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ELECTRON SPIN ECHO STUDIES ON CHLOROPLASTS

SPECTRAL CHARACTERISTICS OF ELECTRON TRANSPORT COMPONENTS AND LIGHT-INDUCED TRANSIENTS

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Summary

Electron-spin resonance echoes are used to study the complex overlapping ESR spectra of whole chloroplasts. By varying the repetition rate of the microwave pulse sequence, delay time, and pulse width, signals with different longitudinal and transverse relaxation times were extracted. We have identified the echo signals due to plastocyanin and ferredoxins. In addition, we have found a strong signal at $g = 4.3$, that possibly arises from distorted cytochrome, and weak signals in the region $g = 6$—9. The strong echo signal at $g = 2.0047$ (Signal II), is made up of at least three 'dark' components that differ in their relaxation times. Upon illumination at 1.2 K several of the echo signals including Signal II show reversible light-induced components. The kinetics of these transients depend on the addition of 3(3,4-dichlorophenyl)-1,1-dimethyl urea. Part of the transients are believed to arise from cyclic electron flow around Photosystem I.

Introduction

ESR has proved itself a valuable tool in the study of electron transport components in photosynthesis (for a recent comprehensive review see Ref. 1). In an earlier report we have illustrated the merits of pulsed electron spin resonance (ESR) or electron spin echo techniques for certain applications [2]. In

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Abbreviations: ESR, electron spin resonance; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethyl urea.

this communication we report on electron spin echo studies of electron transport components in whole chloroplasts. The advantages of electron spin echo above conventional ESR may be enumerated as follows: (i) electron spin echo records the absorption χ'' directly, and thus is especially suited for the detection of the broad ESR signals found in many transition metal containing proteins that participate in electron transport reactions; (ii) it is much easier with ESE than with conventional ESR to discriminate between components of overlapping spectra having different longitudinal and/or transverse relaxation times; (iii) analysis of the echo decay curve yields information on hyperfine couplings that are poorly resolved in inhomogeneously broadened conventional ESR spectra; (iv) microwave excitation occurs with very short pulses, permitting a faster response than attainable with a conventional ESR spectrometer (a response time shorter than 1 μs is commonly attained in electron spin echo spectrometers). In our previous paper some of the applications mentioned above have been illustrated in a study of so-called Signal II [2]. By using electron spin echo to discriminate between species with different T_1 and/or T_2 we have been able to obtain evidence that Signal II is composed of two radical species (tentatively identified as a chromanoxyl and a semiquinone), each of which may be located in a number of different environments.

In this communication, electron spin echo spectra of electron carriers in dark-adapted whole chloroplasts, and in material after illumination at 1.2 K are presented. Many of the electron carrier electron spin echo show reversible changes in their amplitude upon repetitive illumination at 1.2 K by 8 ms light pulses. These changes are partly due to light-induced enhanced relaxation and partly to low temperature electron transport around Photosystem I.

A preliminary account of this work was presented at the 6th International Biophysics Congress, 1978, Kyoto, Japan [3].

Materials and Methods

Preparations and instrumental

Chloroplasts were obtained from market spinach. Leaves were washed and disrupted in a cooled blender for approximately 30 s in a solution of pH 7.8 containing 50 mM *N*-tris(hydroxymethyl)methylglycine (Tricine), 0.4 M sucrose, 10 mM KCl and 2 mM $MgCl_2$. The homogenate was filtered through four layers of nylon cloth and the filtrate briefly centrifuged at up to $8000 \times g$. The chloroplast pellet was resuspended in 1 ml of the same buffer solution and stored at 0° C in the dark before use. In order to avoid crystallization of the sample at low temperature, glycerol was added to the chloroplast suspension to a concentration of 50%.

All samples were exposed during handling for about one minute to weak white light (~ 40 $\mu W/cm^2$), then left in the dark for several minutes, and subsequently frozen in a stream of cooled N_2 gas (~ 100 K). For some experiments 30 μM of the electron transport inhibitor 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added.

Electron spin echo measurements were performed at X band ($\simeq 9$ GHz) at a temperature of 1.2 K. The apparatus, a modification of a Varian V-4502

spectrometer, has been described by Botter et al. [4,5]. The power of the signal oscillator is 1 Watt in pulsed superheterodyne operation. It can be switched by two 30 dB HP3735B PIN modulators actuated by a Bradley 176B pulse generator to a level of 1 μW; the minimum pulse width is 50 ns. The signal reflected from the cavity is first amplified by a Varian VTX 4276JL low noise travelling wave tube amplifier which also serves as a limiter for the driving pulses. Then it is detected through a Varian XBH-7-30-12-50 balanced mixer connected to a Philips 2K25 klystron, acting as a local oscillator and yielding an intermediate frequency (IF) of 30 MHz. The intermediate frequency signal amplified by a Varian ITA-34-30-08-50 IF amplifier is fed into a PAR 160 Boxcar integrator. By a simple change the spectrometer can be converted from electron spin echo mode to conventional ESR operation, without affecting the sample or its temperature. This permits a direct comparison of the conventional ESR spectrum with the electron spin echo spectrum.

