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Characterisation of the low-fluorescent (LF1) mutant of *Scenedesmus* by EPR

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An EPR study of the low fluorescent mutant, LF1, of the green alga, *Scenedesmus obliquus*, has been performed and the following results were obtained. (1) Stable photoinduced charge separation occurs at low temperature forming the characteristic EPR signals from $Q_A^- Fe^{2+}$, the primary semiquinone-iron complex, indicating no major structural modification of the electron acceptor complex in the LF1 mutant. (2) The source of the electron in this reaction at 200 K is a component, probably a chlorophyll molecule, which gives rise to an EPR signal at $g \approx 2.0026$ when photooxidized; in the wild type the S_2 manganese multiline EPR signal is generated under these conditions. (3) Contrary to a previous report (Biochim. Biophys. Acta 682 (1982) 106–114), Signal II is present in the dark in LF1; however, it exhibits different microwave power saturation characteristics from those of Signal II in the wild type. These results show that the LF1 mutant has a functional reaction centre which lacks the redox active manganese of the O_2-evolving enzyme. In addition, as reported previously, cytochrome *b*-559 is in the low-potential, oxidized form in LF1, while it is largely in the high-potential, reduced form in the wild-type. This effect is thought to be only indirectly related to the 34 kDa to the 36 kDa change in D_1 polypeptide size, which is assumed to be responsible for the lack of Mn binding in the LF1 mutant.

The low-fluorescent mutant, LF1, of *Scenedesmus obliquus* is deficient in photosysnthetic oxygen evolution but the activity of the Photosystem II (PS II) reaction centre seems to be normal, as measured by fluorescence induction and DCIP photoreduction, when artificial electron donors are added [1,2]. The cytochrome *b*-559 in this mutant is in its low-potential form and there is less than half the usual manganese content per centre associated with thylakoids isolated from LF1 cells [1,2]. Typical values for the manganese content were reported to be 4.3 ± 0.7 per centre for wild-type *Scenedesmus* while the LF1 mutant contained only 1.6 ± 0.2 manganese per centre [2].

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Abbreviations: D^+, the oxidized form of the component that gives rise to dark stable Signal II; D_1, the approx. 33 kDa polypeptide which binds atrazine herbicides; D_2, the approx. 33 kDa polypeptide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; LF1, a low-fluorescent mutant of *Scenedesmus*; Mes, 4-morpholineethanesulphonic acid; PS I, Photosystem I; PS II, Photosystem II; S_2, a charge-storage state of the oxygen-evolving enzyme; Z, the reduced form of the component responsible for rapidly decaying Signal II.

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These changes in the electron donor side components of PS II in the LF1 mutant are accompanied by an increase in the apparent molecular weight of a PS II polypeptide from 34 to 36 kDa, as determined by polyacrylamide gel electrophoresis [1,2]. This polypeptide was further characterized and it is present in so-called PS II core preparations [3]. Furthermore, it is labelled both by azidoatrazine [4] and by antibodies specific to D_1 (Ref. 5; see also Metz, J.G., Vermaas, W.F.J. and Seibert, M., unpublished data). These results clearly demonstrate that the polypeptide which is modified in the LF1 mutant is the herbicide-binding protein known as D_1 (Refs. 4 and 5; see also Metz, J.G. et al., unpublished data). A donor and acceptor side role for D_1 is in accordance with the view that D_1 represents a membrane spanning reaction-centre protein equivalent to the L subunit in purple bacteria [6].

In this work an EPR study of the LF1 mutant has been performed.

Wild-type and LF1 mutant *Scenedesmus* cells were grown heterotropically in the dark [7]. Thylakoids and PS-II-enriched membranes were prepared as described previously [3], the latter being a modification of the preparation of Kuwabara and Murata [8]. The thylakoids (in 50 mM sodium/potassium phosphate (pH 6.9)/0.3 M sucrose/50 mM NaCl) and the PS-II-enriched membranes (in 25 mM Mes (pH 6.5)/0.4 M sucrose/10 mM NaCl) were frozen and stored at −80 °C until use. Prior to use the thylakoids were thawed and diluted by 33% with a buffer containing 20 mM Hepes (pH 7.0), 0.4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$.

EPR samples were prepared under dim green light in 3 mm internal diameter calibrated quartz EPR tubes. Samples were dark adapted at room temperature for 15 min before being frozen in the dark. The concentrations of chlorophyll was as described in the legends, being between 4 and 9 mg chlorophyll per ml. In some samples of the PS-II-enriched membranes the chloride concentration was adjusted to 100 mM by the addition of NaCl and $CaCl_2$ was added to give a final concentration of 5 mM Ca^{2+}. Other additions were as described in the legends.

