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# Numbers and functions of plastoquinone molecules associated with Photosystem II preparations from *Synechococcus* sp.

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**The contents of plastoquinone and PS II electron carriers ($Q_B$ and Z) of four PS II preparations from the thermophilic cyanobacterium *Synechococcus* sp. were determined. (1) The oxygen-evolving preparations and the PS II reaction center complexes contained about three and two plastoquinone molecules per PS II, respectively. CP2-b, which represents the functional core of the reaction center complex (Yamagishi, A. and Katoh, S. (1985) Biochim. Biophys. Acta 807, 74–80), had a reduced amount of plastoquinone which is just comparable with the $Q_A$ content, whereas no plastoquinone was detected in CP2-c, the antenna chlorophyll-protein containing only the 40 kDa subunit. (2) The four preparations were essentially free from vitamin $K_1$. (3) The occurrence of $Q_B$ and Z in the PS II preparations were determined by measuring oxidation kinetics of $Q_A^-$, as well as effects of 3-(3,4-dichlorophenyl)-1,1-dimethylurea, ferricyanide and benzidine on the $Q_A^-$ oxidation with repetitive flash technique. EPR measurements were also carried out to determine Signal $II_s$ and $II_f$. $Q_B$ and Z were present in the oxygen-evolving preparations, while the PS II reaction center complexes had Z, but no or only a residual amount of $Q_B$. CP2-b lacked both $Q_B$ and Z. (4) $Q_A^-$ was oxidized partly by a back electron transport to $Z^+$ and partly by ferricyanide added in the reaction center complexes. No back reaction between $Q_A^-$ and P-680$^+$ was detected in CP2-b, which instead showed absorption changes indicative of the triplet formation. It is concluded that three plastoquinone molecules (but not vitamin $K_1$) function as $Q_A$, $Q_B$ and Z and, besides them, there is no extra bound plastoquinone molecule which serves as an additional electron donor or acceptor in the vicinity of the PS II reaction center. The results also suggest that $Q_A$, pheophytin and P-680 are located in the interior of the reaction center complexes, but are exposed on removal of the 40 kDa subunit.**

## Introduction

Plastoquinone is a multifunctional electron carrier of photosynthesis in higher plants, algae and cyanobacteria (for reviews, see Refs. 1–3). The hydrophobic quinone molecules present in the fluid lipid phase of the thylakoid membranes serve as mobile electron carriers connecting the PS II reaction center complexes and the cytochrome $b_6$-$f$ complexes and at the same time as transmembrane proton carriers. Several plastoquinone molecules bound to the PS II reaction center complexes play specific roles in the secondary electron transport of PS II. The primary photochemistry of PS II produces oxidized P-680 and reduced pheophytin. $Q_A$, a bound plastoquinone molecule, accepts elec-

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Abbreviations: PS, Photosystem; LDAO, lauryldimethylamine *N*-oxide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; Chl, chlorophyll.

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trons from reduced pheophytin and in turn is oxidized by $Q_B$, another plastoquinone molecule bound to a specific subunit of the PS II reaction center complexes. $Q_B$ is considered to be in equilibrium with the pool quinone molecules with the binding constant, which varies depending upon the redox state of $Q_B$. Oxidized P-680 is reduced by Z which is assumed to give rise to the EPR signal $II_f$ or $II_{vf}$. Several lines of indirect evidence suggest that Z is a bound quinone [4–8].

The plastoquinone contents of several PS II preparations have been reported [9–12]. The interpretation of the plastoquinone to PS II stoichiometry is, however, hampered by the lack of knowledge on the number of plastoquinone molecules functioning on the oxidizing side of PS II. It could be either zero or one, depending upon whether Z is a plastoquinone molecule, or even two if Signal $II_f(II_{vf})$ and Signal $II_s$ arise from different plastoquinone molecules. A further complication arises if an additional plastoquinone acceptor function between pheophytin and $Q_A$ as has been suggested in Ref. 13.

Recently, four PS II preparations have been isolated and partially characterized from the thermophilic cyanobacterium, *Synechococcus* sp. An oxygen-evolving preparation free from PS I was obtained from the thylakoid membranes solubilized with β-octylglucoside [14]. A purified PS II reaction center complex was isolated by means of digitonin-polyacrylamide gel electrophoresis [15]. The reaction center complex consisting of five subunits of 47, 40, 31, 28 and 10 kDa was further resolved by a mild SDS-gel electrophoresis into two complemental chlorophyll-proteins; CP2-b, which is composed of the 47 kDa subunit and the three small subunits and CP2-c, which has only the 40 kDa subunit [15–18]. The reaction center complex and CP2-b are highly active in DCIP photoreduction with diphenylcarbazide, but not with water, as electron donor [15]. $Q_A$ is present in all the preparations except CP2-c, which serves as an intrinsic antenna chlorophyll-protein of the reaction center complex [14,18].

