Reconstitution of the Endogenous Plastoquinone Pool in Photosystem II (PS II) Membrane Fragments, Inside-Out-Vesicles, and PS II Core Complexes from Spinach†

J. Kurreck, A. G. Seeliger, F. Reifarth, M. Karge, and G. Renger*

Max-Volmer-Institut der TU Berlin, Strasse des 17. Juni 135, D-10623 Berlin, Germany

Received May 8, 1995; Revised Manuscript Received October 4, 1995®

ABSTRACT: The possibility of reconstituting a functionally competent endogenous plastoquinone pool in photosystem II (PS II) membrane fragments, inside-out-vesicles (ISO-vesicles), and PS II core complexes was analyzed by measuring (i) the characteristic period four oscillation of the oxygen yield due to excitation of dark-adapted samples with a train of short flashes and (ii) laser flash-induced transients of the relative quantum yield of chlorophyll fluorescence. The data obtained revealed that (a) an endogenous pool capacity comparable to that of intact thylakoids can be restored in PS II membrane fragments and ISO-vesicles by a sonication treatment using native plastoquinone-9 (PQ-9), (b) a more pronounced oxygen oscillation pattern arises in PS II core complexes after application of the same reconstitution procedure, (c) the extent of the endogenous pool restoration at a ratio of 15 quinone molecules per PS II in the reconstitution assay strongly depends on the nature of the quinone molecule [maximum effects can be only achieved with PQ-9, while at the same concentration ubiquinone-45 (UQ-9) is almost inefficient], and (d) a sonication step is required for stable insertion of PQ-9 into PS II preparations. Measurements of the reconstitution degree as a function of the structure of different quinones with selected properties lead to the conclusion that specific binding domains exist in PS II in addition to the Q_B site. These domains exhibit a surprisingly high specificity for the type of quinone that can be bound. On the basis of a comparison of the results obtained, the structure of the quinone head group seems to be more important than the large hydrophobic side chain and/or the general lipophilicity of the compound.

In all oxygen-evolving photoautotrophic organisms, the key steps of light-induced water cleavage into dioxygen and chemically bound hydrogen take place within a multimeric pigment/protein complex referred to as photosystem II (PS II), which is anisotropically integrated into the thylakoid membrane [for recent reviews, see Renger (1993), and Vermaas et al. (1993)]. The overall process catalyzed by PS II comprises three types of reaction sequences: (a) photooxidation of a special chlorophyll *a* component (P680) and subsequent stabilization of the primary charge separation by rapid electron transfer from the pheophytin anion ($Pheo^-$) to a specially bound plastoquinone (Q_A) [for a review, see Renger (1992)], (b) cooperation of four oxidizing redox equivalents within a manganese-containing functional unit, designated as a water-oxidizing complex (WOC), giving rise to oxidation of two water molecules into dioxygen under release of four protons into the lumen [for reviews, see Debus (1992), Rutherford et al. (1992), and Renger (1993)], and (c) plastoquinone reduction to plastoquinol under proton uptake from the stroma [for a review, see Crofts and Wraight (1983)].

Significant progress in the understanding of the structural and functional organization of these processes has been achieved by the isolation of functionally competent PS II preparations. This permitted the application of sophisticated spectroscopic techniques and eliminated the overlapping by other reactions, e.g. those occurring in PS I or the Cyt b_6f complex. The first step in this direction was the development of a procedure by Berthold et al. (1981) that allowed for the isolation of PS II membrane fragments. Later, PS II core complexes were prepared (Ikeuchi et al., 1985; Franzen et al., 1986; Ghanotakis et al., 1987; Haag et al., 1990). At the present time, these latter preparations represent the minimum entity with a fully competent water cleavage activity.

Striking functional similarities (Rutherford, 1986) and structural homologies between bacterial reaction centers and PS II (Trebst, 1986; Michel & Deisenhofer, 1988) led to the suggestion that all redox groups participating in reaction sequences a and c are incorporated into a heterodimer consisting of polypeptides D1 and D2. This idea stimulated efforts to isolate the proposed unit. Nanba and Satoh (1987) were successful in achieving this goal by preparing a complex that contained D1 and D2 together with cytochrome b_{559} (Cyt b559)[1] and was able to perform the primary charge separation. Unfortunately, the plastoquinone acceptor Q_A is lost during the isolation procedure so that in a strict sense these preparations are not complete PS II reaction centers. All attempts to reconstitute Q_A were not sufficiently successful so far.

The loss of Q_A is a serious problem for D1/D2/Cyt b559 complexes, but other types of PS II preparations are also

† This work was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

* Corresponding author. FAX: 49-30-314 211 22.

® Abstract published in *Advance ACS Abstracts,* November 15, 1995.

[1] Abbreviations: Cyt b559, cytochrome b_{559}; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMDBQ, 2,3-dimethyl-5-decyl-*p*-benzoquinone; ISO-vesicles, inside-out-vesicles; Ph-p-BQ, phenyl-*p*-benzoquinone; PQ-9, plastoquinone-9; UQ-9, ubiquinone-45 (coenzym Q-9); WOC, water-oxidizing complex.

0006-2960/95/0434-15721$09.00/0

modified at the level of functional plastoquinone molecules. Although Q_A remains bound and functionally intact in PS II membrane fragments and PS II core complexes, the plastoquinone content is diminished in these preparations as reflected by a reduced effective pool size (Messinger et al., 1993) together with a slightly modified Q_B site in the former (Renger et al., 1986) and a severely disturbed Q_B site in the latter sample type (Gleiter et al., 1993). These effects seriously restrict measurements of the characteristic quarternary oscillation pattern of the oxygen yield with the conventional Joliot-type electrode (Joliot, 1972) to one or at most two periods in PS II membrane fragments [see Messinger et al. (1993) and references therein]. Even more drastic changes are observed in PS II core complexes (Lübbers & Junge, 1990; Haag et al., 1992; Gleiter et al., 1993). Features similar to those in PS II membrane fragments were obtained with detergent free, PS II enriched ISO-vesicles (Messinger et al., 1993). These vesicles with inverted membrane polarity proved to be an invaluable material for several types of studies, especially the immunological determination of the orientation of membrane bound proteins.

The shortcomings owing to small pool size led to attempts of increasing its capacity by incorporation of exogenous quinones into PS II membrane fragments. Experiments were performed with special quinone derivatives like tribromotoluoquinone (TBTQ) which are known to exhibit a comparatively high affinity to PS II (Renger et al., 1987, 1988). However, no significant increase could be achieved. This failure could have been due to using either an inappropriate procedure and/or inefficient quinones. Preliminary experiments with plastoquinone-9 (PQ-9) led to some increase of the pool size (Messinger, 1988). The present study shows that the plastoquinone pool can be successfully reconstituted in PS II membrane fragments and ISO-vesicles by using plastoquinone-9 (PQ-9) as the quinone component and by applying a special sonication treatment. A significant improvement of plastoquinone functions could also be achieved in PS II core complexes. A striking feature emerging from these experiments is the finding that the extent of reconstitution strongly depends on the nature of the quinone; the maximum effect could be only obtained with the native PQ-9, while most of the quinones analyzed are entirely inefficient.

