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Review

Modification of photosystem I reaction center by the extraction and exchange of chlorophylls and quinones

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Abstract

The photosystem (PS) I photosynthetic reaction center was modified through the selective extraction and exchange of chlorophylls and quinones. Extraction of lyophilized photosystem I complex with diethyl ether depleted more than 90% chlorophyll (Chl) molecules bound to the complex, preserving the photochemical electron transfer activity from the primary electron donor P700 to the acceptor chlorophyll A₀. The treatment extracted all the carotenoids and the secondary acceptor phylloquinone (A₁), and produced a PS I reaction center that contains nine molecules of Chls including P700 and A₀, and three Fe-S clusters (F_X, F_A and F_B). The ether-extracted PS I complex showed fast electron transfer from P700 to A₀ as it is, and to FeS clusters if phylloquinone or an appropriate artificial quinone was reconstituted as A₁. The ether-extracted PS I enabled accurate detection of the primary photoreactions with little disturbance from the absorbance changes of the bulk pigments. The quinone reconstitution created the new reactions between the artificial cofactors and the intrinsic components with altered energy gaps. We review the studies done in the ether-extracted PS I complex including chlorophyll forms of the core moiety of PS I, fluorescence of P700, reaction rate between A₀ and reconstituted A₁, and the fast electron transfer from P700 to A₀. Natural exchange of chlorophyll *a* to 710–740 nm absorbing chlorophyll *d* in PS I of the newly found cyanobacteria-like organism *Acaryochloris marina* was also reviewed. Based on the results of exchange studies in different systems, designs of photosynthetic reaction centers are discussed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Electron transfer; Chlorophyll; Quinone; P700; Photosystem I; Reaction center

1. Introduction

The photosystem (PS) I reaction center (RC) of

Abbreviations: A₀, primary electron acceptor of photosystem I; A₁, secondary electron acceptor phylloquinone; Chl, chlorophyll; BChl, bacteriochlorophyll; P700, primary electron donor of photosystem I; PS, photosystem; VK, vitamin K; ws, water saturation

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oxygenic photosynthesis of plants and cyanobacteria is a pigment–protein complex made of two essentially homologous core polypeptides named PsaA and PsaB and makes up ‘so-called heterodimer RC’ [1,2]. The complex contains more than 100 molecules of chlorophyll (Chl) together with carotenoids, phylloquinone and iron-sulfur center F_X. Two additional iron-sulfur centers F_A and F_B are located on the PsaC subunit bound to the RC surface. Fig. 1 (inset) shows the cofactor arrangement in the core moiety of

PS I revealed by the X-ray crystallography of PS I RC of a thermophilic cyanobacterium *Synechococcus elongatus* [3–5]. RC structures having FeS centers are also assumed for the RCs of heliobacteria and green sulfur bacteria that are grouped into type I RC together with PS I RC. The heliobacterial RC, which is a homodimer of two PshA subunit polypeptides, and the green sulfur bacterial RC, which is a homodimer of two PscA polypeptides, bind 25–30 bacteriochlorophylls (BChl) *g* and *a*, respectively (see Table 1 and reviews by Feiler and Hauska [6] and Klukas et al. [5]). All these polypeptides have 11 membrane spanning helices and are partially homologous to each other in the amino acid sequence. The structures with a large number of pigments are thus a common feature of the type I RCs. The majority of the pigments seem to serve as antenna and transfer excitation energy to the primary electron donor situated in the central core moiety. This makes a clear contrast to that of type II RCs of purple bacteria and photosystem II of plant/cyanobacteria that bind only six pigments on the heterodimer made of PufM/PufL or D1/D2 polypeptides, respectively (see review by Satoh [7]).

Fig. 1 shows the energy levels and reaction times of the electron transfer cofactors in the type II RC complex of a purple bacterium *Rhodospira rubra* and in PS I RC of plant/cyanobacteria according to Iwaki and Itoh [8]. The cofactor arrangement in the core moiety of PS I RC seems to be partially homologous to that of the RC of purple bacteria shown in Fig. 1, suggesting the common origin of the two types of RCs [5]. However, the energy levels and the reaction times of the electron transfer cofactors are significantly different from each other. A striking feature of the electron transfer in the *Rb. sphaeroides* RC is the lack of FeS clusters and the reaction of ubiquinone Q_A that stabilizes the reducing power after reaction with bacteriopheophytin *a* with a large energy gap of 0.6 eV (Fig. 1). In PS I, on the other hand, phylloquinone that also functions as the secondary electron acceptor is reduced by the primary electron acceptor chlorophyll *a* (A_0) with a 10 times faster reaction time of 23 ps with almost a half extent of energy gap of 0.35 eV. The reducing power finally produced by the two types of RCs are, therefore, significantly different from each other.

If the type I and II RCs had evolved from a com-

Table 1

Ether treatment of membrane fragments from spinach, cyanobacterium and heliobacterium

Components	H ₂ O content in ether (% saturation)	Organisms		
		Spinach	Cyanobacterium ^a	Heliobacterium ^b
Chlorophyll <i>a</i> remained (%)	0	40	40.5	83
	50	12	13.5	15
	100	4.7	3.8	1.1
Chl ^c /P ^d ratio (mol/mol)	untreated	145	150	25
	0	56	58	21
	50	16	20	14
	100	11	14	46
Quinones/P ratio (mol/mol)	untreated	2.2 ^e	2.9 ^e	2.8 ^f
	50	0	0	0
Carotenoids/P ratio (mol/mol)	untreated	20	25	2.2 ^g
	50	0	0	0

Pigments and quinones were extracted with ether containing water at 0, 50 and 100% saturation, respectively. Data modified from Ikegami and Katoh [16,41], Kobayashi et al. [87] and Kleinherrbrink et al. [75]. The table is made after [10].

^aA thermophilic cyanobacterium, *S. elongatus*.

^b*H. chlorum*.

^cor BChl *g*.

^dor P798.

^eVK₁.

^fVK₂.

^gNeurosporene.