In the present work, the plastoquinone contents of the four *Synechococcus* PS II preparations were measured. The occurrence and abundance of $Q_B$ and Z in the oxygen-evolving preparations, reaction center complexes and CP2-b were determined by spectrophotometric and EPR techniques. Functions of plastoquinone molecules associated with the PS II reaction center will be discussed by relating the numbers of the secondary electron carriers present in the respective preparations with their plastoquinone contents.

## Materials and Methods

The thermophilic cyanobacterium *Synechococcus* sp. was grown at 55°C for two days [19,20]. The PS II reaction center complexes were extracted with β-octylglucoside from the thylakoid membranes and highly purified by digitonin-polyacrylamide gel electrophoresis as described previously [15]. The PS II reaction center complexes lacking the chlorophyll-binding subunit of 40 kDa (CP2-b) and the antenna chlorophyll-protein complexes of PS II which contain only the 40 kDa subunit (CP2-c) were prepared from LDAO extracts of the membranes by means of polyacrylamide gel electrophoresis with 0.05% SDS present only in the reservoir buffer [15]. A PS II oxygen-evolving preparation was obtained by a cushion centrifugation from the thylakoid membranes after solubilization with β-octylglucoside [14]. Spinach oxygen-evolving preparation was prepared by the method of Berthold et al. [21]. Tris-treatment was carried out by incubating the oxygen-evolving preparations with 0.8 M Tris-HCl (pH 8.0) at 0°C for 30 min [22].

Quinones were extracted with 80–90% acetone, separated by an alumina column and determined by spectroscopically measuring the oxidized-minus-reduced difference spectra [23]. Chlorophyll *a* was determined by the method of Mackinney [24].

P-700 and $Q_A$ (X320) were determined by measuring absorption changes at 703 nm and 325 nm, respectively, with a Hitachi 356 spectrophotometer with strong continuous illumination to ensure the complete oxidation of P-700 and complete reduction of $Q_A$, as described previously [18]. Differential extinction coefficients of P-700 and $Q_A$ used were 70 $mM^{-1} \cdot cm^{-1}$ at 703 nm [25] and 13 $mM^{-1} \cdot cm^{-1}$ at 325 nm [26], respectively.

Flash-induced absorption changes were measured with a Union Giken single-beam spectrophotometer as described previously [27]. A dye laser (Phase R DL-1100) with Red 4 (Cresyl Violet) in ethanol was used as the source of intense 659 nm flashes with a half peak-height duration of 300 ns. Flashes were passed through a Toshiba VR-64 filter and the photomultiplier was guarded against the actinic flash with a Corning 4-96 filter. Differential extinction coefficient of $Q_A$ at 415 nm and Z at 440 nm were assumed to be 6.8 $mM^{-1} \cdot cm^{-1}$ and 3.5 $mM^{-1} \cdot cm^{-1}$, respectively, from the difference spectra reported in Ref. 7. P-680 oxidation in the red band region (Fig. 5) was measured with xenon flashes of 5 $\mu$s duration which passed through a Corning 4-96 filter. A monochrometer and a Toshiba VR-61 filter were placed between samples and the photomultiplier. Signal II was determined by EPR spectroscopy as described by Satoh et al. [28]. All measurements were carried out at room temperature (20–25°C).

## Results

### *Quinone contents of PS II preparations*

The contents of plastoquinone and vitamine $K_1$ in the oxygen-evolving preparation, the reaction center complexes, CP2-b and CP2-c are shown in Table I. For the comparison, the quinone content of *Synechococcus* thylakoid membranes determined previously [23] is included in Table I. Spectrophotometric assay of $Q_A$ at 325 nm with continuous illumination showed that the thylakoid membranes contain one $Q_A$ per 356 chlorophyll *a*, in agreement with the chlorophyll *a*-to-PS II reaction center ratio of 400 in cells, which has been estimated from the $O_2$ yield per flash [29]. Because there is one plastoquinone molecule per 54 chlorophyll *a* [23], about seven plastoquinone molecules per PS II reaction center are present in the membranes.

The plastoquinone content of the oxygen-evolving preparations varied somewhat with preparations and the mean value of one plastoquinone per 16.5 chlorophyll *a* was obtained from seven separate preparations. Because $Q_A$ is present per 48 chlorophyll *a* [14], the plastoquinone-to-$Q_A$ ratio is about 3. Oxygen-evolving preparations isolated from spinach chloroplasts also contain about three plastoquinones per PS II [10,12].

The abundance of plastoquinone in the PS II reaction center complexes also fluctuated to some extent. Measurements with four separate preparations gave the average chlorophyll *a*-to-plastoquinone ratio of 19.4. The oxygen-evolving preparations used here still contained small amounts of P-700, whereas the reaction center complexes were completely free from PS I. Thus, the number of plastoquinone molecules associated with the PS II reaction center complexes should be significantly less than that of the oxygen-evolving preparations. Yamagishi and Katoh showed recently that the content of $Q_A$ is lower than that of photoreducible pheophytin in the reaction center complexes [18]. The chlorophyll *a*-to-$Q_A$ ratio of 46 yields the plastoquinone to PS II ratio of 2.4, whereas the ratio of 1.6 is obtained from the chlorophyll *a*-to-pheophytin ratio of 32 [18].

