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Secondary electron transfer reactions of the isolated Photosystem II reaction centre after reconstitution with plastoquinone-9 and diacylglycerolipids

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A Photosystem II (PS II) reaction centre preparation isolated with Triton X-100 and stabilised by transfer to dodecylmaltoside has been used in a reconstitution procedure with diacyl lipids and the naturally occurring PS II quinone, plastoquinone-9 (PQ9). As shown previously, this isolated protein complex consists of the D1, D2, *b*-559 α and β polypeptides, plus the *psb*I gene product. Before reconstitution there was only about 0.004 mol PQ9 and 0.2 mol diacyl lipid per mole chlorophyll *a* associated with the isolated complex. After reconstitution and precipitation by centrifugation the complex was found to have an associated lipid matrix of 1.3 mol PQ9 and 3.7 mol diacyl lipid for each mole of chlorophyll *a*. After reconstitution, the presence of a functional quinone in the PS II reaction centre was indicated by a strong thermoluminescence signal with a peak emission at −60 °C. This was recognised as the previously characterised Z_v signal on the basis of the relationship between emission and excitation temperatures and a complete quenching by ethanol. This signal was essentially unaltered by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and was the same after reconstitution with phosphatidylcholine or a thylakoid lipid extract. Calculation of the activation free energy for the charge separation reaction which gave rise to this signal suggested that this was 0.48 ± 0.01 eV and was the same as for a smaller Z_v signal detected in the isolated complex before reconstitution with PQ9. When duroquinone was used this value was not significantly different, although a much smaller signal was observed. With decylplastoquinone the thermoluminescence peak was as large as with PQ9 but the difference in shape indicated that the activation free energy was significantly higher at 0.55 ± 0.02 eV. Photosynthetic electron transfer in the reconstituted complex was assayed by reduction of 2,6-dichlorophenolindophenol (DCPIP) with diphenylcarbazide (DPC) as an artificial electron donor. A low residual rate was strongly enhanced by reconstitution with PQ9. This activity was greater when reconstitution was with a thylakoid lipid extract than with phosphatidylcholine and was inhibited by addition of DCMU (I_{50} = 0.2 mM).

Introduction

Isolation of the Photosystem II (PS II) reaction centre complex, which consists of the D1, D2, *psb*I and cytochrome *b*-559 proteins, has facilitated a closer analysis of the conversion of light energy to the primary radical pair $P680^+Pheo^-$ (e.g. Refs. 1–4). This primary photochemical reaction is of the upmost importance for the investigation of oxygenic photosynthesis because it generates a redox species which is sufficiently positive to oxidise water. However, the rapid back-reaction to give the ground state P680Pheo means that in itself the formation of the primary radical pair does not give a stable, and therefore useful, energy source which will oxidise water and drive the overall photosynthetic reactions. To stabilise the capture of light energy in PSII depends on the subsequent, and therefore secondary transfer of electrons from $Pheo^-$ to the quinones designated Q_A and Q_B.

Abbreviations PS II, Photosystem II; P680, primary chlorophyll electron donor in PSII; Pheo, pheophytin a the primary electron acceptor in PSII: PQ9, plastoquinone-9; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, diphenylcarbazide; DCPIP, 2,6-dichlorophenolindophenol; DPQ, decylplastoquinone; DQ, duroquinone; Q_A and Q_B, primary and secondary quinone acceptors.

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Experiments with the isolated PSII reaction centre could be valuable for determining the factors which regulate the secondary, as well as the primary, electron transfer events. Unfortunately progress has been hampered in that it has not been possible to isolate a PS II reaction centre that retains significant amounts of quinone. Successful experiments have been carried out with secondary electron transfer to the artificial inorganic compound silicomolybdate [5]. A more desirable approach has, however, been demonstrated by reconstituting quinone-dependent electron transfer activities by addition of quinones, such as decylplastoquinone (DPQ) [6,7] or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) [3,8,9]. Here, we describe a potentially more significant system in which we succeed in the reconstitution of the naturally occurring PS II quinone, plastoquinone-9 (PQ9).