Illumination at 77 K, 200 K and 0 °C was provided by an 800 W projector, the sample being immersed in baths containing liquid N_2, solid CO_2/ethanol and ice water, respectively.

EPR spectra were recorded using a Bruker 200t spectrometer and an Oxford Instruments liquid helium temperature cryostat and control system. The microwave saturation study was performed

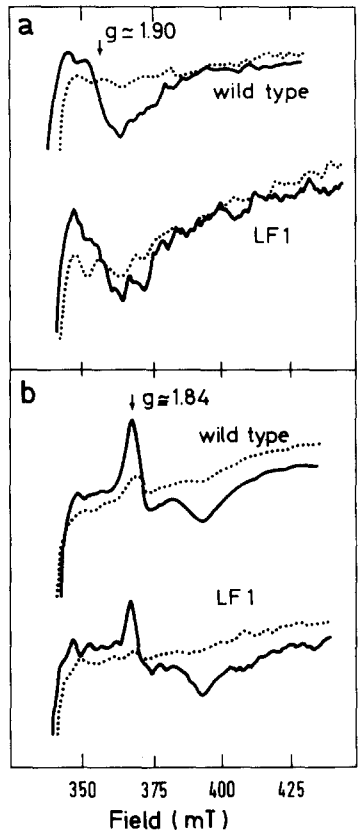

Fig. 1. Photoreduced $Q_A^- Fe^{2+}$ in PS-II-enriched membranes of *Scenedesmus* wild-type and LF1 mutant strains. (a) Samples with no additions were illuminated for 4 min at 200 K, then dark adapted for a further 5 min at 200 K before the EPR spectra (solid lines) were recorded. The broken lines were recorded prior to illumination at 200 K. (b) Samples were incubated for 5 min at 20 °C with 200 mM sodium formate (pH 6.0). Illumination for 25 min was given at 77 K, followed by a dark adaptation period of 5 min at 200 K before the spectra (solid lines) were recorded. The spectra in the dark, prior to illumination, are shown with the broken line. The concentration of chlorophyll in the LF1 samples was 5 mg/ml, while that in the wild type was 9 mg/ml. For comparison, the spectra of LF1 were recorded at twice the gain of those for the wild type. The gains in (a) were 5-times greater than in (b) EPR conditions were as follows: microwave frequency, 9.44 GHz; microwave power, 8 dB down from 200 mW (≈ 32 mW); modulation amplitude, 32 G; temperature, 4.5 K.

using a Bruker 200D-SCR spectrometer equipped with a 90 dB bridge.

Fig. 1A shows that illumination at 200 K of PS-II-enriched membranes of *Scenedesmus* wild type and LF1 mutant results in formation of an EPR signal centred at approx. $g = 1.9$. This light-induced signal is attributed to the semiquinone-iron interaction, $Q_A^- Fe^{2+}$ [9]. More convincing spectra were obtained by photoreducing $Q_A^- Fe^{2+}$ at 77 K in the presence of sodium formate (Fig. 1B). Formate binding modifies the EPR spectrum of $Q_A^- Fe^{2+}$, changing it to a narrow signal with a peak at $g \approx 1.84$, which has significantly increased amplitude [10,11]. Contributions to the spectra in Fig. 1 from photoreduced iron sulphur centres of PS I (for a review, see Ref. 12), were minimised by a period of dark adaptation at 200 K after the low-temperature illumination, during which time the electron on the iron sulphur centres recombines with the positive charge on $P\text{-}700^+$ [9]. It is clear from Fig. 1 that the $Q_A^- Fe^{2+}$ signals are similar in terms of their shape and amplitude in the wild type and the mutant.

In the wild-type membranes, illumination at 200 K results in formation of the S_2 multiline signal (see Ref. 13) which is attributed to a mixed valence manganese cluster [14]. This signal is absent in the LF1 mutant (Fig. 2). It has been noted that the 17 and 23 kDa polypeptides are more loosely bound in the LF1 mutant [4,5]. The absence of these polypeptides leads to lower-binding affinities for calcium and chloride, deficiencies in these ions results in inhibition of O_2 evolution [15]. Such lesions could be responsible for the absence of the multiline signal in PS-II-enriched membranes of LF1 (see Refs. 16 and 17 for recent examples of these effects). However, results similar to those shown in Fig. 2 were obtained in the presence of 5 mM Ca^{2+} and 100 mM Cl^- and also in unfractionated thylakoid membranes (not shown).

