheophytin monomer [8–12]. X (sometimes designated A_2) is thought to be an iron-sulfur center [4,13,14] and Centers A and B (sometimes designated P-430) are iron-sulfur centers which may act in series or in parallel [15–17]. These ideas have developed primarily from optical and EPR studies on preparations which were poised at various redox potentials in order to block the electron transfer at different points along the electron-transport chain. Little is known about the forward kinetics of the primary reactions or whether or not all of the four observed electron acceptors lie along the 'normal' forward electron-transport pathway.

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Attempts to determine the kinetics of the forward electron transport have been made by the application of time-resolved picosecond optical spectroscopy to various types of PS I particles [5,6,18,19]. These studies all suggest that P-700 is oxidized within a few picoseconds of photoexcitation, and evidence is given that A_1 is possibly a chlorophyll-like species which after initial reduction is reoxidized in 50–200 ps.

Another approach to study these reactions has been the application of time-resolved EPR methods [7,12,20–31]. Although the systems studied have been basically similar (whole chloroplasts, whole cells of photosynthetic algae and relatively enriched PS I particles), it is difficult to relate the reported results from the different laboratories to each other, and no coherent picture to explain all the results has yet emerged. We have applied pulsed EPR techniques (time-resolved electron spin echo (ESE) spectroscopy) with time resolution of the order of 30–40 ns [29,30].

In our time-resolved ESE study of the photosynthetic alga *Synechoccus lividus*, we have observed at room temperature spin-polarized signals around g 2.00 within 40 ns of laser excitation [29,30]. These signals show emission and enhanced absorption components. Around the g factor region 2.0023 we have observed an unexpected time-dependent echo phase shift. We refer to these signals as special ESE signals, and to the signals with the expected phase as standard signals. * One plausible explanation of this unexpected result is that we are directly observing dynamic radical-radical interactions which decay with a time constant ($k_{1/e}$) of the order of 170 ns where P^+-700 is one member of the interacting radical pair [30]. In this paper we describe how the redox state of the reaction center affects the special ESE signal. The results support our proposal that the time-dependent echo phase shift is due to dynamic radical interactions taking place during forward electron transport. They strongly suggest that in the untreated photosynthetic systems we are initially observing P^+-700 as it interacts with the reduced acceptor which precedes the Center A-Center B complex, and the decay of the special ESE signal (approx. 170 ns) gives the lifetime of this reduced acceptor as it participates in forward electron transport.

* In a previous report we have called these signals X and Y echoes or signals [30].

Materials and Methods

Chloroplasts were prepared from pea seedlings [32] and were stored at 77 K in 0.4 M sucrose, 20 mM Hepes, pH 7.5, 15 mM NaCl buffer. Freeze-dried deuterated (99.7%) cells of *S. lividus* [33,34] were resuspended in ^2H_2O and passed twice through a French pressure cell to break the cells.

Oxidation-reduction potentiometry was carried out essentially as described in Ref. 35. The test mixture contained 100 mM glycine-KOH, pH 11, 100 mM KCl and 100 μM each of benzyl viologen, methyl viologen, triquat (1,1′-trimethylene-2,2′-dipyridinium dibromide) and tetraquat (1,1′-tetramethylene-2,2′-dipyridinium dibromide). The reaction mixture was maintained at pH 9 or 10 by careful monitoring with a pH electrode and adjusted as necessary throughout the course of the experiments. The redox potential was adjusted by making small additions of sodium dithionite (4% in 100 mM glycine-KOH, pH 11) and potassium ferricyanide (0.2 M in H_2O). In some experiments aminoiminomethane sulfinic acid was used as a reducing agent. In the experiments using deuterated algae reagents were made up in ^2H_2O and KO^2H where possible.

For the ESE experiments the reaction mixture was circulated through a flat cell in the spectrometer cavity. For low-temperature conventional EPR experiments 3 mm internal diameter quartz EPR tubes were filled, anaerobically, with small aliquots of the reaction mixture. Samples were frozen in the dark and stored at 77 K before EPR measurements were carried out.

The ESE spectrometer and the time-resolved technique have been described previously [29,30,36,37]. Low-temperature EPR was carried out using a Varian E9 spectrometer fitted with an Air Products liquid helium cryostat.