To achieve incorporation of such a lipophilic molecule into the protein complex our attention was naturally focused on the possible importance of the amphiphilic diacylglycerolipids which constitute the majority of the hydrophobic domain of the thylakoid membrane. For this reason we explored the possibility of using a lipid reconstitution system. Particular incentive to use this approach comes from the demonstration of the importance of acyl lipids in reconstitution experiments with bacterial photosynthetic reaction centres (e.g. Refs. 10,11) and from our previous studies which showed that detergent and lipid additions can enhance some activities of the isolated PS II reaction centres [7]. Furthermore, the importance of acyl lipids in regulating electron transfer to Q_A and Q_B has been emphasised in several different types of experiment [12–15]. Having found that our PS II reaction centre preparations have a very low diacyl lipid content, at about 0.2 mol per mol chlorophyll *a* (unpublished data), it seemed imperative to establish a reconstitution procedure if we were to improve our studies of quinone function in this isolated complex. Moreover, such reconstitutions would have the additional benefit of giving an experimental system for investigating the role of protein–acyl lipid interactions. We have given a preliminary report [16] of the success of this approach for PQ9 reconstitution and here we present further details. In particular, we have studied the recovery of a thermoluminescence signal indicative of Q_A function, monitored the ability of the isolated reaction centre to reduce 2,6-dichlorophenolindophenol (DCPIP) using diphenylcarbazide (DPC) as an electron donor and checked the sensitivity of these reactions to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

Materials and Methods

The PS II reaction centre was isolated from *Pisum sativum* L. plants with a procedure which involved transfer into the detergent dodecylmaltoside to give a complex which was stable during incubation and assay at room temperature. The isolation protocol has been described in detail previously [6,17] and involved thorough solubilisation of a PS-II-enriched membrane preparation with Triton X-100 and chromatography on a DEAE ion-exchange column. A fraction containing the reaction centre was diluted into a buffer containing 2 mM dodecylmaltoside (*n*-dodecyl-*β*-D-maltoside, c.m.c. 0.1–0.2 mM) and the same chromatography was repeated in the presence of this concentration of detergent.

Phosphatidylcholine was purchased from Sigma (P-5638) and the diacylglycerolipids of chloroplast thylakoids extracted from a membrane preparation [18] by the method of Bligh and Dyer [19], with separation from extracted pigments and neutral lipids by chromatography on DEAE and silicic acid columns essentially as described by Murata et al. [20]. Decylplastoquinone and plastoquinone-9 were gifts from Dr P. Rich, Glynn Research Institute, Bodmin, Cornwall and Hoffman La Roche, Basel, Switzerland, respectively.

Lipid suspensions were prepared by sonication of a dry film of diacylglycerolipid in a buffer containing 1 mM dodecyl maltoside under nitrogen. The detergent/lipid mixture was then added to a reaction centre sample, incubated for 2 min at room temperature in the dark and then diluted 20-fold in buffer without detergent. For thermoluminescence measurements the buffer used was 50 mM Hepes (pH 7.5) and incubation before dilution was carried out in a total volume of 25 μl with reaction centre samples at 0.2 mM chlorophyll *a* and 3 mM acyl lipid, with quinone and DCMU incorporated during preparation of the lipid suspension (i.e. no ethanol was added). For DCPIP reduction assays the buffer was 50 mM Tris-HCl (pH 8.5) and the incubation before dilution was in a total volume of 50 μl with 0.04 mM chlorophyll *a*, 1.5 mM lipid and 10% ethanol containing quinone and herbicide was added to the assay system.

Thermoluminescence curves were obtained as described by Vass et al. [21] with 500 μl samples which contained 5 nmol chlorophyll *a* and were illuminated for 1 min with light at 10 W m^{-2} at −60 °C, cooled rapidly to −95 °C in the dark and then warmed at a constant rate of 20 C° min^{-1}. Assays of light dependent reduction of 0.25 μM DCPIP were carried out with 0.5 mM diphenylcarbazide (DPC) as an electron donor in a 1 ml assay system containing 2 nmol chlorophyll *a* of the reaction centre, using a dual-beam spectrophotometer and an actinic light source as described previously [6].

