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SIMILARITY OF EPR SIGNAL II_f RISE AND P-680$^+$ DECAY KINETICS IN TRIS-WASHED CHLOROPLAST PHOTOSYSTEM II PREPARATIONS AS A FUNCTION OF pH

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The rise time of Signal II_f and the decay time of P-680$^+$ have been measured kinetically as a function of pH by using EPR. The Photosystem II-enriched preparations which were used as samples were derived from spinach chloroplasts, and they evolved oxygen before Tris washing. The onset kinetics of Signal II_f are in agreement, within experimental error, with the fast component of the decay of an EPR signal attributable to P-680$^+$. The signal II_f rise kinetics also show good agreement with published values of the pH dependence of the decay of P-680$^+$ measured optically (Conjeaud, H. and Mathis, P. (1980) Biochim. Biophys. Acta 590, 353–359). These results are consistent with a model where the species Z (or D_1) responsible for Signal II_f is the immediate electron donor to P-680$^+$ in tris-washed Photosystem II fragments.

Introduction

An intermediate electron carrier on the oxidizing side of PS II, most likely a plastoquinone species [1], is usually designated as Z [2,3]. In its oxidized form this species gives rise to EPR Signal II_f in chloroplasts inhibited at the O_2-evolving complex and to Signal II_{vf} in chloroplasts competent in water splitting [2,4,5]. The difference in nomenclature for the two signals arises because the mode of reduction and the decay kinetics of Z^+ differ in these two types of chloroplasts. In O_2-evolving preparations Z^+ is reduced by the O_2-evolving complex in a reaction which occurs in the submillisecond time range [4,6]. When O_2 evolution is inhibited, the reaction is blocked, and Z^+ is rereduced by either endogenous reductants or by exogenous electron donors in a reaction which typically occurs in the 10 ms–1 s time range [7,8].

In Tris-washed chloroplasts, Z was originally postulated as the immediate reductant of the oxidized reaction center chlorophyll, P-680$^+$ [9]. In this model the species Z would be identified with D_1, the donor to P-680$^+$ which has been inferred from optical measurements [10,11]. It is now possible to test this hypothesis directly by measuring the rise kinetics of Signal II_f under conditions where the decay of P-680$^+$ is well characterized. In the work reported here we have measured the rise kinetics of the EPR Signal II_f attributed to Z^+ as well as the decay of an EPR signal that corresponds to P-680$^+$, as determined from the field profile of this kinetic component. A comparison of our results at different pH values with optical data reported for the decay of P-680$^+$ [10,11] shows good agreement and indicates that in Tris-washed chloroplasts Z reduces P-680$^+$ directly.

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CIDEP, chemically induced dynamic electron polarization; PS, photosystem.

Materials and Methods

Spinach chloroplasts were used to prepare O_2-evolving PS II fragments according to the method of Berthold et al. [12]. These fragments were subsequently Tris washed, centrifuged at $40000 \times g$ for 20 min, washed in buffer, centrifuged again at $40000 \times g$ for 20 min, and resuspended in the same buffer. The buffers contained 0.4 M sucrose, 10 mM NaCl, and 50 mM of buffer (Hepes at pH 6.9, Mes at pH 6.0 and 5.6, and succinate-Mes at pH 5.2). The precipitate was resuspended to a total chlorophyll concentration of 4–8 mg/ml, as determined from chlorophyll absorbance at 645 and 663 nm [13]. The sample was then frozen in two separate aliquots until the start of the experiment. Upon thawing, 10 mM ferrocyanide and 10 mM ferricyanide were added as exogenous electron carriers.

During the experiment the sample was flowed through a Scanlon EPR flat cell at a rate of 1 ml/min by a Gilson minipuls II peristaltic pump and then back into an ice-cooled reservoir. The temperature in the flat cell was 24.5 ± 0.5°C as measured by a Fluke 2100 A digital thermometer using a copper-constantan thermocouple. The thermocouple was located inside the flow system at the exit of the flat cell.

