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Photoaffinity labeling of the phylloquinone-binding polypeptides by 2-azidoanthraquinone in photosystem I particles*

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A photoaffinity label, 2-azido-9,10-anthraquinone, binds at the quinone-binding (Q_ϕ) site with high affinity and can substitute for the secondary acceptor, phylloquinone, in photosystem I reaction center of spinach. Phylloquinone-depleted photosystem I particles reconstituted with azido-[^{3}H]anthraquinone were illuminated with UV light and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis. The large core polypeptides (*psaA* and/or *psaB*) were selectively labeled. The labeling was competitively inhibited in the presence of anthraquinone. These results indicate that the Q_ϕ site is located on *psaA* or *psaB* polypeptides.

Photosystem I; Reaction center; Photoaffinity label; Phylloquinone (vitamin K_1); Electron transfer

1. INTRODUCTION

Photosystem (PS) I in cyanobacteria and plants contains phylloquinone (= 2-methyl-3-phytyl-1,4-naphthoquinone = vitamin K_1) which is not contained in PS II [1]. Two molecules of phylloquinone were contained in photoactive preparations of PS I reaction center (RC) complex [2,3]. One of the two phylloquinones functions as the secondary acceptor (Q_ϕ or A_1) [4,5] in the light-induced electron transfer as shown below [6–8].

$$P700 \xrightarrow{h\nu} P700^* \xrightarrow{<14\ ps} A_0 \xrightarrow{35\ ps} Q_\phi \xrightarrow{15\text{–}200\ ns} F_X \rightarrow (F_A/F_B)$$
$$\searrow\ P700^T \xleftarrow{40ns}\ \swarrow$$

P700, A_0, F_X and F_A/F_B are the primary donor chlorophyll *a* dimer, the primary acceptor chlorophyll *a*, the acceptor iron–sulfur center X, and iron–sulfur centers A and B, respectively. The quinone-binding site, designated the Q_ϕ site seems to bind a wide variety of quinone analogues and inhibitors, such as anthraquinones and fluorenones in place of phylloquinone [9–11].

The two 4Fe–4S centers, F_A and F_B are present in the 9-kDa *psaC* polypeptide [12,13]. The 4Fe–4S center F_X has been proposed to be ligated by four cysteine residues, Cys^{573} and Cys^{582} on *psaA* and Cys^{559} and Cys^{568} on *psaB* polypeptides. Electron transfer from P700 to F_X has been shown to normally proceed in the core complex, which is constructed with only *psaA* (83.0 kDa), *psaB* (82.4 kDa) and *psaK* (8.4 kDa) polypeptides [14]. However, the exact location of the Q_ϕ site on the *psaA* or the *psaB* polypeptide is not known.

Marinetti et al. [15] showed that 2-azido-[^{3}H]9,10-anthraquinone (Az[^{3}H]AQ) specifically labels the M subunit of the RC complex isolated from the purple bacterium *Rhodobacter sphaeroides* (R-26). The binding of the primary acceptor ubiquinone, Q_A, to the M subunit was later confirmed by X-ray crystallography [16]. This method was adapted to PS I particles in the present study in order to determine the location of the Q_ϕ site.

2. MATERIALS AND METHODS

PS I particles, derived from spinach chloroplasts were lyophilized, and phylloquinones were extracted from the particles with diethyl ether as described previously [11]. The phylloquinone-depleted PS I particles were solubilized in 50 mM Tris-Cl buffer, pH 7.5, that contained 0.1% Triton X-100 and then undissolved materials were removed by centrifugation. All these procedures were performed at 0–6°C. Unlabeled AzAQ and Az[^{3}H]AQ, prepared as described by Marinetti et at. [15], were purified by thin-layer chromatography on silica gel. AzAQ dissolved in dimethylsulfoxide was used to reconstitute the phylloquinone-depleted PS I particles by overnight incubation in the dark at 0°C. The quinone-depleted and the reconstituted PS I particles were excited by an Nd-YAG laser flash (532 nm, FWHM 10 ns), and the absorption changes were measured at 7°C as described elsewhere [9].