Illumination

Actinic light was provided by a Philips SP or Osram HBO 1000 Watt mercury arc or by an Osram XBO 900 Watt xenon lamp, combined with a filter system consisting of a solution of 100 g/l, $CuSO_4$ (10 cm) and Schott KV 520, Chance-Pilkington HA3 heat absorbing and Balzers Calflex C heat reflecting filters. Light pulses of 8 ms duration were produced by a Compur *m* electronic shutter. The light intensity was adjusted with Schott neutral density filters (NG series) to an intensity of 80 mW/cm^2 at the sample position as measured with a YSI model 65 radiometer. We checked for average sample heating by measuring with conventional ESR the susceptibility at 1.2 K of a paramagnetic dummy sample having similar light absorption characteristics as the chloroplast suspension. No effect of continuous or pulsed illumination was observed. However, even when the average temperature of the sample, which is immersed in superfluid helium, does not rise measurably, local heating may occur as a result of light absorption by the paramagnetic molecules themselves. Electron spin echo is more sensitive to such local effects than conventional ESR because of its exponential dependence on the spin-lattice and spin-spin relaxation rates, which may be strongly temperature dependent. We have measured these relaxation rates in the dark and under continuous illumination. No change in T_2 and a decrease of T_1 ($\sim$5%) was observed. The latter effect may cause an increase in the electron spin echo intensity when the repetition rate is close to T_1^{-1} (see below).

Spin echo generation and its sampling

A brief explanation of the spin echo method [6—8] as we have used it is given in Ref. 2. As illustrated in Fig. 1, an initial 90° pulse of the microwave driving field lasting for a period t_p such that $|\gamma H_1| t_p = \frac{1}{2}\pi$ (γ is the gyromagnetic ratio), rotates the net magnetic moment M to the equatorial $x'y'$ plane in a coordinate system rotating with the resonance frequency about the Zeeman field H_0 (the z' axis). When the driving field has been switched off ($H_1 = 0$), the magnetic moments of the individual spin packets start to dephase in the $x'y'$ plane as a result of different local fields experienced by the individual spins. A 180° pulse applied at time τ after the 90° pulse inverts the accumu-

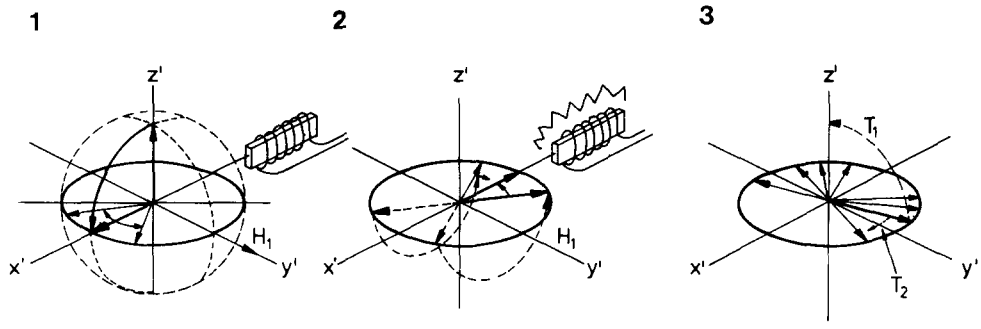

Fig. 1. The formation of the two pulse electron spin-echo in the rotating frame. 1. Initially, all spins are aligned along the z' axis, which is the direction of the static field H_0. When the microwave field H_1 is applied, a 90° pulse rotates the net magnetic moment M into the equatorial $x'y'$ plane. After the removal of H_1, the magnetic moment vector begins to fan out in this plane. 2. At $t = \tau$ the microwave field H_1 is again applied. By a 180° pulse the magnetic moments are rotated quickly by 180° about the direction of H_1 into the same plane. The direction of the fanning movement is now reversed and the magnetic moments are refocussed along the negative x' axis. At $t = 2\tau$ a net resultant magnetization appears along this axis, where the echo signal can be detected by a microwave receiver. 3. The refocussed magnetization along the negative x' axis begins to spread out and decay due to the tranverse spin-spin relaxation (T_2) and the longitudinal spin-lattice relaxation (T_1).

lated phase lags, and after a further period τ the moments are refocussed which causes the transverse magnetization to reappear along the negative x' axis at time 2τ. The 90°—180° pulses are applied with a repetition rate R and the signal is sampled by a Boxcar integrator at $t = 2\tau$. The width of the 90° pulse (in this work 80—200 ns depending on the species studied) defines the shortest time at which the magnetization can be sampled. This time should be distinguished from the shortest time at which an echo can be seen, which amounted to 1.1 μs for this work.

By the initial 90° pulse the system is brought into a non-stationary state and it will tend to return to thermodynamical equilibrium. In the usual Bloch formalism this is described by the two characteristic relaxation times T_1 and T_2. In this description the longitudinal magnetization returns to its equilibrium value at a rate T_1^{-1} and the total transverse magnetization decays to zero with a rate T_2^{-1}.

However, we are not dealing with a system of spins having a single resonance frequency, but instead the absorption line is 'inhomogeneously broadened' and consists of a distribution of 'spin packets' with different resonance frequencies. As a result, the decay of the transverse magnetization with time as sampled in an electron spin echo experiment, cannot be represented by a simple exponential with a rate T_2^{-1}. In fact, the decay of the echo height I as a function of the delay τ will show a modulation pattern that represents the Fourier transform of the absorption line shape [8,9]. Nevertheless, smoothing out the modulation, one may define an effective spin memory time T_M from the echo decay curves via the relation $I(\tau) = I_0 \exp(-2\tau/T_M)$.

The spin lattice relaxation time T_1 is at low temperature much longer than T_2. If the repetition time R^{-1} is made shorter than T_1, the net magnetization along the z axis is reduced and the observed echo intensity decreased. By a stepwise increase in R one, thus, may gradually eliminate those components

of a composite signal that have $T_1^{-1} < R$.

Mims has discussed the complex problem of the relation between the echo intensity and the microwave field strength H_1 for inhomogeneously broadened lines [8]. In accordance with his results we find that for the value of H_1 used in this work, the relative contribution of different paramagnetic species to the echo signals varies with the pulse duration t_p. This effect was employed as an additional means of discriminating between different paramagnetic species.

