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Spectroelectrochemistry of P700 in native photosystem I particles and diethyl ether-treated thylakoid membranes from spinach and *Thermosynechococcus elongatus*

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Abstract The redox potentials ($E^{\circ'}$) of P700 in intact and diethyl ether-treated thylakoid membranes as well as native photosystem (PS) I particles from spinach and *Thermosynechococcus elongatus* have been measured by a spectroelectrochemistry with an error range of ±2–3 mV. Stepwise removal of antenna pigments by ether treatment caused distinct shifts of the $E^{\circ'}$ value with increasing degree of water saturation in ether; *negatively* from +471 to +428 mV for spinach, but *positively* from +423 to +436 mV for *T. elongatus*. Such a contrasting behavior is discussed by invoking the mode of action of ether on the microenvironments around P700.

Keywords: Photosystem I; P700; Redox potential; Chl/P700 ratio; Ether treatment

1. Introduction

The primary electron donor of photosystem (PS) I, P700, consists of a heterodimer of chlorophyll (Chl) *a* and *a*′ (the $C13^2$-epimer of Chl *a*) [1] and generates a strong reducing power upon photo-excitation. One of the key factors that drive PS I photochemistry is the redox potential (denoted by $E^{\circ'}$ hereafter) of P700. Until recently, however, the reported $E^{\circ'}$ value exhibited a heavy scatter from +375 to +525 mV vs. SHE [2], due partly to the inaccuracy of potential control in the conventional chemical redox titration. This problem has been overcome to a satisfactory extent by the introduction of a spectroelectrochemical technique using an optically transparent thin-layer electrode (OTTLE) cell [3–5], where the potential can be strictly controlled and the redox equilibrium is rapidly attained in the cell. By such measurements we were able to determine the $E^{\circ'}$ value of P700 to within ±2 mV and demonstrated for the first time that the $E^{\circ'}$ value is species-dependent, being +470 mV for spinach and +423 mV for the trimeric PS I of a thermophilic cyanobacterium *Thermosynechococcus elongatus* [3,4].

The $E^{\circ'}$ value of P700 is by as much as 400 mV lower than that of monomeric Chl *a* or *a*′ in organic solvents [6]. In addition to such a huge shift, originating mainly in the Chl *a*/*a*′ dimerization, the $E^{\circ'}$ value has been supposed to undergo a subtle change from the microenvironments of the heterodimer, through electrostatic interaction, hydrogen bonding and dielectric properties of the surrounding medium or protein network [7–9].

The redox potential of P700 has often been examined for the so-called PS I preparation, in which peripheral proteins and antenna pigments are partially removed by treating a sample with either a detergent or an organic solvent. Ikegami and coworkers [10–12] succeeded in preparing PS I samples with a very small number of antenna Chls, by extracting them from thylakoid membranes or PS I particles of spinach and *T. elongatus* with diethyl ether containing a small amount of water. They preliminarily reported that the $E^{\circ'}$ value for *T. elongatus*, as determined by chemical titration, was lowered by ether treatment, namely by partial removal of antenna Chls. However, the cause for such a potential shift remains ambiguous to date.

To elucidate this point, and to systematically study the effect of diethyl ether treatment (removal of antenna pigments) on the redox behavior of P700, in this work we have measured the $E^{\circ'}$ value, by the thin-layer cell spectroelectrochemistry, of intact and ether-treated thylakoid membranes and native PS I particles (PS I—LHC I for spinach, and PS I trimer for *T. elongatus*). The $E^{\circ'}$ values for the two species showed a shift in the opposite direction, and the experimental results are discussed in terms of a microenvironmental change around P700 due to diethyl ether treatment.

2. Materials and methods

2.1. Sample preparation

Thylakoid membranes and PS I samples were prepared from spinach and *T. elongatus* according to Wynn and Malkin [13] and our previous work [14]. For the ether treatment, thylakoid membranes were washed with a large amount of distilled water, were lyophilized and then extracted twice with diethyl ether containing water at 0%, 25%, 50% and 75% saturation, to yield P700-enriched particles. They were washed twice with a Tris–HCl buffer (pH 8.0) containing 10 mM NaCl and

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Abbreviations: PS I, photosystem I; Chl, chlorophyll; LHC, light-harvesting complex; OTTLE, optically transparent thin-layer electrode; HPLC, high-performance liquid chromatography; Pc, plastocyanin; Cyt, cytochrome

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0.025% dodecyl maltoside (DM), and then dissolved in the same buffer with 0.2% DM. After removing insoluble greenish-white materials by centrifugation, the blue-green supernatant was used for measurements. Photo-oxidation and chemical re-reduction ensured that P700 was kept in an active form for all the preparations.

2.2. Sample characterization

The Chl *a*/P700 molar ratio was determined from oxidized-minus-reduced difference spectra, either through photo- and/or ferricyanide-oxidation followed by ascorbate-reduction, or electrochemical oxidation/reduction, using the P700 extinction coefficient of 64 mM^{-1} cm^{-1} at 700 nm at around 4 °C.

To determine the quantities of Chl *a* and Chl *b* in spinach, samples were analyzed by reversed-phase high-performance liquid chromatography (HPLC) [14], and the ratio of Chl *a*/*b* in spinach were estimated in 80% aqueous acetone according to Porra et al. [15].

For the electrochemical oxidation/reduction, the sample particles were submitted to potentials of +650 and +50 mV for 1 min each. Absorbance bleaching at around 700 nm and 680 nm was observed for all the particles. The ratio of Chl/P700 was evaluated by assuming a common extinction coefficient for $P700^+$ at ca. 700 nm and for PS I at the Q_y band. This procedure yielded the ratio of Chl (*a* + *b*)/P700 in spinach and Chl *a*/P700 in *T. elongatus*.