Results

Using the time-resolved ESE technique we have observed within 40 ns of laser excitation an EPR

signal in pea chloroplasts. The signal appears to be the same as that which we have observed in whole cells of the photosynthetic alga *S. lividus* (normal protonated) [30]. Most notably, it exhibits a time-dependent special ESE signal. In fact, in the chloroplasts as in the protonated alga it is very difficult to observe a standard ESE signal above the noise. This is in contrast to observations in fully deuterated algae where both special and standard ESE signals are easily observed (see below) [29,30]. * The signal (Fig. 1) exhibits characteristics of P^+-700 with respect to g factor and line width. Also, the ESE envelope modulation which serves as a fingerprint for radical species [38,39] exhibited by the transient special ESE signal is the same as that observed for the stable P^+-700 radical which was generated by chemical oxidation in the alga *S. lividus*.

We have followed the intensity of these signals as a function of ambient redox potential. Fig. 1 shows that the intensity of the special ESE signal is reduced as the potential is lowered over the range -480 to -600 mV. The effect on signal intensity is partially reversible when the potential is raised again. So the reduction in intensity at lower potentials cannot be attributed to destruction of the sample. The reduction of signal intensity occurs over the range where Center A ($E_m \simeq -550$ mV) and Center B ($E_m \simeq -585$ mV) [15–17,26] undergo reduction. In order to confirm that Centers A and B were reduced under the conditions of the experiment the conventional EPR signals from this complex [15] were monitored at 10 K in samples taken anaerobically from the poising vessel. As predicted from the literature, at the lowest potential we monitored Center A was almost totally reduced while Center B was still partially oxidized. Since some special ESE signal was still present under these conditions, it is suggested that the decrease in the intensity of this signal is related to the reduction of Center B. At all potentials examined it was difficult to observe any standard ESE signal above the noise.

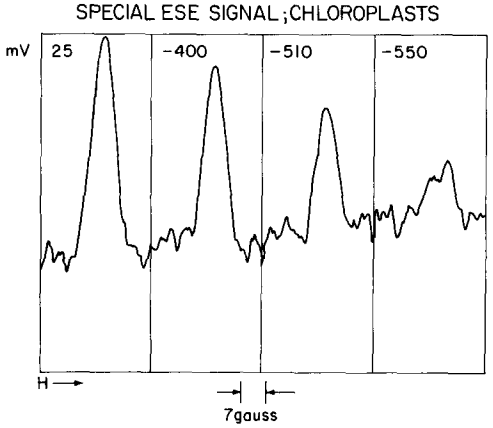

Fig. 1. Special ESE signal as a function of magnetic field (H) within approx. 40 ns of laser excitation (10 ns pulse width N_2 laser) at different ambient redox potentials (pH 9.0). The time between the two microwave pulses τ was 730 ns. The g factor is 2.0023 and line width 7.7 G.

Since it is difficult to observe routinely the standard ESE signals in protonated algae and chloroplasts, experiments were carried out using broken cells of deuterated *S. lividus* in order to follow the effect of redox potential on both the special and standard ESE signals. The special ESE signal in deuterated *S. lividus* has the same potential dependence as that exhibited by chloroplasts but the effect is more fully reversible (Fig. 2). In fact, with this system we were able to monitor the reversibility of the signals by cycling the redox potential (high → low → high) three times before the signal completely deteriorated. The decrease in the extent of the special ESE signal also appears to correspond to the chemical reduction of the Center A-Center B complex as monitored by low-temperature EPR.

Because the standard ESE signal exhibits fast kinetic responses at the field value labeled 3 in Fig. 2 (within approx. 80 ns of laser excitation the signal goes from emission to absorption [29,30]), spectra of this signal were recorded at two different delay times with respect to the laser pulse (Fig. 2). It can be seen that the signal intensity is

* We note that in normal protonated algae we have observed a weak standard ESE signal with similar overall shape (although broadened) to that observed in the deuterated algae (Fig. 2) and therefore it appears that this standard ESE signal corresponds to that reportedly observed at low temperatures by time-resolved continuous-wave EPR methods [7,25]. We have also observed this signal in normal protonated algae at room temperature by direct absorption detection techniques [31].