Association of lipid and quinone with the reaction centre preparation after the incubation/dilution procedure was assessed by centrifugation at 350 000 × *g* for 20 min and analysis of the acyl lipid and quinone content of the pellets using gas-liquid chromatography

[18] and reverse-phase high-performance liquid chromatography (HPLC) respectively. The HPLC analysis was carried out on a Spherisorb ODS-reverse phase column with an isocratic solvent system of acetonitrile/methanol (3 : 2, v/v) at 1.0 ml min^{-1}, with spectrophotometric quantification using an 8 μl flow cell monitored at 255 nm for PQ9 and 663 nm for chlorophyll *a*. Peak areas for samples were compared with those of standards prepared at known concentration using extinction coefficients of 15.2 mM^{-1} cm^{-1} for PQ9 in ethanol (255 nm), and 70.8 (665 nm) and 82.6 (662 nm) mM^{-1} cm^{-1} for chlorophyll *a* [22] in methanol and acetone, respectively.

Results

The use of dodecylmaltoside in the final phase of the procedure for isolating the PS II reaction centre gave preparations which could be used without thermal damage in reconstitution protocols involving incubation and manipulation at room temperature. A good indication of the damage which can occur in the isolated PS II reaction centre is a blue shift in the wavelength of maximum absorbance in the red region of the absorbance spectrum [16,23] and this characteristic was used to monitor the relative stabilities of samples in dodecylmaltoside and Triton X-100. The former had a maximum absorbance which was 0.5 nm less than the reaction centres in Triton X-100 but were much more stable when incubated at 20 °C. Furthermore, the enhanced stability of the dodecylmaltoside preparations was also apparent after reconstitution with thylakoid lipid.

Assessment of the extent of the association of lipid and quinone with the reaction centre was carried out by quantitative analysis of the lipid and quinone recovered from pellets produced by centrifugal precipitation of the entire reaction centre population in reconstitution systems which contained standard amounts of lipid and quinone but various quantities of the protein complex. From the linear relationship between amount of lipid (or quinone) precipitated and the amount of reaction centre used in the reconstitution it was calculated that for each mole of chlorophyll *a* there were in the region of 1.3 mol PQ9 and 3.7 mol diacyl lipid. This represents a substantial increase above about 0.004 mol PQ9 and 0.2 mol diacyl lipid detected in samples not subjected to the reconstitution procedure. In Table I the close similarity of the highly unsaturated fatty acid composition in the lipid extract and the lipid recovered from reconstituted reaction centres suggests that there was no significant lipid degradation during the reconstitution incubation.

The PS II reaction centre samples which were prepared in dodecylmaltoside gave thermoluminescence curves (Fig. 1a) which were similar to those previously

TABLE I

Fatty acid composition of the lipid extract of chloroplast thylakoids, the suspension of this lipid in 50 mM Tris (pH 8.5), and the lipid in the reconstituted PS II reaction centre (RC) preparation

	mol% fatty acids and methyl esters				
	16:0	18:0	18:1	18:2	18:3
Thylakoid extract	8	3	1	6	81
Lipid suspension	10	5	4	4	76
Reconst. PS II RC	9	4	4	4	78

published for reaction centres prepared in Triton X-100 [24]. The high luminescence above 0 °C was attributable to detergent–pigment interactions as noted for the reaction centre [24] and larger PS II preparations [25] isolated using Triton X-100. This part of the thermoluminescence signal was increased on addition of thylakoid lipids and this change, plus the relative sizes of emissions, meant that two small bands between −30 and 0 °C were only just discernible and not necessarily attributable to previously identified signals such as the 'A' band. Furthermore, any apparent changes in these small bands could not be regarded as significant because of interference from alteration of the larger emission centred on temperatures above 0 °C. As with the PS II reaction centres in Triton X-100 there was also a small but clear signal in the region of −60 °C. This was hardly detectable in reaction centre samples subjected to reconstitution in diacylglycerolipids (Fig. 1b) but was strongly enhanced (about 10-fold) if PQ9 was included in the reconstitution system (Fig. 2). This band was identified as the Z_v peak on the basis of previous characterisation by Vass et al. [26] with thylakoid preparations in which water oxidation was inhibited by