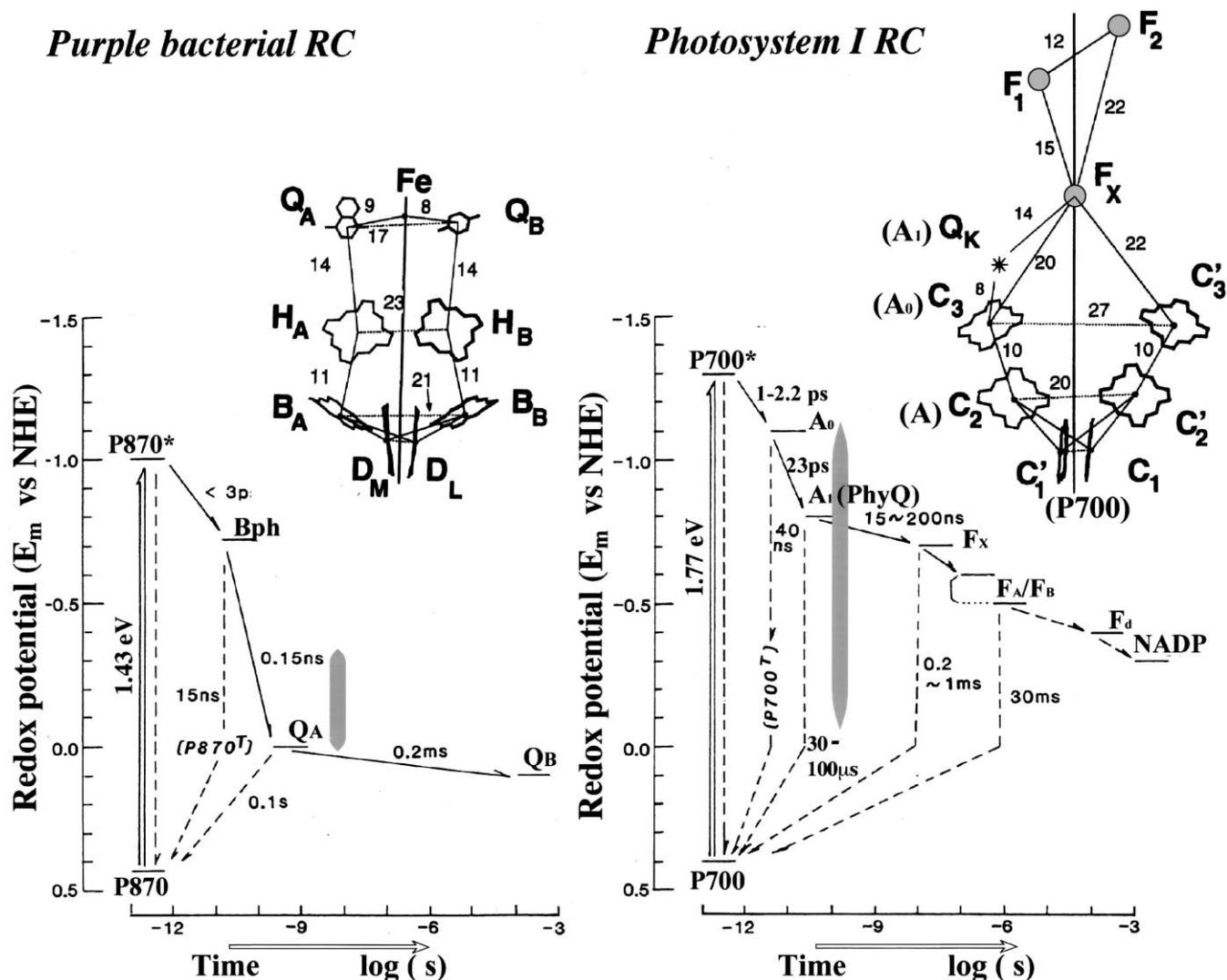


Fig. 1. Energy levels of electron transfer components plotted against reaction times of the reaction center complexes of purple bacteria and photosystem I. Energy levels are shown against the logarithms of the reaction times represented in a unit of seconds [8]. Left, in RC complex of *Rb. sphaeroides*. Right, in photosystem I. Gray zones show the ranges of redox levels of Q_A [37] and A_1 [36], respectively, modified by the reconstitution of artificial quinone/quinonoid compounds. Insert structures represent the arrangements of cofactors in RC complex of *Blastochloris viridis* (*Rhodospseudomonas viridis*) and *S. elongatus* PS I drawn after [5]. Numbers indicate distance between cofactors. D, B, H and Q represent donor BChl *a*, accessory BChl *a*, bacteriopheophytin *a* and electron acceptor ubiquinones, respectively. C_{1-3} and Q_K represent Chl *a* and phylloquinone, respectively.

mon ancestral RC in the early era of photosynthetic evolution as suggested [5], then we might be able to exchange some of their features artificially. Although the means for the alteration of RCs are still very limited, the ether extraction initiated by Ikegami and Katoh [9] has been a powerful modification method of PS I RC as reviewed recently [10]. Ether extraction allows the modification of PS I RC from two aspects: (1) the extraction of most of the periph-

eral antenna pigments bound to PS I RC as originally indicated by Ikegami and Katoh [9] and (2) the replacement of the electron acceptor phylloquinone by the artificial quinones as initiated by the works of Iwaki et al. [11]. The severe extraction of antenna pigments can be attained only by the ether extraction method [10]. The PS I acceptor phylloquinone was extracted by ether extraction by Itoh et al. [12], and by a hexane/methanol mixture by Biggins and Ma-

this [13] without damaging FeS clusters. The variety of quinone analogues was shown to be introduced into PS I RC after extraction with ether [11] or hexane/methanol by Biggins [14] and by Snyder et al. [15]. The PS I phylloquinone was further shown to be exchangeable by the incubation of PS I RC at elevated temperatures by Rustandi et al. [16] and Ostafin and Weber [17] and by the *in vivo* system using a phylloquinone-depleted mutant of cyanobacteria by Johnson et al. [18] and Zybailov et al. [19]. Although each method of quinone exchange differs to some extent, they gave almost consistent results as for the location of the quinone binding site [19–23]. The exchange with artificial quinones after ether extraction produced PS I RC containing a variety of quinone/quinonoid molecules that cover a wide range of redox level as shown by a gray zone in Fig. 1, exactly at the A_1 site. The redox potential value of reconstituted A_1 shows a 3 times wider range than that attained in the exchange of Q_A ubiquinone in *Rb. sphaeroides* RC and is one of the widest among those ever attained by the artificial modification of biological electron transfer systems.

This review focuses on the studies of the extraction and exchange of Chl and quinones carried out mainly on the ether-extracted PS I RC. Please see a recent review [10] for the details of the ether extraction method itself and reviews [1–6] as well as those in this special issue for the other details of the PS I RC. Natural exchange of chlorophyll in PS I RC of the recently identified oxygenic organism *Acaryochloris marina* [24] is also discussed. Based on the results obtained by the quinone and chlorophyll exchange studies, the basic design of the reaction center is also discussed.

2. Modification of PS I RC by the extraction of chlorophylls

2.1. Overview of ether extraction

Elimination of the large number of antenna chlorophylls from PS I might be a possible way to convert PS I to mimic type II RCs. The large number of Chls on the type I RC complex, on the other hand, has been one of the major difficulties in the spectroscopic studies of electron and energy transfer (see reviews

on PS I by Brettel [2], Golbeck [1,25] and Malkin [26]). Selective eliminations of antenna pigments have been attempted by treatment with detergents (see review by Malkin [26]) or with organic solvents [9]. Although the detergent treatment reduced the number of Chls bound to RC to about 30 Chls/P700, it also destroyed iron-sulfur centers [27]. The extraction method with diethyl ether containing a small amount of water or other organic solvent invented by Ikegami [9] was more successful in decreasing the number of Chls and produced the PS I RC with a total number of Chls as low as 9/P700 (Fig. 2), preserving apparently intact charge separation activity [28,29]. Ten years after the invention of the ether extraction method, Itoh et al. [12] found that

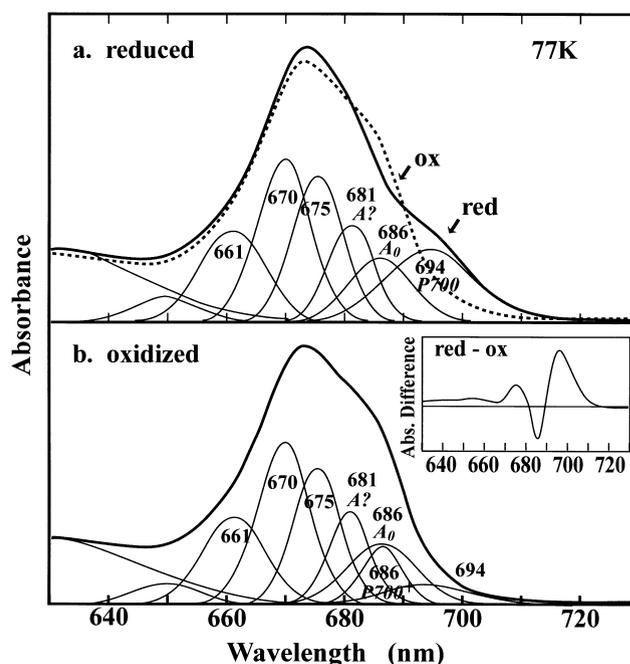


Fig. 2. 77 K absorption spectra of ether/acetalddehyde-extracted PS I RC that has nine Chl *a*/P700. PS I particles from spinach were extracted with a mixture of dried ether (47.5%), water-saturated ether (47.5%) and acetalddehyde (5%). The spectra in the reduced state (red) were measured in the presence of 5 mM ascorbate (a) and those in the oxidized state (ox) in the presence of 0.2 mM ferricyanide (b) at 77 K in the presence of 60% glycerol. Thick solid lines represent the observed data and thin lines represent the components' absorption bands of each chlorophyll form estimated by a Gaussian deconvolution program. The absorption band due to A_0 was introduced according to the result of difference absorption measurement. The peaks of major component bands were also adjusted to interpret fluorescence and CD results. Insert presents the reduced-minus-oxidized difference spectrum. Drawn after [39].

the ether-extracted PS I RC lacks phylloquinone and requires the reconstitution of an appropriate quinone or quinonoid compound to regain its full activity.

The ether-extracted PS I demonstrated that the pigments extractable from the RC complex are necessary neither for the charge separation nor for the gross structural integrity of RC, suggesting their location apart from the essential core of the RC [9,10]. The X-ray analysis of crystal structure [3–5] indicated the distant locations of antenna Chls forming a large circle around the pigment-sparse central core moiety in which six Chl *a* molecules (core Chls) are held at the interphase between PsaA and PsaB polypeptides (see Fig. 1). The core Chls are arranged in a C2 symmetry forming two almost equivalent branches that resemble those of BChl *a* and bacteriopheophytin *a* in the purple bacterial RC complex [30]. The special pair of Chl *a* (P700) gives an electron to the electron acceptor Chl *a* (A_0) via accessory monomer Chl *a* (*A*), and then to the secondary acceptor phylloquinone (A_1), the iron-sulfur center F_X , and to the iron-sulfur centers F_A and F_B situated on the peripheral PsaC protein. Only one of two branches of cofactors seems to be active in the electron transfer from P700 to F_X since only one phylloquinone is necessary for the full activity of the electron transfer as shown by reconstitution [31] and extraction [27,32]. The other branch might be inactive or function with low efficiency as pointed out by Joliot and Joliot [33]. Two other Chls, designated connecting Chl *a*, also exist at symmetrical positions outside of the core Chls shown in Fig. 1, and seem to mediate excitation energy transfer from the Chls in the peripheral circle [3–5]. However, the spectral features and locations of Chl molecules other than P700 and A_0 in the PS I RC have not been assigned yet. We also tried to assign some of them in this review.