Results

Electron spin echo spectra

The two-pulse Hahn echo height as a function of magnetic field gives the absorptive part of the magnetic susceptibility, which corresponds to the integral of the customary ESR derivative signal. Three spectra of fresh spinach chloroplasts, taken with different pulse width and (or) repetition rate, are shown in Fig. 2. The observed spectra are spread over a wide range in magnetic field from 500 to 4500 Gauss and show greatly different relative intensities of the various components depending on the conditions. It follows that whenever

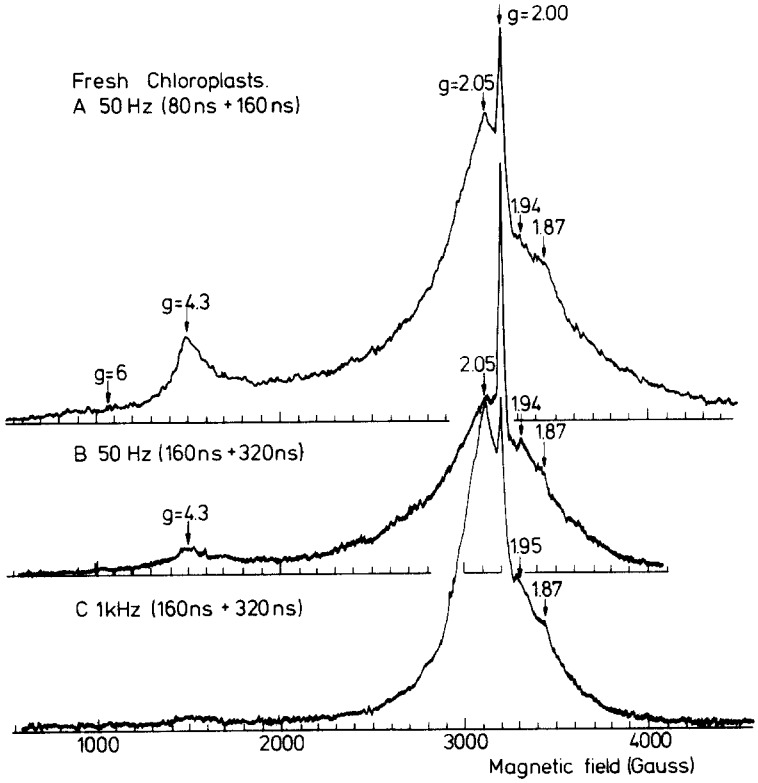

Fig. 2. Electron spin echo spectra in the dark of fresh untreated spinach chloroplasts at 1.2 K. Chlorophyll concentration 3 mg/ml. Instrument settings: scan rate 200 Gauss/min; microwave peak power 1 W; repetition rate in A and B 50 Hz, in C 1 kHz; duration for 90° pulse in A 80 ns, in B and C 160 ns; delay time (τ) 1.2 μs.

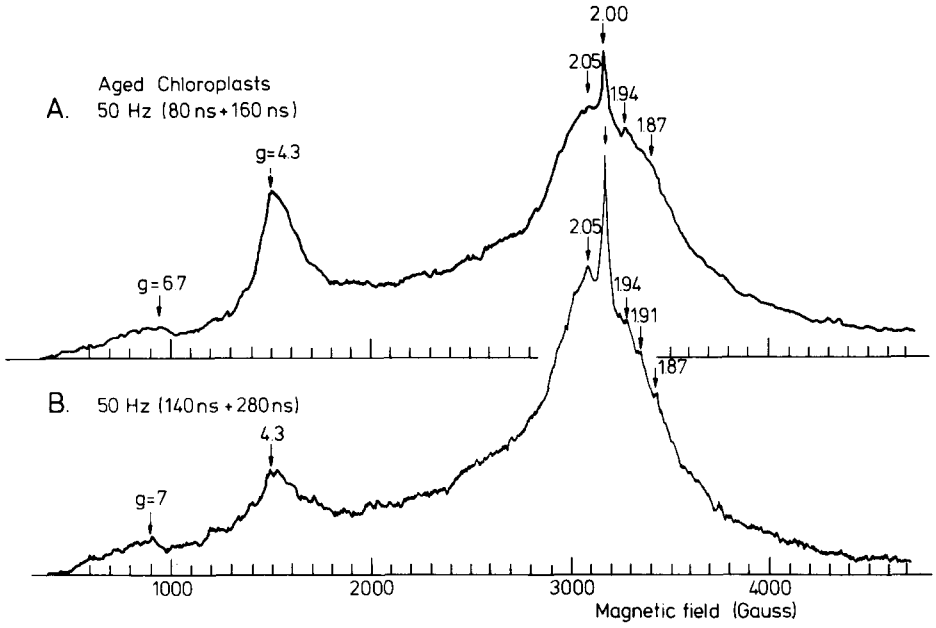

Fig. 3. Electron spin echo spectra of aged chloroplasts at 1.2 K. The sample in sucrose buffer solution had been kept in the dark at −5° C for 30 days. After melting, glycerol was added (50% v/v). Then, the sample was quickly frozen to 77 K. Chlorophyll concentration 3 mg/ml. Instrument settings: scan rate 500 Gauss/min; repetition rate 50 Hz; duration for 90° pulse in A 80 ns, in B 140 ns; delay time 1.2 μs.