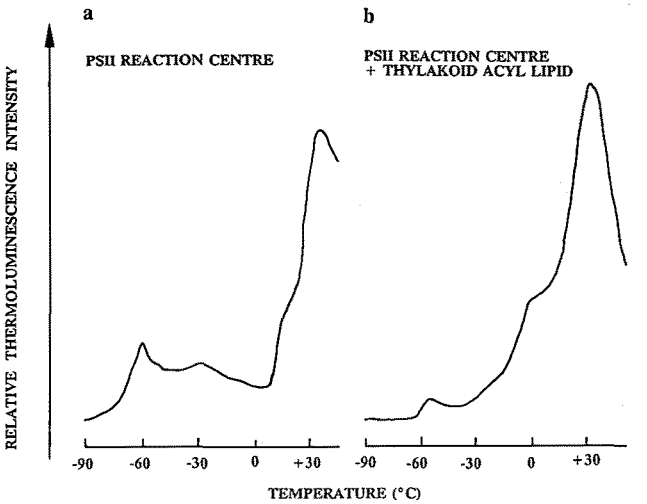

Fig. 1. Effect of diacyl lipid on thermoluminescence from the isolated PS II reaction centre. Curves were obtained from samples prepared in dodecylmaltoside, incubated for 2 min in 1 mM dodecylmaltoside, 20 mM Hepes (pH 7.5) and then diluted into detergent-free buffer, incubated (a) without and (b) with 3 mM thylakoid lipid extract.

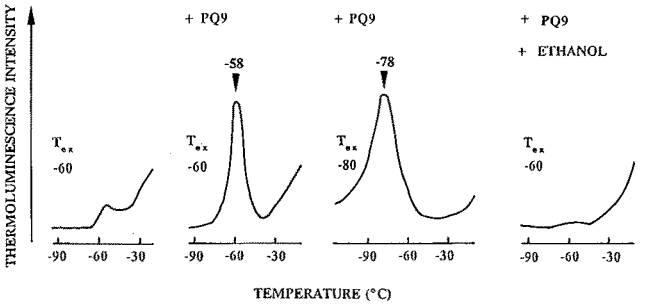

Fig. 2. Effect of PQ9 addition, excitation temperature (T_{ex}) and ethanol addition on the thermoluminescence signal between −90 and −30°C from the isolated PS II reaction centre reconstituted with thylakoid lipids. PQ9 was added to 400 μM during incubation (20 μM after dilution to give the final assay volume). When used, ethanol was added after dilution to give 4% (v/v) concentration.

Tris washing. As also shown in Fig. 2, the temperature of this emission peak varied with the excitation temperature and the band was completely quenched by addition of ethanol. The size of this Z_v band was dependent on the amount of PQ9 added and a maximum signal was obtained at about 20 μM concentration in the final suspension with a 50% maximum at 1 μM (Fig. 3). Both the size and temperature for peak emission were not affected significantly by either substituting phosphatidylcholine for the thylakoid lipid extract (Fig. 4) or introducing DCMU into the lipid reconstitution system (Fig. 5). As shown previously [16], it was also possible to introduce alternative quinones into the reconstitution procedure and still observe a Z_v signal, although peak heights and widths were altered. These differences give different values of calculated activation

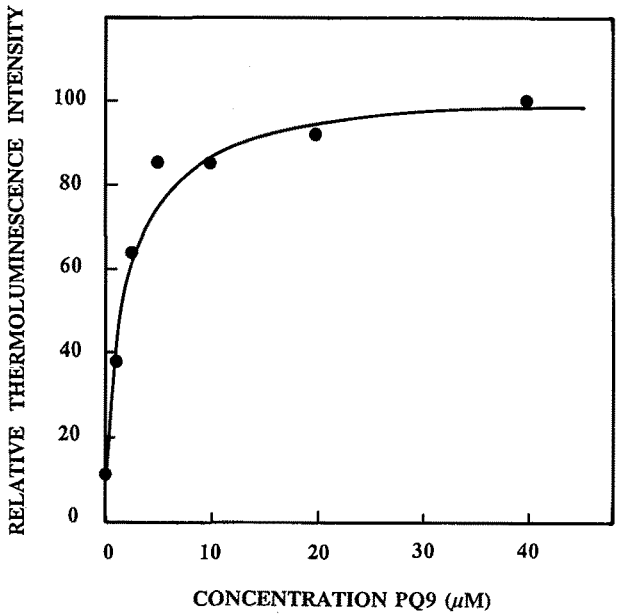

Fig. 3. Effect of PQ9 concentration on the thermoluminescence intensity obtained with PS II reaction centre samples. Conditions were as described in Materials and Methods and quinone concentrations are given for the final assay volume.