The $Q_A$ content of CP2-b is only slightly less than that of the PS II reaction center complexes based on the total chlorophyll *a*, but is about a half that of the intact complexes on the basis of chlorophyll *a* associated with the 47 kDa subunit [18]. Plastoquinone was present in an amount just comparable with the $Q_A$ content. No plastoquinone was detected in CP2-c, which serves as an antenna of the reaction center complexes.

We showed recently that *Synechococcus* thylakoid membranes contain about three vitamin

TABLE I

QUINONE CONTENTS OF *SYNECHOCOCCUS* THYLAKOID MEMBRANES AND PS II PREPARATIONS

PQ, plastoquinone; $VK_1$, vitamin $K_1$.

| | Thylakoid membranes | Oxygen-evolving preparations | Reaction center complexes | CP2-b |
|---|---|---|---|---|
| Chl *a*/PQ | 54 [23] | 16.5 ± 6.4 | 19.4 ± 3.9 | 66.8 ± 9.0 |
| Chl *a*/$Q_A$ | 356 | 47.5 ± 6.8 [14] | 46 ± 13 [18] | 57 ± 5 [18] |
| PQ/$Q_A$ | 6.6 | 2.9 | 2.4 | 1.2 |
| $VK_1/Q_A$ | 7.4 [23] | 0.23 | 0 | 0 |

$K_1$ per P-700 and that a major fraction of the vitamin molecules is associated with the PS I reaction center complexes [23]. Table I shows that the PS II reaction center complexes are essentially free from vitamin $K_1$. Small amounts of vitamin $K_1$ detected in the oxygen-evolving preparations are ascribed to contamination of the PS I complexes.

The above results show that the number of plastoquinone molecule per $Q_A$ decreases in the order of the oxygen-evolving preparations, the reaction center complexes, CP2-b and CP2-c. In the following, we have investigated secondary electron carriers functioning in the PS II preparations (except CP2-c) to compare with their plastoquinone contents.

*Oxygen-evolving preparations*

To examine the occurrence of $Q_B$ in the oxygen-evolving preparations, we measured oxidation kinetics of photoreduced $Q_A$ with the repetitive flash spectroscopy. The flash excitation caused a rapid absorption increase at 415 nm as shown in Fig. 1a. The decay kinetics were biphasic, showing two first-order components with half-times of 1 ms (37–50%) and 6 ms (50–63%). The difference spectrum for the total absorption changes which shows peaks at 415 and 442 nm and bleachings at 430 and 550 nm (Fig. 2) agrees with that for the $Q_A$ reduction [7,30,31]. The slow decay component determined 2.4 ms after flashes had a similar spectrum. Thus, the fast and slow decay components are both ascribed to oxidation of $Q_A^-$. The amount of photoreduced $Q_A$ which was determined at 415 nm with flashes corresponds to more than 90% of $Q_A$ measured at 325 nm with a strong continuous light [14].

The addition of a low concentration of ferricyanide was necessary for the $Q_A$ photoresponse to be observed under repetitive flash excitation. Otherwise, $Q_A$ would remain in the reduced state after initial several flashes. The rates of the fast and slow decay components were, however, independent of the ferricyanide concentrations added (Fig. 3). The results exclude a direct chemical oxidation of $Q_A^-$ by ferricyanide and suggest the occurrence of another electron acceptor which oxidizes $Q_A^-$ and then gives its electrons to ferricyanide during the flash intervals.

Fig. 1, trace b, shows that $Q_A^-$ oxidation was strongly inhibited by DCMU. This is evidence that $Q_A^-$ is oxidized by $Q_B$, but not by a back reaction because DCMU inhibits electron transport from $Q_A^-$ to $Q_B$ by competing with $Q_B$ for the $Q_B$-binding site [2,3]. Note that the fast and slow components are both suppressed by DCMU. This indicates that the two decay components are due to oxidation of $Q_A^-$ by $Q_B$, although the cause for the biphasic kinetics is not known. More detailed studies on the oxidation kinetics of $Q_A^-$ are in progress.

