5′-Monohydroxyphylloquinone as a Component of Photosystem I

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Thylakoids and photosystem I (PS I) preparations from *Euglena gracilis* and from the cyanobacterium *Anacystis nidulans* were analyzed for their prenylquinone content. Both organisms contained 5′-monohydroxyphylloquinone as the dominant quinone of PS I, and not phylloquinone as other organisms. A stoichiometry of about two 5′-monohydroxyphylloquinone per P700 (the primary electron donor of PS I) was determined for *Anacystis*. However, both the PS I preparation and the thylakoids from *Euglena* contained only one 5′-monohydroxyphylloquinone per P700. Phylloquinone and an unidentified naphthoquinone were present in trace amounts.

Introduction

Phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone) has, in recent years, been shown to be a component of photosystem I (PS I) of chloroplasts and cyanobacteria [1–4], with a stoichiometry of two per PS I (P700). Both quinone molecules copurify with the so-called P700-reaction center, a photochemically active subcomplex of PS I which contains only two kinds of polypeptides, with a molecular mass of about 80 kDa each (apparent molecular mass about 65 kDa on SDS-PAGE). Of the five known bound electron acceptors of PS I (see ref. [5] for a review), three early electron acceptors (center A_0, center A_1 and the Fe–S center X) are also located on the P700-reaction center [6–8]. Phylloquinone is probably center A_1, as recently discussed [9].

Not all green plants and cyanobacteria contain phylloquinone in high amounts; it was reported to be absent from *Euglena* [10]. We have analyzed the quinone content of photosystem I of two such species, *Euglena gracilis* and a strain of the cyanobacterium *Anacystis nidulans*. In both organisms, the principal quinone associated with PS I was identified as 5′-monohydroxyphylloquinone, a derivative of phylloquinone that carries an hydroxyl group at C-5 of the phytyl side chain. *Euglena* appears to be exceptional in as far as the stoichiometry of the naphthoquinone to P700 is one, and not two as usual [1–4]. This stoichiometry was also found in thylakoids of *Euglena* and is, thus, not an artifact of the isolation procedure for the photosystem.

Abbreviations and trivial names: 5′-HOP, 5′-monohydroxyphylloquinone; phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone; P700, primary electron donor of photosystem I; PS I, photosystem I; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Triton X-100, octylphenoxy polyethoxyethanol.

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Materials and Methods

Strains and culture conditions

Euglena gracilis Klebs (strain Z) was obtained from Dr. U. Johannigmeyer, University of Bochum, F.R.G. It was grown in 20 l glass jars filled with 15 l of either an acidic (pH 3.4) photoautotrophic medium [11] or a photoheterotrophic medium (Medium A of Greenblatt and Schiff [12]). The cultures were sparged with sterile air containing 2% (v/v) CO_2 and illuminated with fluorescent tubes ("Universal-white" from Osram, F.R.G.) of about 3000 Lux at the surface of the cultures. The temperature was maintained at 25 °C. *Anacystis nidulans* (P. Richt) from the Algal Collection at the Institute of Applied Microbiology, University of Tokyo, was obtained from Dr. T. Omata, RIKEN, Saitama, Japan. It was grown photoautotrophically in Medium C of Kratz und Myers [13] under the conditions described above.

Euglena cells were harvested by centrifugation, washed once and resuspended in 40 mM Tris (pH 8.1 with HCl), 5 mM $MgCl_2$, 0.33 M mannitol and used immediately. *Anacystis* cells were harvested by centrifugation, washed and resuspended in 50 mM Tris

(pH 7.5), 50 mM NaCl and either used immediately or stored frozen at −20 °C.

Preparation of thylakoids, of photosystem I and of the P700-reaction center

All steps were performed in dim light at 0 to 5 °C. Cells were broken by passage through a French pressure cell at 100 MPa. All solutions containing Tris buffer were adjusted to pH 8.1 with HCl.