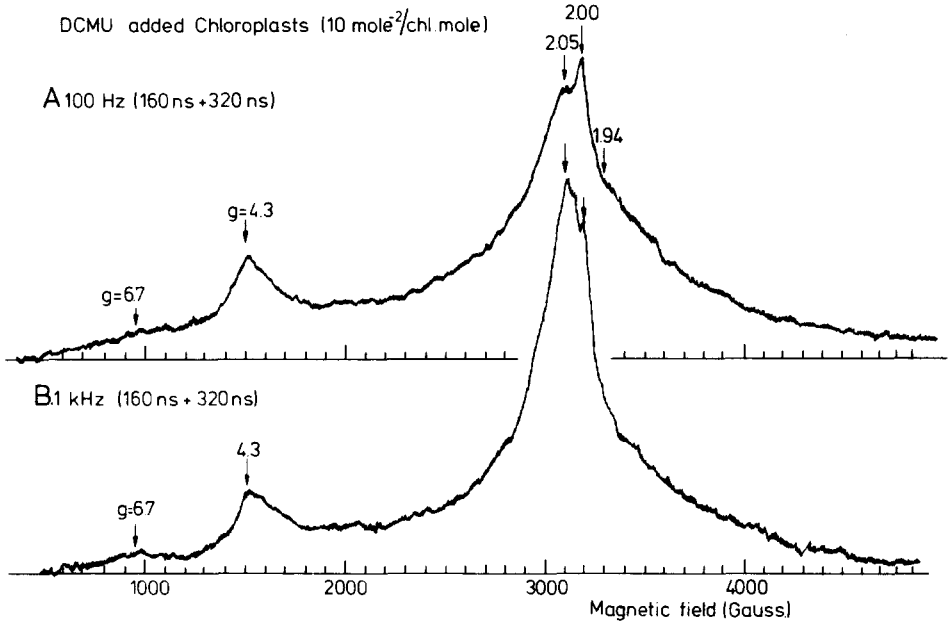

Fig. 4. Electron spin echo spectra at 1.2 K of chloroplasts treated with DCMU. Chlorophyll concentration 2.5 mg/ml; DCMU concentration 30 μM. Instrument settings: scan rate 500 Gauss/min; repetition rate in A 100 Hz, in B 1 kHz; duration for 90° pulse 160 ns; delay time 1.2 μs.

comparing relative intensities of several paramagnetic species, one must take the electron spin echo measuring conditions into account. Since a repetition rate R in excess of the relaxation rate T_1^{-1} of a given component leads to a reduction of its contribution to the echo intensity, the fast repetition rate spectrum contains a smaller number of signal species than the slow repetition spectrum.

At magnetic fields around 3200 Gauss one can distinguish three different signals in the spectra of Fig. 2, with g-values of about 2.05, 2.0047 (Signal II [2]) and 1.94. Signal II is more intense for low R and wider pulses. This means that this signal includes a species with a relatively long T_1 and large inhomogeneous width. The three signals show different behaviour under varying R (Fig. 2). At R = 50 Hz also low field signals at g = 4.3 and 6 are observed. At R = 1 kHz these signals are very weak.

The signal at g = 2.05 is attributed to oxidized plastocyanin, the ESR signal of which is characteristed by $g_\perp \simeq 2.05$ and $g_{/\!/} \simeq 2.2$ to 2.3 [10—13]. The electron spin echo signal which shows a peak and a shoulder at g = 1.94 and 1.87 is ascribed to reduced ferredoxin [11,13—16]. Corresponding conventional ESR signals were observed in the dark in untreated spinach chloroplasts [11,13].

As shown in Figs. 3 and 4, the addition of DCMU, or aging, strongly enhances the low field signal at g = 4.3 and to a somewhat lesser degree that at $g \approx 6$ (Fig. 4a). Warden found low field signals at g = 5.66, 6.12 and 6.74 in spinach chloroplasts in the presence of 5 mM ferricyanide [17]; from potentiometric analysis he assigned the signal at g = 6.12 to cytochrome b-559. The low field signals in Figs. 2—4 possibly include a small contribution from oxidized cytochrome b-559. The spectra in Fig. 4b and in Fig. 2c can be compared directly since they were taken with the same sampling conditions. As follows from the variation with the sampling rate in these figures, the DCMU treatment causes a species to appear at g = 4.3 which has a shorter T_1 than that originally present.

The strong narrow signal at g = 2.0047 is due exclusively to Signal II, in spite of the fact that the same sample gives a strong Signal I in the conventional ESR spectrum. The reason for the absence of Signal I is its short T_2. Recently, employing a faster response form of our spectrometer we have been able to observe P-700$^+$ having a strongly modulated phase-memory decay curve with a T_2 of 0.5 μs, in good agreement with recent data obtained by Thurnauer et al. [18]. For this T_2 we would detect only 4% of P-700$^+$ at the shortest delay time (1.1 μs) attainable during this work.

Spin-lattice relaxation times

The spin-lattice relaxation times T_1 of the various magnetic components in fresh chloroplasts were measured using a three-pulse sequence method [8]. The first 180° pulse changes the magnetization vector from M_0 to $-M_0$ along the z axis. During the time interval Δt this negative magnetization relaxes toward the original positive direction. The Hahn echo pulse sequence that follows probes the value of the longitudinal magnetization at the time Δt via the echo height. Logarithmic plots of the echo intensities versus Δt observed at 3100 and 3300 Gauss are given in Fig. 5 together with a com-

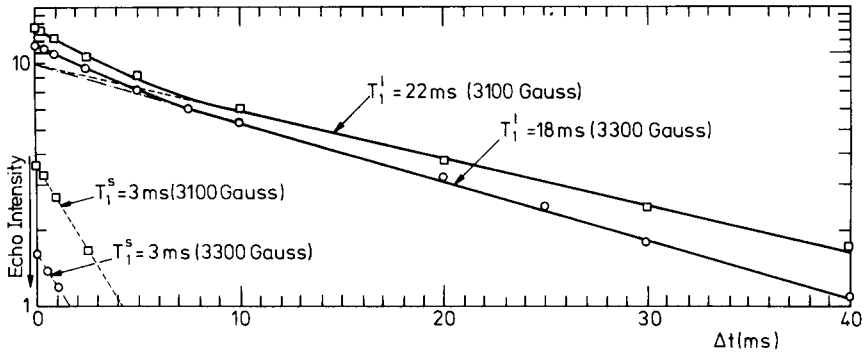

Fig. 5. Echo intensity as a function of the time interval Δt, following a (180°-Δt-90°-180°) pulse sequence at 1.2 K at 3100 Gauss (plastocyanin region) and 3300 Gauss (ferredoxin region). Components of the spin-lattice relaxation time T_1 are indicated in the figure. Instrument settings: repetition rate 10 Hz; duration for 90° pulse 100 ns; delay time 1.2 μs.

ponent analysis based on the assumption that the intensity recovery can be described by a sum of exponentials. The measurements were done at a repetition rate of 10 Hz in order to improve signal to noise ratio for long Δt. At R = 10 Hz components with T_1 longer than about 200 ms are eliminated.