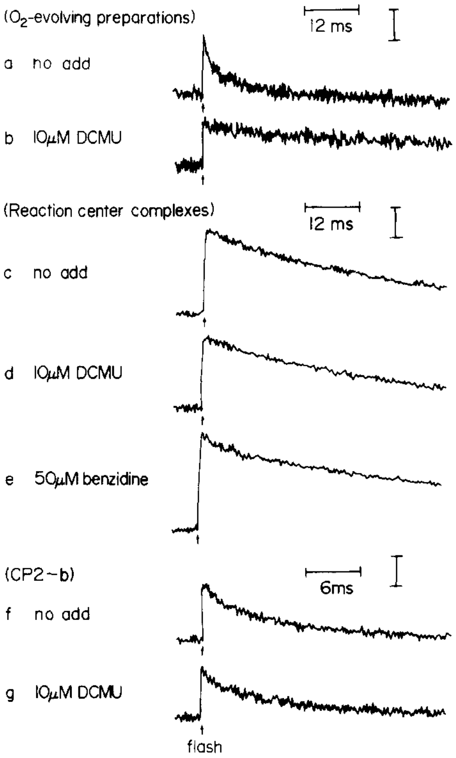

Fig. 1. Flash-induced absorption changes at 415 nm in the oxygen-evolving preparations and the reaction center complexes and at 410 nm in CP2-b. (a) and (b), oxygen-evolving preparations (15 μg Chl/ml) suspended in 1 M sucrose/10 mM NaCl/5 mM $MgCl_2$/50 mM Hepes/NaOH (pH 7.5)/0.1 mM ferricyanide; (c)–(e), reaction center complexes (15 μg Chl/ml) suspended in 50 mM Tris-HCl (pH 7.5)/0.1 mM ferricyanide; (f) and (g), CP2-b (10 μg Chl/ml) in 50 mM Tris-HCl (pH 7.5)/0.1 mM ferricyanide. Additions were indicated in the figure. Laser flashes were fired at 1 Hz, and 40 signals were averaged. A vertical bar corresponds to absorption changes of $1.0 \cdot 10^{-3}$ for (a)–(e) and $5.0 \cdot 10^{-4}$ for (f) and (g).

The half-times of $Q_A^-$ oxidation by $Q_B$ were 0.1–0.6 ms in ordinary plants [32,33]. The slow rates of the $Q_A^-$ oxidation in the *Synechococcus*

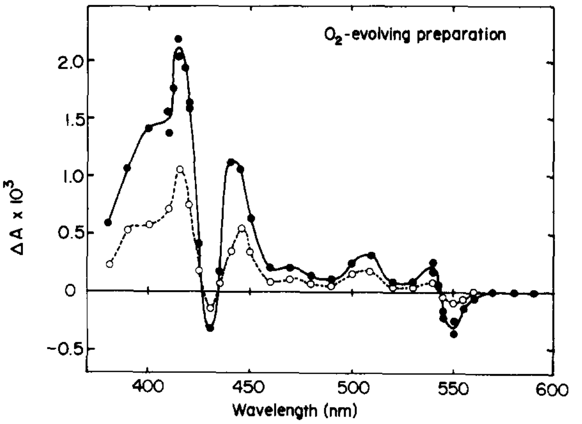

Fig. 2. Difference spectra of flash-induced absorption changes in the oxygen-evolving preparations. Experimental conditions were as in Fig. 1a. Solid circles, total absorption changes; open circles, absorption changes determined 2.4 ms after the flash excitation.

preparations can be ascribed to the assay temperature (25°C), which is much below the growth temperature of 55°C for the thermophilic cyanobacterium. Thus, all the results obtained are consistent with the view that $Q_B$ is present in an amount sufficient to oxidize $Q_A^-$ in the oxygen-evolving preparations.

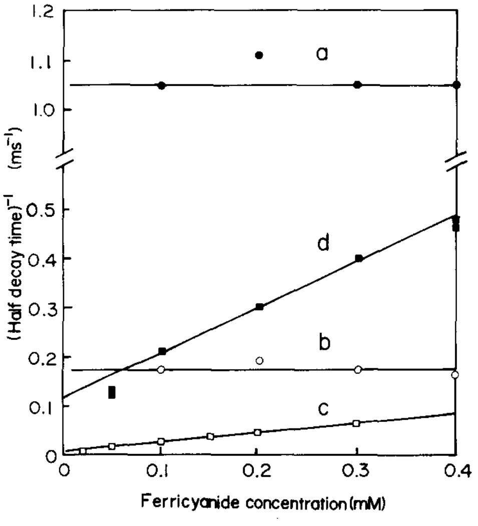

Fig. 3. Dependence of rates of $Q_A^-$ oxidation in three PS II preparations on ferricyanide concentrations. Experimental conditions were as in Fig. 1, except that ferricyanide concentration was varied. (a) and (b), fast and slow decay components in the oxygen-evolving preparations, respectively; (c) reaction center complexes; (d) CP2-b.

### *PS II reaction center complexes*

The difference spectrum shown in Fig. 4A indicates that flash-induced absorption changes in the PS II reaction center complexes are mostly due to $Q_A$ reduction, although high 440 nm and 395 nm peaks relative to the 415 nm peak indicate an overlapping absorption change (see Fig. 4B). The reoxidation of $Q_A^-$ determined at 415 nm was monophasic and its half-time of several tens of milliseconds was significantly larger than that in the oxygen-evolving preparations (Fig. 1c). This, together with its insensitivity to DCMU (Fig. 1d), clearly demonstrate that $Q_A^-$ is not oxidized by $Q_B$ in this preparation.