The cofactors in the reducing side of photosystem I (A_0 , phylloquinone and FeS centers) differ from those (pheophytins and quinones) of type II RCs and produce significantly higher reducing power compared to the latter. Direct evidence for the participation of phylloquinone in PS I electron transfer was revealed by the extraction and reconstitution of phylloquinone by the ether extraction of spinach PS I [12], or by the hexane–methanol extraction of cya-

nobacterial PS I as shown by Biggins and Mathis [13]. The success of reconstitution opened a chance for Iwaki and co-workers [11,34–36] to directly test the electron transfer mechanism in the PS I reaction center through the reconstitution of artificial quinones or quinonoid compounds with a variety of molecular structure and redox properties in a way similar to those done in the detergent-treated purple bacterial RC by Gunner et al. [37]. The antenna-depleted PS I also suits for the precise measurement of the primary charge separation as revealed by a recent observation of the ultra-fast process studied by Kumazaki et al. [38], who detected the rapid electron transfer from P700 to *A* and to A_0 with 0.8 ps and 2–3 ps reaction times, respectively.

The ether extraction and quinone reconstitution studies have indicated that more than 90% of Chls bound to PS I RC can be depleted and that various artificial compounds can replace the role of low potential phylloquinone. The PS I RC with a smaller content of Chl and a positive potential quinone then is expected to show a functional property similar to that of type II RC. The newly found organism *A. marina* [24] further presented us a natural example of exchange of chlorophyll since this organism uses chlorophyll *d* both for antenna and the primary electron donor chlorophyll P740, which absorbs at 700–740 nm.

2.2. Experimental procedure of the ether extraction method

Various organic solvents with high dielectric constants extract Chl from PS I RC, but usually with poor selectivity [39]. Diethyl ether, designated ‘ether’ hereafter, was most effective in the selective extraction of pigments from PS I [9]. A remarkable feature of ether is to dissolve a small amount of water. The effective dielectric constant of ether thus can be varied by changing the water saturation (ws) level as reviewed recently [10]. We can extract more Chl with ether at the higher ws level from the fully dried PS I preparation. Ether containing a small amount of acetaldehyde (5%) more extensively extracted chlorophylls as shown in Fig. 2 [29], although the RC structure became unstable after this harsh treatment.

2.2.1. Preparation of diethyl ether at different ws levels

The ws level can be freely adjusted by mixing desiccant-dried and water-saturated ether together at a different ratio. To prepare 100% ws diethyl ether, add 50 ml distilled water to a newly opened 500 ml bottle of diethyl ether. The 100% ws ether contains about 1% (v/v) water at 4°C. Add 100 g anhydrous Na₂SO₄ to another 500 ml bottle of ether to prepare 0% ws diethyl ether. By mixing appropriate volumes of diethyl ether from the two bottles, diethyl ether at the desired ws level can be prepared just before the extraction.

2.2.2. Preparation of PS I RC or membranes suitable for ether extraction

Photosynthetic membranes or PS I particles or RC complex obtained by detergent treatment should be repeatedly washed with distilled water to fully remove salt and detergent, and then lyophilized completely (e.g. 50 h) before ether extraction. Complete drying through a careful long lyophilization (usually more than 2 days) is a key for reproducible extraction. Aliquots of the lyophilized materials were packed in folded paper to give about 10 mg for each packet and stored in an N₂ gas filled glass tube at –80°C until use.

The activities and yields of the materials after ether extraction were highly dependent on the detergents used for their preparation. The pigments in material treated with Triton X-100 or dodecyl maltoside were not efficiently extracted and resulted in less enrichment of P700. Detergents that could not be fully removed by subsequent washings seem to prevent the effective extraction of pigments. Digitonin was found to be the best detergent to be used for the isolation of PS I particles used for ether extraction.

The efficiency of pigment extraction by ether extraction also varied depending on the source of materials or organisms (Table 2). P700 in PS I RC of the mesophilic cyanobacterium *Synechococcus* PCC6301 [40] or the thermophilic cyanobacterium *S. elongatus* [41] was less resistant to ether treatment than that in spinach PS I particles. The ether treatment of the PS I particles of these organisms thus resulted in less enrichment of P700.

2.2.3. Protocol of ether extraction

To prevent a change in the ws level of ether, attention should be paid to the humidity and temperature (less than 5°C) during the whole extraction procedure. About 10 mg of the lyophilized materials was put in a metal centrifugation tube capped with aluminum foil and 30 ml of diethyl ether at the desired ws levels at 5°C were added. The materials were carefully suspended in ether with a small painting brush (its resistance against ether should be checked before use!). The pigment extracted into ether supernatant was then removed by centrifugation. The procedure was repeated by adding another 30 ml ether to the precipitate. The paint-green colored ether-extracted materials were then dried and suspended in 0.05 M Tris–Cl buffer at pH 7.5–10. If necessary, the extracted materials were solubilized with a buffer containing 0.05% Triton X-100 or sucrose monolaurate for 10 min and then insoluble materials were removed by centrifugation. The extracted materials can further be purified on a protein basis by fractionation with ammonium sulfate and by ultracentrifugation on a sucrose density gradient to yield PS I core complex made of PsaA/B, C polypeptides as shown by Ikegami and Ke [42,43]. During the solubilization or purification of the ether-extracted materials, attention should be paid to the decrease of heat instability as the extracted Chl (unstable above 10°C) increases.

2.3. Components extracted by ether treatment

2.3.1. Chlorophylls

Table 1 summarizes the effects of ether extraction on various components in PS I and PS II. The molar ratio of total Chl *a*/P700 in the ether-extracted PS I particles decreased as the ws level in ether increases [9]. A ratio of 11 was attained by the treatment of digitonin-treated spinach PS I particles with 80% ws ether. At a higher ws level the ratio did not change anymore due to the parallel loss of P700 (Table 1). Using a mixture of ether and acetaldehyde instead of water [39], PS I particles with a total Chl *a*/P700 ratio of 9 were prepared (Fig. 2). The higher resistance of P700 to the extraction suggests their environment in the PS I core to be somewhat different from those of peripheral antenna Chls. Six to eight Chls other than P700 were also more resistant and

might be either in the core moiety or in some other special environment. Ether at a low ws level preferentially extracts 700–710 nm forms of Chl *a* (Chl-705) (Fig. 2) [43,44] that give characteristic fluorescence peaks at around 730 nm.

Most of Chls *a* and *b* and pheophytin *a* bound to light harvesting complex (LHC) II or PS II RC complex were also easily extracted by the ether treatment of thylakoid membranes, so that the PS II components could not be enriched (Ikegami, unpublished data). Chl *b* in LHCI contained in PS I particles was resistant to the 0% ws ether extraction and the extraction with the 80% ws ether produced a PS I RC with a Chl *b*/P700 ratio of less than unity [42].

2.3.2. Phylloquinone

Two molecules of phylloquinone bound to PS I RC, one of which functions as the secondary acceptor A_1 , were extracted with ether at any ws level. The phylloquinone can be fully reconstituted into the ether-extracted spinach PS I particles [12]. Similar results were obtained by the extraction of PS I RC with a hexane/methanol mixture in cyanobacterial PS I by Biggins and Mathis [13].

2.3.3. Iron-sulfur centers and polypeptides

Ether extraction does not extract F_X and F_A/F_B [12,32], although their EPR signals were a little modified presumably due to the structural changes of proteins induced by the loss of Chls and lipids. No removal of more than 10 subunit polypeptides was detected after ether treatment of the PS I complex prepared with digitonin [43,45]. No critical damage of PsaA/B or PsaC polypeptides seemed to be induced by ether extraction since the high activity of photoinduced electron transfer from P700 to A_1 -phylloquinone, F_X and F_A/F_B was recovered by the reconstitution of one phylloquinone/PS I RC [46].

2.3.4. Carotenoids

Carotenoids were completely extracted with ether at any ws level [9,41], including a 15-*cis*- β -carotene that is suggested to be present in the PS I core [47]. In the ether-extracted PS I that has no carotenoids and phylloquinone, light excitation produces the triplet state of P700 ($P700^T$) that was formed by the charge recombination between $P700^+$ and A_0^- at a yield of about 30% at 230 K. The lifetime of $P700^T$

(40 μ s) was further elongated to 600 μ s by the removal of oxygen. This seems to be a loss of $P700^T$ quenching by carotenoids since the $P700^T$ lifetime was short (6 μ s) and insensitive to the oxygen concentration in intact PS I RC [32]. Most lipids are extracted with ether, but a few percent of lipids are still bound to the RC (Ikegami, unpublished results).

3. Functional studies of the ether-extracted PS I

3.1. Spectral profiles of P700 and A_0

P700 remains photochemically active even after ether treatment, suggesting that the ether-extracted PS I contained most of the Chls in the central core at their original locations. The reduced-minus-oxidized difference spectrum of P700 was determined either by chemical reduction/oxidation with ascorbate/ferricyanide or by light-induced absorption change (Fig. 2). A_0 was determined by laser flash photolysis of the ether-extracted PS I by Mathis et al. [48], in which A_0^- had a long lifetime of 40 ns due to the loss of A_1 phylloquinone, and decayed in the charge recombination reaction with $P700^+$ [48,49]. The difference spectrum of A_0^-/A_0 obtained as the difference between the transient spectrum after laser excitation and that of $P700^+/P700$ measured under continuous illumination showed a narrow bleaching of A_0 around 690 nm at 250 K. Most of P700 and A_0 were photoactive. The extinction coefficient of A_0 was estimated to be 80 $\text{mM}^{-1} \text{cm}^{-1}$. Since no strong derivative-shaped circular dichroism (CD) signal was detected around 690 nm in this preparation except that of P700 [50], A_0 can be assumed to have a monomer-like character.