Despite the lack of a photoinduced multiline signal in LF1, it is clear that stable charge separation takes place upon illumination at low temperature, since $Q_A^- Fe^{2+}$ is formed (Figs. 1 and 2). The inset in Fig. 2 shows that cytochrome *b*-559 (see Ref. 18) in LF1 is fully oxidized in the dark. In the wild type much less cytochrome is oxidized in the dark and a small amount of cytochrome is

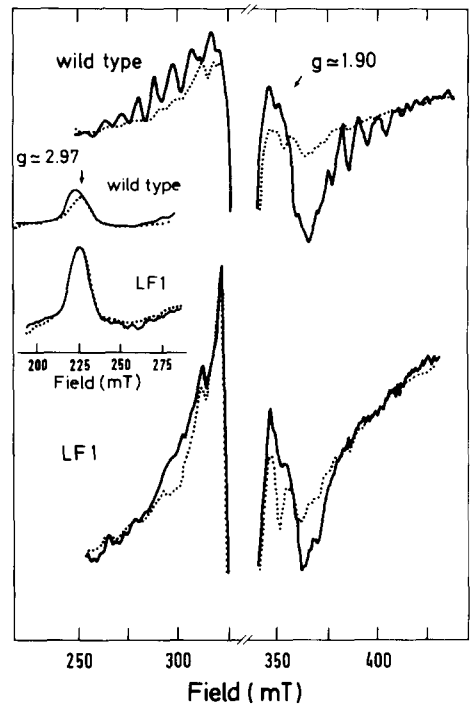

Fig. 2. EPR spectra in PS-II-enriched membranes of *Scenedesmus* wild type and LF1 after 200K illumination for 4 min followed by 5 min of dark adaptation at 200 K (solid lines). The broken lines are spectra which were recorded in dark-adapted samples prior to illumination. The LF1 sample (5 mg Chl/ml) was recorded at twice the gain of the wild type sample (9 mg Chl/ml). EPR conditions were as in Fig. 1, except the temperature was 8 K. Inset: EPR spectra of oxidized cytochrome *b*-559 recorded in the same samples as in the main figure. EPR conditions were as in Fig. 1, except the temperature was 15 K and the microwave power was 15 dB ($\approx$ 6.32 mW).

photooxidized under these conditions, presumably at the expense of multiline signal formation. Illumination at 77 K results in a much larger fraction of cytochrome *b*-559 going photooxidized in the wild type (not shown).

The source of the electron involved in $Q_A^- Fe^{2+}$ reduction in LF1 at 200 K (and at 77 K) seems likely to be the component, presumably a chlorophyll molecule [19,20], which gives rise to a free radical signal at $g \approx 2.0026$ photoinduced under these conditions (Fig. 3B, solid line). Very little of this signal is photoinduced in the wild type under these conditions (Fig. 3A, solid line). This component is oxidized in PS II from higher plants under

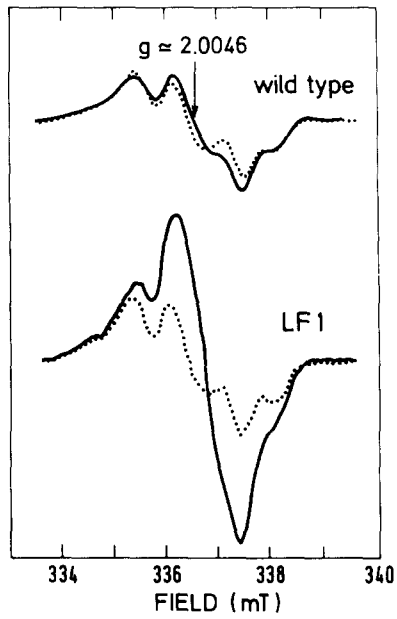

Fig. 3. EPR spectra in the $g = 2.00$ region in PS-II-enriched membranes of *Scenedesmus* wild type and LF1 strains before (broken lines) and after (solid lines) 4 min of illumination at 200 K. Samples were dark adapted at 200 K for 5 min immediately after illumination to minimize contributions from PS I. The LF1 sample (5 mg Chl/ml) was recorded at twice the gain of the wild-type sample (9 mg Chl/ml). EPR conditions were as follows: microwave power, 60 dB; temperature, 15 K; modulation amplitude, 2 G.

conditions in which the oxygen-evolving enzyme is non-functional (e.g., temperatures below 130 K) and when cytochrome *b*-559 is oxidized [20].