MATERIALS AND METHODS

Inside-out-vesicles were prepared from market spinach as described in Åkerlund and Anderson (1983) with some modifications. After the final isolation step, the ISO-vesicles were resuspended in 5 mM sodium phosphate buffer (pH 7.4), 500 mM sucrose, and 10 mM NaCl at chlorophyll concentrations of about 3 mg/mL. PS II membrane fragments were isolated from spinach according to the procedure of Berthold et al. (1981) with slight modifications (Völker et al., 1985). After the final isolation step, the PS II membrane fragments were resuspended in a weakly buffered medium [10 mM MES/NaOH (pH 6.5), 15 mM NaCl, 4 mM $MgCl_2$, and 400 mM sucrose] to chlorophyll concentrations of about 5 mg/mL. Preparations of PS II core complexes with high oxygen evolution capacity were obtained as outlined in Haag et al. (1990). Analyses of the polypeptide pattern by polyacrylamide gel electrophoresis (PAGE) reveal that the complex contained CP47, CP43, D1, D2, the α- and β-subunits of Cyt b559, the extrinsic 33 kDa protein, and some smaller polypeptides. All samples were frozen in small aliquots in liquid nitrogen and stored at −80 °C until use.

Plastoquinone-9 (PQ-9) was prepared according to a method described by MacMillan et al. (1995) with some modifications. Spinach chloroplasts were treated in dim green light with a 1:1 mixture of acetone and methanol. The pigments were then extracted into a hydrophobic organic phase (heptane). The PQ-9 was finally purified by high-performance liquid chromatography (HPLC). A nucleosil $100C_{18}$ column (pore size 7 μm, 16 mm × 120 mm) and a solvent mixture of methanol and tetrahydrofuran (9:1) were used. PQ-9 was dried on a vacuum line and redissolved in ethanol. Finally, it was spectroscopically identified (UV−vis and mass spectroscopy). Phenyl-*p*-benzoquinone and 2,3-dimethyl-5-decyl-*p*-benzoquinone (DMDBQ) were obtained from Sigma, and ubiquinone-45 (coenzym Q-9), designated as UQ-9 in this paper, was obtained from Fluka.

For the reconstitution of PS II membrane fragments and ISO-vesicles, the samples were diluted to a final concentration of 1 mM chlorophyll in buffer A [10 mM NaCl, 5 mM $MgCl_2$, and 50 mM MES/NaOH (pH 6.5)]. Core complexes were used at a concentration of 100 μM chlorophyll. Quinones were added to give a final concentration of 60 μM. This amount corresponds with an average quinone:PS II stoichiometry of about 15 (PS II membrane fragments and ISO-vesicles) and 30 (PS II core complexes). The quinone content was increased by a factor of 10 in some check experiments (see Results). The content of ethanol was kept below 2%. After incubation for 30 min on ice, the samples were sonicated for 15 min in an ice-cooled Brandelin Sonorex Super RK255 sonication bath. In order to eliminate unspecific effects caused by this treatment, control samples were prepared by using exactly the same procedure except with omission of the quinone. These samples are referred to as "treated control" and are used in all experiments for comparison. Test measurements revealed that the loss of oxygen evolution capacity caused by the sonication was negligibly small (see Results). Likewise, the relaxation kinetics of the flash-induced fluorescence quantum yield remained virtually unaffected (data not shown).

To check if the ISO-vesicles were still closed after sonication, the decay kinetics of the electrochromic 515 nm absorption change was measured with a conventional single beam pulse spectrophotometer as desribed in Junge and Witt (1968).

The oxygen evolution capacity was determined in all preparations before and after sonication with a Clark-type electrode as described in Renger (1972). The suspension contained the sample (50 μg of chlorophyll/mL), 10 mM NaCl, and 20 mM MES/NaOH (pH 6.5) and, in the case of PS II core complexes, 30 mM $CaCl_2$. $K_3[Fe(CN)_6]$ (1 mM) and Ph-p-BQ (200 μM) were used as acceptors for PS II membrane fragments and ISO-vesicles, and DCBQ (500 μM) for core complexes. The rates and the average yield per flash of oxygen evolution did not decrease by more than 10% in the sonicated samples.

Flash-induced O_2 oscillation patterns were measured with a modified Joliot-type electrode (Joliot, 1972) as described in Messinger and Renger (1990) and Gleiter et al. (1993). The measurements of this study were performed at an electrode temperature of 10 °C and a chlorophyll concentration of 1 mg/mL for samples of PS II membrane fragments

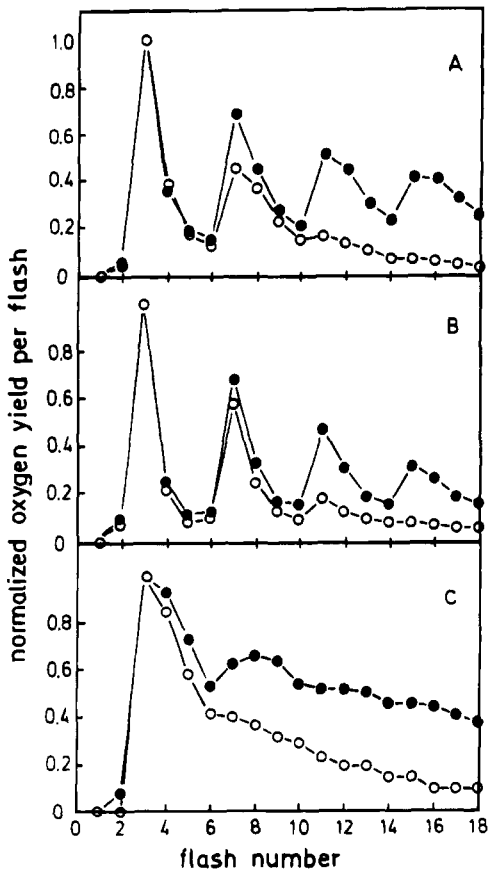

FIGURE 1: Oxygen yield per flash as a function of flash number in dark-adapted PS II membrane fragments (A), ISO-vesicles (B), and PS II core complexes (C): (O) treated control (see Materials and Methods) and (●) PQ-9-reconstituted samples.

and ISO-vesicles. Ten microliters of these suspensions was rapidly transferred to the Pt electrode and incubated for 2 min to achieve precipitation and thermal equilibration. Measurements with core particles required a sedimentation with 250 mg/mL PEG 6000 in order to cover the surface of the electrode with the resulting paste. The polarization (−650 mV) was switched on about 30 s before excitation with a train of short [full width half-maximum (FWHM) = 3 μs] saturating Xenon flashes separated by a dark time of 500 ms. The flow buffer contained 50 mM MES/NaOH (pH 6.5), 20 mM $CaCl_2$, and 10 mM $MgCl_2$ in the case of PS II membrane fragments and ISO-vesicles and 50 mM MES/NaOH (pH 6.5), 10 mM NaCl, and 20 mM $CaCl_2$ in the case of PS II core complexes.

The probabilities of misses and double hits and the S_i populations before flash excitation were determined by a least-squares fit method within the framework of an extended Kok model [see Kok et al. (1970), and for extension, see Messinger et al. (1991)].

Flash-induced changes of the fluorescence quantum yield were monitored with home-built equipment as described in Gleiter et al. (1990).

RESULTS

Oscillation Patterns of Oxygen Yield in Control and Plastoquinone-9-Treated Samples. Figure 1 shows typical oscillation patterns of the oxygen yield as a function of the flash number in dark-adapted control and PQ-9-reconstituted PS II membrane fragments (A), ISO-vesicles (B), and core particles (C). All samples reconstituted with PQ-9 revealed a markedly pronounced and prolonged period four oscillation of the flash-induced oxygen yield compared with those of the control. In order to eliminate the possibility of nonspecific effects due to sample treatment including a sonication step, the control samples were prepared under the same conditions but without PQ-9 addition (see Materials and Methods). A comparison of these controls with untreated samples reveals that no significant differences are caused by the sonication treatment without PQ-9. The traces of these control samples (open symbols) exhibit the typical features known from the literature (Messinger et al., 1993; Lübbers & Junge, 1990; Haag et al., 1992; Gleiter et al., 1993); (i) the extent of the signals fades out after two periods in PS II membrane fragments and ISO-vesicles, and (ii) the normalized oxygen yield per flash continuously declines after the third flash without pronounced oscillations in PS II core complexes.