In ordinary PS I preparations, the absorption band of P700 in the whole spectrum cannot be recognized because of the large amount of other Chls. In the PS I particles obtained by the extraction with 80% ws ether or an acetaldehyde–ether mixture, the contribution of P700 absorption bands was clearly recognizable in the whole spectrum (Fig. 2). A deconvolution of the spectra measured in the 9Chl/P700 preparation under the P700-reduced and -oxidized conditions revealed the difference spectrum of P700 (Fig. 2) with peaks at 694 and 687 nm, respectively, at 77 K [39].

The wide bandwidth of P700 indicates it to be a special pair of Chl *a*. The spectrum of the oxidized P700 (gray zone in Fig. 2b) estimated by assuming almost invariant absorption bands for the other Chls, showed a peak at 687 nm with a narrower bandwidth. The main and the satellite negative peaks in the P700⁺/P700 difference spectrum thus were simulated as the difference between the disappearance of the reduced form and the appearance of a new band accompanied with the oxidized form of P700 and a small shift of 681 and 670 nm Chl forms. The results thus suggest the oxidized P700 to be responsible for the 687 nm Qy absorption band. In the oxidized state of P700, ENDOR studies indicated that about 80% of unpaired electronic spin is located on one of the two Chls *a* [51,52]. The narrow 687 nm band that appeared in the P700 oxidized state thus is ascribed to the absorption of P700⁺ in which electronic coupling between the two constituent Chls *a* is weak and the unpaired spin density is almost localized on one Chl *a*. Another interpretation for the narrow 687 nm band estimated in the P700 oxidized condition might be that the 687 nm band represents a part of electrochromic shift of Chls (681 or 686 nm form) nearby P700 as was the case with the accessory BChls in the purple bacterial RC complex. However, simulation of the spectrum was rather difficult in this line. The dimeric nature of P700 was also clearly shown by the derivative-shaped difference CD spectrum of P700 obtained in the 11Chl/P700 particles [50] that confirmed the CD measurement in the ordinary PS I preparations [53,54]. It was also confirmed by the low temperature resonance Raman study of P700 measured by Moenne-Loccoz et al. [55], measurements of Stark effect by Krawczyk and Ikegami [56] or the weaker magnetic CD signal of P700 measured by Nozawa et al. [57] in the 11Chl/P700 PS I preparation.

Several Chl *a* derivatives (enol form [58], chlorinated Chl *a* [59] or stereo isomer Chl *a'* [60]) have been proposed as the chemical identity of P700. HPLC analysis of pigments by Kobayashi et al. [61] and Maeda et al. [62] detected about one Chl *a'*/P700, but no enol form or chlorinated Chl *a*, in the 11Chl/P700 particles. This directed Chl *a'* to be a component of P700.

3.2. Roles of Chls remained in the ether-extracted PS I particles

The absorption spectrum of 11Chl/P700 particles obtained by 80% ws ether can be fitted by the sum of four major Gaussian bands for Chls, that is, Chl-660, Chl-670, Chl-675, Chl-682 and Chl-686 with the areas in a ratio of about 1:3 (or 2):2:2:1, in addition to the two Chls (Chl-694) corresponding to P700 [50]. Curve analysis of the absorption spectra at 77 K of the ether-acetaldehyde extracted 9Chl/P700 particles (Fig. 2) obtained by Ikegami et al. [39] showed that the ratio of the area of each Gaussian band was nearly 1:2:2:1:1:2 for Chl forms with peaks at 661, 670, 675, 681, 686 (A₀) and 694 nm (P700). The depletion of two more Chls was attained by the ether-acetaldehyde mixture, one in Chl-682 and the other in Chl *a*-670, compared with the 11Chl/P700 particles. The depletion does not seem to affect the efficiency of primary charge separation.

Chl-686 is the most probable candidate for A₀, since the reduced-minus-oxidized difference spectrum of A₀ was shown to give a peak at 686 nm in the ether-extracted PS I [36,48]. The Qy absorption axis of Chl-686 was estimated to be tilted about 50° with respect to the membrane surface by linear dichroism study [65]. In the PS I structure, the counterpart of A₀ (A'₀) is known to be located at a site almost symmetrical to that of A₀ [3–5]. We can assign only one molecule of Chl-686 in the 77 K absorption spectrum so that its counterpart A'₀ seems to give a peak at different wavelength due to the different amino acid ligand or might be lost. A short wavelength form Chl-670 then can be a candidate for A'₀ since it was photobleached at 210 K under strong continuous illumination in the presence of dithionite in the 11Chl/P700 particles [42]. Chl-670 has a property similar to 'chlorophyll *a*-670' that was shown to be reduced in the photoaccumulation experiment in the cyanobacterial PS I complex under strong reducing conditions and once proposed to be a candidate for A₀ [63].

Chl-682 is a probable candidate for the accessory Chls (A) that are adjacent to P700 since it shows a red shift upon P700⁺ oxidation. Its counterpart A' would have a similar spectrum. The porphyrin ring of Chl-675 is shown to be oriented almost perpendicular to that of P700 by linear dichroism studies of

Breton and Ikegami [64] and Itoh et al. [65]. A small portion of Chl-675 was shown to be bleached upon the reduction with ferrocyanide, showing a redox midpoint potential a little more negative than that of P700 [28]. The large variety of the amplitude of the satellite band in the difference spectrum of P700 found in different preparations seems to be explained by the different contribution of this Chl on the P700 difference spectrum. The fluorescence lifetime (FI-679) of Chl-675 was 5–7 ns [66] (see Fig. 3), indicating no transfer of the excitation energy from this Chl to P700 [67]. Therefore, it is not yet clear whether Chl-675 is an intrinsic Chl form that was decoupled energetically from the other chlorophylls by the ether extraction or simply a Chl form produced by the ether treatment.

Chls other than Chl-675 on the 11Chl/P700 particles were found to efficiently equilibrate excitation energy with P700 within 1 ps [38] so that all these Chls seem to be situated in close vicinity to P700. Chlorophyll forms produced as the denaturation products of the intrinsic forms might also contribute to some extent to Chl-670 and/or Chl-662, because the degradation products of P700 give the 672 nm Chl form after treatment with SDS or heat (Iwaki et al., unpublished data).

3.3. Fluorescence of 11Chl/P700 particles and delayed fluorescence of P700

Ether at low ws level preferentially extracts 700–710 nm forms of Chl *a* (e.g. Chl-705) (as seen in Fig. 2) [41,43,67] that is known to give characteristic fluorescence peaks at around 730 nm at 77 K [68–70]. The excitation energy from the short wavelength chlorophylls seems to be trapped on the Chl-705 and, then, to P700 by uphill energy transfer [71–73]. Chl-705 was assumed to be located at the periphery of cyanobacterial PS I RC (cf. [73]). The removal of Chl-705 by ether extraction did not significantly affect the efficiency of primary charge separation in PS I.

The 11Chl/P700 particles emit fluorescence with major peaks at 679 (FI-679) and 695 nm (FI-695) at 280 K. The yield of FI-695 depends on the redox states of both P700 and the electron acceptors [44]. The emission peak of the long wavelength fluorescence shifted from 695 to 702 nm on cooling from

290 to 77 K as shown in Fig. 3 [66,67]. FI-695 can be detectable only when P700 is reduced. The intensity measured under continuous illumination increased a little more as the FeS clusters were reduced and significantly decreased by the prolonged illumination in the presence of dithionite [43]. As shown in Fig. 3, FI-695/702 shows multiexponential decay with the longest decay time of 30/100 ns at 280/77 K. The slow decay phase is ascribable to the delayed fluorescence produced in the charge recombination reaction between $P700^+$ and A_0^- that has a similar range of lifetime [66]. Iwaki et al. [67] analyzed excitation and emission spectra of FI-695/702 and showed that the peak shift occurs in parallel with the red shift of the absorption peak of P700 from 692 to 698 nm. No Chl absorbs at a wavelength longer than P700 in this