Thylakoids and photosystem I from *Euglena*

The supernatant of a 1 min centrifugation at $500 \times g$ was centrifuged for 30 min at $23{,}000 \times g$, the pellet suspended in 20 mM Tris, 5 mM $MgCl_2$, centrifuged (1 h at $23{,}000 \times g$), the pellet resuspended in 0.15 M NaCl, centrifuged (30 min at $27{,}000 \times g$), resuspended in 2 M NaBr, 0.33 M mannitol, 20 mM Tris, stirred for 30 min, diluted two-fold with water and centrifuged 1 h at $27{,}000 \times g$. This last step was repeated once, the resulting pellet suspended in 10 mM Tris, centrifuged for 1 h at $27{,}000 \times g$ and the pellet resuspended in 40 mM Tris, 5 mM $MgCl_2$, 0.33 M mannitol. Occasionally, the thylakoid preparation was further purified by centrifugation on a linear gradient of Percoll (Pharmacia, Sweden) in 40 mM Tris as described [14]. For the isolation of PS I, the thylakoids were adjusted to 1 mg/ml of chlorophyll, 2% (v/v) of Triton X-100, and stirred for 18 h. The supernatant of a 30 min centrifugation at $48{,}000 \times g$ was applied to a column of DE-52 (Whatman, England; volume 1.5 ml per mg chlorophyll) equilibrated with 20 mM Tris, 0.1% Triton, 0.25 mM EDTA, and the photosystem eluted with a linear gradient from 0.0 to 0.4 M NaCl in this buffer. Fractions with the highest content of P700 were combined and dialyzed against 20 mM Tris.

Thylakoids and PS I from *Anacystis nidulans*

The supernatant of a low-speed centrifugation (10 min at $3000 \times g$) was subjected to ultracentrifugation (1 h at $100{,}000 \times g$). The membran pellet consisting mainly of thylakoids was washed once in 50 mM Tris, 0.1 M NaCl (1 h centrifugation at $100{,}000 \times g$) and resuspended in 0.1 M sodium phosphate (pH 7.5). To isolate PS I, the membranes were adjusted to 0.5 mg/ml of chlorophyll, 0.2% Triton, stirred for 30 min and centrifuged for 1 h at $100{,}000 \times g$. The pellet was suspended in 0.1 M phosphate (pH 7.5), 1% Triton, 0.5 M KCl to about 1 mg/ml of chlorophyll, stirred for 2 h and centrifuged at $48{,}000 \times g$ for 2 h, the supernatant dialyzed against 20 mM phosphate (pH 7.5), 0.1% Triton, 0.25 mM EDTA and applied to a column of DE-52 equilibrated with this buffer. The photosystem was eluted with a linear phosphate gradient from 20 to 200 mM (pH 7.5, containing 0.1% Triton, 0.25 mM EDTA). P700-containing fractions were combined, made 0.3% in sodium desoxycholate, and a saturated solution of $(NH_4)_2SO_4$ was added to 35% saturation. The pellet was suspended in and dialyzed against 20 mM Tris, 0.1% Triton and applied to a sucrose step gradient (6 steps from 10 to 35% sucrose in this buffer), from which PS I was recovered after 15 h centrifugation at 38,000 rpm (Ti 60-rotor from Beckman) and dialyzed.

Preparation of P700-reaction centers

A published procedure [15] was followed for both organisms, which employs a mild SDS treatment and sucrose density gradient centrifugation.

Chlorophyll was quantified as in [16] for *Anacystis* and as in [17] for *Euglena*. P700 was quantified by optical redox difference spectroscopy (ascorbate-reduced minus ferricyanide-oxidized) as in [18] using the extinction coefficients given by Hiyama and Ke [19] for the peak near 700 nm. Membranes were solubilized at a chlorophyll concentration of about 40 μg/ml in 0.1 M Tris, 0.2% Triton X-100, 0.5 M KCl for 15 min at room temperature before measurement.

EPR spectra were recorded on a Bruker ER 220 D X-band spectrometer equipped with a double resonance cavity essentially as described [20]. The 1,1-diphenyl-2-picrylhydrazyl radical was used as a standard.

Quinone analysis

Total lipids were solubilized from the various samples by repeated extraction [21] and applied to TLC plates (silica gel 60 F_{254} from Merck). In the case of *Anacystis*, plates were developed with benzene [22], inspected under UV light and the bands with R_f = 0.05 (5′-HOP) and 0.47 (phylloquinone and plastoquinone-9) were eluted with methanol. In the case of *Euglena*, this TLC system did not purify 5′-HOP well. Instead, the plates were developed with 30% (v/v) diethyl ether in petroleum ether (40–60 °C, Merck). The bands corresponding to 5′-HOP, phylloquinone and plastoquinone-9 (R_f values of 0.20,

0.54 and 0.58, respectively) were eluted. The quinones were further purified and quantified by reversed phase HPLC. Phylloquinone and plastoquinone were analyzed as in [3]. 5′-HOP was eluted from the same column with methanol : H_2O (90:10 by volume) with a retention time of 12 min. For the quantification of 5′-HOP, the extinction coefficient given for phylloquinone (14.9 $mM^{-1}cm^{-1}$ at 270 nm, ref. [23]) was used. Both naphthoquinones have identical UV spectra. Mass spectra were recorded on a MAT 112 S instrument.