These phenomena can be consistently explained by the limited pool size in the former type of samples, while more severe modifications of the PS II acceptor side occur in the latter type. Strikingly different oscillation patterns are observed in samples that were treated with PQ-9 (see traces with closed symbols). It has to be emphasized that the reconstitution experiments were performed at rather low quinone concentrations (15 or 30 molecules per PS II) in order to unravel possible specific effects. In the case of PS II membrane fragments, a pronounced oscillation pattern which resembles that of isolated spinach thylakoids is observed [see Messinger et al. (1993)]. The evaluation of the data within the framework of the extended Kok model [Kok et al., 1970; for extension, see Messinger et al. (1991)] leads to following values of the probabilities of misses (α) and double hits (β) and the initial (symbolized by index "0") population of the redox states S_i within the WOC before the flash train: $\alpha = 0.10$, $\beta = 0.02$, $[S_0]_0 \approx 0.07$, and $[S_1]_0 = 0.82-0.85$. A slight improvement of the fit of the experimental data including the oxygen yield of flash numbers 1−12 is obtained by introduction of an activity parameter δ [see Kebekus et al. (1995)]. The δ values are in the range of 0.98−0.99, i.e. very close to 1. This finding shows that, except for somewhat smaller β values owing to a retarded Q_A^- reoxidation [for a discussion, see Messinger et al. (1993)], the PQ-9 reconstituted PS II membrane fragments exhibit a functional pool size similar to that of thylakoids. It has to be emphasized that a satisfying fit of the oscillation pattern of PQ-9-reconstituted PS II membrane fragments within the framework of the Kok model with constant α and β values in all redox transitions required the assumption that the WOC attains with a low probability ($\approx$0.10) the redox state S_{-1}. Although a priori the existence of S_{-1} cannot be excluded, it seems more likely that this is due to using the simplifiying Kok model as a basis of the fit procedure. The latter idea is supported by the finding that the assumption of a minor initial S_{-1} population also improves in some cases the fit of the oscillation pattern of thylakoids (J. Messinger and G. Renger, unpublished results). A consideration of possible redox equilibria at both the donor and acceptor side led to the conclusion that the α values should depend on the flash number [see Renger and Hanssum (1988)]. An extension and detailed analysis of this idea has shown that this can be expected to be the case [see Shinkarev & Wraight

(1993)]. If one takes into account this effect, the small initial S_{-1} population can be eliminated (data not shown). The apparent small initial $[S_0]_0$ population can be ascribed to the fast S_2/S_3 reduction by Y_D (Babcock & Sauer, 1973; Velthuys & Visser, 1975; Vermaas et al., 1984; Messinger & Renger, 1993).

At first glance, the traces of Figure 1B might suggest that an analogous PQ-9 reconstitution is achieved in ISO-vesicles. However, a closer inspection shows that after two quaternary periods the oxygen yield normalized to the third flash decreases significantly. This effect bears some difficulties in evaluation of the oscillation pattern within the framework of the Kok model. If one takes into account this decrease by an additional parameter δ as described in Kebekus et al. (1995), the evaluation of the data leads to Kok parameters very similar to those for PQ-9-reconstituted PS II membrane fragments, except for slightly lower α and higher β values (data not shown). The former effect is probably due to the shift of the redox equilibrium $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ toward the right side (thus giving rise to similar values as in thylakoids), and the latter originates from faster Q_A^- reoxidation in ISO-vesicles [see Messinger et al. (1993)]. These differences between PS II membrane fragments and ISO-vesicles are ascribed to modifications of the Q_B site caused by the Triton X-100 treatment of the former sample type.

Using the same fit procedure for the description of the oscillation pattern measured in PQ-9-reconstituted PS II core complexes results in markedly different Kok parameters compared with those in PS II membrane fragments and ISO-vesicles; the α values are almost doubled ($\alpha \approx 0.23$), probably due to a shift of the redox equilibria at the acceptor side toward Q_A^-. Alternatively, the possibility has to be considered that a significant fraction of Q_A^- could not be reoxidized during the dark time between the flashes due to a markedly disturbed Q_B site. A variation of the flash frequency can be performed only in a limited range because of the accelerated decay of S_2 and S_3 in PS II core complexes (Gleiter et al., 1993; van Leuween et al., 1993). Therefore, the contribution of this effect to the α values could not be unambiguously clarified. The values of β remain small as expected (vide supra). With respect to the dark population of the redox states of the WOC, a significant increase of $[S_{-1}]_0$ (up to 0.25) and $[S_0]_0$ (to about 0.20) is obtained concomitant with a decrease of $[S_1]_0$ (0.55−0.60). Although a direct proof is lacking, it seems most likely that the comparatively high population of $[S_{-1}]_0$ and $[S_0]_0$ is owed to a less tight shielding of the donor side toward reductive decay of oxidizing equivalents in PS II core complexes (Gleiter et al., 1993; van Leeuwen et al., 1993).

Reconstitution Conditions and Efficiency of Other Quinones. The data reported so far have clearly shown that a significant reconstitution of a functionally competent endogenous pool can be achieved with PQ-9 as quinone component. Now questions arise as to the mode of incorporation and the binding strength of PQ-9. Figure 2 compares the oscillation patterns measured in untreated PS II membrane fragments and in samples incubated in the dark (30 min) with PQ-9 on ice without and with an additional reconstitution step comprising a 15 min sonication (see Materials and Methods) and in a PQ-9-reconstituted sample after an additional washing procedure. The results obtained reveal that incubation with PQ-9 alone is not sufficient to restore the pool capacity. A proper incorporation of PQ-9 into the membrane fragments requires a sonication treatment. After this reconstitution step, the PQ-9 is rather tightly bound and is not markedly affected by a subsequent washing with buffer solution. Generally, the same features were observed for ISO-vesicles and PS II core complexes (data not shown).

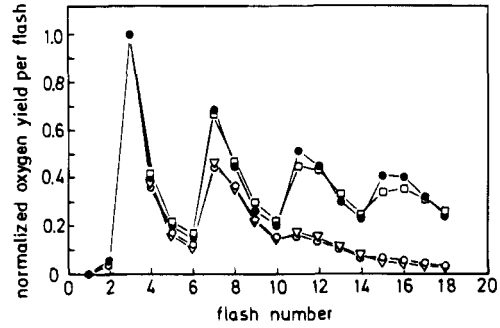

FIGURE 2: Oxygen yield per flash as a function of flash number in dark-adapted PS II membrane fragments: (○) treated control (see Materials and Methods), (●) PQ-9-reconstituted samples, (▽) incubation with PQ-9 without the sonication step, (□) PQ-9-reconstituted samples washed with buffer solution before the measurement.