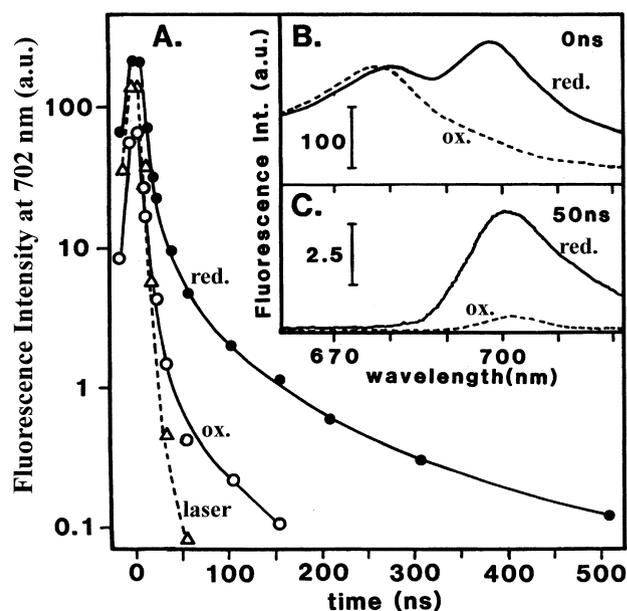


Fig. 3. Fluorescence decay in the 11Chl/P700 PS I particles at 77 K. (A) Time course of fluorescence intensity after laser excitation. (B,C) Time-resolved fluorescence spectra measured at 0 and 50 ns after the peak time of laser excitation, respectively under P700-reduced (red) and oxidized (ox) conditions. Vertical bars indicate the units of fluorescence intensity. 11Chl/P700 particles were dissolved in Tris-Cl, pH 7.0, 60% glycerol in the presence of 5 mM ascorbate (red) or 1 mM potassium ferrocyanide (ox). Each time-resolved fluorescence spectrum was measured with a spectrograph made of a combination of a 10 ns gating image intensifier, diode array and 30 cm monochromator. The gate of the image intensifier was opened at various times after excitation with a 10 ns 532 nm laser. Drawn after [66].

preparation so that the delayed fluorescence seems to be emitted by P700 itself [67]. Reconstitution of phyloquinone suppressed longer lived components of Fl-695 [66] as expected from its function to oxidize A_0^- with 23 ps. The PS I particles extracted with 0% w/v ether had a 30Chl/P700 ratio and contained a portion of long wavelength chlorophylls. This preparation emitted the delayed fluorescence at 720 nm instead of 702 nm at 77 K, indicating the energy transfer from P700 to the longer wavelength Chls (Itoh et al., unpublished results).

3.4. Dependence of heat stability of the reaction center on Chl content

PS I RC is known to show high stability against heat treatment. Heat treatment at 80°C for 5 min inactivates half of P700 in spinach PS I particles. Hoshina et al. [74] showed that heat treatment of PS I at 55°C and 65°C for 5 min in the presence of 50% ethylene glycol selectively destroyed F_A/F_B and F_X , respectively. The heat stability decreased along with the extraction of pigments from the PS I particles. The half-inactivation temperature of the spinach 11 Chl/P700 particles was about 32°C [75]. In thermophilic cyanobacterial PS I, heat treatment at 95°C for 5 min inactivated half of P700 and the half-inactivation temperature was lowered down to 37°C after ether extraction [76]. Therefore, the heat stability of PS I RC seems to be lost along with the depletion of antenna Chl *a* as easily assumed from its structure containing many Chls between the membrane spanning helices [5].

This was further evidenced by the recovery of the heat stability by the addition of Chl *a* and lipids to Chl-depleted particles [75]. Simple addition of Chl *a* had no appreciable effect on the heat stability. Interestingly, the heat stability of P700 was restored by the concomitant addition of phosphatidylglycerol (PG), or monogalactosyl diacylglycerol (MGDG) with less effectiveness, whereas phosphatidylcholine, phosphatidylinositol and digalactosyl diacylglycerol had only a minor effect. The restoration of the heat stability was proportional to the amount of rebound Chl *a* molecules that are also active as antenna pigments [76]. PG and MGDG, therefore, were predicted to bind at the specific sites around the reaction

center polypeptides to form a structure required for the proper binding of Chl *a* [75].

3.5. Excitation energy transfer and electron transfer in the ether-extracted PS I

The ether-extracted PS I particles have been a useful system to investigate the transfer of excitation energy due to the lack of an energy transfer process among peripheral antenna pigments. The loss of the secondary acceptor phyloquinone also stabilizes A_0^- and facilitates the study of energy transfer to P700. Direct excitation of P700 is also available.

Iwaki et al. [67] measured fluorescence excitation and emission spectra of 11Chl/P700 particles under the P700-reduced and -oxidized state at 77 K and indicated that Chl-675 did not transfer energy to P700 and that the other chlorophylls efficiently transfer energy to P700. Upon the ps excitation of the ether-extracted PS I particles at a flux density of less than one photon/P700, Kumazaki et al. [77] showed that the excitation energy was equilibrated among most of the Chls within 0.5 ps, and then used in the charge separation reaction between P700 and A_0 that occurs with a rate constant of 6.5 ps at 278 K. The results indicated that the charge separation is the rate-limiting step. Upon the selective excitation of P700, the excitation energy is transferred from P700 uphill to the other Chls within 1 ps, concomitant with the charge separation with an estimated time constant of 2.2 ps as suggested by the fluorescence up-conversion measurements [78]. The rate is close to the intrinsic (i.e. fastest) rate of the charge separation between P700 and A_0 that can be theoretically calculated. Kumazaki et al. [38] recently observed that about one third of the anion radical (A_0^- or A^-) absorption band at 740 nm rose with a rate constant of 0.8 ps at 280 K after selective excitation of P700. The time constant is significantly shorter than that of the primary charge separation in PS II and purple bacterial reaction centers (about 3.0 ps at 290 K) reported by Klug et al. [79] or Parson [80]. This fast rate was theoretically interpreted by assuming a strong electronic coupling between P700 and the nearest Chl (A) situated at an edge-to-edge distance of 4 Å with the assumption of optimal adjustment of energy gap and reorganization

energy according to Marcus' electron transfer theory [81]. The 4 Å edge-to-edge distance is shorter than the distance between the accessory BChl and the special pair (5.4–5.5 Å) in purple bacterial RCs shown in Fig. 1, where the primary charge separation proceeds with a rate constant of 2.5 ps [80]. The 0.8 ps phase thus seems to be ascribed to the electron transfer from P700 to A [38].

Chlorophylls re-added to the ether-extracted PS I RC were also shown to transfer excitation energy to P700 [41,82]. As described in the section on the heat stability of RC, the energy transfer from the re-added Chl becomes more efficient upon the co-addition of suitable lipids like PG. It is, therefore, suggested that the re-added Chls occupy some of the intrinsic Chl binding sites in PS I. The energy transfer rate and Chl binding site, however, have not been determined yet.

3.6. Ether extraction in other reaction centers

3.6.1. PS II reaction center

Ether extraction was also applied to study the arrangement of pigment in the PS II (D1-D2-cytochrome *b*-559) RC complex. Tomo et al. [83] showed that treatment with 30–50% water-saturated ether removed one (Chl-677) of six Chl *a* molecules and one (Car-489) of two β -carotenes selectively in the RC, so that 50% of the photoactivity remained. Reconstitution of artificial dibromothymoquinone (but not of intrinsic plastoquinone) into the Q_A site of purified PS II D1-D2-cytochrome *b*-559 RC complex, which lost intrinsic plastoquinone during the isolation procedure, recovered the stable oxidation of P680 [84].

3.6.2. *Heliobacterium* reaction center

The reaction center BChl *g* dimer (P798) in the type I RC complex of *Heliobacterium chlorum* was not sufficiently resistant to ether extraction [85] so that the total BChl *g*/P798 ratio was decreased to half by the ether extraction (see Table 1). The primary electron acceptor A₀ in this bacterium, which is presumably Chl *a*-like pigment with an absorption maximum at 670 nm [86,87], was resistant to ether extraction (unpublished data).

Menaquinone (vitamin K₂ (VK₂)) is the only quinone found in the *Heliobacterium* [88] and was ex-

tracted by ether extraction. After ether treatment, the kinetics of the flash-induced absorption change of P798 was not significantly altered, i.e. the photooxidized P798 decayed mostly in the ms time range as shown in the control membranes [85]. Therefore, menaquinone might not be an indispensable cofactor in the electron transfer chain of *Heliobacterium*, which is in contrast to the role of phyloquinone in PS I.

4. Modification of PS I by quinone exchange

4.1. Extraction of phyloquinone by ether extraction

Takahashi et al. [89] and Schoeder and Lockau [90] indicated the presence of two molecules of phyloquinone (VK₁) in PS I RC. One of them can be extracted with *n*-hexane or *n*-heptane with almost no effect on PS I electron transfer [27,32] so that its function is not known yet. The depletion of the remaining phyloquinone by detergent resulted in the inhibition of electron transfer to FeS centers. The two phyloquinones were completely extracted by 0–80% w/w ether (Table 1) or by a hexane/methanol mixture [13]. The extraction resulted in the elimination of the A₁⁻ EPR signal and blocked the electron flow from A₀ to the Fe-S centers [12]. This stabilized a radical pair state (P700⁺ A₀⁻) which decayed with a 30 ns decay time forming the P700^T state with a yield of 30%. The reconstitution of one phyloquinone fully recovered the activity [46]. These results suggested that electron transfer occurs only through one branch of cofactors in the core moiety of the PS I RC shown in Fig. 1.

4.2. Reconstitution of artificial quinones at the phyloquinone binding site

Iwaki and coworkers showed that a wide variety of artificial quinones (benzo-, naphtho-, anthraquinones) and quinonoid compounds (fluorenones, anthraldehyde or phenanthroline) bound at the A₁ site after the extraction of intrinsic phyloquinones by ether treatment [11,35,46]. Most of the quinone reconstitution studies were done with the 40–50% w/w ether extracted PS I that contains 13–20 Chl/P700 since the more severe extraction of Chl destabilized the RC structure.