Fig. 3 (broken lines) also shows that Signal II (D^+) is present in the dark in both the wild-type and the LF1 mutant membranes. Fig. 4a and b and 4 d and e show that the dark Signal II has markedly different microwave power saturation characteristics in the two kinds of sample. It is more easily saturated in the LF1 mutant than it is in the wild type. From the plots in Fig. 4c and 4f the $P_{1/2}$ values can be estimated: thylakoids, LF1: 29.5 dB (224 μW); wild type: 26.25 dB (474 μW); PS-II-enriched membranes, LF1: 31.75 dB (134 μW), wild type: 27.5 dB (356 μW). This difference is similar to that seen earlier between Tris-washed and untreated material from higher plants (Ref. 21; see also Styring, S. and Rutherford, A.W., unpublished data). The effect is attributed to coupling between D^+ and the manganese of the O_2-evolving enzyme in the wild type which is absent in the LF1 mutant due to lack of the manganese.

In earlier work in which *Scenedesmus* wild type and LF1 were studied, it was reported that dark Signal II was very much reduced in amplitude in LF1 [22]. This discrepancy with the results reported here can be at least partially explained by the power saturation characteristics shown in Fig. 4. In the earlier work the spectra were recorded at 100 μW ($\approx$ 33 dB) and at 13 K. Fig. 4 shows that, at this power, Signal II is saturated in all of the samples even at 40 K. At lower temperatures the signals become even more easily saturated. In Fig. 4B the different power saturation behaviour in the two strains results in up to a 41% decrease in amplitude in Signal II in LF1 relative to the wild type in saturated conditions. This effect, combined with the broadening of the hyperfine structure which occurs under very saturated conditions, could account for the spectra in Ref. 22.

From this study it can be concluded that the reaction centre photochemistry in the LF1 mutant, as measured by the low-temperature formation of the $Q_A^- Fe^{2+}$ signal is unmodified. This is significant, since it is now known that the LF1 mutation results in a modification of the D_1, herbicide binding polypeptide [4,5]. The $Q_A^- Fe^{2+}$, semiquinone-iron interaction is very sensitive to changes in its local environment; the binding of herbicides to the Q_B-site, for example, results in marked spectral differences (reviewed in Ref. 11). The virtually identical $Q_A^- Fe^{2+}$ signals in the wild type and LF1 mutant may be taken as evidence that, even though the D_1 polypeptide is modified, the electron acceptor side of this polypeptide is not significantly perturbed.

The presence of dark Signal II in LF1, contrary to an earlier report [22], indicates that the donor side of the reaction centre is not greatly modified. This observation is also important, since the previously reported absence of D^+ in the LF1 mutant was difficult to reconcile with the currently emerging view that D and Z are tyrosine residues symmetrically located on the D_1 and D_2 polypeptides (see Ref. 25 and for a discussion Ref. 26).

The absence of the manganese multiline signal and manganese coupling to dark Signal II are evidence that the LF1 mutant lacks the redox active manganese of the O_2-evolving enzyme. These