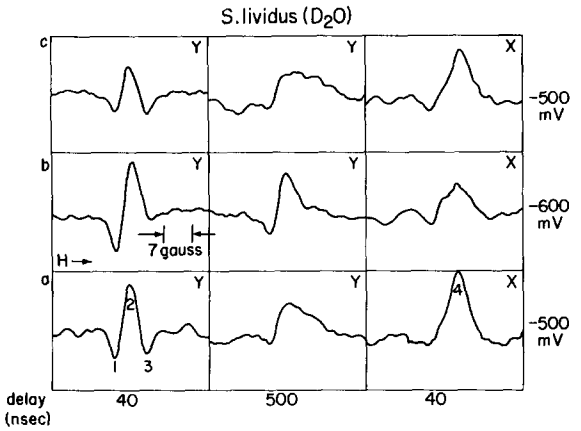

Fig. 2. Standard (Y) and special (X) ESE signals observed in fully deuterated *S. lividus* (pH 10) at different ambient potentials (the potential was lowered and raised in the sequence a,b,c) and different delay times with respect to the laser pulse. τ is 360 ns. The g factors are 2.0062 and 2.0042 at fields 1 and 2, respectively, and 2.0023 at fields 3 and 4.

reduced at this field value (labeled 3) at both delay times (delay approx. 40 ns and delay approx. 500 ns) when the potential is lowered from -500 to -600 mV. The intensities of the low-field responses (field values 1 and 2) are increased slightly as the potential is lowered. The results shown in Fig. 2 together with preliminary kinetic traces suggest that the decay times of the low-field responses are affected by the ambient potential, becoming longer at lower potential. The decay time of the special ESE signal appears unaffected.

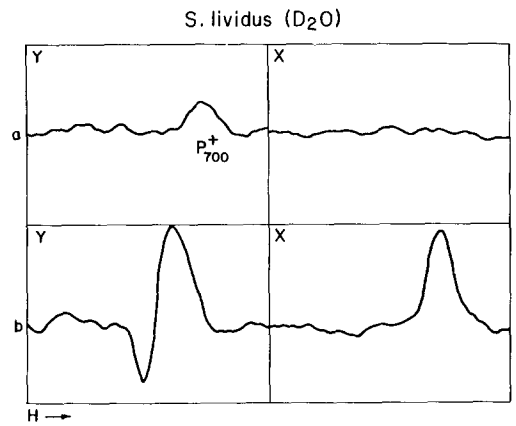

Fig. 3. The standard (Y) and special (X) ESE signals observed in fully deuterated cells of *S. lividus* before (b) and after (a) oxidation with potassium ferricyanide.

Fig. 3 shows that at potentials where P-700 is chemically oxidized, both the special and total standard ESE signals are lost. The latter is replaced by a stable P^+-700 signal. *

The effects shown in Fig. 2 are the same using 589 nm laser excitation instead of the 337 nm light used to obtain Figs. 1 and 2.

Discussion

The observation of the time-dependent special ESE signal ('out-of-phase' signal) is an unexpected result in ESE spectroscopy. We have proposed [30] that the effect is due to a mechanism which can be described by a scheme (below) which invokes radical-radical interaction (both anisotropic and isotropic) as quantified by the constant J.

P^+-700$\cdots R_1^- R_2$	J_1	probe pulse
P^+-700$\cdots R_1 R_2^-$	J_2	echo
		$J_2 \ll J_1$

After the laser pulse and at the time of the first microwave pulse (probe pulse) P^+-700 is present as a partner in an interacting radical pair. The other component of the pair is a reduced early acceptor R_1^-. The interaction energy is J_1. Then by the time of observation of the signal (echo) which is about 700–800 ns after the probe pulse, these particular interactions have decayed as the electron has been transferred to the next acceptor, R_2. Since R_2 is farther from P^+-700 than R_1, the interaction energy of the second radical pair J_2 is relatively small. The decay time of the special ESE signal then gives the lifetime of the interacting pair or the rate of electron transfer from R_1 to R_2. This is observed to be of the order of 170 ns [29,30].

We have carried out quantum-mechanical calculations which describe this system and have shown that this mechanism will produce a time-dependent echo phase shift (Norris, J.R. and Thurnauer, M.C., unpublished data). The analysis also sets limits on the lifetime of the radical pair necessary to give a special ESE signal. In terms of

* We have taken the chemical oxidation results together with the observed line width, g factor and echo envelope modulation as evidence that the signals observed around g 2.0023 (both special and standard ESE) are due to PS I reactions.