TABLE II

Calculated activation free energies of Z_v thermoluminescence bands derived from signals obtained after reconstitution of PS II reaction centres with different quinones. See Ref. 21 for method of calculation

Quinone	Activation free energy (eV)
None	0.473 ± 0.040
Plastoquinone	0.476 ± 0.006
Duroquinone	0.467 ± 0.080
Decylplastoquinone	0.549 ± 0.015

free energies for the charge separation state (Table II), indicating a characteristic redox potential difference for the donor-acceptor pair which is dependent on the quinone used. With PQ9 this value is almost identical to that for the signal from the reaction centre before reconstitution. Similarly, the relatively small band obtained with duroquinone gives a value close to the original. However, addition of DPQ results in a shift to significantly more positive values by about 0.075 eV.

The capacity for secondary electron transfer was further investigated by assays of quinone-dependent DCPIP reduction with DPC as an electron donor. In reaction centre samples not subjected to the reconstitution procedure it was hardly possible to detect light-de-

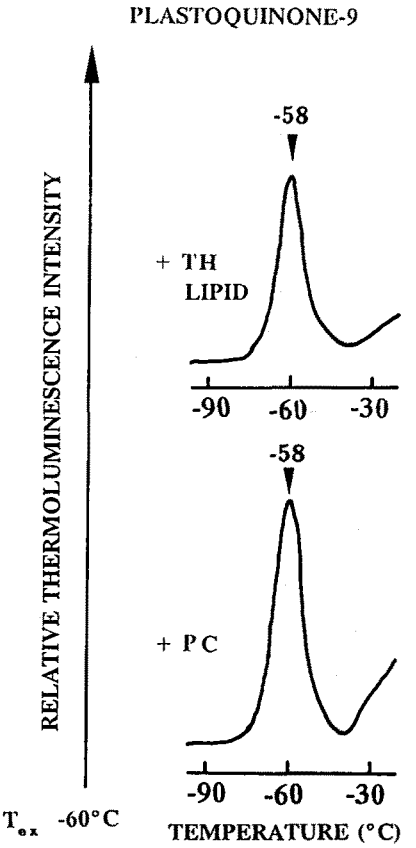

Fig. 4. Comparison of thermoluminescence signals from PS II reaction centre samples reconstituted with PQ9 and either phosphatidylcholine (PC) or thylakoid (TH) lipids.

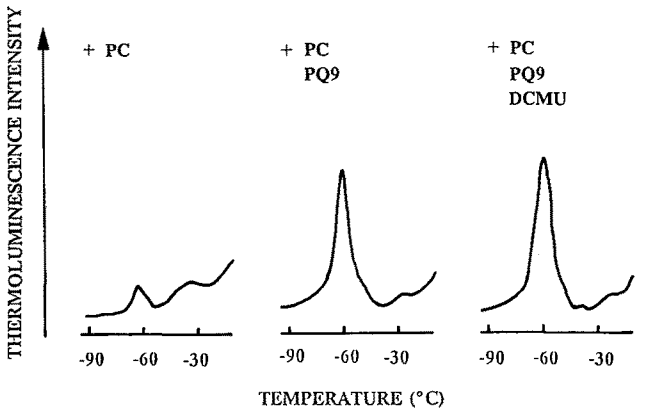

Fig. 5. Effect of DCMU addition on the thermoluminescence signal from PS II reaction centre samples reconstituted with PQ9 and phosphatidylcholine (PC).