As stated above, the plastoquinone content of the reaction center complexes varied somewhat with preparations. The monophasic and DCMU-insensitive decays were observed with the prepara-

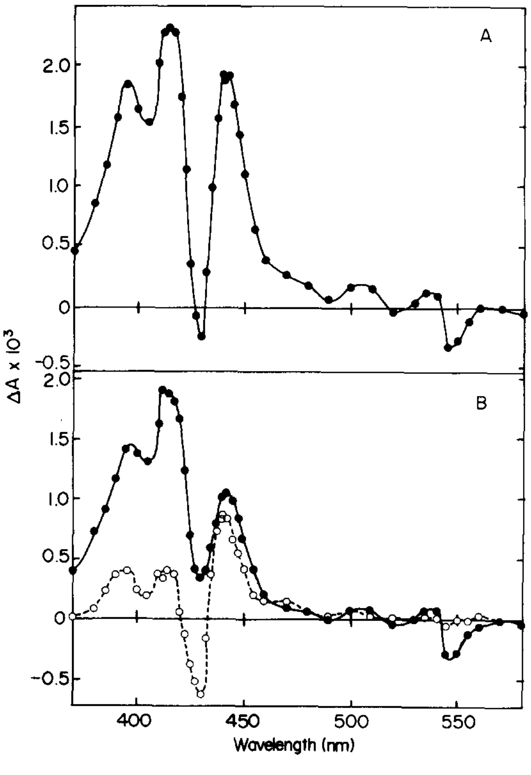

Fig. 4. Difference spectra of flash-induced absorption changes in reaction center complexes. Experimental conditions were the same as Fig. 1c. (A) No addition; (B) 50 μM benzidine were added and ferricyanide concentration was reduced to 50 μM. Open circles, fast decay phase; solid circles, slow decay phase.

tions containing relatively less plastoquinone. In other preparations which had the plastoquinone-to-$Q_A$ ratios considerably larger than 2, the major decay component was preceded by a small fast decay which is sensitive to DCMU (not shown), suggesting the presence of small amounts of $Q_B$.

The slow $Q_A^-$ oxidation is partly ascribed to the chemical oxidation of $Q_A^-$ by ferricyanide added because the oxidation rate was slightly but appreciately increased with increasing concentration of ferricyanide (Fig. 3c). That $Q_A^-$ is also oxidized by a back electron transfer to an oxidant produced on the water side of PS II was, however, indicated from experiments, in which the effects of benzidine, an electron donor to $Z^+$ [34], were examined. Fig. 1e shows that benzidine induced biphasic decay kinetics: the fast decay component was accelerated with increasing concentrations of benzidine, whereas the rate of the slow component was linear to the ferricyanide concentration (data not shown). The difference spectrum of the fast decay component determined in the presence of benzidine agrees with that of Z oxidation [7,8], while the spectrum of the slow component indicates the $Q_A$ reduction (Fig. 4B). The amounts of $Q_A$ reduced and Z oxidized by flashes both corresponded to one per 60 chlorophyll *a*. We conclude, therefore, that the PS II reaction center complexes contain Z which, when oxidized, accepts electrons partly from $Q_A^-$. Benzidine prevents the back electron transport by feeding electrons to $Z^+$ more rapidly than the back electron transport from $Q_A^-$. A second order rate constant for the $Z^+$ reduction by benzidine was estimated to be $2.0 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. A rate constant of $7 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ has been reported with a spinach preparation [35].

### *CP2-b*

The decay kinetics of photoresponses at 410 nm were monophasic (Fig. 1f) and totally insensitive to DCMU in CP2-b (Fig. 1g). $Q_A^-$ seems to be mainly oxidized by ferricyanide in this preparation because the decay rate was strongly accelerated with increasing concentration of the oxidant added (Fig. 3d).

The difference spectrum shows, besides absorption changes indicative of the $Q_A$ reduction, pronounced negative peaks at 430 and 673 nm which can be attributed to P-680 oxidation (Fig. 5). The blue-shifted red band of P-680 may be ascribed to an effect of the detergent, analogous to the blue-shifted P-700 red band in detergent-treated preparations. The addition of benzidine altered the spectrum to that for $Q_A$ reduction alone by eliminating P-680 oxidation (Fig. 5). This is due to a very fast reduction of P-680$^+$ by benzidine: the rate of the P-680$^+$ reduction was linear with concentrations of benzidine added, and a second-order rate constant between P-680$^+$ and benzidine was estimated to be $2.7 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ in CP2-b, which

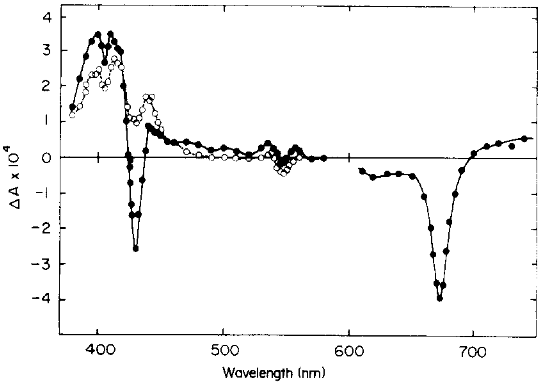

Fig. 5. Difference spectra of flash-induced absorption changes in CP2-b. Experimental conditions were as in Fig. 1f, except that ferricyanide concentration was 20 μM. Solid circles, no addition; open circles, 0.1 mM benzidine was added.