4.2.1. Recovery of F_A/F_B reduction by reconstituted A_1

The photoreduction of reconstituted quinone occurs both at room and cryogenic temperatures. Some of them mediated the electron transfer between A_0^- and F_X if their redox potential (E_m) values of Q^-/Q in situ at the A_1 site are appropriate [11,34,35], as seen in Fig. 4 (top) that represents the slow 30–100 ms re-reduction kinetics of flash induced $P700^+$ or reduction/oxidation of F_A/F_B monitored at an isosbestic wavelength of $P700$ (407 nm) [91]. The quinones/quinonoids with E_m values more positive than that of F_X^-/F_X (around -720 mV with respect to normal hydrogen electrode (NHE) [1,2]) were unable to reduce F_X , and directly reduced $P700^+$ with a reaction time of 100–200 μ s at room temperature as well as at 77 K. The abilities of quinones/quinonoids to accept electrons from A_0^- or to reduce F_X or $P700^+$ did apparently not depend on their molecular structures such as number of aromatic rings, alkyl tail length, addition of halogens, amino or hydroxyl side groups etc., but significantly depended on their redox properties in situ at the A_1 site. Compounds like dibromonaphthoquinone or trinitrofluorene-9-one that have significantly positive redox potential values, also efficiently functioned as electron acceptors to A_0^- but did not reduce FeS centers. It was surprising that the electrons were directly transferred to $P700^+$ from these compounds, i.e. the positive change of the redox potential of A_1 resulted in the switching of the direction of the electron transfer from the normal outward one (to F_X) to the opposite inward one (to $P700$) inside the RC.

4.2.2. Uphill energy electron transfer between F_A/F_B^- and $P700^+$ mediated by A_1

Even when F_A/F_B reduction was recovered by the reconstitution of low potential quinones, the re-reduction rate of $P700^+$ as well as the reoxidation rate of F_A/F_B^- varied depending on the redox potential of the reconstituted quinone as seen in Fig. 4. In other words, the rate of electron transfer from F_A/F_B^- to $P700^+$ varied depending on the redox potential of A_1 , which apparently does not participate in the reaction. PS I reconstituted with quinones like methyl-naphthoquinone (vitamin K_3), menaquinone (vitamin K_2) or phyloquinone gave a rather fast re-reduction rate of $P700^+$ with reaction times of a

few tens of milliseconds as reported in intact PS I (30 ms). The rate was significantly slower with low potential quinones such as anthraquinone or amino-anthraquinone that gave several hundreds ms time constants for both the decay of $P700^+$ re-reduction and the F_A/F_B^- reoxidation.

The variation of the rate shows that the redox potential of A_1 affects the net reaction rate between F_A/F_B^- and $P700^+$. As shown elsewhere in detail (S. Itoh, in preparation), this can be explained if F_A/F_B^- react with $P700^+$ mediated by A_1 . The pathway is assumed to be composed of three sequential reactions: F_A/F_B^- to F_X , F_X to A_1 and A_1 to $P700^+$, and the rate of the uphill energy reaction from F_X to A_1 will be affected by the change in the redox potential of A_1 . The lower the redox potential of A_1 (i.e. the larger the uphill energy gap of the electron transfer reaction from F_X to A_1), the slower the expected net electron transfer rate from F_A/F_B^- to $P700^+$. This mechanism also interprets the result of in vivo plastoquinone reconstitution into PS I [18,19,93] in which a faster reaction rate between F_A/F_B^- and $P700^+$ was suggested. The above A_1 -mediated backward electron transfer mechanism suggests that the reaction of F_X^- with $P700^+$ is mediated by A_1 . The mechanism, then, predicts that the rate of direct, long distance electron transfer between F_X^- and $P700^+$ does not proceed with the 1 ms time constant which was measured in the isolated PS I core complex (see reviews 1 and 2 or [74]). The rate can be assumed to be significantly slower (around 1 s as seen with the F_A/F_B^- reaction rate). The assumed rate agrees with the one predicted by Moser–Dutton's empirical distance–rate equation [96] with the currently available distance and energy gap values.

4.2.3. Quinone reconstitution by different methods

Most of the artificial quinones that were reconstituted to PS I after the hexane/methanol extraction by Biggins [14] were shown to be unable to recover reduction of NADP. This may indicate some difference from the ether extraction method that showed almost normal reduction of FeS clusters with a variety of low potential quinones, although NADP reduction was not measured in the ether-extracted PS I. However, the quinones reconstituted after the hexane/methanol extraction also showed a typical A_1^- type

EPR signal and fast reaction time as was clearly shown by Sieckmann et al. [92]. Rustandi et al. [16] and Ostafin and Wever [17] showed quinone exchange by the incubation of PS I with artificial quinones at elevated temperatures and showed characteristic A_1^- EPR signals of deuterated quinones, although other features of the PS I RC after the exchange were not studied [16,17]. The possible source of discrepancy between different experiments might come from the difference in materials or experimental conditions (membranes, core complex, RC, plants, cyanobacteria, difference in detergent, organic solvent etc.). It is also important that special attention should be paid to the regulation of the bulk concentration of quinones used for the reconstitution in the precise evaluation of the reduction rate of FeS clusters, since extra quinones will serve as the external electron acceptor to FeS clusters [91]. Attention should also be paid to the extraction of non-quinone compounds such as Chls, carotenoids, or lipids. In the ether extraction we can fortunately control the extent of extraction of pigments by the use of low water content and can avoid unnecessary damage of the RC as shown in this review. This kind of control is rather difficult in the other methods. Nevertheless, various quinones seem to be reconstituted at the A_1 site in PS I RC almost properly with different methods.

Reconstitution of plastoquinone into the A_1 site was also realized in a mutant of cyanobacteria that has a defect in the phyloquinone biosynthesis pathway [18,19,93]. Plastoquinone, that normally functions in PS II or in cytochrome b_6/f complex, recovered the function of A_1 although at a somewhat lower efficiency. The lower efficiency seems to be interpreted by the more positive redox potential of plastoquinone at the A_1 site compared to that of phyloquinone, as is the case of 3-methylnaphthoquinone (vitamin K_3) that regained a smaller extent of FeS reduction due to its high redox potential [36,91]. The *in vitro* reconstitution of plastoquinone was reported to be difficult by Iwaki and Itoh [11]. However, the *in vivo* method seems to give a better reconstitution efficiency for the low affinity quinones, probably due to the difference in Chl and lipid contents, although the quinones to be used in this method may be limited by the permeability of membranes or the flexibility of the biosynthesis pathway [18].

4.3. Redox potential values of Q^-/Q *in situ* at the A_1 site

The redox potential values (E_m) *in situ* of the reconstituted quinones were estimated by measurements of the electron transfer kinetics of P700 and FeS clusters in the quinone reconstituted PS I reaction centers [11,34,91]. The quinones that exhibit redox potential values in the organic solvent (i.e. half-wave $E_{1/2}$ redox potential values measured electrochemically in dimethylformamide (DMF)) equal to -400 mV were shown to reduce equal amounts of P700⁺ and FeS clusters after reduction by an electron from A_0^- , as was the case with 2-methylnaphthoquinone (vitamin K_3). Iwaki and Itoh [91] showed that the quinones with more negative $E_{1/2}$ values reduced a higher amount of F_X and those with a more positive $E_{1/2}$ hardly reduced FeS clusters but reduced P700⁺ directly. Therefore, the quinone with an $E_{1/2}$ value of -400 mV in DMF was shown to exhibit an E_m value *in situ* at the A_1 site comparable to that of the F_X^-/F_X that is around -720 mV. On the other hand, quinones with extremely low $E_{1/2}$ values could not be reduced by A_0^- , suggesting their E_m to be comparable to or more negative than that of A_0 (Fig. 4 bottom).

Based on these observations Iwaki and Itoh [91] estimated the relation between $E_{1/2}$ and E_m values of quinone/quinonoid compounds. The E_m of phyloquinone⁻/phyloquinone *in situ*, which has never been measured directly, was estimated to be -820 mV based on the $E_{1/2}$ of phyloquinone⁻/phyloquinone in DMF that is -500 mV with an estimated shift of -320 mV [8]. The -320 mV difference between the quinone redox potentials in DMF and in PS I protein indicates that the A_1 site has microenvironments with an extremely low dielectric nature that destabilizes Q^- . The estimation assumed the electron transfer rate between F_X and quinones to be rapid enough to allow equilibration before reacting with F_A/F_B or P700. The estimation of E_m of phyloquinone in intact PS I, therefore, might be a little biased because of the dynamic equilibration due to the fast electron transfer to F_A/F_B as discussed by Iwaki and Itoh [91]. By evaluating the dynamic equilibrium based on the kinetics in the reconstituted systems, they proposed a little more accurate empirical relation: $E_m = 0.72 \times (E_{1/2} \text{ (DMF)} - 408 \text{ mV})$.

Then, the E_m of phylloquinone and the downhill energy gap between phylloquinone and F_X can be estimated to be -786 mV and 66 meV, respectively. It might, however, also be possible that the E_m of phylloquinone in the reconstituted system is also shifted somewhat from that in the intact system due to structural modification through the loss of structural components such as PG, which are known to be required for the proper function of antenna Chls [76,82].