Results and Discussion

The chlorophyll to P700 ratios of thylakoids, PS I and P700-reaction centers isolated from *Anacystis* and from *Euglena* are given in Table I. The ratio for the PS I preparation from *Euglena* is typical for preparations from which the antenna (LHC I) has been removed [24]. This is also reflected in the Chl *a* to Chl *b* ratio (Table I). The photosystem from *Anacystis* contained dominant polypeptides with apparent molecular masses of 64, 18.5, 18, 15.2 and 14.8 kDa (as determined by SDS-PAGE, not shown), *i.e.* the polypeptides usually found in PS I preparations from cyanobacteria (see ref. [24]). Photosystem I from *Euglena* contained additional polypeptides of 47, 41 and 29 kDa, of which the 47 and 41 kDa proteins (in addition to those at 65 kDa [15, 25]) were retained in the P700-reaction center. It is, at present, not known whether these additional polypeptides are contaminants, degradation products or true components of PS I of *Euglena*.

The quinone analysis of the various samples is shown in Table II. For both *Anacystis* and *Euglena*, the naphthoquinone present in high amounts was identified as 5′-monohydroxyphylloquinone, as already shown for whole cells [10]; its mass spectrum was identical to that previously reported ([10], data not shown).

Table I. Purification of photosystem I and the P700-reaction center from *Anacystis nidulans* and *Euglena gracilis*. The purification steps are described in Materials and Methods. Chlorophyll *a*/chlorophyll *b* ratios were determined as in [34].

Step	Chl/P700 [mol/mol] *Anacystis*	*Euglena*	Chl *a*/Chl *b* *Euglena*
Thylakoids	167	526	9.8
Photosystem I	108	64	63
P700-reaction center	67	65	n.d.

n.d., not determined; Chl, chlorophyll.

Table II. Quinone to P700 stoichiometries of thylakoid membranes and photosystem I preparations from *Anacystis nidulans* and *Euglena gracilis*. Uncorrected values are given. The yield of the individual quinones, carried through the whole assay procedure (see Materials and Methods), was 80% in each case. The results for *Euglena* are from a photoautotrophic culture; photoheterotrophic cultures gave similar results.

Sample	Quinone/P700 [mol/mol] 5′-Monohydroxy-phylloquinone	Phyllo-quinone	Plasto-quinone-9
Anacystis			
Thylakoids	2.9	0.37	4.6
Photosystem I	2.05	0.35	0.40
P700-reaction center	1.0	0.07	<0.05
Euglena			
Thylakoids	0.83	0.03[a]	7.7
Photosystem I	0.78	0.03[a]	<0.05
P700-reaction center	0.92	<0.05	<0.05

[a] Determined in a different experiment (see text).

The quinone content of thylakoids of *Anacystis* has been determined by Omata and Murata [22], who reported proportions very similar to those given in Table II. As was observed [3] for phylloquinone in the case of another cyanobacterium, *Anabaena variabilis*, thylakoids of *Anacystis* contain 5′-HOP in excess to that recovered with PS I (Table II); as postulated for phylloquinone [26], this "extra" 5′-HOP may have a function in respiratory electron transport, which is, at least in part, located on the cyanobacterial thylakoids (see ref. [27]). About two molecules of 5′-HOP co-purify with PS I, as was found for phylloquinone in other organisms [1–4]. Phylloquinone is also present in PS I of *Anacystis*, but in substoichiometric amounts (less that one per PS I). It is, therefore, unlikely to have a functional role. The mild SDS-treatment used to prepare P700-reaction centers [15] removed one of the 5′-HOP molecules (Table II), whereas in spinach and *Anabaena variabilis* both phylloquinones were recovered in that subcomplex [3]. Since one of the naphthoquinone molecules is more loosely bound to PS I than the other one [4, 28], it is probably this loosely bound one which is lost.