pendent DCPIP reduction. With samples reconstituted with lipid, but no quinone, there was a very slight stimulation of the rate. However, addition of PQ9 to the reconstitution procedure did stimulate the activity, but this was only clearly significant and reliably reproducible when both lipid and PQ9 were used (Fig. 6). These rates were typically 200 μmol (mg Chl)$^{-1}$ h^{-1} when thylakoid lipids were used, but 90 μmol (mg Chl^{-1}) h^{-1} with phosphatidylcholine. The dependence on the amount of PQ9 added is shown in Fig. 7 and the optimum PQ9 concentration in the final assay volume was about 10 to 15 μM. This contrasts with about

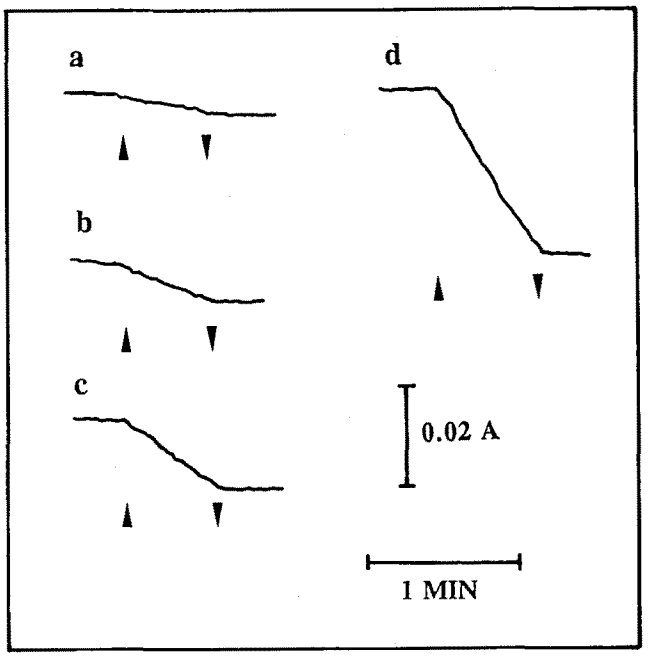

Fig. 6. Spectrophotometric absorbance measurements at 590 nm used to monitor light- and DPC-dependent reduction of DCPIP by the isolated PS II reaction centre samples after the reconstitution procedure. Additions to the reconstitution incubation were: (a) none, (b) thylakoid lipid, (c) phosphatidylcholine with 10 μM PQ9 (d) thylakoid lipid with 10 μM PQ9.

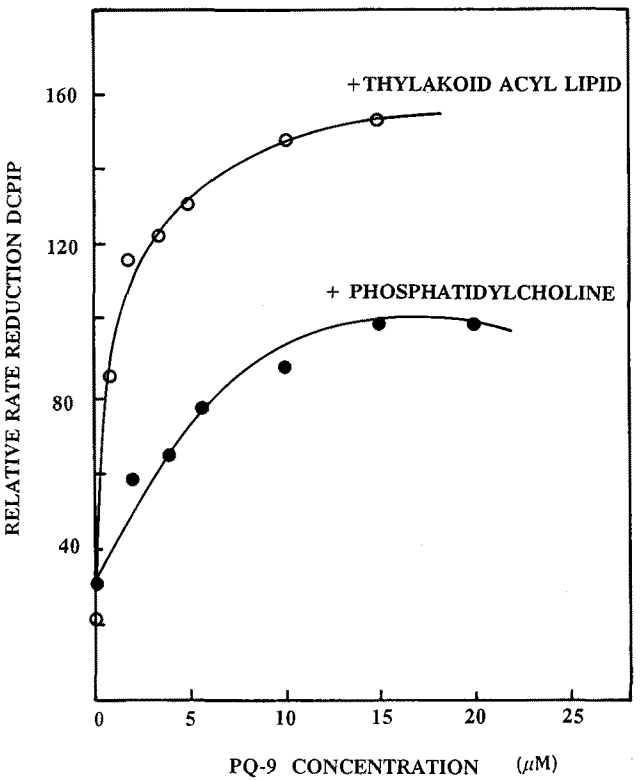

Fig. 7. Effect of PQ9 concentration on the light- and DPC-dependent reduction of DCPIP by the isolated PS II reaction centre reconstituted in either thylakoid lipid (open circles) or phosphatidylcholine (closed circles). Quinone concentrations are given for the final assay volume.