The protein composition was examined by SDS–polyacrylamide gel (15%) electrophoresis. The samples were dissolved in a 50 mM Tris–HCl buffer (pH 6.8) containing 2% SDS for 10 min at 95 °C. The BWM calibration kit (Amersham Biosciences) was employed for molecular weight estimation.

2.3. Spectroelectrochemistry

The $E^{\circ'}$ value of P700 was determined spectroelectrochemically at 4 °C by monitoring redox-induced absorbance changes at around 700 nm and 808 nm resulting from accumulation and re-reduction of $P700^+$. The construction of the OTTLE cell (optical path length: ca. 180 μM, volume: <200 μL) with a gold mesh working electrode, a platinum wire counter electrode and an Ag/AgCl reference electrode was detailed previously [4]. The electrode potentials are hereafter reported against the standard hydrogen electrode, SHE (+199 mV vs. Ag/AgCl in sat. KCl).

Thylakoid membranes or a series of PS I preparations from spinach and *T. elongatus* were suspended in a buffer solution containing 50 mM Tris–HCl (pH 8.0), 0.1–0.2 M KCl, 0.3% DM and three redox mediators: 10 μM *N*-methyl phenazonium methosulfate (PMS, $E^{\circ'}$ = +80 mV), 20 μM tetrachlorobenzoquinone (Cl_4-BQ, $E^{\circ'}$ = +260 mV) and 150 μM 1,1′-ferrocene dimethanol (Fc-DM, $E^{\circ'}$ = +476 mV). Water-insoluble mediators (Cl_4-BQ and Fc-DM) were added by diluting a 50-fold concentrated stock solution containing 10% DM.

The ether-treated samples from spinach were sensitive to the ionic strength of solution; even dipping a normally fabricated salt bridge into a sample caused its aggregation and thereby hampered the measurement. Hence a salt bridge with 0.1 M (less than 1/80 of normal concentration) of KCl was used for the ether-treated samples, after verifying that it exerted a negligible effect on the liquid junction potential.

3. Results

3.1. Characteristics of samples with different Chl/P700 ratios

Table 1 summarizes the molecular compositions of thylakoid membranes and a series of PS I preparations from spinach and *T. elongatus* submitted to measurements in the present work.

The antenna pigments were removed progressively with increasing water saturation in diethyl ether, as seen in the Chl *a*/P700 (spinach) and Chl *a*/P700 (*T. elongatus*) molar ratios. As was noted also by Ikegami et al. [11], the antenna pigments in *T. elongatus* were slightly more resistant to the ether treatment, resulting in lesser enrichment of P700 than in spinach treated with ether of the same water saturation degree; roughly 95% Chl molecules were extracted at 75% water saturation for spinach thylakoid membranes, while the figure was about 85% for *T. elongatus*. Chl *b* molecules bound to light-harvesting complex (LHC) in spinach PS I were resistant to dry ether extraction, whereas most of the Chl *b* molecules were extracted with ether at 75% water saturation, yielding a sample with a Chl *b*/P700 ratio of ca. 2.

Fig. 1 displays visible absorption spectra of the samples prepared. In accordance with the finding by Ikegami et al. [11], diethyl ether at higher degree of water saturation preferentially extracted antenna Chl molecules absorbing at 700–710 nm, and this tended to shift the absorption maximum of the Q_y band to shorter wavelengths for samples from both species. For spinach (left column), a clear shoulder band at 650 nm in thylakoid membranes treated with 0%, 25% and 50% water-saturated ether is due to Chl *b*, and the absorbance became insignificant after treatment with 75% water-saturated ether, where a marked band at 418 nm originates in cytochrome (Cyt). For *T. elongatus* (right column), the broad absorption in the 480–550 nm range in ether-treated thylakoid membranes might come from carotenoids [16]. The presence of these impurities in ether-treated samples are in line with the SDS–PAGE analyses described below.

3.2. Protein composition of PS I preparations

Fig. 2 shows SDS–PAGE patterns of the samples prepared from spinach and *T. elongatus*. The pattern for the spinach native PS I (PS I–LHC I, Lane 7) is almost identical with that in literature [13], and contains two high molecular weight polypeptides (58 and 62 kDa) and several low molecular weight subunits as well as the entire component of bound electron

Table 1
Molecular compositions of thylakoid membranes and PS I preparations from spinach and *Thermosynechococcus elongatus*

	Chl (*a* + *b*)/P700[a] (spinach)	Chl *a*/P700[d] (spinach)	Chl *a*/*b*[e] (spinach)	Chl *a*/P700[a] (*T. elongatus*)
Thylakoid	374 ± 3	241 ± 8[b]	3.1 ± 0.2	109 ± 2[b], 110
Native PS I	131 ± 3	115 ± 3[c]	8.2 ± 0.2	96[c], 99[c]
0% Sat.	124 ± 2	75, 78	2.4 ± 0.5	52.2 ± 2.2
25% Sat.	94.0 ± 3.0	39, 41	2.2 ± 0.5	34.4 ± 1.3
50% Sat.	57.2 ± 3.4	28, 27	2.1 ± 0.5	26.6 ± 1.4
75% Sat.	19.2 ± 1.5	15, 17	6.3 ± 0.2	18.1 ± 0.9

A value with ±S.D. is a mean of 3–5 independent runs. "Native PS I" denotes PS I–LHC I for spinach and PS I trimer for *Thermosynechococcus elongatus*.
[a]Quantified by electrochemical oxidation/reduction.
[b]Deduced from 660-nm traces of reversed-phase HPLC.
[c]Quantified by photo-oxidation.
[d]Quantified by chemical oxidation/reduction.
[e]Estimated by the method of Porra et al. [14] in 80% aqueous acetone.