The recovery curves observed for the signals at 3100 and 3300 Gauss are nearly exponential in the time region beyond 6 ms, yielding characteristic times T_1 of 22 and 18 ms respectively. For both curves the fast component that remains after subtracting the contribution due to the slower component has a T_1 value of about 3 ms. The contribution of these fast relaxing components to the total echo intensity is about 30% and 15%, at 3100 and 3300 Gauss respectively. As shown in Fig. 2, the signal at 3100 Gauss attributed to plastocyanin is quite intense in the 1 kHz spectrum which mainly samples species with T_1 shorter than a few ms. The signal at 3300 Gauss attributed to ferredoxin is rather weak in the same spectrum. Therefore, the 3 ms com-

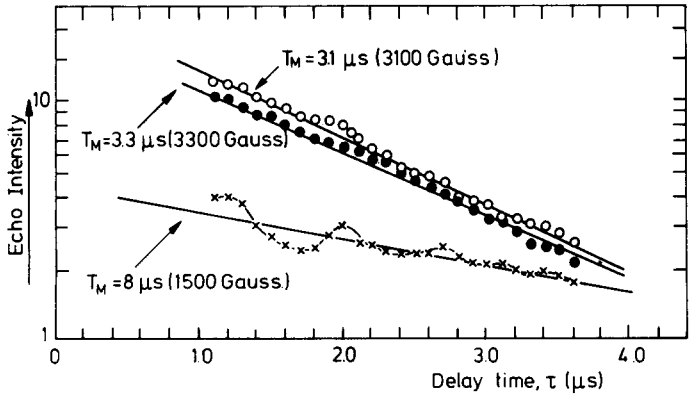

Fig. 6. Echo intensity as a function of delay time τ at 1.2 K of the signals at g = 1.94 (3100 Gauss), g = 2.05 (3300 Gauss) and g = 4.3 (1500 Gauss). The relative intensities of the decay curves are normalized. Instrument settings: repetition rate 50 Hz; duration for 90° pulse 80 ns.

ponent present in the recovery curve at 3300 Gauss may include a small part of plastocyanin, of which the tail extends somewhat beyond 3300 Gauss.

The recovery curve measured for Signal II at 3200 Gauss is non-exponential yielding three components with a T_1 of 0.6, 3 and 20 ms, indicating that in the dark Signal II has at least three components with different spin lattice relaxation times [2].

Modulations in the echo decay envelope contain information on the hyperfine interactions. Hence, this phenomenon is in principle an important tool for the assignment of echo signals [8,19]. Previous work showed that the decay envelope of Signal II exhibited a different modulation pattern, when measured at different repetition rates [2]. This indicates that different T_1 components of Signal II have a different spatial distribution of the unpaired electron, suggesting that these components of Signal II represent at least two different radicals.

Fig. 6 shows the decay envelopes of the broad electron spin echo signals at 3100 and 3300 Gauss. The echo envelopes give nearly the same phase-