The more unusual case is *Euglena* (Table II). Thylakoids, photosystem I and the P700-reaction center contain only about one 5′-HOP per P700. Because of

this surprising result, the thylakoids and PS I were carefully analyzed for naphthoquinones not only by TLC (see Methods), but also by applying large amounts of lipid extracts to columns of aluminum oxide that were eluted stepwise with increasing amounts of diethyl ether in petroleum ether (40–60 °C), similar to the method described by Threlfall and Goodwin [29]. The individual fractions were assayed for naphthoquinones by TLC and HPLC (as in Methods) and by UV-redox difference spectroscopy. In addition to 5′-HOP (which eluted at 10% diethyl ether), two other naphthoquinones were detected, both eluted with 5% diethyl ether. On silica gel 60 plates developed with 30% diethyl ether in petroleum ether, they had R_f values of 0.54 (identified as phylloquinone) and of 0.50 (structure unknown, tentatively identified as menaquinone-6 on the basis of its mass spectrum). Both quinones are present in very low amounts (about 0.03 of each per P700, see Table II), which is probably the reason why they had not been detected previously [10]. Other naphthoquinones were not found.

Since the photosystem of *Euglena* shows this 1:1 stoichiometry, we recorded, and tried to quantify, the EPR spectra of its early electron acceptors A_0 and A_1, reduced by a photoaccumulation technique [30–32], as detailed in [20]. The spectra are shown in Fig. 1. The sample in the EPR tube was first illuminated at −78 °C until the spectrum was maximal. Its features are typical of the asymmetric EPR spectrum of center A_1, with a *g*-value of 2.0043 and a peak-to-peak line width of 1.02 mT (trace A_1^-). It should be noted here, that there is some doubt about the assignment of this EPR spectrum (see [9, 20]). Further illumination as before, but at −44 °C, resulted in the spectrum of trace $A_1^- + A_0^-$, with a more symmetric line shape, a *g*-value of 2.0033 and a line width of 1.35 mT. This spectrum is believed to reflect reduced centers A_1 plus A_0. Integration of the spectra (traces A_1^- and $A_1^- + A_0^-$, recorded at low microwave power, *cf.* ref. [30]) showed EPR centers A_1 and A_0 to be present in a ratio of about 1:1, as in PS I samples from spinach [30], which contain two phylloquinone molecules.

Several explanations are possible for the stoichiometry of one naphthoquinone per PS I in *Euglena*. (i) Two types of PS I are present, one with two naphthoquinones, the other one, of unknown function, without naphthoquinones. (ii) The second quinone is a benzoquinone. We did, however, not detect plastoquinone in the PS I preparations. (iii) The stoichiometry is truly 1:1. This is not so unlikely. As extraction [4, 28] as well as UV destruction [33] experiments have shown, the presence of only one naphthoquinone is sufficient for photosynthetic reduction of soluble ferredoxin.

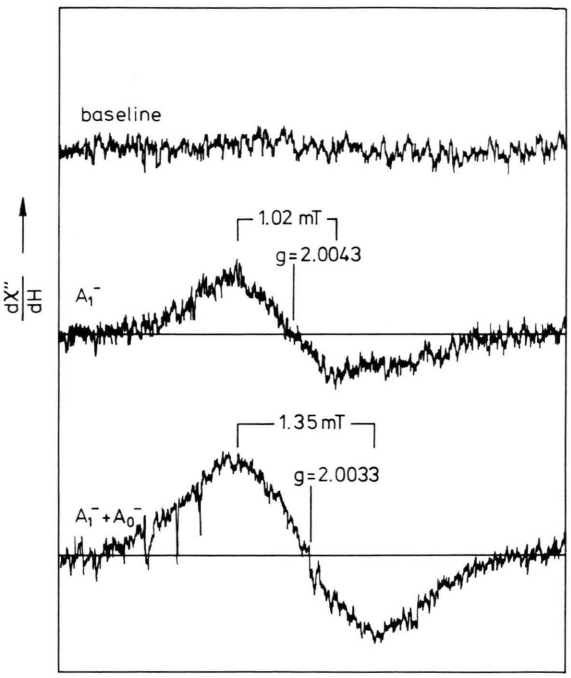

Fig. 1. X-band EPR spectra, in the $g = 2.0$ region, of photosystem I from *Euglena gracilis* illuminated at cryogenic temperatures. For technical details, see ref. [20]. Baseline: illuminated at 0 °C (to reduce the Fe–S centers), then frozen in the dark. A_1^-: illuminated further at −78 °C. $A_1^- + A_0^-$: illuminated further at −44 °C. The samples contained 7.5 μM photosystem I (P700) in 0.1 M glycine (pH 10.2 with NaOH), 60% glycerol, 10 mM sodium dithionite. Conditions of EPR spectroscopy: microwave power, 63 μW; microwave frequency, 9.48 GHz; field modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; scan range shown, 5 mT; field center, 338 mT; instrument gain, 1×10^6; T = 77 K.

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