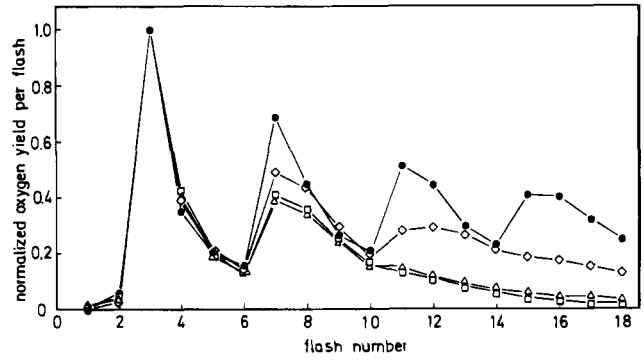

FIGURE 3: Oxygen yield per flash as a function of flash number in dark adapted PS II membrane fragments. Reconstitution with PQ-9 (●), DMDBQ (◇), UQ-9 (△), and Ph-p-BQ (□).

It has to be emphasized that the sonication step does not affect the average oxygen yield per flash as a measure of the number of intact WOCs (see Materials and Methods). This procedure is really rather mild because even the membrane permeability of ISO-vesicles is not markedly changed as shown by an almost invariant relaxation kinetics (half-life time on the order of 100 ms) of the flash-induced 515 nm absorption change (data not shown) that reflects the transmembrane electric potential difference owing to a delocalized electrochromic effect (Junge & Witt, 1968; Emrich et al., 1969). Therefore, the ISO-vesicles used in this study contain an inner aqueous phase that is separated from the outer phase by a membrane barrier.

After it was unambiguously shown that a successful reconstitution can be achieved with PQ-9, the question arises if there exists a specificity for the type of quinone used. In order to address this problem, the following compounds were selected: phenyl-*p*-benzoquinone (Ph-p-BQ) as a highly lipophilic species, 2,3-dimethyl-5-decyl-*p*-benzoquinone (DMDBQ) as a compound with the same head group as PQ-9 but a shortened hydrophobic tail, and UQ-9 which has the same tail but a different head group. Figure 3 shows oscillation patterns of the flash-induced oxygen yield measured in samples that were treated with the same reconstitution procedure described for PQ-9 but using the above-

mentioned quinones instead of PQ-9. Surprisingly, marked differences were observed. None of the tested quinones were as efficient as PQ-9. The lipophilic Ph-p-BQ was virtually without any effect under the reconstitution conditions at a ratio of 15 molecules per PS II. Unexpectedly, UQ-9 could also not restore the plastoquinone pool activity. On the other hand, a partial effect was obtained with DMDBQ. These findings provide convincing evidence for a comparatively high structural selectivity and readily explain our previous failure to achieve a significant increase of the pool size by using quinones other than PQ-9 (Renger et al., 1989). If one considers the properties of the different quinones studied, the present results suggest that the structure of the head group seems to be more important than the length of the hydrophobic tail and/or the general lipophilicity of the compound (see Discussion).

Flash-Induced Changes of the Normalized Fluorescence Yield in Control and PQ-9-Treated Samples. In intact thylakoids, the reoxidation of Q_A^- is dominated by the electron transfer to Q_B or Q_B^- in the Q_B site. The kinetics of these reactions are in the time domain of a few hundred microseconds (Robinson & Crofts, 1983; Weiss & Renger, 1984a). A minor component with markedly slower kinetics is ascribed to diffusion-limited processes, i.e. Q_A^- reoxidation by a plastoquinone molecule that migrates to an empty Q_B site. In PS II membrane fragments, these kinetics are somehow retarded, and in PS II core complexes, they are significantly slowed down [for recent comparative studies, see Haag et al. (1992) Gleiter et al. (1993)]. It therefore appeared that analyzing the effect of a PQ-9 reconstitution on the overall kinetics of Q_A^- reoxidation would be worthwhile. The time course of this process was determined by measuring time-resolved transient fluorescence yield changes induced by a laser flash. The samples were excited by a train of laser flashes (FWHM = 3 ns, λ = 532 nm) at a frequency of 2 Hz, and the flash-induced time dependent yield of chlorophyll fluorescence was monitored in the time range of 500 ms as the emission caused by LED pulses of varying frequencies, as described in Haag et al. (1992). The results obtained in the 10 ms range after the first and second actinic laser flash are summarized in Figure 4. A qualitative inspection of the data reveals that in ISO-vesicles the decay of the transient fluorescence yield induced by the first and second flash is dominated by fast kinetics in the millisecond range. These kinetics are less pronounced in PS II membrane fragments and comprise only a minor fraction in PS II core complexes. The normalized extent of the fast kinetics reflects the integrity of the Q_B site in terms of plastoquinone binding. Therefore, the data of Figure 4 reveal that the Q_B site is mostly intact in ISO-vesicles that were prepared without any detergent treatment step [for a more detailed discussion of Triton X-100 effects on the Q_B site, see Messinger et al. (1993) and references therein] and strongly modified in PS II core complexes. In the PQ-9-reconstituted samples, the extent of the fast decay seems to be slightly diminished except for the relaxation after the first flash in PS II core complexes.

In order to illustrate the increase of the endogenous PQ pool, Figure 5 shows the traces of the transient fluorescence yield induced by the first, second, twelfth, and fourty-fifth flash of the trains of saturating laser flashes measured in PS II membrane fragments. In the treated control (top part),

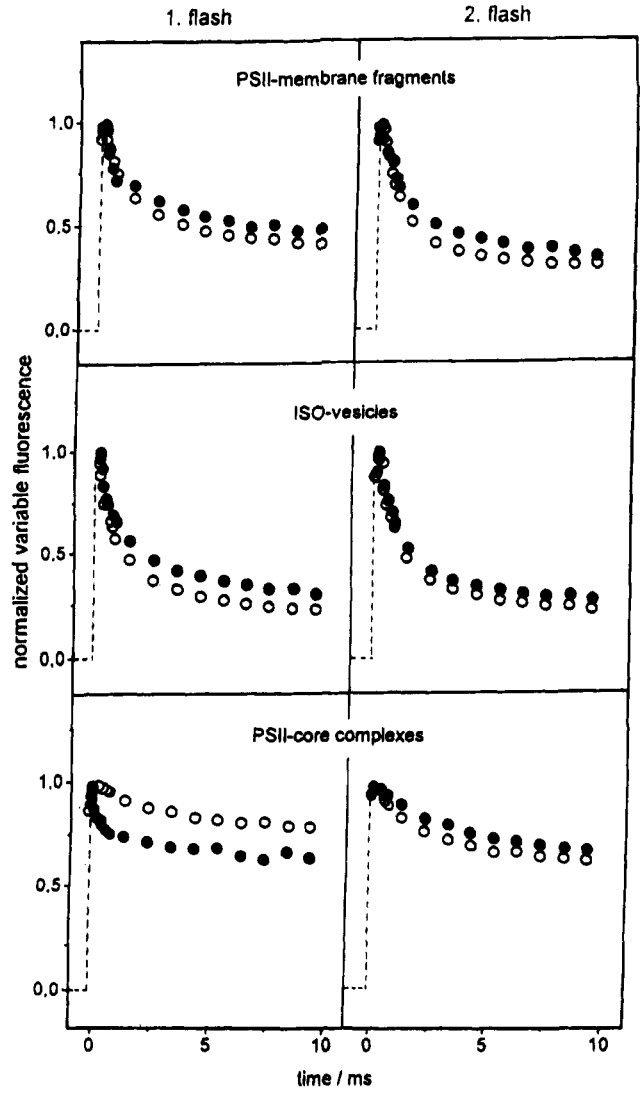

FIGURE 4: Time course of the relative fluorescence quantum yield induced by the first and second flash of a train of laser flashes in dark-adapted PS II membrane fragments, ISO-vesicles, and PS II core complexes: (○) treated control (see Materials and Methods) and (●) PQ-9-reconstituted samples. Dark time between the first and second flash is 500 ms (other experimental conditions, see Materials and Methods).

the fluorescence yield induced by the twelfth flash does not relax in the 10 ms time domain because the endogenous PQ pool is exhausted and Q_A^- has to be reoxidized via the much slower reactions with the PS II donor side. The same feature is observed after the forty-fifth flash. A strikingly different pattern arises in PQ-9-reconstituted PS II membrane fragments. In this case, the relaxation kinetics of the fluorescence yield after the twelfth flash are virtually the same as after the first flash, while after the forty-fifth flash, practically no decay is observed as in the treated control.

