Quantitation of plastoquinone-9 in photosystem II reaction center particles

Chemical identification of the primary quinone, electron acceptor Q_A

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Quantitative analysis of PQ-9 was performed on photosystem II reaction center particles isolated from *Chlamydomonas reinhardtii*. These particles show light-driven electron transfer from secondary donor Z to primary quinone acceptor Q_A. If both Z and Q_A were PQ-9, we would expect to find 2 PQ-9/RC. Quantitative determinations indicate an average of 1.16 ± 0.14 PQ-9/RC. As Q_A is not covalently bound to the RC (shown by Q_A reversible extraction and reconstitution) and as Q_A is known through optical spectroscopy to be a plastoquinone, we attribute the PQ-9 to Q_A and conclude that Z is not a PQH_2-9.

Photosystem II *Plastoquinone-9* *Primary acceptor* *Secondary donor* *HPLC* *TLC*

1. INTRODUCTION

The photosystem II reaction center has a secondary donor, Z, responsible in its oxidized form (Z^+) for an EPR signal called 'signal II vf' (very fast) in oxygen-evolving thylakoid membranes and 'signal II f' (fast) when the oxygen-evolving site is inactivated [1].

There is at the moment some controversy about the chemical nature of Z. Strong EPR evidence for a plastoquinol was obtained by extraction and reconstitution of deuterated plastoquinol [2]. The radical formed upon oxidation of Z has been proposed to be a plastochromanoxyl radical [2], a plastosemiquinone anion [3] and more recently a plastosemiquinone cation ($QH_2^{\cdot+}$). Evidence for a semiquinone cation is based on the highly positive midpoint potential of the redox couple $QH_2^{\cdot+}/QH_2$ (e.g. E_o ($QH_2^{\cdot+}/QH_2$) > 920 mV for the trimethyl benzoquinol) [4]; on the hyperfine structure of the EPR spectrum of signal II [5] and its stimulation using model compounds; and on the optical difference spectrum Z^+–Z in the UV and blue [6,7].

Neither the EPR nor the optical spectrum is sufficient to unambigiously identify the chemical nature of Z^+. It is for this reason that we have resorted to the chemical means.

Here we describe quantitative measurements of the PQ-9 content of active PS II particles isolated from *Chlamydomonas reinhardtii*. These PS II particles contain an abbreviated electron transfer chain with Z as terminal donor and the primary quinone Q_A as terminal acceptor. Acceptor Q_B is completely absent according to functional studies. The finding of close to 1 PQ-9/PS II RC means that Q_A and Z cannot both be PQ-9.

2. MATERIALS AND METHODS

PS II particles were prepared according to Diner and Wollman [8], from 2 mutant strains of *Chla-*

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Abbreviations: PQ-9, plastoquinone-9 (9 corresponds to the number of isoprenoids units); PS, photosystem; RC, reaction center

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mydomonas reinhardtii isolated by Bennoun: a double mutant strain F.54-14, lacking chloroplast ATPase and PS I RCs; a double mutant strain B.F.4.M.18, lacking PS I RCs, largely devoid of PS I peripheral antenna and of light harvesting complex. For this strain, the procedure of Diner and Wollman [8] was modified; purified thylakoid membranes, at a final concentration of 0.3 mg Chl/ml, were solubilized at 4°C for 1 h in 20 mM Mes (pH 5.9), 0.5% Triton X-100 and 1.25% digitonin.

The chlorophyll concentration was determined according to Vernon et al. [9].

The Q_A photoreduction in the PS II particles was measured by flash-induced absorbance changes at 325 nm using a flash-detection spectrophotometer similar to that described by Joliot et al. [10]. PS II particles (10 μg Chl/ml) were resuspended in 50 mM Hepes (pH 7.5) in the presence of 10 μM $K_3[Fe(CN)_6]$ and incubated in the dark for 3 min, after which 2 mM hydroxylamine was added. After 3 min, a series of 14 short saturating xenon flashes was given (10 Hz) and the final light minus dark $\Delta A_{325\ nm}$ measured.

The presence of the artifical donor hydroxylamine assures reduction of Z^+, excluding any contribution of this donor to the absorbance changes at 325 nm. An extinction coefficient $\Delta\epsilon_{325\ nm}$ for Q_A minus Q_A of 12.5 $mM^{-1} \cdot cm^{-1}$ [11] was used for the calculation of the absolute concentration of photoreducible Q_A in the PS II particles.