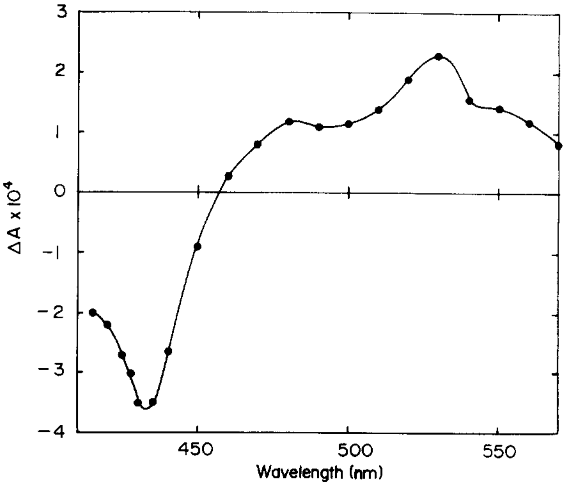

Fig. 6. Difference spectrum of flash-induced absorption changes with a half-decay time of about 10 μs in CP2-b. Experimental conditions were as in Fig. 1f, except a faster recording time was used.

is 10-times larger than the rate constant between $Z^+$ and benzidine in the reaction center complexes. Obviously, P-680 is more exposed in CP2-b than in the reaction center complexes. The results indicate that $Q_B$ and Z are not present in CP2-b so that, while $Q_A^-$ is oxidized by ferricyanide, P-680 remains in the oxidized state, unless benzidine is added.

A back electron transport from $Q_A^-$ to P-$680^+$ with a half-time of 100–200 $\mu$s is known to take place when a rapid reduction of P-$680^+$ by Z is prevented [36]. The measurement of the absorption changes at faster recording times failed to reveal such a back reaction in CP2-b. Instead, photoresponses which decay with half-times of 10 $\mu$s or less were observed. The difference spectrum for the fast-decaying absorption changes shows a bleaching at 430 nm and positive bands at 480 and 530 nm (Fig. 6). The spectrum resembles that for the formation of triplets in PS I reaction center complexes (CPl-e), in which a 430 nm bleaching is ascribed to the P-700 triplet and a 530 nm band to a triplet of $\beta$-carotene [27]. Analogously, the 430 nm bleaching observed here may be ascribed to the P-680 triplet produced as a result of the charge recombination between reduced pheophytin and P-$680^+$ in a fraction of CP2-b which has no $Q_A$ [37] and the 530 nm band to a carotenoid triplet generated by triplet–triplet energy transfer from excited antenna chlorophyll *a*.

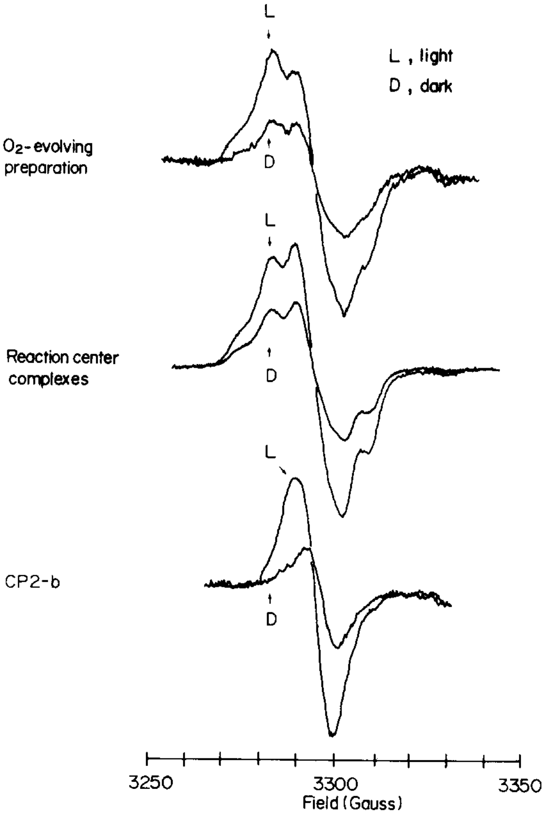

Fig. 7. EPR signals in three PS II preparations. Oxygen-evolving preparations were incubated with 0.8 M Tris-HCl (pH 8.0) for 30 min at 0°C, span down and suspended in 1 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 50 mM HEPES/NaOH (pH 7.5). Reaction center complexes and CP2-b were suspended in 50 mM Tris/HCl (pH 7.5). Instrument conditions: modulation amplitude, 4 G; time constant, 100 ms; power, 20 mW; gains, $2 \cdot 10^3$ for the oxygen-evolving preparations (1.74 mg Chl/ml), $5 \cdot 10^2$ for the reaction center complexes (5.08 mg Chl/ml) and $2 \cdot 10^3$ for CP2-b (2.70 mg Chl/ml); scan rate, 50 G/min; temperature, 20°C.