100 μM for optimum additions of duroquinone, or DPQ, to reaction centre preparations without lipid [16]. In Table III the effect of addition of increasing concentrations of DCMU is shown. As reported previously [7], there was a partial inhibition by DCMU of DCPIP reduction in a non-lipid reconstitution when the quinone was DPQ rather than PQ9. When the reaction centres were reconstituted with lipid and PQ9 the effect of DCMU was much greater, with almost complete inhibition being achieved with phosphatidylcholine present. Although this is a significantly more sensitive response

TABLE III

Effect of DCMU additions on the light- and DPC-dependent reduction of DCPIP by the isolated PS II reaction centre with 50 μM DPQ or reconstituted with PQ9 and either phosphatidylcholine (PC) or thylakoid lipids (TL)

Relative ratios are given as percentage of controls without DCMU addition.

	μM DCMU			
	50	125	250	350
DPQ	88	96	72	67
PQ9+PC	81	73	22	8
PQ9+TL	95	62	41	21

to DCMU than in the absence of lipid, the 100 to 200 μM concentration needed for 50% inhibition is nevertheless a far higher concentration than that needed to inhibit activity of thylakoid membranes (less than 1 μM) and indicates that full recovery of herbicide binding capacity may require further manipulation of the reconstitution system.

Discussion

Our success with the partial reconstitution of PQ9 into the isolated PS II reaction centre emphasises the importance of diacyl lipids in the interaction of PQ9 with the Q_A and Q_B site of PS II. Two different measurements were made of quinone function and the role of lipids appeared to be distinctly different in each case. Phosphatidylcholine, and a thylakoid lipid extract, were equally effective in reinstating the quinone-dependent Z_v thermoluminescence signal. On the other hand, thylakoid lipids were much more effective than phosphatidylcholine when the PQ9-dependent electron transfer to DCPIP was assayed. This difference in lipid specificity may be due to the fact that only the Q_A, and not the Q_B site, is involved in the charge recombination event which gives rise to the Z_v signal, whereas both Q_A and a functional Q_B site are probably needed for the light-dependent reduction of DCPIP.

Measurement of thermoluminescence has proved a convenient and reliable assay for charge separation reactions involving the quinones within PS II. In thylakoids much of the work has focused on the study of thermoluminescence signals related to the water oxidation reactions such as the B band from charge recombination between the Q_B^- and S_2 state. However, the large number of thermoluminescence bands and the complexity of signals produced [27] has restricted the usefulness of the technique for some studies. The Z_v band is clearly observed only in preparations which lack the water oxidation reactions [21,28]. The well-defined signal in the reconstituted PQ9/reaction centre system seems to suggest that this technique will be valuable in future studies involving quinone reconstitution and function. There is general agreement that Q_A^- is involved in giving this signal, but the nature of the oxidant has been a matter of controversy, with some believing that it is the secondary donor D^+ [29] while others favour $P680^+$ [30]. Although there is indirect evidence for functional activity of Z in the isolated PS II reaction centre [8], no strong EPR signal indicative of the photoaccumulation of Z^+ has been detected [31,32]. This suggests that $P680^+$ might be involved in the recombination thermoluminescence giving rise to the Z_v signal. It is clear that our results can not be taken as strong evidence for the existence of Z^+ or D^+ in the isolated reaction centre. In the present study, the size of the Z_v signal was compared with those previously observed with other PS II preparations and we conclude that of the reaction centres used for reconstitution with quinones, up to 20% are involved in the emission phenomenon. The failure to achieve 100% could be due to a combination of many factors. These probably include the presence of incomplete Q_A sites which are incapable of reconstitution, a reversible quinone interaction which leaves empty Q_A sites at any one time and an inability to trap the charge separation state in the full number of centres capable of forming Q_A^-. The activation energy for the charge separation which was calculated from the Z_v band is close to that obtained for the same band in isolated thylakoids [21]. Interestingly, when DPQ was used, a significant shift in this value was found, although the similarity in size of signal indicated that a similar number of reaction centres were involved.