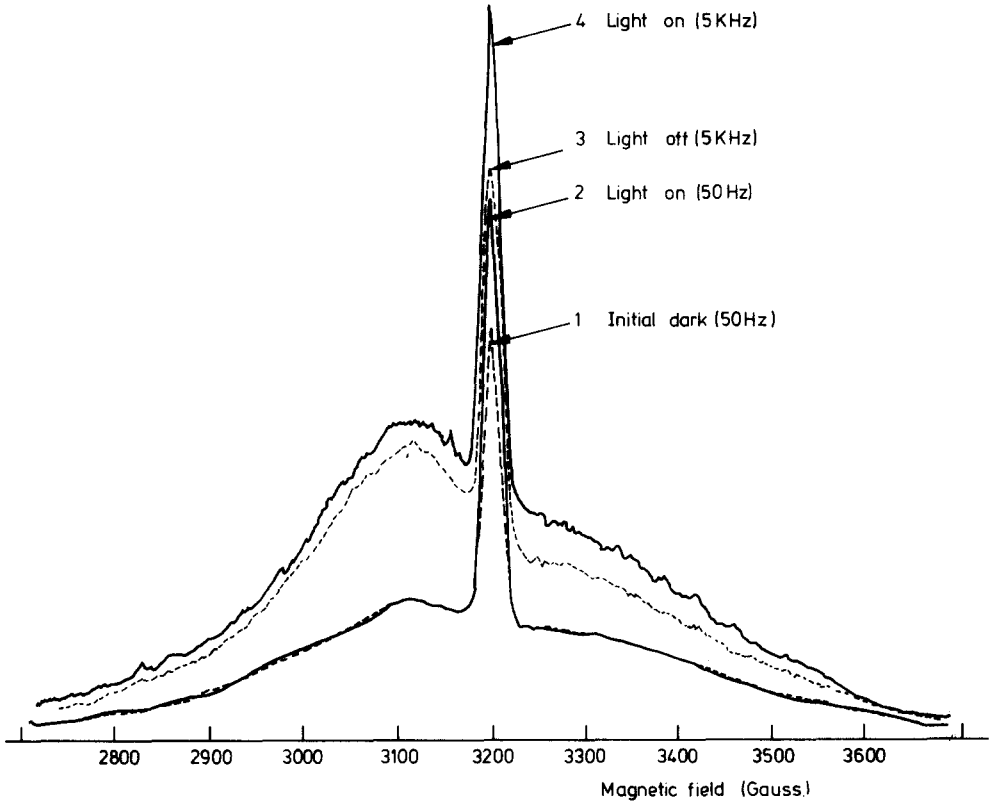

Fig. 7. Effects of illumination on the high field signals. (1) Initial dark spectrum of dark adapted sample. Repetition rate 50 Hz. Duration for 90° pulse 160 ns. (2) Illumination by weak light increases the Signal II intensity, which remains constant after cessation of illumination, with the same sampling condition as (1). (3) The same sample as (2) was measured at 5 kHz repetition rate. Duration for 90° pulse 200 ns. (4) Illumination increased the signal intensities reversibly over the whole region at the 5 kHz sampling rate.

memory time T_M of 3.1 and 3.3 μs, respectively. For echoes taken at 1500 Gauss, a distinct modulation in the envelope is observed. Smoothing leads to a relatively long memory time of 8 μs.

Effect of continuous and pulsed illumination

After taking the 'dark' spectra, the samples were illuminated for 5 min at 1.2 K. The effects of this illumination on the echo spectrum are shown for two different repetition rates of 50 Hz and 5 kHz in Fig. 7. The intensity of Signal II for R = 50 Hz is seen to increase by 40% of the initial 'dark' intensity. For this sampling rate, the change in intensity is irreversible. On the other hand, a light-induced reversible change of Signal II and the metalloprotein signals were observed for R = 5 kHz. The intensity change at 3100 Gauss (plastocyanin region) is rather smaller than that observed at 3300 Gauss (ferredoxin region).

After successive 8 ms periods of illumination, transient signals in DCMU treated chloroplasts were observed at magnetic fields from 1000 to 3600 Gauss with an echo sampling rate of 1 kHz. The repetition rate of the 8 ms illumina-

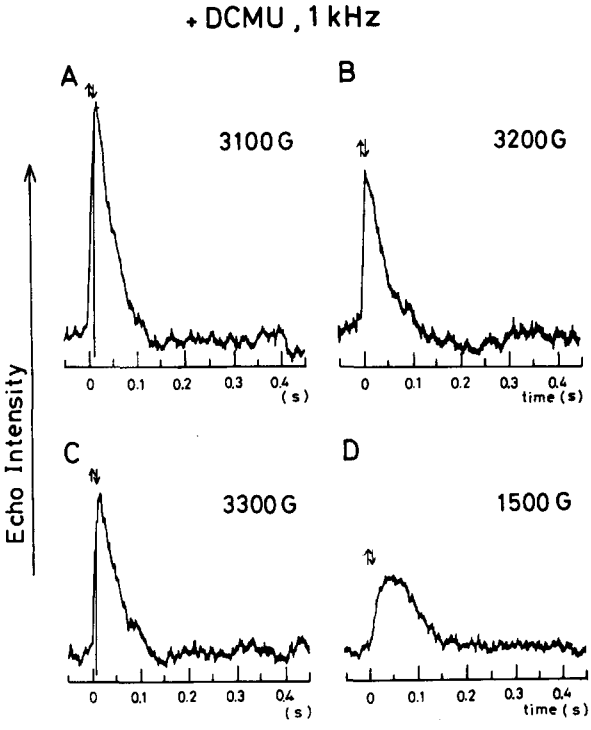

Fig. 8. Light-induced transients in DCMU-treated chloroplasts. A, B, C and D represent transients in the plastocyanin region, Signal II, the ferredoxin type iron-sulfur protein region, and the high spin iron protein region, respectively. Upward and downward arrow indicates the beginning and end of an 8 ms lightpulse, respectively. Measuring conditions: averaging 512 times; duration of exciting light 8 ms; light energy at the sample position 120 mW/cm^2 (350—700 nm); repetition rate of illumination 1 Hz; microwave peak power 1 W; echo sampling rate as indicated; duration for 90° microwave pulse 160 ns; delay time of 180° 0.6 μs; temperature 1.2 K. Chlorophyll concentration 2.5 mg/ml; DCMU concentration 30 μM.

tion was 1 Hz, which excludes reversible processes with a time constant longer than a few seconds. The strongest transient signals were observed at 3100 Gauss (Fig. 8,A). It is attributed to plastocyanin on the basis of its intensity and g-value (2.05). A logarithmic plot of its decay versus time initially does not yield a linear relation, but for longer times the decay constant amounts to 28 ms. The transient signal at 3300 Gauss (Fig. 8,C) is too strong to ascribe it to the tail of the plastocyanin signal, because the intensities of the transient signals at 3100 and 3300 Gauss are quite similar. Therefore, the 3300 G transient is assigned to one or more iron-sulfur proteins [13—16]. Its decay is almost exponential with a decay constant of 35 ms. In DCMU treated chloroplasts, the electron spin echo signal of Signal II is very weak (Fig. 4) and consequently the transient measured at 3200 Gauss (Fig. 8,B) is mostly made up of contributions of plastocyanin and ferredoxin).