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Abbreviations: AzAQ, 2-azido-9,10-anthraquinone; CBB, Coomassie brilliant blue; LDAO, lauryldimethylamine oxide; P700, the primary donor chlorophyll *a*; PS, photosystem; Q_ϕ, the secondary acceptor quinone; RC, reaction center; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

The solution of quinone-depleted PS I particles (equivalent to 0.5 μM P700) was incubated with 0.2 μM Az[^{3}H]AQ in the absence and in the presence of 27 μM unlabeled 9,10-anthraquinone (AQ) at 5°C for 24 h in the dark. Then it was illuminated with light from a germicidal lamp at 0°C for 30 min. Unreacted quinones were removed by centrifugation and washing in 50 mM Tris-Cl, pH 8.0. Digestion of the labeled PS I particles was achieved by incubation with lysyl endopeptidase from *Achromobacter lyticus* (Wako Chemicals Co.) for 30 min at 30°C. The photoaffinity-labeled PS I particles were then lysed in 10% β-mercaptoethanol and 2% sodium dodecylsulfate and subjected to electrophoresis in 12–22% polyacrylamide gradient gel with a stacking gel.

The ubiquinone-depleted RC complex from *Rb. sphaeroides* (R-26) was prepared by treatment with 2% LDAO and 10 mM *o*-phenanthroline for 6 h at 25°C as described elsewhere [15]. The ubiquinone-depleted RC complex (6 μM) was also illuminated by UV-light in the presence of 0.43 μM Az[^{3}H]AQ, as described above for PS I particles, in a reaction medium that contains 0.5% LDAO.

3. RESULTS AND DISCUSSION

3.1. *Reconstitution of AzAQ as the secondary acceptor in PS I RC*

The laser excitation of the quinone-depleted PS I particles induced only a small absorption change at 696 nm over the μs–ms time range, since most of the $P700^+$ reacted with A_0^- within less than 1 μs as a result of the rapid (40 ns) charge recombination (Fig. 1, insert) [4,17]. The extent of the absorption change at 696 nm was increased with increases in the concentration of AzAQ used for reconstitution (Fig. 1). This phenomenon is due to the suppression of the rapid charge recombination by the electron transfer from A_0^- to AzAQ-reconstituted PS I particles, the reduction of F_A/F_B was also restored when checked by the measurement of 'P430' signal by the flash photolysis as seen in the case of PS I RC reconstituted by phylloquinone [5] or artificial compound [11] (not shown). One molecule of AzAQ per RC was sufficient to mediate the electron transfer from A_0^- to F_A/F_B presumably via F_X. These results confirm that AzAQ binds specifically at the Q_φ site with high affinity and functions as the acceptor Q_φ in the PS I RC.

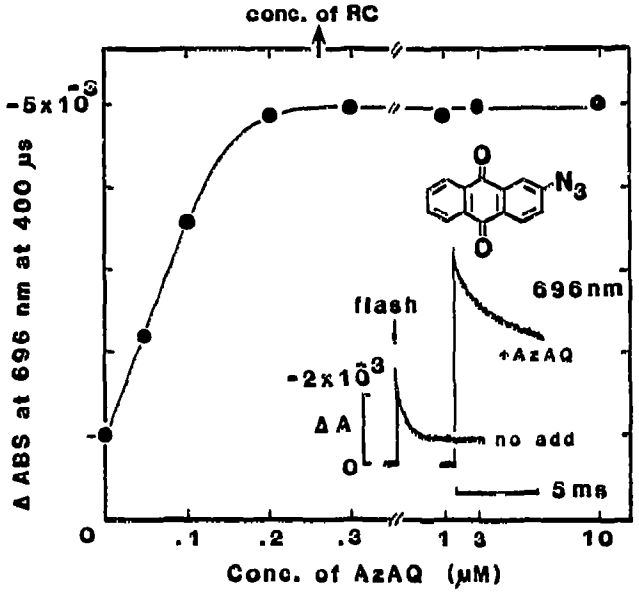

Fig. 1. Dependence of absorption changes of P700 after laser-flash excitation on the concentration of AzAQ added to phylloquinone-depleted PS I particles. The extent at 400 μs after the flash was plotted to minimize the contribution of the triplet state of P700. (Insert) Decay kinetics of $P700^+$ measured at 696 nm in the absence and presence of 0.2 μM AzAQ. The structural formula of AzAQ is also shown. Reaction mixtures contained 0.2 μM P700, 10 mM sodium ascorbate, 0.5 μM *N*-ethyldibenzopyrazine ethyl sulfate and 50 mM Tris-Cl buffer, pH 7.5.