A quantitative evaluation of the results was performed on the basis of the following equation for the nonlinear relationship between normalized fluorescence quantum yield and population of state Q_A^- (Joliot & Joliot, 1964):

$$F_{var,rel}(t) = b[Q_A^-(t)] + (1 - b)\frac{(1 - p)[Q_A^-(t)]}{1 - p[Q_A^-(t)]} \quad (1)$$

where $F_{var,rel}(t)$ is the variable fluorescence at time t normal-

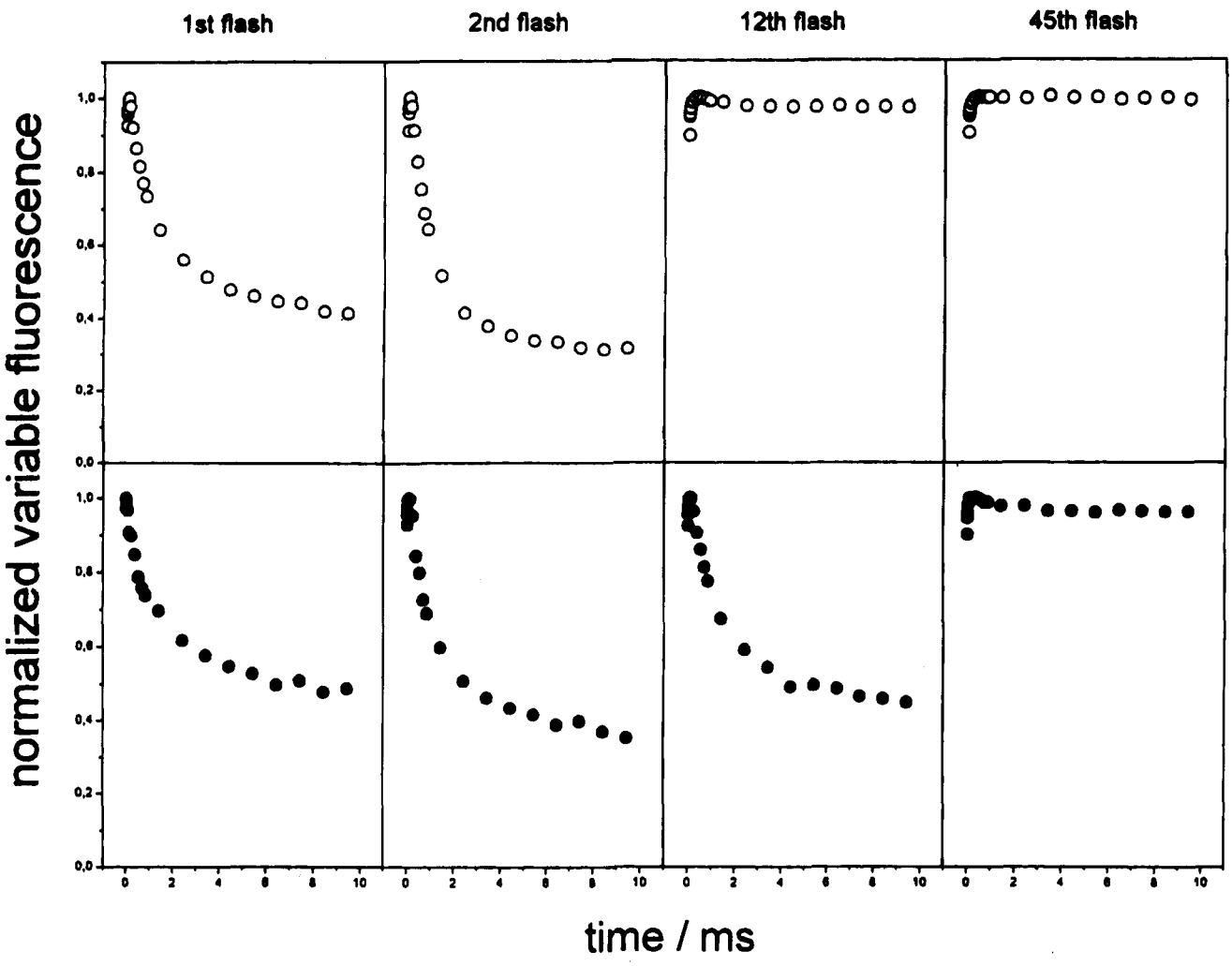

FIGURE 5: Time course of the relative fluorescence quantum yield induced by first, second, twelfth, and forty-fifth flash of a train of saturating laser flashes in dark-adapted PS II membrane fragements: (○) treated control (see Materials and Methods) and (●) PQ-9-reconstituted samples. Dark time between the first and second flash is 500 ms (other experimental conditions, see Materials and Methods).

ized to its extrapolated maximum level ($F_{var,max} = F_{max} - F_0$) and $1 - b$ = fraction of PS II units (PSU) which are connected via inter-PS II excitation energy transfer with probability p. The possibility of a dimeric structure of PS II as discussed by Boekema et al. (1994) has not been explicitly considered in eq 1. In the case of PS II membrane fragments and ISO-vesicles, values of $b = 0.3$ and $p = 0.5$ were used for the fit, while for PS II core complexes, $b = 1$ and $p = 0$ are most reliable [see Gleiter et al. (1993)]. The normalized time dependent Q_A^- populations, $[Q_A^-(t)]$, exhibit a multiphasic decay kinetics that can be described by the general formula

$$[Q_A^-(t)] = \sum_{i=1}^{3} a_i \exp(-t/\tau_i) + a(t = 500 \text{ ms}) \qquad (2)$$

The lifetimes τ_i, amplitudes a_i, and the fraction of $[Q_A^-(t)]$ remaining 500 ms after the actinic flash, $a(t = 500$ ms), gathered from a numerical best fit analysis of the experimental results by using eqs 1 and 2 are summarized in Table 1.

The fast component (normalized amplitude a_1, lifetime τ_1) of the overall $[Q_A^-(t)]$ decay is owed to electron transfer from Q_A^- to a Q_B site occupied with Q_B or Q_B^-. As expected, the extent of this reaction has its largest values in ISO-vesicles with its virtually intact Q_B site (due to omission of detergent treatment) and its lowest values in PS II core complexes where the Q_B site is severely modified (Gleiter et al., 1993). Likewise, in all samples, the a_1 values are higher after the second flash. This is understandable because Q_B^- is more stably bound than Q_B, and after dark incubation, most of the Q_B attains the oxidized quinone state [for a discussion, see Rutherford et al. (1984)]. Therefore, the probability of an occupied Q_B site is higher after the second flash. The amplitudes a_1 are expected to increase upon PQ-9 reconstitution as a consequence of an increased occupation probability. This anticipated phenomenon is observed in PS II core complexes. However, surprisingly, an opposite effect was found in PS II membrane fragments and ISO-vesicles. At present, no convincing explanation can be offered for this latter finding. The Q_A^- reoxidation kinetics are not markedly changed by PQ-9 reconstitution in PS II membrane fragments and ISO-vesicles. On the other hand, these kinetics become accelerated by 1 order of magnitude in dark-adapted PS II core complexes (see traces of the first flash). In this case, the kinetics are even faster than in intact thylakoids [for a recent study, see Renger et al. (1995)].