Without addition of quinone, DCPIP does not act as an effective electron acceptor from the isolated PS II reaction centre. On the other hand, with the quinone reconstitution system DCPIP reduction gave an assay of net photosynthetic electron transfer from the artificial donor DPC which was convenient to monitor spectrophotometrically and was as useful for assaying net electron transfer in the reaction centre as the reduction of silicomolybdate reported previously [5]. Another consequence of silicomolybdate acting as a secondary electron acceptor and stabiliser of charge separation in the isolated reaction centre was previously shown to be the reduction of the light-induced formation of a P680 triplet state [31] and protection from photodamage [33]. The quinone in our reconstitution system appears to have a similar effect, giving protection against light damage (data not shown). This indicates that damage in some circumstances could be a direct consequence of malfunction in the secondary electron transfer reactions of the quinones.

PQ9 is insoluble in aqueous media and therefore the primary role of the diacyl lipids in the reconstitution system must be assumed to be provision of a matrix in which this quinone is soluble. The mixing of acyl lipid and the hydrophobic reaction centre proteins during the incubation and dilution procedure will be expected to facilitate the insertion of the PQ9 into quinone binding sites. Indeed, the extraction of a small residual Q_A fraction by acyl lipid might explain the apparent decrease in the small Z_v signal when reconstitution was carried out with acyl lipid but no quinone. In line with this unspecific role of acyl lipid as a medium ensuring quinone solubility, the reconstitution of the tightly bound Q_A detected by the Z_v thermoluminescence signal seemed to be unaffected by the use of phosphatidylcholine or a thylakoid lipid extract. However, the marked difference between these two lipid preparations for the DCPIP reduction assay suggests that a second role of the lipid was involved in giving this activity. In fact, the insensitivity of the Z_v band but marked sensi-

tivity of DCPIP reduction to DCMU suggested that in the latter case the Q_B site was involved. Therefore, a second role for the reconstituted lipid matrix might be to contribute to the structure of the Q_B site and probably provide a matrix in which there is a quinone pool freely available for exchange at this site. Certainly, our results show that thylakoid lipids are better suited to this role than phosphatidylcholine, which is a lipid not abundant in, or possibly even absent from [34], the thylakoid membrane. Our previous analyses indicated that the thylakoid lipid, phosphatidylglycerol, is involved in specific PS II–lipid interactions, possibly at the Q_B site, because an amino acid substitution near this Q_B site which gave resistance to PS II herbicides also resulted in significant changes in the fatty acid composition of this lipid in isolated PS II fractions [35]. The functional significance of the lipids associated with PS II was also suggested by the persistence of sulphoquinovosyldiacylglycerol [36] and monogalactosyldiacylglycerol [37] during lipid depletion and purification of PS II protein complexes.

The suggested involvement of acyl lipid in facilitating Q_A-binding and functionally activating the Q_B site depends on the reconstitution system having produced a lipid-quinone-protein matrix. The centrifugation after reconstitution showed that such a complex could be separated from free lipid and quinone. The mole ratios determined for these components can be expressed as 8 mol PQ9 and 22 mol diacyl lipid for the 6 mol of chlorophyll *a* shown to be present [38,39] in each reaction centre, defined as containing 2 pheophytin *a*. This gives a convenient system for studying lipid–protein interactions, but it can not be concluded that the complex represents a reconstitution which is fully representative of the in vivo state. Detergent molecules were present in the incubation and, even after the dilution, some probably adhere to the protein or are intermixed with the lipid molecules. Indeed, the absence of neighbouring hydrophobic proteins which occur in the PS II complex in vivo emphasises that this PS II reaction centre reconstitution is an artificial system. Nevertheless, it promises to be extremely useful for studying acyl lipid and quinone interactions with the reaction centre, as well as interactions between reaction centre and other hydrophobic proteins. The association of other PS II proteins with the reaction centre is being studied, particularly with consideration of the concept of close homogy between the structures of the PS II and purple bacterial reaction centres. In the latter, the H-subunit plays a significant role in stabilisation and function of Q_A and Q_B. However, no equivalent protein has been found in PS II. Our work reported here seems to indicate that the Q_B site can function without proteins other than those in the isolated reaction centre. This could be taken as support for the notion that the *psb*I gene product in the isolated PS II reaction centre could have a role similar to the H-subunit [40]. However, to give validity to such speculation requires further work possibly involving reconstitution procedures along the lines of those reported in this paper.

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