4.4. Energy gap dependence of the ps–ns electron transfer rate from A_0^- to A_1

The rate of electron transfer from A_0^- to A_1 was measured by a ps–ns transient absorption spectroscopy in the quinone reconstituted PS I reaction cen-

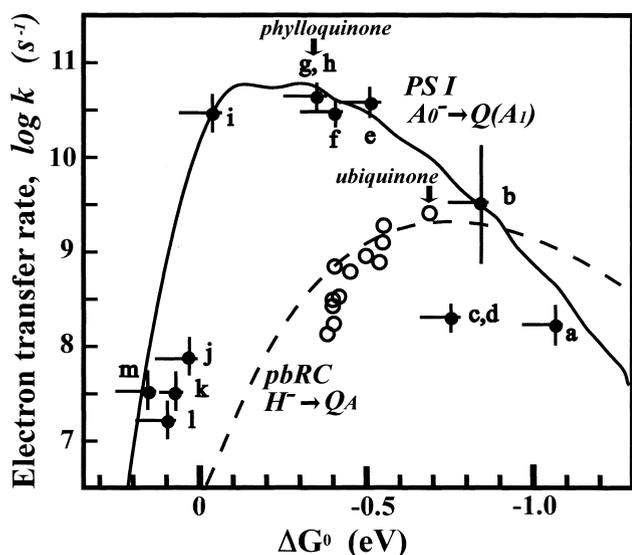


Fig. 5. Dependence of electron transfer rate constant k on the energy gap values in the reaction between the reduced primary acceptor A_0^- (Chl-690) and the reconstituted quinone/quinonoid compounds (●). Each point represents the rate measured from the absorption change of A_0^- re-oxidation in 20–30Chl/P700 PS I reconstituted with each compound used in Fig. 4 (top). Bars indicate error ranges in the estimation. Chemicals used for reconstitutions are as follows: a, 2,4,5,7-tetranitro-FL; b, 2,4,7-trinitro-FL; c, 2,3-dibromo-NQ; d, 2,3-dichloro-NQ; e, 3-anilino-2-chloro-NQ; f, 2-methyl-NQ; g, menaquinone-4; h, phylloquinone; i, 2-amino-AQ; j, 1,2-diamino-AQ; k, anthraldehyde; l, 9-FL; m, 2,6-diamino-AQ. FL, NQ and AQ represent fluorene-9-one, 1,4-naphthoquinone and 9,10-anthraquinone, respectively. ○, data obtained in *Rb. sphaeroides* RC complex reconstituted with artificial quinones. The figure was drawn after [36].

ters by Kumazaki et al. [94] and Iwaki et al. [36]. Iwaki et al. [36] selected various reconstituted quinones that give a free energy change (ΔG^0) of -1.1 to 0.2 eV for the reaction between A_0 and reconstituted A_1 (see Fig. 1). The maximum rate constant (k) of $4.35 \times 10^{-10} \text{ s}^{-1}$ (23 ps) was obtained in the reaction center which contained intrinsic phylloquinone or its analogue menaquinone (shown by an arrow in Fig. 5), in which ΔG^0 is -0.34 eV, in agreement with the rate obtained in the PS I complex with intrinsic phylloquinone and a higher number of antenna Chl a by Hastings et al. [70,95]. The electron transfer occurred with slower rates ($t_{1/e} = 30$ ps–60 ns) with the quinones/quinonoids that gave ΔG^0 higher or lower than that of phylloquinone. The $\log k$ vs. ΔG^0 plot thus showed an asymmetrical bell shape (Fig. 5) with a maximum at the ΔG^0 value of intrinsic phylloquinone (indicated by arrows in the figure) over three orders of rate constants and over 1.3 eV of ΔG^0 , as expected from the electron transfer theory proposed by Marcus [81].

$$k = \frac{2\pi}{h} V^2 (4\pi\lambda_{\text{total}} k_B T)^{-1/2} \exp \left[-\frac{(\lambda_{\text{total}} + \Delta G^0)^2}{4\lambda_{\text{total}} k_B T} \right] \quad (1)$$

$$k = \frac{2\pi}{h} V^2 (4\pi\lambda_{\text{out}} k_B T)^{-1/2} \times \sum_{n=0}^{\infty} \left[\frac{e^{-S} S^n}{n!} \right] \exp \left[-\frac{(\lambda_{\text{out}} + \Delta G^0 + n\hbar\omega)^2}{4\lambda_{\text{out}} k_B T} \right] \quad (2)$$

$$S = \frac{\lambda_{\text{in}}}{\hbar\omega} \quad (3)$$

$$\lambda_{\text{total}} = \lambda_{\text{in}} + \lambda_{\text{out}} \quad (4)$$

where V is the electronic coupling matrix element that depends on the distance between the reactant molecules. λ_{out} , λ_{in} and λ_{total} represent the reorganization energies of outer medium, reactant molecules and their sum, respectively. k_B is the Boltzmann factor. Other symbols represent ordinary physical parameters. The rate is expected to be optimum at $-\Delta G^0 = \lambda_{\text{total}}$. The ΔG^0 dependence in Fig. 5 was analyzed using a quantum mechanically extended expression (Eq. 2) assuming that a single high frequency vibrational mode (inner sphere, $\hbar\omega \gg k_B T$)

and low frequency modes (outer sphere, $h\omega \ll k_B T$) are associated in the reorganization [36].

Iwaki et al. [36] estimated the structural features of the environments of reactant molecules based on the obtained parameters in Table 2: (1) the edge-to-edge distance between A_0 and A_1 is estimated to be 7.8 Å from the optimum rate in Fig. 6, i.e. that with phyloquinone according to the empirical distance–rate equation proposed by Moser et al. [96], (2) the reorganization energy of medium (protein) λ_{out} of the A_0^- and A_1 sites is estimated to be 0.12 eV from the fitting parameters, and (3) the effective dielectric constant around A_0 and quinone is estimated to be 2.4 based on this λ_{out} . These features, especially the small reorganization energy that matches the ΔG^0 value of the reaction, contribute to the ultra-rapid, 23 ps reaction of phyloquinone. The proteinaceous environments around A_0 and A_1 , then, appear to be highly optimized through the evolution. The ΔG^0 range obtained in the quinone-reconstituted PS I RC shown in Fig. 5 (and the gray zone in Fig. 1) is one of the widest among those ever studied in the biological electron transfer system (see [96]) and directly indicated the slow-down of the rate in the inverted region (at ΔG^0 values between -0.5 and -1.1) as theoretically predicted.

The ΔG^0 dependence, however, was significantly different from the corresponding one obtained in the reaction between bacteriopheophytin⁻ and Q_A that was measured by Gunner et al. [37] in the quinone-reconstituted RC complex of the purple bacterium *Rb. sphaeroides*. In the latter case (also plotted in Fig. 5), the intrinsic ubiquinone also gave the optimum rate but with a 10 times slower rate of 200 ps with a larger 0.7 eV reorganization energy. The slower optimum rate can be interpreted to reflect the lower V value that comes from the longer distance of 10 Å between bacteriopheophytin and Q_A , and the large

reorganization energy comes from that of medium as seen from Table 2 [37]. Therefore, both types of RC complexes are highly optimized with their intrinsic electron transfer components and maintained $-\Delta G^0 = \lambda$ condition, respectively, at a different optimum $-\Delta G^0$ value by the modifications of distance and protein structure around quinones. The low dielectric constant that was estimated for PS I also interprets the extremely negative E_m in situ of phyloquinone [36]. The two types of RCs seem to have optimized the reaction to give different reducing power through the evolutionary modification of amino acid residues nearby quinones.

4.5. Energy gap between A_1 and F_X and temperature dependence of electron transfer

Along with the change of quinone, the reaction rate with F_X seemed to vary. The reduction rate of F_X is expected to increase/decrease along with the negative/positive shift of E_m of reconstituted quinone as the reaction proceeds at the normal energy gap region. This is seen in experiments in which most of the reconstituted quinones with $E_{1/2}$ values more negative than that of phyloquinone fully reduced F_A/F_B while those with more positive $E_{1/2}$ values gave less or no reduction of F_A/F_B [91]. The change in reaction rate was actually shown in a recent study of an in vitro plastoquinone-reconstituted system [19].

4.5.1. Reaction at 77 K and the redox potential of A_1

The reaction rate between P700 and the reduced quinones/quinonoids at the A_1 site was measured at 283 K [11,34,91] and 77 K [97]. Re-reduction times of P700⁺ by A_1^- in PS I containing the high redox potential quinones were 180 μs at 283 and 77 K [91] as known from the reaction of intact PS I containing

Table 2

ΔG^0 values and parameters used for the curve fitting in Fig. 5: charge shift reactions of $P700^+A_0^- \rightarrow P700^+Q^-$ in PS I and $P^+H^- \rightarrow P^+Q_A^-$ in *Rb. sphaeroides* RCs are compared

Reaction center	ΔG^0 (eV) ^a	λ_{total} (eV)	λ_{out} (eV)	λ_{in} (eV)	ϵ_s	V (cm ⁻¹)	R^b (Å)
PS I	-0.34	0.30	0.12	0.18	2.4	14	7.8
<i>Rb. sphaeroides</i>	-0.65	0.80	0.60	0.20	3.7	3	10 ^c

^aFree energy changes in the intrinsic RCs [26,27].