The kinetics with τ_3 values of a few hundreds of milliseconds are ascribed to recombination reactions of Q_A^- with the PS II donor side. In PS II deprived of its oxygen

Table 1: Time Constants of Q_A^- Reoxidation, Fraction of Q_A Remaining Reduced after 500 ms in Dark-Adapted Treated Control (See Materials and Methods), Symbolized by tc and PQ-9-Reconstituted PS II Membrane Fragments (PQ-9 PS II mf), ISO-Vesicles (PQ-9 ISO), and PS II Core Complexes (PQ-9 PS II core) after the First (Top Part) and Second Laser Flash (Bottom Part)

	relaxation kinetics						
	fast		middle		slow		"terminal"
sample	a_1	τ_1 (ms)	a_2	τ_2 (ms)	a_3	τ_3 (ms)	a (t = 500 ms)
First Flash							
PS II mf (tc)	0.34	1.10	0.22	10	0.19	100	0.25
PQ-9 PS II mf	0.21	0.61	0.28	7.2	0.13	95	0.39
ISO-vesicles (tc)	0.46	0.84	0.28	5.2	0.09	100	0.17
PQ-9 ISO-vesicles	0.33	0.79	0.32	6.4	0.10	120	0.25
PS II core (tc)	0.10	1.41	0.15	8.6	0.27	110	0.48
PS-9 PS II core	0.22	0.11	0.19	6.2	0.17	135	0.42
Second Flash							
PS II mf (tc)	0.56	1.2	0.11	17.0	0.09	130	0.25
PQ-9 PS II mf	0.38	1.0	0.20	5.5	0.08	90	0.34
ISO-vesicles (tc)	0.61	1.3	0.11	10	0.09	90	0.19
PQ-9 ISO-vesicles	0.56	1.4	0.11	9.5	0.10	105	0.23
PS II core (tc)	0.31	2.2	0.09	11.5	0.18	125	0.43
PS-9 PS II core	0.27	3.2	0.11	15	0.20	110	0.42

evolution capacity, this reaction is characterized by $t_{1/2}$ values of about 60−100 ms (Weiss & Renger, 1984b), while reoxidation of Q_A^- with redox states S_2 and S_3 of an intact WOC takes place with half-life times of 1−2 s (Bennoun, 1970; Renger & Weiss, 1982). Therefore, it seems reasonable to assume that a_3 is a rough measure of centers that are impaired at both the acceptor (Q_A^- reoxidation by Q_B) and the donor side (WOC). The present data do not permit a more detailed analysis (it is not the aim of this study to analyze these reactions). At room temperature, the level of Q_A^- staying reduced at a sufficiently long dark time after the flash (a few seconds) reflects the equilibrium constant K_{eq} of the reaction $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$ provided that Q_B is fully oxidized before the actinic flash and all Q_B sites are at least transiently occupied during the period of signal monitoring. Furthermore, recombination reactions with the donor side have to be eliminated. These conditions are not satisfied in the experiments presented here, and therefore, possible effects on K_{eq} will not be discussed. Regardless of this particular point, the above-mentioned assignment of the different components of the $[Q_A^-]$ decay reveals that the sum of the normalized amplitudes a_1 and a_2 over the whole train of actinic laser flashes provides a measure of the pool size. Accordingly, in the absence of exogenous acceptors, the sum $a_1 + a_2$ should drop down to values close to 0 during excitation with a train of actinic laser pulses. Figure 6 shows the results obtained in PS II membrane fragments reconstituted with different types of quinones. In control and Ph-p-BQ-treated samples, the extent of $a_1 + a_2$ sharply declines after the fourth flash and approaches values very close to 0 after the tenth flash. This finding is in perfect agreement with measurements of the oxygen yield oscillation pattern (see Figure 3), indicating that Ph-p-BQ is totally inefficient in reconstituting a functional quinone pool at a ratio of 15 molecules per PS II in the reconstitution assay (see Discussion). In contrast, a markedly different pattern is observed in PQ-9-reconstituted PS II membrane fragments (open circles). In this case, the sum $a_1 + a_2$ remains virtually constant up to the fourteenth flash and then exhibits a very similar sharp decline as is observed in the control at a much smaller flash number.

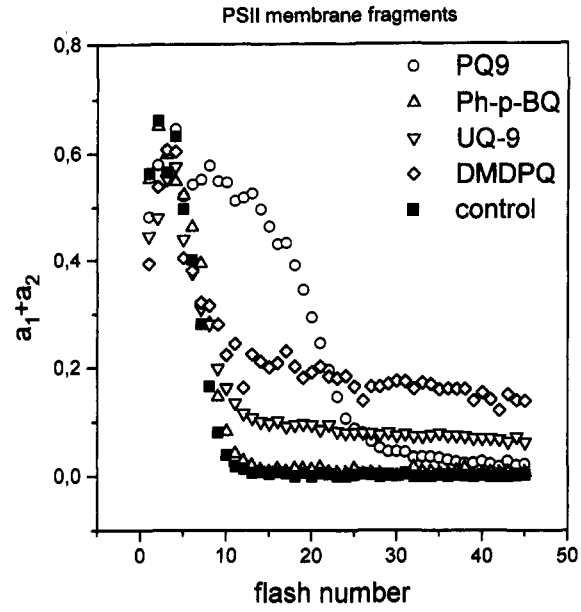

FIGURE 6: Sum of the normalized amplitudes $a_1 + a_2$ of the $[Q_A^-]$ decay as a function of flash number in treated control (■) (see Materials and Methods) and samples reconstituted with PQ-9 (○), Ph-p-BQ (△), UQ-9 (▽), and DMDBQ (◇). Dark time between the flashes 500 ms (other experimental conditions, see Materials and Methods).

On the basis of these data, the "natural" average pool size per PS II of the membrane fragments used in this study is estimated to be on the order of 2−3 PQ molecules. This capacity can be significantly increased by a suitable reconstitution procedure using PQ-9. A typical value of about 14 electrons obtained for the total pool capacity indicates that 4−5 PQ-9 molecules per PS II can be specifically inserted into the membrane fragements. Similar values were obtained for ISO-vesicles.

In contrast to the clear all- or nontype reconstitution with PQ-9 and Ph-p-Q, respectively, the quinones UQ-9 and DMDBQ give rise to mixed-type features. At first, the steep decline starts at the same flash number as that of the control and Ph-p-BQ-reconstituted sample. However, the value of $a_1 + a_2$ does not reach the 0 level even after 45 flashes. Two different types of effects can be considered to cause this phenomenon: (a) a partial reoxidation of the reduced quinones during the dark time between the flashes and/or (b) a sample heterogeneity, i.e. a smaller fraction of PS II that can be afforded with a rather large pool and a major fraction that is unable to use these quinones. In order to check for the possibility of O_2 acting as oxidant for reduced quinone, fluorescence measurements were performed in DMDBQ-reconstituted PS II membrane fragments under strictly anaerobic conditions achieved by the enzymatic assay with glucose, glucose oxidase, and catalase. These measurements led to virtually the same result as seen under aerobic conditions; i.e. O_2 is not the species that could support the presumed quinone regeneration. This conclusion is supported by a similar dependence on the quinone type for restoring the oxygen yield oscillation pattern (compare Figures 3 and 6) because the partial pressure of O_2 is vanishingly small at the surface of the Joliot-type electrode. Although the existence of another oxidant cannot be entirely excluded, it seems more likely that the binding of DMDBQ

and to a minor extent of UQ-9 is heterogeneous. Further experiments are required to clarify this particular point.

