Photo-Induced Electron Transfer in Chlorophyll Containing Liposomes

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Liposomes (lipid, DL-α-dipalmitoyl-phosphatidylcholine) containing chlorophyll a (ratio lipid to chlorophyll 30:1) exhibit an absorption maximum at 670 nm. Upon oxidation with iodine these liposomes yield a chlorophyll radical that shows a ESR signal with a line width (peak to peak) $\Delta Y = \sim 0.1$ G and a *g*-value of 2.0022, consistent with the presence of monomeric chlorophyll. Under anaerobic conditions, in the absence of acceptors, no light induced ESR signal is observed, whereas under aerobic conditions the chlorophyll free radical is generated in the light. Acceptors having access to the lipid membrane, like Fe^{3+}-pyrophosphate and methylviologen, give rise to chlorophyll radical formation, or, like quinones, form a semiquinone radical in the light. Non-permeable acceptors, such as ferricyanide, NADP, FMN and cytochrome *c* do not act as acceptors and no chlorophyll radicals can be produced by light. Furthermore, light dependent and *completely reversible* electron transfer from N,N,N′,N′-tetramethyl-*p*-phenylenediamine to ubiquinone can be demonstrated. Liposomes containing chlorophyll, therefore, can serve as a model system for photosynthetic electron transport.

In algae and green plants the light-dependent reactions of photosynthesis occur in a special membrane system, the thylakoids, which consist of a unit bilayer membrane and include pigments and protein particles [1]. Bilayer systems can be easily generated in the laboratory, either in the form of single bilayers, multiple bilayers, or spherical bilayer vesicles (liposomes). The photosynthetic pigment chlorophyll can be readily incorporated into the bilayer (for a recent review, see ref. 2). Such systems are worthy of special attention as models for part of the photosynthetic apparatus. Besides questions about the location and properties of chlorophyll in the membrane, it is of particular interest to ascertain whether electron transfer – as it occurs *in vivo* – can be achieved. In a recent review, Läuger *et al.* [2] have summed up the present situation. Whereas photoelectric phenomena in lipid bilayers containing chlorophyll have been reported by a number of observers [3–5], there are only few reports on electron transfer reactions in these systems observed without applying an external voltage [6–11].

ESR spectroscopy is a highly sensitive method for detecting the free radicals that may result from electron transfer reactions. This technique has been very recently applied by Tomkiewicz and Corker [10, 11], who reported light driven chlorophyll radical formation in chlorophyll containing liposomes in the presence of ferricyanide, samarium and europium trichloride, and an iron-sulfur protein from *Rhodospirillum rubrum* at low temperatures. We wish to report in this paper some new results on the light-induced formation of free radicals in chlorophyll a-containing liposomes at room temperature. Acceptors, such as are commonly used in photosynthetic electron transport, give rise to radical formation, either of chlorophyll or of the electron acceptor used, if these are lipophilic. If the acceptor is hydrophilic, no free radicals are observed. Furthermore, reversible electron transfer from a donor to an acceptor, sensitized by chlorophyll, has been demonstrated.

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Abbreviations: MV, methyl viologen; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine; UQ-30, ubiquinone-30, 2,3-dimethoxy-5-methyl-6 (all *trans*)-farnesyl-farnesyl-1,4-benzoquinone.

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Materials and Methods

1. Reagents and preparation of liposomes

DL-α-dipalmitoyl-phosphatidylcholine, ubiquinone-30 (coenzyme Q_6), NADP, FMN and cytochrome c (from horse heart) were obtained from Sigma. *p*-benzoquinone and 2,5-dimethyl-*p*-benzoquinone were sublimed prior to use. Chlorophyll a was prepared according to Strain and Svec [12].

Liposomes were prepared according to the procedure of Deamer *et al.* [13] by evaporating chloro-

form solutions of DL-α-dipalmitoyl-phosphatidylcholine and chlorophyll a. The resulting lipid film was sonicated for about 1 min with a buffer solution containing 0.1 M sodium phosphate, 0.1 M sodium pyrophosphate and 1 mM sodium EDTA, pH 7.0 (adjusted with sulfuric acid). A Branson Sonifier, Model J 17 V, was used at a power setting of 30 W. The temperature was allowed to rise to 40 – 50 °C. All operations were carried out under strictly anaerobic conditions in a nitrogen atmosphere. Liposomes were freshly prepared for every experiment and used within 5 hours. For experiments with lipophilic quinones, these were dissolved in the starting chloroform solution containing lipid and chlorophyll a prior to evaporation, whereas all other additions were made after the liposomes were formed.