An extensive analysis by high pressure liquid chromatography (HPLC) and thin layer chromatography (TLC) was performed on organic solvent extracts of PS II particles. PS II particles were repeatedly extracted at room temperature using various solvents (acetone, methanol, chloroform-methanol) and gave identical determinations of PQ-9. The results presented here were obtained using acetone extraction of PS II particles (200 μg chlorophyll) in the presence of 1 μmol DCPIP/mg Chl. The particles were extracted (× 4) with acetone (4 ml) by shaking (by hand) at room temperature for 3 min followed by centrifugation of 12000 × *g* for 2 min. The acetone extracts were pooled and evaporated to dryness.

The residue as dissolved into *n*-heptane (~ 1 ml) and passed through a silica gel filter (Sep-Pak, Waters Associates). The Sep-Pak was washed with 30 ml CH_2Cl_2-*n*-heptane (4:6, v/v). Chlorophyll and most of the carotenoids were adsorbed [12]. The eluant was evaporated to dryness using a rotary evaporator, the residue was dissolved in *n*-heptane and applied to an HPLC column or onto a thin-layer plate.

The HPLC was performed on either a Waters μ-Porasil column (silica 3.9 × 30 mm) with *n*-heptane-0.1% dioxan [13] as eluant at 1.5 ml/min or a Waters Bonda Pak C_{18} (reverse phase, 3.9 × 30 mm) with methanol-isopropanol (3:1, v/v) as eluant at 1.5 ml/min [12]. After each HPLC run, the μ-Porasil column was washed with CH_2Cl_2-1% isopropanol (90 ml) to remove polar contaminants and then reequilibrated with *n*-heptane-0.1% dioxane (180 ml). The degree of activation of the μ-Porasil column was a critical factor in determining the mobility of PQ-9. PQ-9 was detected by its absorption at 255 nm using a Waters LC spectrophotometer (Lambda-Max model 480). The PQ-9 peak size was calibrated using stock solutions of PQ-9 in *n*-heptane in which the quinone concentration had been determined by oxidized minus $NaBH_4$ reduced difference spectra in UV ($\Delta\epsilon_{255\ nm}$ = 14.8 $mM^{-1} \cdot cm^{-1}$) [14].

The standard PQ-9 was generously provided by Hoffman-LaRoche and prepurified by TLC prior to use.

TLC was performed on either Whatman KC18F reverse-phase plates with acetone-water (95:5, v/v) as eluant or Merck silica 60F254 concentration zone plates with benzene as eluant. The quinones were stained using leucomethylene blue. PQ-9 was eluted from the thin-layer plate with ethanol and quantitated as above by the oxidized minus $NaBH_4$ reduced difference spectrum.

The yield of the PQ-9 quantitation procedure was determined by adding a known quantity of radioactive PQ-9 to the PS II particles at the first step of the extraction. The radioactive PQ-9 was prepared as described by Diner et al. [15]. For the complete extraction and analytical procedure, the recovery of the radioactive PQ-9 added to PS II particles was 87.5% for the HPLC method and 97% for the TLC method.

The mass spectra of the extracted quinone and standard PQ-9 were performed on a Nermag R 10-10 B mass spectrophotometer using the following operating conditions: positive ions, chemical ionisation (reagent gas NH_3), inlet via desorption probe.

3. RESULTS

Fig.1 shows an elution profile of an acetone extract of PS II particles on an HPLC μ-Porasil column. The peak indicated by the arrow appears with the same retention time as the purified standard PQ-9 (18.7 min, 28 ml). The silica μ-Porasil column showed higher resolution than the reverse-

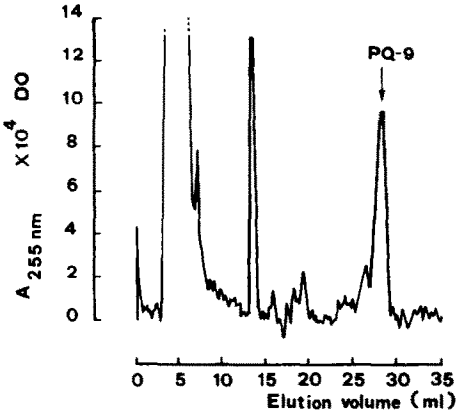

Fig.1. Typical elution of a μ-Porasil silica gel column of an acetone extract of PS II particles previously passes through a silica gel Sep-Pak filter (Waters) (see section 2).