*Signal II*

The EPR signal $II_f$ is considered to arise from $Z^+$ [35,38–40]. EPR experiments were also carried out to examine the distribution and abundance of Z among the PS II preparations. The oxygen-evolving preparations, which had been treated with 0.8 M Tris-HCl (pH 8) to block electron transfer from water to Z, showed Signal $II_s$ in the dark and, upon illumination, the signal amplitude was nearly doubled, indicating the formation of Signal

TABLE II

RELATIVE AMPLITUDES OF SIGNAL $II_s$ AND $II_f$ IN PS II PREPARATIONS FROM SPINACH AND *SYNECHOCOCCUS*

| Materials | Preparations | Relative signal amplitude | |
|---|---|---|---|
| | | Signal $II_s$<br>RC II | Signal $II_f$<br>RC II |
| Spinach | Tris-treated oxygen-evolving preparations | 1.00 [d] | 1.46 [b] |
| *Synechococcus* | Tris-treated oxygen-evolving preparations | 1.09 [c] | 1.12 [c] |
| *Synechococcus* | Reaction center complexes | 1.67 [c]<br>1.16 [d] | 1.62 [c]<br>1.12 [d] |
| *Synechococcus* | CP2-b | 0 | 0 |

[a] Signal $II_s$ amplitude per 250 chlorophylls was taken as 1.00 [21].
[b] Contained a contribution from P-$700^+$.
[c] Relative signal amplitude per $Q_A$.
[d] Relative signal amplitude per photoreducible pheophytin.

$II_f$ (Fig. 7). The effect of illumination was much less in the untreated oxygen-evolving preparations, where $Z^+$ can be very rapidly reduced with electrons from water (not shown).

In order to quantify the spin concentration, we compared the Signal $II_s$ and $II_f$ of *Synechococcus* preparations with Signal $II_s$ of Tris-washed spinach oxygen-evolving PS II preparations of Berthold et al. [21,35], with which the spin concentration of Signal II relative to chlorophyll *a* or an oxygen-evolving unit have been well established. Unfortunately, the spinach preparation used here contained a small amount of P-700, which interferes with the estimation of Signal $II_f$. Table II shows that the amplitudes of Signal $II_s$ and $II_f$ per PS II in the *Synechococcus* oxygen-evolving preparations and the PS II reaction center complexes were roughly comparable with the amplitude of Signal $II_s$ in the spinach preparations. Neither Signal $II_s$ nor Signal $II_f$ was detected in CP2-b. Instead, CP2-b showed a light-induced signal of a free radical type, which may be ascribed to P-680$^+$, although its peak-to-peak width ($\Delta H$) of 10.0 G (or 10.5 G after correction for a dark signal) is slightly broader than that reported for P-680$^+$ [41,42]. The results are consistent with the spectrophotometric studies and indicate that a stoichiometric amount of Z is present in the oxygen-evolving preparation and the PS II reaction center complexes but not in CP2-b.

## Discussion

The three *Synechococcus* PS II preparations which contain different numbers of plastoquinone molecules per PS II reaction center are also different in the compositions of secondary electron transport of PS II. The stoichiometric quantities of $Q_A$, $Q_B$ and Z are present in the oxygen-evolving preparations which have three plastoquinone molecules per $Q_A$. The PS II reaction center complex preparations that contain about two plastoquinone molecules per PS II have $Q_A$ and Z. Thus there is a good correspondence between the numbers of bound plastoquinone molecules and the numbers of the PS II secondary electron carriers present in the two PS II preparations, suggesting that not only $Q_A$ and $Q_B$, but also Z is plastoquinone.

CP2-b lacks both $Q_B$ and Z. Comparison of the stoichiometry between the reaction center complex and CP2-b further provides a strong support for the view that Z is plastoquinone. The $Q_A$ content of CP2-b is about a half that in the reaction center complexes on the basis of chlorophyll *a* associated with the 47 kDa subunit [18]. When corrected for the decrease of plastoquinone due to the lowered $Q_A$ content, the loss of Z is accompanied by the release of one plastoquinone molecule from the reaction center complex. This provides an additional support for the conclusion that Z is a bound plastoquinone molecule.

Optical spectroscopy has suggested that $Z^+$ is a quinone cation radical [7,8]. The difference spectrum of Z oxidization was more similar to the hydrosemiquinone-cation-minus-quinol-difference spectrum of a vitamin-$K_1$ analogue than that of a plastoquinone analogue [8]. The finding that no vitamin $K_1$ is present in the reaction center complexes which have Z, however, conclusively rules out the possibility that Z is a bound vitamin $K_1$ molecule. Spectral properties of the cation radical would be considerably modified by the binding of the quinone molecule to a specific protein.

The EPR signal $II_f$ is considered to arise from $Z^+$ [35,38–40]. The occurrence of Signal $II_f$ in the reaction center complexes which are shown to contain a functional Z by flash photolysis, but not in CP2-b which lacks Z, supports this view.