2. *Optical and ESR measurements*

Visible spectra were recorded with a Cary 14 spectrometer equipped with Model 1411 diffuse reflectance accessory to correct for scattering. ESR measurements were made with a Varian E 9 X-band spectrophotometer connected to a Nicolet Model 1072 time-averaging computer. Normally, 8 30-sec scans were collected in each experiment. Illumination of the ESR cavity was provided by a 300 W Eimac xenon arc lamp (Model X 6263), which could be modulated for kinetic experiments. Light was passed through a 5 cm water filter and a 2424 Corning sharp cut filter (588 – 599 nm).

Results

The liposomes used in the work reported here consisted of DL-α-dipalmitoyl-phosphatidylcholine (45 mM) and chlorophyll a (1.5 mM) (in a ratio of lipid to chlorophyll of 30:1). The liposomes were dispersed in 0.1 M sodium phosphate, 0.1 M sodium pyrophosphate, and 1 mM sodium EDTA, at pH 7.0, and exhibited a characteristic sharp absorption maximum in the red region at 670 nm. We use the designation chlorophyll-liposomes for liposome system prepared as just described.

Oxidation by iodine-potassium iodide at room temperature generates a chlorophyll radical giving rise to an ESR signal (Fig. 1) without hyperfine structure, a line width of ΔH 9.1 ($\pm$0.5) G and a g-value of 2.0022 $\pm$ 0.0003. Upon illumination with red light, the ESR signal height increases more than two-fold. Turning the light off causes the signal to return completely to its previous dark level.

Before studies with acceptors were undertaken, the ESR activity of chlorophyll-liposomes was investigated without any addition under anaerobic and aerobic conditions. Under anaerobic conditions (Fig. 2) there is neither a dark nor a prominent light signal. However, if air is allowed access to the sample inside the tube for one minute only, again there is no dark signal, but now a large ESR signal is generated in the light. The 8.3 G line width again indicates the formation of the monomer chlorophyll$^{+\cdot}$ radical. This sight induced radical formation is completely reversible, *i. e.*, in a subsequent dark period no ESR signal can be observed. It is important, therefore, to exclude oxygen in all experiments where donor and acceptor systems are to be evaluated.

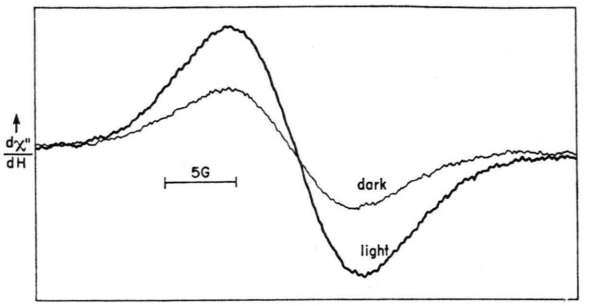

Fig. 1. Dark/light ESR spectrum of chlorophyll-liposomes upon oxidation by iodine-potassium iodide at 20 °C. The concentration of DL-α-dipalmitoyl-phosphatidylcholine was 45 mM, that of chlorophyll a 1.5 mM. The ESR spectrum was recorded at a frequency of 9.49 GHz, modulation frequency 100 kHz, modulation amplitude 2.0 G and power setting 10 mW. The concentration of iodine/potassium iodide was 10 mM.

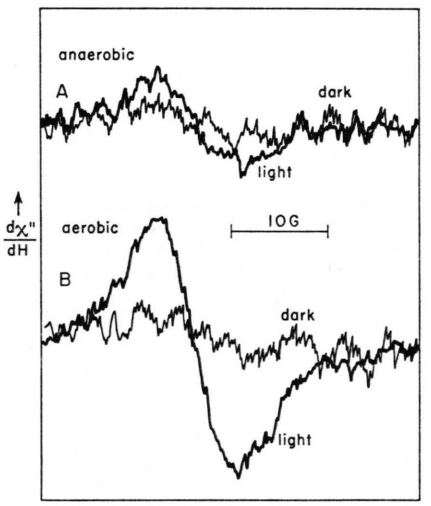

Fig. 2. Dark/light ESR spectrum of chlorophyll-liposomes under anaerobic (A) and aerobic (B) conditions. No addition. Other conditions like in Fig. 1.