Table 1

Quantitation of PQ-9 in PS II particles using different chromatographic separation methods

Mutant	Chl/Q_A (molar ratio)	PQ-9/PS II particle (molar ratio)		
		HPLC μ-Porasil	HPLC C_{18}	TLC
F.54-14	61	1.28	1.05	
		0.97	1.04	
F.54-14	48	1.30		
		1.12		
F.54-14	48			1.07
				1.15
				1.04
B.F.4-14	38	1.38		
		1.35		

The analysis of PS II particle extracts indicates an average of 1.16 ± 0.14 PQ-9/active RC (photoreducible Q_A) based on a total of 11 determinations on 4 different PS II particle preparations and using 3 different chromatographic separation methods

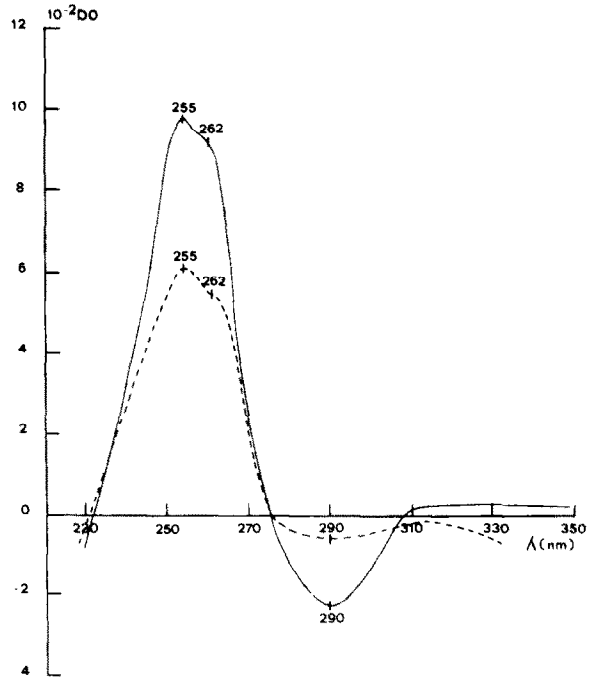

Fig.2. Oxidized minus $NaBH_4$ reduced difference spectrum in absolute ethanol of the purified standard PQ-9 (5 μg/ml) (solid line) and of the quinone extracted from the PS II RC (dashed line).

phase C_{18} column. The retention time of the extracted quinone was 5.4 min identical to that of purified standard PQ-9.

The oxidized minus reduced difference spectra of the extracted quinone and standard PQ-9 are also quite similar, showing the same isosbestic points at 276 and 232 nm and the same maxima at 255 and 262 nm (fig.2).

The mass spectra are also identical for the extracted quinone and the standard PQ-9 showing an intense cluster peak at *m/z* 766, 767, 768, 769 due to the reduction of the quinone, to the addition of the reagent gas ($[M+NH_4]^+$, $[M+2+NH_4]^+$) and to their isotopes, and a minor cluster at 749, 750, 751 ($[M+H]^+$, $[M+2+H]^+$).

4. DISCUSSION

Based on 1 photoreducible PQ/PS II RC, we have measured one spin/RC arising from signal II, 0.7 spins arising in the light from signal II f and 0.3 spins arising from a long-lived signal II species [16].

If both Z and Q_A were PQ-9, we would expect to find 2 PQ-9/RC; our results indicate 1.16 ± 0.14. Q_A is not covalently bound to the RC as indicated by reports of reversible-solvent extraction and reconstitution of PQ-9 [2] and by our own results involving reversible-detergent extraction and reconstitution of PQ-9 (Diner, B. and Popot, J.L., unpublished). As Q_A is known through optical spectroscopy to be a plastoquinone we attribute the PQ-9 to Q_A and conclude that the donor Z is not a PQ-9 in *C. reinhardtii*. The value somewhat greater than 1 PQ-9/RC could arise from low-level contamination of inactive centers, of Q_B or of cytochrome b_6/f complex.

Omato et al. [17] have reported 1.85 PQ-9/active PS II particles isolated from spinach. These authors attributed 1 PQ-9 to Q_A and one to Z. However the absence of signal II spin quantitation and uncertainty about the presence of Q_B weaken this assignment.

In conclusion, our results show the existence of only one PQ-9/PS II particle that we attribute to the primary acceptor Q_A, a non-covalently bound plastoquinone. Our results indicate that donor Z which gives rise to signal II f is not a PQ-9, but does not exclude that Z could be a covalently bound form of PQ (difficult to extract), a more polar form of this quinone or an altogether different quinone.

The strongest experimental evidence in favor of attributing Z to a plastoquinol is that of Kohl and Wood [2] whose extraction and reconstitution studies using deuterated quinol indicated a narrowing of the reconstituted signal II. It is likely that these authors detected signal II s a slower relaxing species probably located on a side pathway rather than on the main chain linking the PS II RC to the O_2-evolving site. As it is likely that signal II s and II f probably arise from the same species, it would be desirable to reconfirm the critical experiment of Kohl and Wood [2].

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