In the transient signal at 1500 Gauss (Fig. 8,D), one can clearly see a lag in

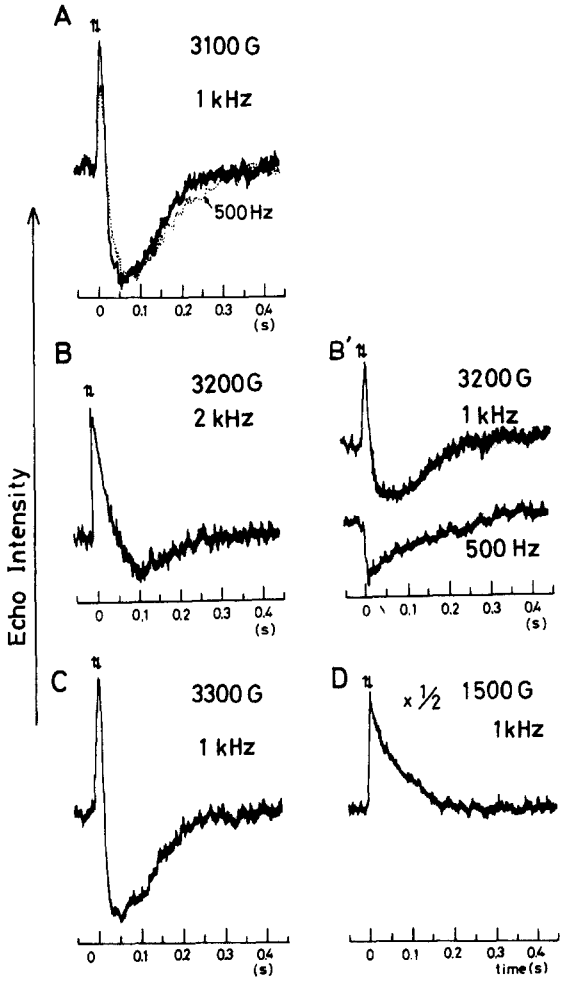

Fig. 9. Light-induced transients in non-treated chloroplasts. A, B, C and D as in Fig. 8. Light energy at the sample position 40 mW/cm^2, chlorophyll concentration 3 mg/ml, other conditions as in Fig. 8.

the rise. The maximum intensity appears about 40 ms after illumination. At that time about half of the transient signal of plastocyanin has already decayed. The exponential rise time is about 30 ms and the characteristic decay time constant amounts to 35 ms. Thus, to within the experimental error, the 1500 Gauss signal is characterized by a risetime that is equal to the decay time (28 ms) of the plastocyanin signal and by a decay time equal to that observed for the iron-sulfur protein at 3300 Gauss.

The transient signals in untreated samples exhibit a complex behaviour which depends on the echo sampling rate (Fig. 9). Here, the most striking phenomenon is the steep decrease that follows an initial increase in all signals except that at 1500 Gauss, where a positive but no negative transient is observed (Fig. 9,D). The negative transients decay with a characteristic time of about 80 ms.

Discussion

Spectral assignments

As expected, the echo signals obtained in chloroplasts arise from a number of paramagnetic species. By a comparison with the results of optical and ESR measurements at low temperature a number of likely candidates for the species giving rise to the electron spin echo signals may be suggested. In this report we will discuss the signals outside the $g \simeq 2.00$—2.01 region. An account of our electron spin echo experiments on Signal II centered at $g = 2.0047$ is given in Ref. 2.

In untreated chloroplasts light-inducible conventional ESR signals at $g = 1.86$, 1.94 and 2.05 have been observed that were ascribed to reduced ferredoxin [11,13—16]. Our dark spectrum of the region to high field of $g = 2$ clearly shows shoulders at $g = 1.87$ and 1.94. One should recall that by electron spin echo one measures the total absorption, whereas in conventional ESR broad anisotropic lines merely yield signals at the turning points at g_x, g_y and g_z. This means that all ferredoxins which were reduced prior to freezing will contribute to the electron spin echo intensity around $g = 1.9$. As seen in Fig. 7, illumination does not enhance the intensity at $g = 1.9$ as measured with $R = 50$ Hz, but at $R = 5$ kHz, an appreciable enhancement is produced. This increase is partly reversible. Apparently, only the contribution of the fast-relaxing ferredoxins is enhanced by illumination. We believe that this contribution is due to one of the primary acceptors of Photosystem I, the so-called species A [11,15]. Another acceptor of Photosystem I, the ferredoxin species B, with g values of 2.05, 1.89 and 1.92, is difficult to produce by light in untreated chloroplasts [14—16]. We have seen its electron spin echo signals in samples prereduced with dithionite, which also showed stronger peaks at $g = 1.87$ and 1.94 than observed in Fig. 7 (data not shown). Because of the uncertainties in amplitude of ferredoxin type signals arising from primary acceptors and soluble ferredoxin, it is difficult to quantify the relative contribution of A to the total electron spin echo amplitude around $g = 1.9$.

The electron spin echo signal peaking at $g = 2.05$ is attributed to plastocyanin. The conventional ESR spectrum of this copper containing protein arises from an axially symmetric g-tensor with $g_\perp = 2.05$ and $g_{/\!/} = 2.2$—2.3, i.e.

the bulk of the intensity in the electron spin echo absorption mode spectrum of plastocyanin should be found at $g = 2.05$. A small contribution may arise from the low field tail of the ferredoxin powder spectrum, which extends just beyond $g = 2.05$. In spinach chloroplasts, plastocyanin is known to occur in dimeric form, each subunit containing one Cu atom. As we have seen in Fig. 7, at $g = 2.05$, two T_1 components are found with T_1 of 3 and 22 ms. These two species may arise from the two Cu^{2+} ions, which may have a different spin lattice relaxation owing to environmental influences, or they may correspond to different types of plastocyanin dimers with different T_1.