Table I. Light-induced radical formation in chlorophyll a containing liposomes in the presence of various acceptors. The concentration of lipid was 45 mM, that of chlorophyll a 1.5 mM and those of the acceptors added as indicated. Experiments wer run anaerobically at 20 °C.

Compound	Conc. [mM]	Redox potential [a] E_0' [mV]	Linewidth ΔH [G]
Nonquinoid			
$K_3[Fe(CN)_6]$	2	+410	–
cytochrome c	1	+254	–
Fe^{3+}-pyrophosphate	1	−140	8.0
FMN	2	−211	–
NADP	2	−324	–
MV	2	−440	9.9
Quinoid			
p-benzoquinone	7	+293	1.3/5.7/10.7
2,5-dimethyl-*p*-benzoquinone	7	+176	–
UQ-30	3	+100	–
2-hydroxynaphthoquinone	2	−154	3.7/11.7
anthraquinone-2-sulfonic acid	2	−250	3.8/8.7

[a] Redox potentials have been taken from refs 14–16.

Table I lists various compounds that were tested for their ability to cause light induced ESR signals in chlorophyll-liposomes. The compounds are divided into non-quinoid and quinoid compounds on the basis of their different response. Of the non-quinoid substances tested only Fe^{3+}-pyrophosphate and MV give rise to an ESR signal in the light, which is similar in line width and *g*-value to that observed in the presence of oxygen. This signal again is completely reversible in the dark. The other compounds, ferricyanide, cytochrome c, FMN and NADP, are inactive.

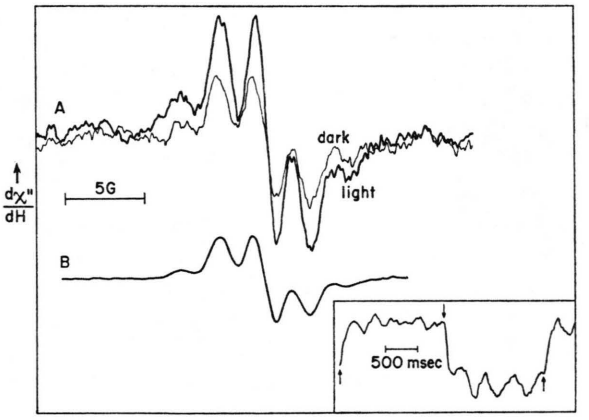

Fig. 3. Dark/light ESR spectrum of chlorophyll-liposomes in the presence of 7 mM *p*-benzoquinone (A). ESR spectrum of *p*-benzosemiquinone in ethanol/water (B). Insert: kinetics of formation and decay of *p*-benzosemiquinone radical in chlorophyll-liposomes. Time constant ESR spectrometer 30 msec, time averaging computer 10 msec, light modulation 0.303 Hz, 128 scans, The magnetic field was set on the center peak in A. Upward arrow means light on, downward arrow light off.

The quinoid substances behave in somewhat different fashion. Here only semiquinone radicals are formed upon illumination. Fig. 3A shows dark/light ESR spectra of chlorophyll-liposomes into which *p*-benzoquinone has been incorporated. The 5-line spectrum is clearly due to *p*-benzosemiquinone anion radical, as can be judged from Fig. 3B in which the semiquinone radical generated in ethanol/water by chemical reduction is shown. A certain amount of semiquinone already present in the dark is obviously produced by chemical reduction. The signal in the liposome system, however, can be considerably increased by light irradiation. In this ESR spectrum no spectral component is present which can be ascribed to a chlorophyll radical. The insert in Fig. 3 shows the kinetics of the formation of semiquinone radical in the light. Rise and decay times are about the same (100 msec) and demonstrate the complete reversibility of the electron transfer process. 2-Hydroxynaphthoquinone and anthraquinone-2-sulfonic acid behave in the same way (see Table I). Finally, UQ-30 and 2,5-dimethyl-*p*-benzoquinone do not give rise to any ESR signal in the absence of an added electron donor.