Our results, however, indicate that there is only a single plastoquinone molecule which can be attributed to Z. This is significant because there are two Signal II species, i.e., Signal $II_f$ (or Signal $II_{vf}$ in preparations active in oxygen evolution), which is related to an electron donor to P-680 in the main pathway, and Signal $II_s$ which is considered to arise from a donor in a side path [43]. An important consequence of the present work is that Signal $II_f$ (or Signal $II_{vf}$) and Signal $II_s$ cannot be ascribed to two different plastoquinone molecules. Because it is difficult to imagine how the two signals could arise from a single quinone molecule, our results indicate that either one or both of Singal $II_s$ and Signal $II_f$ is not related to plastoquinone.

It is to be mentioned here that the above arguments are based on the quinone contents determined by the assay procedure, which cannot detect a quinone molecule covalently bound to proteins. A possibility remains that one of the two signals arises from a covalently bound quinone.

Our results show that two plastoquinone molecules function as $Q_A$ and $Q_B$ on the reducing side of the PS II reaction center. However, there is no extra plastoquinone (or vitamin $K_1$) molecule which can serve as an additional bound electron acceptor of PS II.

$Q_B$ functions as a two electron gate [1–3]. $Q_B^-$ strongly binds to a specific site of the reaction center complexes, whereas the binding constants for $Q_B$ and $Q_B^{2-}$ are considered to be low. As such, it is expected that a large fraction of the binding sites would be vacant in the dark. However, $Q_B$ is found in the oxygen-evolving preparations which lack a plastoquinone pool. A small amount of $Q_B$ is still associated with even PS II reaction center complexes. The results show that $Q_B$ binds to the specific site more strongly than expected above, unless the oxygen-evolving preparations contain most of $Q_B$ in the semiquinone anion form. More has to be learned about the binding and valence state of $Q_B$ in the PS II preparations.

CP2-b is a PS II complex which has the shortest span of electron-transport chain so far reported. The chain consists of only three electron carriers, P-680, pheophytin and $Q_A$, if a carrier which is assumed to locate between P-680 and pheophytin is not taken into account [44]. The high DCIP photoreduction activity of the CP2-b with diphenylcarbazide as electron donor [15] indicates that $Q_A$ has the principal importance in the stabilization of the charges separated by the primary photochemistry. The absence of Z from this complex, which lacks the 40 kDa chlorophyll-binding subunit [15,18] suggests that Z is located on the 40 kDa chlorophyll-binding subunit. No plastoquinone was, however, detected in the isolated 40 kDa subunit (CP2-c). It is therefore equally plausible that the SDS treatment used for the separation of the 40 kDa subunit from the reaction center complex has solubilized Z, which is located on some other subunit.

Previous experiments showed that $Q_A$ and pheophytin are readily accessible to exogenously added redox reagents in CP2-b [15]. The flash spectroscopy employed in the present work provides more quantitative information on the reactivity of the bound electron carriers with redox substances added. Bezidine reduces $Z^+$, but apparently not P-680$^+$, in the reaction center complexes, whereas P-680$^+$ in CP2-b is reduced by benzidine more rapidly than $Z^+$ in the reaction center complexes. Ferricyanide rapidly oxidizes $Q_A^-$ in CP2-b, but only slowly in the reaction center complexes. The results implicate that all the PS II electron carriers are located inside the PS II reaction center complex and hence separated from the outer aqueous phase by protein barriers. They are exposed on removal of the 40 kDa chlorophyll-binding subunit, or partial releases of the smaller subunits, from the reaction center complexes and become accessible to reagents added.

A back electron transport from $Q_A^-$ to P-680$^+$ with a half-time of about 200 $\mu$s occurs in the oxygen-evolving preparation when electron transfer from Z to P-680$^+$ is blocked by removal of $Ca^{2+}$ [45]. However, no rapid back reaction between $Q_A^-$ and P-680$^+$ occurs in CP2-b, which lacks Z. CP2-b also showed an altered absorption spectrum and ePR signal of P-680$^+$. These may be attributed to the removal of Z, or a modification of the environment surrounding P-680 which is caused by the changes in the subunit structure.

Finally, the size of the plastoquinone pool in the cyanobacterium can be estimated from the intramembrane distribution of plastoquinone molecules. The thylakoid membranes contain about seven plastoquinone molecules per PS II reaction center, of which three molecules are associated with the reaction center complexes. Thus four plastoquinone molecules are considered to be located in the fluid lipid phase of the membranes and function as mobile electron carriers. The size of the acceptor pool before the DBMIB-inhibition site has previously been estimated to be 12-electron equivalents by measuring the area over the fluorescence induction curves in the cells [46]. When corrected for electrons stored in $Q_A$ and $Q_B$, the plastoquinone pool size of nine electron equivalents is obtained. Thus the two independent methods give a pool sized of 4–5 plastoquinone molecules per PS II reaction center.

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