3.2. *Photoaffinity labeling of the quinone-binding site*

The PS I particles were labeled with Az[^{3}H]AQ by UV illumination, and the labeled polypeptides were analyzed by SDS/PAGE. The gels stained with CBB are shown in the left lanes in A and B in Fig. 2. The *psaA* and *psaB* polypeptides, forming one broad band at 67 kDa, were specifically labeled. The labeling of the *psaA/psaB* bands was suppressed in the presence of excess AQ during the UV illumination (right lane in Fig. 2B).

The regions at the top of gels and a band at 2.5 kDa also appeared to be labeled (in the right lanes in A and B). These bands still remained in the presence of excess AQ (the right lane in B) and seem to be non-specific. The bands at the top of gels in A and B presumably correspond to aggregated polypeptides since they were eliminated after digestion with lysyl endopeptidase (in the left lane in C). The aggregates might have adsorbed water-insoluble Az[^{3}H]AQ or its degradates.

Under the same conditions, the M subunit of the RC complex of the purple bacterium, *Rb. sphaeroides* (R-26), was exclusively labeled with Az[^{3}H]AQ (Fig. 2, lanes in D). This result confirms that Az[^{3}H]AQ binds specifically at the Q_A site [15]. The above results indicate that the experimental procedure used here is adequate for the labeling of the quinone-binding site in the PS I RC.

3.3. *Candidates for the azido-attached fragment*

When the PS I particles were digested with lysyl endopeptidase, labeled proteins were only recovered at positions of 14.5, 9 and > 4 kDa. Candidate segments covalently attached to Az[^{3}H]AQ can be limited to the following on the basis of amino acid sequences and the secondary structures of *psaA* and *psaB* polypeptides, proposed by Kirsch et al. [18] (Fig. 3). Some portions of these two polypeptides give fragments with the molecular weights greater than 18 kDa as indicated by dotted lines in Fig. 3, and they cannot be candidates. The other regions corresponding to less than 15 kDa can be candidates. Prolonged digestion decreased the intensity of the band of 14.5 and 9 kDa with a concomitant increase in the intensity of the band of less than 4

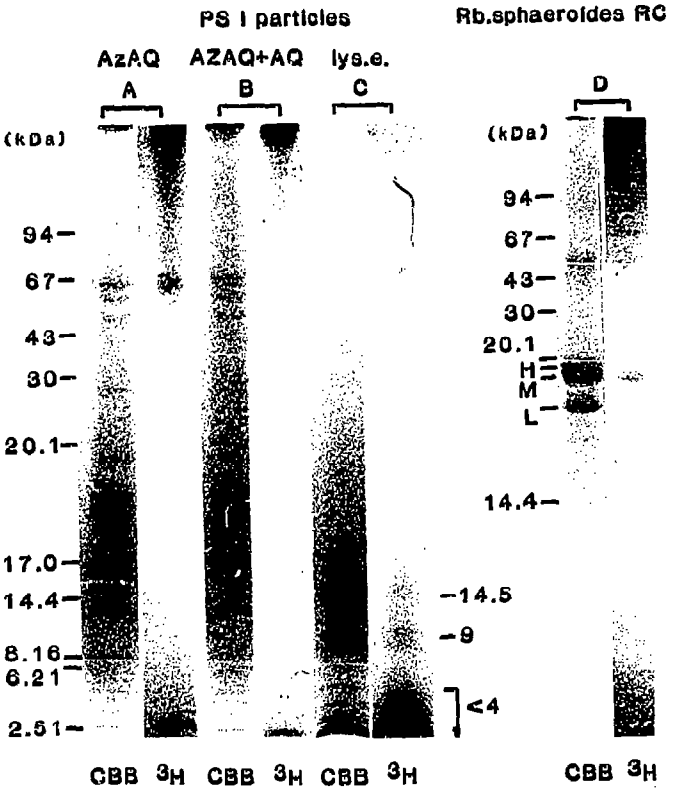

Fig. 2. Patterns after SDS-PAGE of spinach PS I particles and the RC of the purple bacterium, *Rb. sphaeroides* (R-26), after labeling with Az[^{3}H]AQ. Lanes in A and B represent PS I particles reconstituted with Az[^{3}H]AQ in the absence and presence of unlabeled AQ, respectively. Lanes in C represent the Az[^{3}H]AQ-reconstituted PS I particles after digestion with lysyl endopeptidase. Lanes in D represent the RC of *Rb. sphaeroides* (R-26) reconstituted with Az[^{3}H]AQ. The left and right lanes in A–D show CBB-stained gels and their fluorograms, respectively.