Signals at $g = 4.3$ are not uncommonly found in biological material. Their origin is usually not specified; the g value indicates that the signal is due to high-spin paramagnetic ions, e.g. iron, in a tetrahedral or distorted octahedral environment [19]. The signal is especially strong in aged samples and also enhanced in samples to which DCMU has been added (Figs. 3 and 4). This fact, and its behaviour under pulsed illumination (see below), suggests that this signal does not originate from non-specific, protein-bound, iron but from some electron transport component and that the electron transfer to this compound is hindered in aged chloroplasts. A candidate for the $g = 4.3$ signal is a ferric hemoprotein that has undergone a conformational change [20]. The ESR spectra of ferric hemeproteins are sensitive to such a change, as evidenced by the experiments of Yonetani and Schleyer [21]. The hemeprotein might be cytochrome *b*-6 because this cytochrome is readily autooxidized at room temperature in the dark [22]. Cytochrome *f* seems to be excluded, because it is not trapped in its oxidized state by slow cooling, nor photo-oxidized at low temperature. DCMU inhibits the reduction of photo-oxidized cytochrome *f* in the interval between preillumination and freezing to 77 K [22]. This may explain the intensity increment over the wide region from $g = 6$ to $g = 2$ in the spectrum of the sample with DCMU.

The possibility that the $g = 4.3$ signal is due to rubredoxin is unlikely. Peisach et al. [23] have shown that the $g = 4.3$ resonance of rubredoxin in *Pseudomonas oleovorans* is an excited state transition and that at temperatures below 2 K the ground state transition at $g = 9.4$ becomes predominant. In contrast, from Figs. 2—4 it is clear that in our case even at 1.2 K the amplitude of the $g = 4.3$ signal is much larger than the intensity at $g \sim 9$.

The signal at $g = 2.0047$ is clearly due to Signal II [2]. Considering the conditions of sample preparation and cooling, the 'dark' signal observed at 1.2 K belongs mostly to SII_u (the stable component of Signal II) whereas the light-induced signal at $g = 2.0047$ is most likely SII_s (a light-induceable slowly decaying component of Signal II). At 1.2 K we have found five components of Signal II, which have different T_1 and different photoactivity. The origin of the various components of SII is discussed in our earlier report on electron spin echo of Signal II [2]. The various T_1 values probably arise from differences in environmental structure, e.g. a varying distance to transition ions, resulting from variations in the location of plastoquinone in the frozen pool of plastoquinones mediating electron transport from Photosystem II to Photosystem I.

Light-induced transients

All electron spin echo signals exhibit a light-induced rise in electron spin

echo amplitude, that is dependent to a variable extent on the sampling rate. This change may result from a temporary faster T_1 arising from local heating, from light-induced electron transport giving rise to an increase in concentration of one or more T_1 components of the various species, or from a combination of both effects. As the electron spin echo intensity is proportional to $1 - \exp(-1/RT_1)$, a decrease in T_1 resulting from heating will result in an increase in signal intensity at fixed R. We have observed that T_1 may change as much as 5% upon illumination at 1.2 K with white light at an intensity of 80 mW/cm^2. Using our measured values of the T_1 values and their relative contributions to the spin lattice relaxation, one finds that a 5% decrease in all components (a worst case) leads to an electron spin echo signal increase of 0.5—6.5, 1.5—5.0 and 2.0—5.0% at repetition rates between 50 and 5000 Hz for Signal II, plastocyanin and ferredoxin, respectively. This means that even when the sample is cooled in superfluid helium appreciable positive changes in electron spin echo may occur owing to local heating effects. However, the lag in the rise observed at 1500 Gauss (Fig. 8,D) indicates that at 1500 Gauss the change is caused by electron transport, and not by a heating effect. For the other signals we must reserve an opinion to what extent the rise in electron spin echo amplitude is caused by electron transport until more work is done on samples in which one or more redox components are eliminated by redox titration or fractionation. At any rate, it is clear that whenever background electron spin echo signals are present, the effects of local heating should be taken into account when studying with electron spin echo the time-dependent behaviour of light-induced radicals at low temperature.

Following a light-induced fast rise in amplitude, samples not treated with DCMU show a decrease in amplitude below the original level. In principle, a negative change in electron spin echo amplitude upon illumination may arise because of a temperature induced decrease in T_2. The electron spin echo intensity is proportional to $\exp(-2\tau\ T_2^{-1})$, so that at fixed τ a decrease in T_2 will result in a decrease in signal intensity. We have measured T_2 in the dark and under illumination at 1.2 K with white light (80 mW/cm^2). No change in T_2 was observed. This, together with the fact that in DCMU-treated samples (Fig. 8) the negative changes in electron spin echo intensity are absent, suggests that the decrease in samples not treated with DCMU (Fig. 9) results from low temperature electron transport. Such reversible transport must arise either from a cyclic reaction, or from a series of back reactions. The recovery time, about 80 ms, makes the latter possibility less likely, as the back reaction between the first ferredoxin-type acceptor (X) and $P\text{-}700^+$ takes about 0.7 s at 10 K [24]. Since at cryogenic temperatures, light induced absorption changes related to Photosystem II are irreversible [25—27], electron transport reactions should occur in Photosytem I, so that the postulated cyclic flow should involve parts of the linear chain of Photosystem I, together with one or more cyclic carriers represented by the $g = 4.3$ signal (possibly cytochrome *b*-6) and Signal II (plastoquinone), respectively [28]. Very recently, Velthuys [29] has shown that not only is cytochrome *b*-6 involved in cyclic electron flow in Photosystem I [30—32], but that its oxydation/reduction is correlated with concomitant reduction/oxydation of plasto(hydro)quinone. This lends additional credence to our proposal [28] that even at low temperature cyclic flow in

Photosystem I involving cytochrome *b*-6, plastoquinone and plastocyanin is possible, be it with low efficiency. The inhibitory effect of DCMU on the cyclic flow at low temperature is unexplained at present. Again, more work needs to be done on samples in which parts of the cyclic redox chain are eliminated, to chart low temperature cyclic electron flow in Photosystem I.

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