DISCUSSION

In general, four different types of PQ-9 functions are discernible in the linear electron transport pathway from H_2O to $NADP^+$ in all oxygen-evolving photosynthetic organisms. The PQ-9 reactions take place at particular binding sites. (a) PQ-9 noncovalently bound to a site located mainly in polypeptide D2 and referred to as Q_A is the electron acceptor for rapid reoxidation of $Pheo^-$ that is indispensable for stabilization of the primary charge separation in PS II [for a review, see Renger (1992)]. (b) A PQ-9 molecule transiently bound to a specific pocket in polypeptide D1 (Q_B site) becomes reduced to plastoquinol (PQH_2) via a sequence of two univalent redox steps driven by Q_A^- as reductant [for a review, see Crofts and Wraight (1983)]. (c) The PQ-9 pool consisting of mobile PQ-9 molecules, which are inserted into the thylakoid membrane, provides the functional link between PS II and the cytochrome b_6/f complex, probably in a diffusion-controlled manner (Joliot et al., 1992; Lavergne et al., 1992; Blackwell et al., 1993). (d) PQH_2 formed at PS II is transferred to the cytochrome b_6/f complex and bound at specific site(s) where it acts as an electron donor for the ultimate reduction of plastocyanine. The oxidized PQ-9 is released from this complex and can return as an electron acceptor to the Q_B site of PS II [for a review, see Cramer et al. (1991)]. Reactions of the cytochrome b_6/f complex will not be discussed in this report.

PQ-9 in the Binding Sites of Q_A and Q_B in PS II. The binding sites of Q_A and Q_B are assumed to exhibit striking structural similarities with the well-characterized corresponding sites of ubi(mena)quinone in anoxygenic purple bacteria (Trebst, 1986; Michel & Deisenhofer, 1988). In spite of this general homology, some differences are expected to exist with respect to certain properties because different types of quinones are used as functional components in PS II and purple bacteria. Other differences are probably due to particular structural features of the protein matrix and the lack of the H-type subunit in PS II [for a discussion, see Trebst (1991)]. According to their quite different functions, Q_A and Q_B are characterized by distinct properties; Q_A is firmly bound and normally acts only as a univalent redox couple comprising the quinone and semiquinone form (Witt, 1973). On the other hand, Q_B is only transiently bound and becomes released after its two-step reduction to the quinol form [for review, see Crofts and Wraight (1983)].

In reaction centers of the anoxygenic purple bacterium *Rhodobacter sphaeroides*, Q_A (UQ-10) can be replaced by a large variety of synthetic quinones [for a review, see Gunner and Dutton (1989)]. In marked contrast, in PS II, only UQ-9 was found to be able to partially restore the function of the native PQ-9 in Q_A while other quinones are inactive (Diner et al., 1988). The Q_A site of the samples used in the present study remained intact as shown by the virtually unaffected capacity of stable charge separation which is the prerequisite of oxygen evolution (Renger & Eckert, 1980). Therefore, the quinone effects reported in this study do not originate from modified properties of Q_A.

With respect to the Q_B site, it is important to note that this pocket is not highly specific for binding of only the native PQ-9 but it is able to interact with several exogenous electron acceptors, including different quinones, and to bind a large number of compounds that prevent Q_A^- reoxidation, thereby acting as powerful herbicides [for a review, see Oettmeier (1992)]. The affinity of benzoquinones for the Q_B site has been previously analyzed in thylakoids (Soll & Oettmeier, 1984) and PS II preparations from the thermophilic cyanobacterium *Synechococcus elongatus* (Satoh et al., 1992). Both studies revealed that the efficiency of acting as an electron acceptor depends on several parameters like redox potential, steric factor(s), and hydrophobic properties of the exogenous quinones. The results presented here confirm our previous conclusion (Haag et al., 1992; Gleiter et al., 1993) that the Q_B site is severely modified in PS II core complexes but the reoxidation of Q_A^- by exogenous acceptors is not severely impaired as reflected by high rates of oxygen evolution under saturating light (Haag et al., 1990). The specific reconstitution procedure does not restore the properties of a native Q_B site in PS II core complexes, as is shown by a comparison of their relaxation kinetics with those of ISO-vesicles (see Figure 4). Therefore, the Q_B site seems not to be of central relevance for the formation of an enlarged PQ-9 pool (vide infra).

Endogenous PQ Pool and Its Reconstitution in PS II Membrane Fragments, ISO-Vesicles, and PS II Core Complexes. Values between 7 and 40 were reported for the average number of mobile PQ-9 molecules per electron transport chain in cyanobacterial cells and chloroplasts [for a review, see Hauska and Hurt (1982)]. On the basis of measurements of fluorescence induction curves in the absence and presence of DCMU, typical values of 5−7 PQ-9 molecules per PS II are obtained for the size of a functional pool in isolated spinach thylakoids [see Renger and Schulze (1985) and references therein]. In PS II membrane fragments, ISO-vesicles, and PS II core complexes, the average number of functionally active endogenous plastoquinone molecules per PS II is drastically reduced compared with those of isolated thylakoids. This is illustrated by (i) the marked decrease of the oxygen yield in dark-adapted samples illuminated with a train of single turnover flashes (see Figure 1) and (ii) the sharp decline of the extent of fast Q_A^- reoxidation after a few flashes monitored via transient fluorescence quantum yield changes (see Figures 5 and 6). The origin of the diminished pool size in PS II membrane fragments and ISO-vesicles is not yet clarified. It is obviously not primarily caused by the detergent (Triton X-100) treatment because the isolation of ISO-vesicles does not comprise such a step. Likewise, the loss of cytochrome b_6/f complexes cannot account for the extent of the decrease of pool capacity. One rational explanation could be offered by the assumption that the lateral distribution in the thylakoid membrane is different for PS II complexes and PQ-9 molecules so that in the isolated grana membrane fraction the PQ-9/PS II ratio is smaller than in broken chloroplasts. Further detailed investigations are required to prove this idea (this particular problem is beyond the scope of the topic of the present study). Regardless of the underlying mechanism, the diminished PQ pool size bears some shortcomings of these samples, e.g. if measurements have to be performed in the absence of exogenous electron acceptors. Therefore, the central aim of this study was the attempt to "load" PS II membrane fragments, ISO-vesicles, and PS II core complexes with an endogenous quinone pool of significant size and the

capacity to act as an efficient acceptor for Q_A^- reoxidation. To our knowledge, systematic studies on this problem are lacking.

The results obtained here provide clear evidence that the size of an endogenous and functionally competent quinone pool can be significantly increased in PS II membrane fragments, ISO-vesicles, and PS II core complexes by application of a special reconstitution procedure that comprises a suitable sonication step and the use of natural PQ-9 as a quinone component. Surprisingly, the extent of pool reconstitution strongly depends on the nature of the quinone-type molecule. Experiments with trypsin-treated thylakoids (Renger, 1976) indicate that the reoxidation of Q_A^- by quinones requires an intact Q_B site. On the basis of this conclusion, the striking specificity can be explained by two generally different mechanisms or a combination of both. (a) All lipophilic quinones are nonspecifically associated with the lipid phase of the membrane and/or the detergent belt of PS II core complexes so that the specific efficiency of a particular quinone in restoring a functional competent pool is dominated by its binding and turnover at the Q_B site. (b) The quinones associated with the different PS II preparations by the reconstitution assay are all able to interact with the Q_B site and to mediate an efficient Q_A^- reoxidation, but the number of exchangeable quinone molecules that can be additionally inserted strongly depends on specific structural determinants.