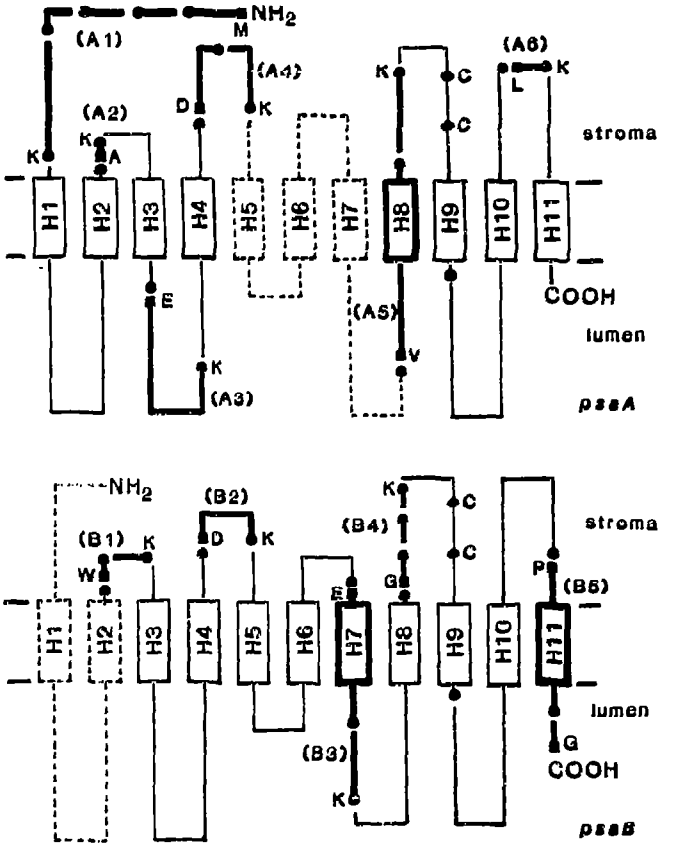

Fig. 3. Candidate fragments for the binding site of AzAQ on *psaA* and *psaB* polypeptides. Hydrophobic trans-membrane α-helices (H1–H11) are drawn in boxes as proposed by Kirsch et al. [18]. The fragments with C-terminal lysines are represented by thick lines (<4 kDa), thin lines (>4 kDa and <15 kDa) and dotted lines (>18 kDa).

kDa (not shown). In the amino acid sequence of *psaA*, the following regions contain fragments of less than 4 kDa after full digestion with lysyl endopeptidase: A1 (Met^{1}...Lys^{38}), A2 (Ala^{181}...Lys^{184}), A3 (Glu^{233}...Lys^{268}), A4 (Asp^{321}...Lys^{330}), A5 (Val^{519}...Lys^{564}) and A6 (Leu^{706}...Lys^{707}) (thick lines in the upper panel in Fig. 3). The candidates in *psaB* are, B1 (Trp^{161}...Lys^{169}), B2 (Asp^{303}...Lys^{320}), B3 (Glu^{416}...Lys^{467}), B4 (Glu^{537}...Lys^{551}) and B5 (Pro^{697}...Gly^{734}) (thick lines in the lower panel in Fig. 3.

The Q_ϕ quinone is expected to be close to the membrane surface on the stroma side, and presumably in the vicinity of the F_X to account for the rapid electron transfer rate. Segment A5 or B4, then, appears to be the most plausible candidate. These segments are close to the presumed F_X regions [6], which are indicated by the position of cysteines (C) in Fig. 3. The segments partially overlap with the proposed leucine zipper motifs [19], which cover the H8 helices and their extrusions to the stroma side. The sequences of amino acids in the regions, from H8 to H9 helices, tend to be conserved in a variety of organisms [6].

Iwaki and Itoh interpreted the mechanism of the molecular recognition of quinones by PS I RC protein by assuming that the acceptor quinone binds reversibly at only one site in the RC protein [20]. The present results strongly support their assumption and further indicate that the high affinity Q_ϕ-binding site exists on *psaA*/*psaB* polypeptides. The small transmembrane-polypeptides, *psaI*, *psaJ* and *psaK* do not seem to be close enough to be labeled by azidoanthraquinone, although they are associated with the PS I core complex [6,14,21] and were proposed to bind the prosthetic groups [21]. Our preliminary results with PS I particles from *Chlamydomonas reinhardtii* indicated that Az[^{3}H]AQ mainly labels the band of *psaA* rather than that of *psaB* (not shown). Identification of the azido-attached fragment is now in progress.

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