There is, however, clear evidence of electron transfer in chlorophyll-liposomes from TMPD (E_0' = +270 mV [17]) as donor to UQ-30 (E_0' = +100 mV) as acceptor, when both these compounds are present in the liposome system in addition to chlorophyll a. TMPD upon oxidation yields a stable aromatic carbon radical, characterized by a complex multiline ESR spectrum. Adjusting the magnetic field to one of the intense center peaks of the ESR signal, the rise and decay of $TMPD^{+\cdot}$ formation in the system, described above, can be followed (Fig. 4B). On turning the light on, a large amount of $TMPD^{+\cdot}$ is generated, which reaches a maximum after about 2 min and eventually levels off. On switching the light off, $TMPD^{+\cdot}$ is re-reduced and the signal height returns to its original level within about 10 min. The electron transfer reaction appears completely reversible, for on turning the light on again the concentration of $TMPD^{+\cdot}$ reaches the same value (as estimated from the ESR peak size)

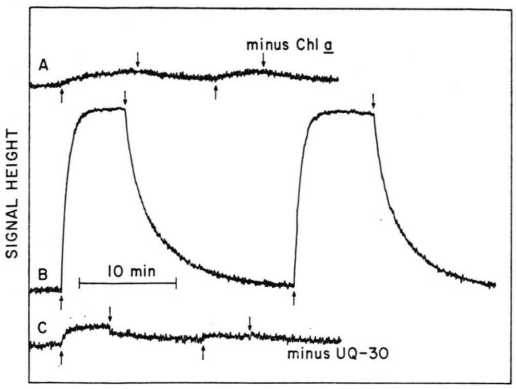

Fig. 4. Generation and decay of $TMPD^{+\cdot}$ in chlorophyll-liposomes with UQ-30 as an acceptor (B). The concentration of TMPD is 2 mM, that of UQ-30 3 mM. The magnetic field is adjusted to one of the center peaks of the $TMPD^{+}$ signal. Upward arrow means light on, downward arrow light off. A, chlorophyll a, C, UQ-30 omitted. Other conditions like in Fig. 1.

as in the preceding light period and decays back to the same level after turning off the light. To prove that all three components, chlorophyll a, UQ-30 and TMPD, are needed to perform the reaction shown in Fig. 4A, chlorophyll a, and in Fig. 4C UQ-30 have been omitted. In both cases, only negligible changes of $TMPD^{+\cdot}$ concentration occur upon illumination.

Discussion

Chlorophyll-liposomes, at a ratio of lipid to chlorophyll 30:1, exhibit an absorption maximum in the red region of the spectrum at 670 nm, in accordance to previous work [7, 8, 10, 11, 18]. The absorption at 670 nm indicates the presence of monomeric chlorophyll [19]. This is further supported by the ESR spectrum of $chlorophyll^{+\cdot}$ free radicals, generated in the liposomes by oxidation with iodine, where the line width of $\Delta H = 9.1$ G is almost the same as observed for monomeric $chlorophyll^{+\cdot}$ in organic solvents ($\Delta H = 9.3 \pm 0.3$ G) [20, 21]. These results do not provide evidence for the presence of dimeric or oligomeric chlorophyll species in chlorophyll-liposomes, as recently proposed by Lee [18]. The increase in size of the ESR signal in the light is an indication of a light driven reaction involving chlorophyll, which will now be discussed.

As demonstrated by the experimental results, there is no ESR signal at all in the light if no electron acceptor is provided. Oxygen can function as an acceptor because the presence of air evokes a large chlorophyll radical signal. It is important, therefore, to again stress that any work with electron donors and acceptors in chlorophyll-liposome systems must be performed anaerobically.