PS I, this means that if P^+-700 $\cdots$ R_1^- which is produced essentially simultaneously with the laser pulse has decayed to P^+-700 $\cdots$ R_2^- by the time of application of the probe microwave pulse, no special signal will be observed. The standard ESE signal should be that of the P^+-700 radical with a decay time determined by the spin-lattice relaxation time T_1 and the chemical lifetime. If P^+-700 $\cdots$ R_1^- lives longer than the ESE pulse sequence (approx. 700–1500 ns in this case), then again no special ESE signal will be observed. Then the nature of the standard ESE signal depends on the particular radical pair (the identity of R_1^-, the lifetime of the pair and the possible existence of paramagnetic neighbors in the system). It should be mentioned that it is only the standard signal which can be compared to the continuous-wave time-resolved EPR experiments [7,12,20–28,31].

The results presented here show that the intensity of the special ESE signal is significantly decreased over the redox range where the Center A-Center B complex is chemically reduced. In particular, it appears that the decrease in signal intensity correlates with Center B reduction. Considering the current thinking on PS I acceptors (see Introduction) and assuming that X and the Center A-Center B complex participate in forward electron transport as monitored by EPR, this implies that electron transfer from X (R_1) to the Center A-Center B complex (R_2) is blocked, thus altering the lifetime of the pair state P^+-700A_1X^- as compared to that when the Center A-Center B complex is not reduced. In terms of the discussion above, our observations suggest that in the reduced system the pair lifetime no longer falls within the limits necessary to give the unexpected special ESE signal. Therefore, the special ESE signal (observed when the Center A-Center B complex is not reduced) is the result of the decay of the electron-electron interactions in the pair P^+-700A_1X^- as the electron moves from X^- to the Center A-Center B complex. The rate of this step is given by the decay of the special ESE signal or 170 ns.

Several values have been reported for the rate of this reaction at room temperature. A previous report stated that the Center A-Center B cluster (referred to as P-430) is reduced in a time inferior to 100 ns [40]. This number was obtained by the observation of transient optical absorption changes at 430 nm with an instrument time resolution of 100 ns. The absorption changes at this wavelength, however, are due not only to Centers A and B but also to X and P-700. Also, an estimate for the lifetime of the pair P^+-700A_1X^- ranging from 2 to 30 ns has been given [12]. Recently, it was reported that the Center A-Center B cluster (P-430) is reduced at room temperature in approx. 200 ps, however, directly by A_1^- rather than X [6]. This is in contrast to an earlier study [4] in which it was suggested that at least in the temperature range 5–277 K both A_1 and X participate in electron transfer from P-700 to Centers A and B.

Ultimately, it should be possible to sort out some of these discrepancies by a careful examination of the effect of ambient redox potential on the standard ESE signal including a detailed kinetic study. With a reported 250 μs back-reaction between P^+-700 and X^- [3], we would expect to be able to easily observe relatively long-lived standard ESE signals due to P^+-700A_1X^- when Centers A and B are reduced. * On the other hand, a 10 ns decay component of P^+-700 has been reported when Centers A and B have been reduced [5,6], and this short lifetime would preclude observation of a signal by the techniques used here. Our primary observation on the standard ESE signal is that at field value 3 (Fig. 2), the signal intensity is decreased as the Center A-Center B complex is reduced, ** and it appears that at field values 1 and 2 it increases. Therefore, we have as yet no clearcut relationship between the two low-field (1 and 2) and the high-field (3) responses in Fig. 2. We are presently carrying out experiments designed to clarify this point.

The work presented in this paper does not help to decide whether Center A and Center B function

* This assumes that a short T_1 or phase memory time does not prevent observation of the pair.

** We note that our results are in agreement with those of Warden and Adrianowycz [26]. They have titrated the transient P^+-700 signal observed by continuous-wave time-resolved EPR methods and conclude that a significant portion of the chemically induced electron spin polarization in PS I arises from the interaction of P^+-700 with Center B. They have not reported observation of low-field emissive and absorptive components, however.

in series or in parallel (assuming that they both participate in normal forward electron transport). It does suggest, however, that if they function in series then Center A accepts subsequently to Center B. A detailed kinetic investigation and the effect of lowering the temperature [41] may allow us to shed some light on this problem.

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