The former type of mechanism is assumed to be responsible for the efficiency of tailed naphthoquinones in establishing a pool capacity in proteoliposomes containing isolated reaction centers of the anoxygenic purple bacterium *R. sphaeroides* (Moser et al., 1987). It is therefore tempting to ascribe the high structural specificity of quinone pool reconstitution in PS II entirely to the properties of the Q_B site. However, for the following reasons, it seems necessary to consider also the second mechanism; most of the quinones analyzed are potential PS II electron acceptors that mediate a high rate of oxygen evolution if added at sufficient concentration, and it is not easy to understand why these species should not be able to turn over rapidly when bound to PS II preparations. In this case, a question as to the localization of these additional PQ-9 molecules arises . In general, either the lipid phase or specific binding sites at proteins are potential candidates. In the former case, the lipophilicity of the quinones is expected to be of central relevance, while in the latter case, more sophisticated structural effects could be the dominating factor. The idea of a more specific interaction of PQ-9 pool molecules with proteins was previously suggested on the basis of fluorescence measurements in trypsin-treated ISO-vesicles (Völker & Renger, 1984). The data of the present study seem to be in favor with this proposal for two reasons. (i) The total lipid content per P680 was recently reported to be 150 and 10 in PS II membrane fragments and PS II core complexes, respectively (Murata et al., 1990). Therefore, in the latter sample type, only 2 (3) lipids per PQ-9 are present at a total pool size of 5 (3) molecules. This ratio is hardly reconcilable with models that consider the PQ pool to be an intercalation of PQ-9 into the lipid bilayer fraction of the thylakoid membrane according to the rules of a simple two-phase Nernst distribution (but this does not exclude the possibilty of quinone binding to a phase of detergent molecules). (ii)

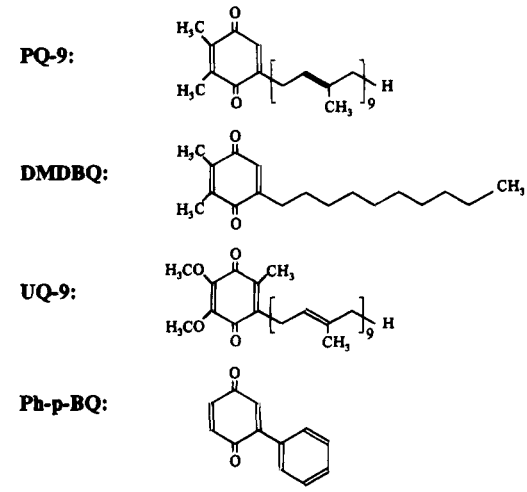

FIGURE 7: Chemical structures of PQ-9, DMDBQ, UQ-9, and Ph-p-BQ.

PS II membrane fragments (and also ISO-vesicles) contain a large part of the grana thylakoid membrane and thus much more lipids per reconstituted PQ-9 (about 30, vide supra). Therefore, in this case, the insertion of PQ-9 into a lipid phase would be possible. However, this appears not to be the dominating feature because the extent of reconstitution of a functionally competent endogenous quinone pool in PS II membrane fragments was found to exhibit a high degree of specificity; i.e. at a molecular ratio of 15 quinone molecules per PS II in the reconstitution assay, all compounds other than PQ-9 are much less efficient or even without any effect. This idea is in line with previous failures to enhance the acceptor pool capacity with *p*-benzoquinone derivatives (Renger et al., 1989). Except for PQ-9, under the reconstitution conditions used here, the best results were obtained with DMDBQ, whereas UQ-9 gave rise to only marginal effects and Ph-p-BQ was totally inefficient. A small effect of Ph-p-BQ was observed (data not shown) at a 10-fold concentration (150:1) resembling that of DMDBQ at the usually applied 15:1 ratio (vide supra).

The structures of the quinones used in this study are shown in Figure 7. Two interesting conclusions can be gathered from a comparison of the structural properties with the efficiency of these compounds to restore a functionally competent endogenous quinone pool. (i) The structure of the redox active head group is much more important than that of the long side chain. (ii) The general lipophilicity of the quinones seems to be of minor relevance. Therefore, more specific sites located at proteins appear to be the most likely candidates for binding of PQ-9 molecules that form the endogenous pool. At first glance, the Q_B site appears to be an attractive candidate, but at most, one quinone can be bound [for a review, see Crofts and Wraight (1983)]. The fluorescence data reveal that the occupancy probability of this site is already significant in the treated control (see Materials and Methods) as reflected by the magnitude of a_1, i.e. at most less than one additional PQ-9 could be bound. Surprisingly, the a_1 values are even slightly smaller in PQ-9-reconstituted PS II membrane fragments and ISO-vesicles than in the control; i.e. the Q_B site is not involved in binding of the additional PQ-9 inserted by the reconstitution procedure. Therefore, additional binding domains are responsible for the specific association of these PQ-9 molecules. In the case of a possible association of PQ-9 to proteins, several

candidates have to be considered because PS II membrane fragments and ISO-vesicles contain 20−30 polypeptides [for a review, see Vermaas et al. (1993)]. Among them, CP47 appears to be a potential candidate because it was shown to contain a binding domain for PQ-9 (Satoh et al., 1986). It remains to be clarified whether CP47 plays an essential role in binding of PQ-9 molecules of the functionally competent endogenous pool. The possible role of other proteins of PS II remains to be analyzed.

The idea of specific binding areas of PQ-9 has another interesting implication. It is known that several PS II complexes share a common PQ pool in thylakoids (Siggel et al., 1972). An analogous feature was observed in PS II membrane fragments (Renger et al., 1989). This phenomenon was ascribed to a "lake" of PQ molecules that can react with a large number of PS II complexes and permit a diffusion-limited functional connection with the cytochrome b_6/f complex [see Haehnel (1984) and Cramer et al. (1991)]. Recently, this view was modified by showing that a clustering of PS II and cytochrome b_6/f complexes is a better model for understanding the electron transfer properties (Lavergne et al., 1991, 1992; Joliot et al., 1992). Therefore, it seems reasonable to assume that the interaction via a common PQ pool probably comprises a contact of different PS II complexes. This idea is consistent with the suggestion of this study on a possible binding of PQ-9 to proteins. It has to be emphasized that in this case the PQ-9 molecules attached to the proposed proteinacous binding domains have to be rapidly exchangeable and much less tightly bound than that at the Q_B site in order to establish an efficient "delocalized" pool. If this condition is not satisfied for other quinones, a functionally competent endogenous pool cannot be formed at low concentrations of these species. Further detailed studies are required to unravel the interaction of PQ-9 molecules with proteins and lipids of the thylakoid membrane.

Regardless of this mechanistically important problem, the reconstitution of the PQ pool in PS II membrane fragments and ISO-vesicles is of practical relevance because it provides a most suitable material for study of the oscillation pattern of the WOC (O_2 evolution, protolytic reactions, S_i state transitions) in the absence of exogenous electron acceptors. This will be outlined in a forthcoming paper.

ACKNOWLEDGMENT

The authors thank Drs. K.-D. Irrgang, F. MacMillan, and J. Messinger for valuable discussions and critical reading of the manuscript, Dr. D. Schröder for mass spectroscopic identification of PQ-9, and S. Hohm-Veit for drawing the figures. The invaluable technical assistance by B. Lange is gratefully acknowledged.

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BI9510338