For a variety of acceptors commonly used as electron acceptors for photosystem I in photosynthetic electron transport [22], only Fe^{3+}-pyrophosphate, MV and quinones gave a response either in the formation of chlorophyll free radical or semiquinone radical. We have been unable to observe a chlorophyll free radical at room temperature in the presence of ferricyanide, either in the dark or in the light, as reported by Tomkiewicz and Corker [10, 11]. The ESR signal, however, as described by these authors has been extremely weak. As to cytochrome c, our results are contradictory to those of Chapman and Fast [7], who reported cytochrome c reduction (followed spectrophotometrically) in chlorophyll-liposomes. The purity of the best chlorophyll used by Chapman and Fast, however, was only 87% [7], and thus, any cytochrome c reduction observed could easily be due to reducing impurities with donor properties. In repeating the experiments of Chapman and Fast, Nicholls *et al.* [9] could not observe any cytochrome c reduction but did so if trimethylhydroquinone has been added.

The different behavior of the acceptors tested appears to be not so much a question of the redox potential of the substance but one of polarity properties. Läuger *et al.* [2] have pointed out that there exist three possibilities for the location of the porphyrin head group of chlorophyll in the bilayer of liposomes: a. the porphyrin rings may be located in the aqueous phase outside the polar groups of the lipids; b. they may be present between the polar head groups; and c. the macrocyclic ring may be buried in the hydrocarbon layer of the membrane (for a more detailed discussion see ref. 23). From the evidence available, Läuger *et al.* [2] favor (b). This view is supported by our results. If the porphyrin rings do protrude into the water phase outside the polar head groups of the lipids, they should be capable of interacting with hydrophilic acceptors such as ferricyanide, FMN, NADP and cytochrome c. Since these substances do not interact, the macrocyclic rings of the chlorophyll must be hidden in the membrane and accessible only to compounds which easily can penetrate it (like MV and TMPD), or are themselves located in the membrane system because of their high lipophilicity

(like quinones). In this context, the behavior of Fe^{3+}-pyrophosphate seems to be exceptional since it has to be considered as hydrophilic. The lipid used in the experiments contains one phosphate group per molecule, and thus may facilitate the permeation of the iron complex to some defined space of the membrane and thus facilitate interaction with the chlorophyll.

In the case of *p*-benzoquinone as an electron acceptor, only the semiquinone anion radical can be observed, but no chlorophyll radical. In experiments with the system chlorophyll a/*p*-benzoquinone in organic solvents, Tollin and co-workers [24–27] and Umrikhina *et al.* [28], however, never were able to observe a chlorophyll radical at room temperature. Such a signal can only be seen when the temperature is lowered to about $-60\,^{\circ}C$ [27, 28]. Tollin and co-workers [23–26] have extensively discussed mechanisms for formation and decay of chlorophyll and semiquinone radicals, and the same considerations will probably apply to the chlorophyll-liposome system also. One possibility, according to their considerations, a complex between an excited chlorophyll molecule and quinone is formed, which undergoes electron transfer. The resulting radical complex is stable at low temperatures but breaks apart as the temperature is raised. The decay of the semiquinone radical is slow enough that it can be detected, whereas the chlorophyll radical decomposes so fast that the slower response time of the ESR instrument makes it impossible to detect.

It is somewhat puzzling that 2,5-dimethyl-*p*-benzoquinone ($E_0' = +176$ mV) and UQ-30 ($E_0' = +100$ mV) do not give rise to any sort of ESR signal in the light, whereas other quinones with lower or higher redox potentials and about the same lipophilicity do. This observation is in agreement with those of Tollin and co-workers [29]. These workers were unable to observe semiquinone radicals with some substituted *p*-benzoquinones, or only very weak signals after extensive time-averaging [29].

Finally, with the couple TMPD/UQ-30 a reversible electron flow from the donor TMPD (widely used as electron donor for photosystem I in photosynthetic electron transport [22]) to the acceptor UQ-30 could be demonstrated. In this system, electron transport, as it occurs in photosynthesis, though not irreversible, is achieved. At present it is not possible to decide whether in the electron transport from TMPD to UQ-30 a chlorophyll cation or anion radical is involved. The steady state, which is reached after several minutes of illumination, might be due to changes in the redox potential of the system or charge accumulation which prevents further interaction of the components.

The chlorophyll-liposome system described here is well defined, simple to prepare and highly reproducible. It would appear to have many advantages for further investigation in electron transport phenomena mediated by light, and should yield useful insights into the mechanisms of photosynthesis and light energy conversion.

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