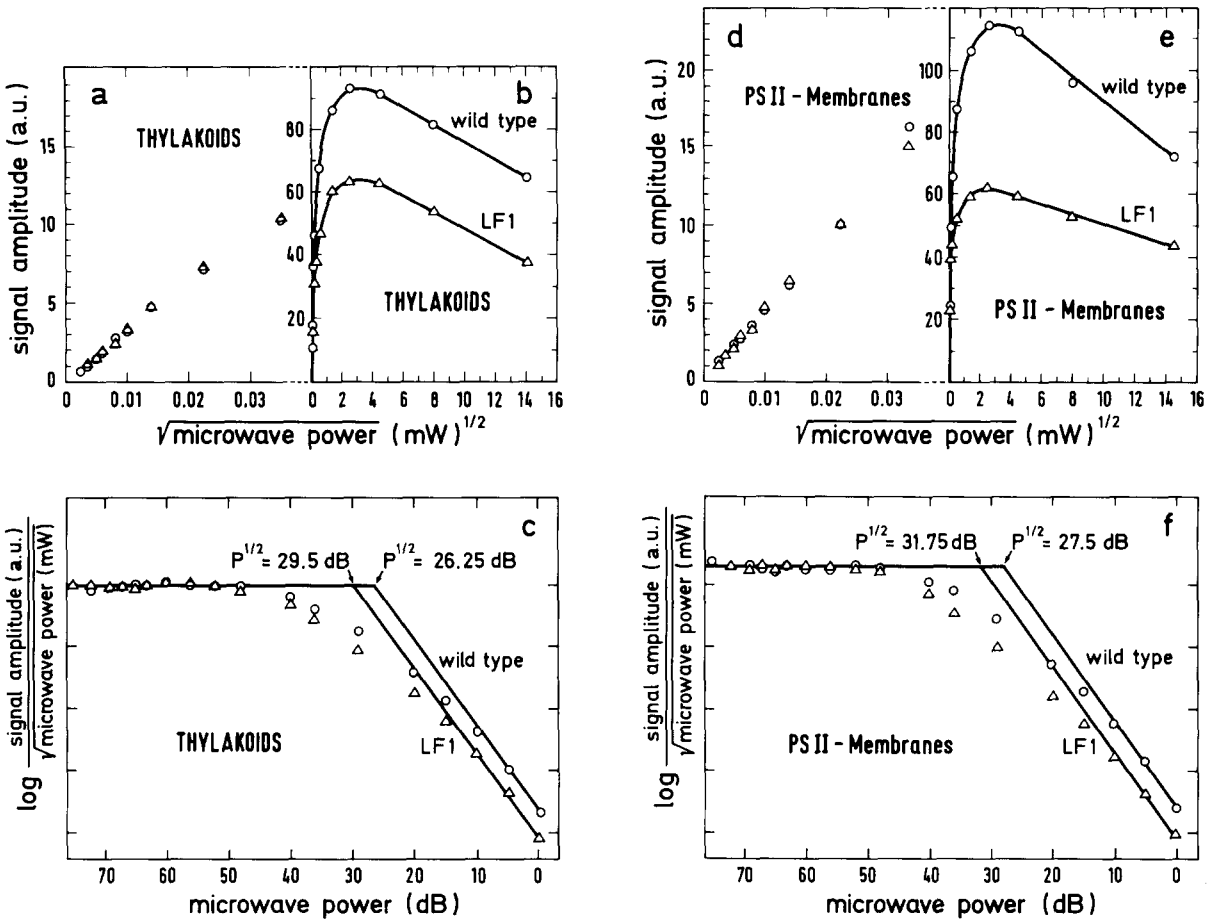

Fig. 4. A microwave power saturation study of dark Signal II in LF1 (triangles) and wild type (circles) *Scenedesmus*. (a–c) thylakoids (7.5 mg Chl/ml); (d–f) PS-II-enriched membranes (LF1, 5 mg Chl/ml); wild type (9 mg Chl/ml). For d–f the amplitudes were normalized to a value measured at a non-saturating power. (a) and (d) show data on an expanded scale at low powers; (b) and (e) shows the effect of higher powers; (c) and (f) show the same data as in (a and b) and (d and e), respectively, but plotted to obtain the $P_{1/2}$ values. EPR conditions were as in Fig. 3, except that the temperature was 40 K and the modulation amplitude was 2.8 G. 0 db corresponds to 200 mW.

results are an extension of the previous studies of the LF1 mutant in which the absence of O_2 evolution was correllated with the lowered manganese content. Here, the absence of the S_2 multiline signal demonstrates the lack of any capacity to accumulate positive charges in LF1. In addition the lower $P_{1/2}$ value for dark Signal II observed here in LF1 is evidence that the manganese cluster of the oxygen-evolving enzyme is absent (Styring, S., and Rutherford, A.W., unpublished data). The lack of manganese binding in LF1 is thought to be due to the increased size of the D_1 polypeptide as observed by the 34 kDa to 36 kDa change in apparent molecular mass on polyacrylamide electrophoresis gels [5]. Implicit in this explanation is that D_1 and D_2, which are thought to be the reaction centre polypeptides [6], form the binding site for the manganese of the O_2-evolving enzyme. However, the alternative explanation, that the manganese is lost as a result of a secondary effect of the D_1 modification (e.g., the weaker binding of the extrinsic polypeptides) cannot be ruled out.

The EPR data here also confirm the earlier observations that cytochrome *b*-559 is in the low-

potential form in the LF1 mutant [1,2]. It is unlikely that this is due to a direct perturbation of the cytochrome by the abnormal D_1 polypeptide, since the low-potential form of the cytochrome is also found in the LF2 mutant of *Scenedesmus*, which lacks manganese due to temperature-sensitive manganese transport into the cell [2]. The low-potential form of the cytochrome could represent an indirect effect of the LF1 lesion, if, for example, a low-potential to high-potential change in the cytochrome takes place during incorporation of manganese into the O_2-evolving enzyme (see Refs. 23 and 24).

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