Before kinetic measurements were initiated, an X-band field-swept spectrum was taken to check the amplitude of the light-induced Signal II. This was also done to check for the absence of Signal I. The absence of Signal I is important, because in preliminary rise time experiments, we found that the CIDEP signal from PS I [14] obscures the rise time of Signal II_f. Once the spectrum was taken, the field position was set to the maximum of the lowest-field hyperfine component of Signal II (g 2.010). The decay kinetics of Signal II_f were measured to determined an appropriate flash repetition rate in the rise time experiments. Typical values of this decay time were 30–150 ms and consequently a flash repetition rate of 2 Hz was used. During the Signal II_f rise time and P-680$^+$ decay measurements, the sample integrity was checked every 10000 passes by measuring the reversible light-induced increase in Signal II upon continuous illumination at this field position.

One of the two aliquots was used for a Signal II_f rise time measurement at the maximum of the low-field hyperfine component of Signal II_f (g 2.010). The other aliquot, treated and monitored as previously mentioned, was used to measure the decay kinetics of P-680$^+$ by increasing the magnetic field by 12 G (g 2.003).

A Varian E-109 EPR spectrometer modified for 1 MHz field modulation was used for the measurements [15]. The instrument response time was limited to about 2 μs or longer. When field-swept spectra were taken, an output low-pass RC filter of 1 s time constant was used on the 1 MHz receiver, and the output signal was fed into a chart recorder. When kinetic traces were obtained, the output signal of the receiver was digitized by a Nicolet Explorer IIIA oscilloscope and accumulated in a signal averager which was designed and constructed by Mr. Gary Smith in our laboratory. A diode detecting the laser flash was used to trigger the oscilloscope to eliminate errors due to delays in the firing circuit. For the kinetic traces the output was filtered by a 0.5 μs low-pass filter and digitized at 200 ns/point. In all cases 4096 points were collected.

The laser used is a modified flashlamp pumped dye laser (Phase-R Corp. DL-1400), which provided 0.3–0.5-μs nonsaturating light pulses at 640 nm with energies of 5–8 mJ/pulse at the sample in the EPR cavity. Rhodamine 640 (Exciton, Dayton, OH, U.S.A.) in methanol was the dye in the laser for all of the traces. The signal-averaged trace was sent upon completion to a VAX 11/780 computer for data analysis and display.

Results

Examples of the kinetic traces and theoretical fits obtained can be seen in Fig. 1. The experimental conditions are as noted in the figure and in the figure legend. The fits of the data were performed by using ZXSSQ, a least-squares fitting routine in the IMSL library [16]. The $Z^{\pm}$ rise times were calculated by fitting each experimental trace to a theoretical expression of the form:

$$\text{If } t < t_0,\ Y(t) = X(1)$$

$$\text{If } t \geq t_0,\ Y(t) = X(1) + X(2)\exp[(t - t_0)/X(3)]$$

where $Y(t)$ is the calculated EPR signal amplitude

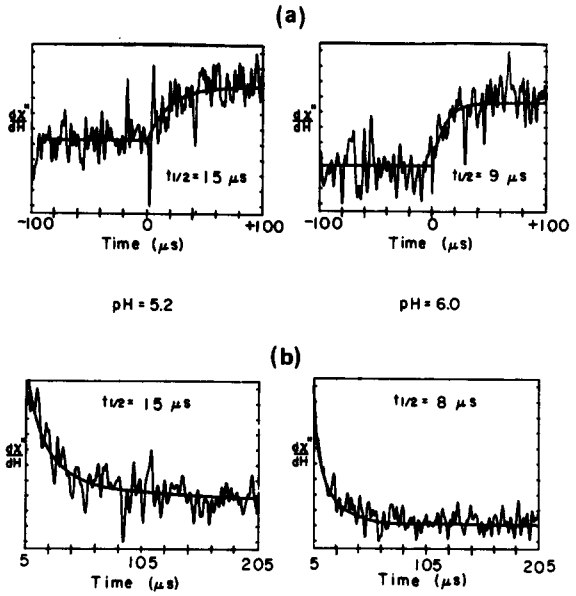

Fig. 1. Transient kinetic traces of EPR signals photoinduced in Tris-washed PS II particles from spinach chloroplasts. Temperature, 24.5 ± 0.5°C; samples suspended in 0.4 M sucrose, 10 mM NaCl, 50 mM buffer (Mes at pH 6.0 and succinate-Mes at pH 5.2), 10 mM potassium ferricyanide, 10 mM potassium ferrocyanide. EPR instrument conditions: microwave power, 20 mW; modulation amplitude, 2.3 G; field modulation at 1 MHz; output time constant, 0.5 μs. (a) Rise kinetics and computer-generated least-squares fit of Signal II_f measured at the low-field hyperfine component (g 2.010); pH 5.2 trace, averages 40000 passes; pH 6.0 trace, 50000 passes. (b) Decay kinetics and fit of EPR signal attributed to P-680$^+$ measured at 12 G higher field (g 2.003); pH 5.2 trace, 10000 passes; pH 6.0 trace, 20000 passes.

at time t, t_0 corresponds to the time at which the laser was fired, $X(1)$ and $X(2)$ are the initial and final EPR signal amplitudes, respectively, and $X(3)$ is the inverse first-order rate constant for the rise of signal II_f.

The fast decay times of the signal attributable to P-680$^+$ were obtained in a similar fashion. However, a fit of the entire curve (including the prerecorded baseline) was complicated by two factors. One is the 2 μs rise time of the instrument. The other factor is the contribution of Signal II_f to the P-680$^+$ decay curve, leading to a different baseline after the flash from that before. Hence, the kinetic information was extracted by fitting the decay to either a single or a double exponential, depending on the amplitude of the contribution from the back-reaction of P-680$^+$ Q$^-$. The kinetics

TABLE I

PRIMARY ELECTRON-TRANSFER KINETICS OF PS II IN TRIS-WASHED PREPARATIONS FROM CHLOROPLASTS

The precision of the kinetic measurements using EPR is estimated to be 2 μs.

EPR signals (this work)			Optical signals (Ref. 11)	
pH	$t_{1/2}$ (μs)		pH	$t_{1/2}$ (μs)
	Signal II	P-680		P-680
5.2	15	15	5.0	14
5.6	10	9	–	–
6.0	8 [a]	8 [a]	6.0	7.2
6.9	≤ 4	–	7.0	4.6

[a] Average of two separate experiments.

of this back-reaction have been found to have a $t_{1/2}$ of 100–200 μs and to be pH indepenent [11, 17]. The amplitude of the contribution from the back-reaction increased with decreasing pH due to inactivation of the donation from Z to P-680$^+$. This slow component is apparent in the P-680$^+$ decay trace at pH 5.2 in Fig. 1. A summary of the data analysis along with a comparison of P-680$^+$ rereduction kinetics measured by Reinman et al. [11] can be found in Table I.

Discussion

The data of Table I show good agreement between the decay of the reaction center chlorophyll absorbance change and the rise time of the Z$^+$ free radical EPR signal (Signal II_f), indicating that the two processes are coupled; i.e., that in Tris-washed chloroplasts Z is oxidized as P-680$^+$ is reduced. An interesting aspect of these data is that the Z and P-680 kinetics are largely independent of the chloroplast or particle preparation, which is consistent with a model having these two reaction partners in the same thylakoid membrane complex. Experimental results reported for small PS II particles which were treated with urea [18] support this conjecture, as do recent results for the behavior of Signal II_f in reaction centers prepared from *Chlamydomonas reinhardii* (Babcock, G.T., Ghanotakis, D.F., Ke, B. and Diner, B.A., unpublished data).

Our conclusions about the rise of Signal II_f and the coupled reduction of P-680$^+$ in Tris-washed chloroplasts are difficult at present to extrapolate to preparations with intact O_2 evolution. Measurements of fluorescence yield changes in *Chlorella vulgaris* under repetitive-flash conditions showed a 400 ns kinetic component attributed to the decay of P-680$^+$ [19]. The rise of Signal II_{vf} in intact spinach chloroplasts has been measured at neutral pH, and a 20 μs half-time was reported [20]. These results were obtained, however, in chloroplasts under conditions where we have observed a weak contribution from the CIDEP signal of P-700$^+$, and it is quite possible that this phenomenon obscured the true rise of Signal II_{vf}. Experiments being developed to reexamine the Signal II_{vf} kinetics in O_2-evolving PS II particles [12] should resolve this uncertainty.

The results that we have obtained with Tris-washed PS II particles are summarized as follows:

Donor → Z → P-680 → Q → PS I

Inhibition of O_2 evolution by Tris blocks the normal reduction of Z^+ by the O_2-evolving complex and leads to the appearance of Signal II_f. The photogeneration of this radical accompanies the rereduction of P-680$^+$ which, in the Tris-inhibited preparation, is dependent upon the pH of the particle suspension.

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