^bEdge-to-edge distance between donor and acceptor.

^cEstimated by X-ray crystallography.

phylloquinone at 77 K [1,2]. Exceptions were two derivatives of fluorene-9-one that showed a 2 times faster rate [91]. The fast rate might be related to their slightly different position in the PS I RC [21]. On the other hand, the quinones with E_m values significantly more negative than that of phylloquinone seemed to reduce F_A/F_B at 77 K since the extent of flash-oxidized P700 decreased after repetitive illumination [97].

4.5.2. Energy gap between A_1 -phylloquinone and F_X

In the PS I RC reconstituted with phylloquinone or menaquinone, the slow re-reduction phase of P700, which represents the FeS reduction, decreased significantly at 77 K, remaining the 180 μ s direct reaction phase between $P700^+$ and A_1^- [97] as reported in intact PS I [1,2,25]. This type of temperature dependence thus is related to the redox property of phylloquinone (i.e. to the energy gap between A_1 and F_X) [97]. Although the energy gap between A_1 -phylloquinone and F_X was assumed to be 66 meV by Iwaki and Itoh [91] by the quinone exchange method, it was also postulated to be smaller or rather uphill [2]. If it is uphill, then the suppression of the reduction of F_A/F_B on cooling is easily understood as the decrease of thermal activation. However, by the uphill mechanism it is rather difficult to interpret the full reduction of F_X and its slow 1 ms reoxidation by $P700^+$ in the isolated core complex that lacks F_A/F_B at room temperature [2,25,74], since without F_A/F_B , electrons will mainly reside on phylloquinone and will be transferred to $P700^+$ with a faster 180 μ s reaction time.

The F_X reduction in the core complex seems to be better interpreted by assuming a downhill energy reaction, in which the 1 ms reaction of F_X^- with $P700^+$ is mediated by the uphill reaction of F_X^- with A_1 , since we can assume that one tenth of the electrons resided on A_1 in equilibrium with F_X with a 60 meV uphill energy gap and, accordingly, that the reaction rate was almost one tenth of the one between the fully reduced A_1 and $P700^+$ as discussed in Section 4.2.2. The rate of the forward downhill electron transfer from A_1 to F_X that has a few tens of mV activation energy, then will decrease/increase along with the negative/positive shift of E_m of A_1 and will show different temperature dependence depending on the energy gap value as observed [97]. The

key to understand the temperature-dependent kinetics of P700 in intact PS I thus seems to be the fast temperature-independent reaction of A_1^- with $P700^+$ and the slow reaction rates of F_X^- and F_A/F_B^- with $P700^+$, respectively.

4.6. Binding affinities of quinones at the A_1 site

Iwaki and Itoh [34] estimated the binding energies of the quinones to the phylloquinone-extracted RC (A_1 site) from their dissociation constants (K_d) by monitoring the recovery of electron transfer from P700 to the reconstituted quinones. The K_d value was determined from the extent of the quinone-reconstituted PS I RC after overnight incubation at varied bulk concentrations of each quinone. The K_d value varied depending on the quinone molecular structure that includes: (1) stabilization by hydrocarbon chains required for the hydrophobic interactions, (2) stabilization with two or three aromatic rings that give hydrophobic and π - π interactions, (3) 2–3 kcal/mol stabilization per one carbonyl group, (4) a few kcal/mol destabilization by halogens added to the quinone rings presumably due to the electrostatic interaction with negative charges or dipoles on nearby amino acids, and (5) stabilization/destabilization by addition of amino or hydroxy groups depending on the positions on the aromatic rings. Features 1–3 of the A_1 quinone binding site predicted from the affinity analysis are now seen in the three-dimensional structure of PS I.

Fig. 6 plots the logarithms of $1/K_d$ against the partition coefficient value (P) of the reconstituted quinones between water and cyclohexane [34]. The P values were kindly provided by P.L. Dutton and used to represent the hydrophobicity of quinone to evaluate the contribution of the interaction of quinones specific at the A_1 site.

The intrinsic phylloquinone or its natural analogue menaquinone was shown to bind most strongly, giving the extremely low K_d of less than 10^{-8} M. Various phenanthrolines, antimycin and myxothiazol, which are known to bind at the quinone binding site in the Q_B site of purple bacterial RC or photosystem II of plant/cyanobacteria, or in the cytochrome b - c or b_6 - f complexes, also competitively inhibited the quinone binding at the A_1 site [46]. The compounds with three to four aromatic rings,

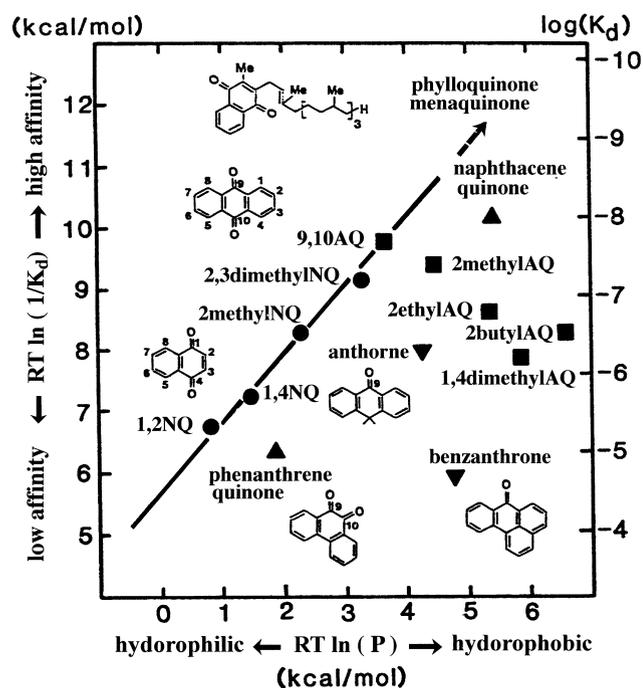


Fig. 6. Binding affinity of quinone/quinonoid compounds to the A_1 site vs. their hydrophobicity. The dissociation constant of the compounds at the A_1 site was estimated by the amount of recovery of flash-induced $P700^+$ extent after the overnight incubation of 16Chl/P700 ether-extracted PS I. Vertical axis represents the logarithms of $(1/K_d)$ in kcal/mol unit. Horizontal axis represents the hydrophobicity of each compound measured as the partition coefficient value (P) between water and cyclohexane expressed in kcal/mol. Note that naphthoquinones with simple methyl side groups show a linear straight line, while the anthraquinones with the bulky side groups, larger compounds, monocarbonyl compounds give binding affinities lower than that expected from their hydrophobicities. The P values are kindly provided by P.L. Dutton. The figure is drawn after [34].

such as anthraquinone, naphthacenequinone or phenanthrenequinone, showed relatively high binding energies ($K_d = 10^{-5}$ – 10^{-8} M^{-1}), suggesting that the site provides a thin and broad space for quinones presumably sandwiched by aromatic amino acid residues, which enable the π – π interaction with the quinone rings. The π – π interaction is expected to destabilize a negative electronic charge on phyloquinone and seems to be the key for the extremely negative E_m of phyloquinone at the A_1 site. Addition of halogens decreased the binding and the effects of amino group attachment varied depending on the position on the aromatic rings [34].

Plastoquinone, which functions in the PS II RC,

was not effective, suggesting its weak affinity at the A_1 site [11]. However, Johnson et al. [18] and Zybalov et al. [19] recently showed binding of plastoquinone at the A_1 site in *in vivo* natural reconstitution in a phyloquinone-deficient mutant of cyanobacteria. This indicates the underestimation of the activity of plastoquinone in the *in vitro* reconstitution. However, menaquinone as well as phyloquinone gave efficient reconstitutions with high affinities in *in vitro* experiments [34,46]. The low efficiency of plastoquinone in the *in vitro* experiment thus seems to come from its weak affinity at the A_1 site.

4.7. Location of quinone/quinonoid reconstituted to PS I RC and active electron transfer branch

By photoaffinity labeling using a tritium-labeled azidoanthraquinone, Iwaki et al. [45] demonstrated that the reconstituted quinone is specifically bound to the core PsaA/B polypeptides. Enzymatic digestion suggested two possible binding sites on the polypeptides. Interestingly, the covalently bound (cold) azidoanthraquinone also functioned as the electron acceptor with a high affinity.

Pulsed electron spin echo envelope modulation (ESEEM) experiments showed that the artificial quinones/quinonoids as well as intrinsic phyloquinone properly bind at the A_1 site. The distance between spins on $P700^+$ and A_1^- were estimated to be 25–26 Å in the PS I reaction centers reconstituted with 12 compounds including phyloquinone, dibromonaphthoquinone, anthraquinone and fluorenones by Dzuba et al. [20] and Iwaki et al. [21]. Fluorenones gave a slightly different but meaningful deviation (a few tenths of Å shorter distance). The results were consistent with the report of van der Est et al. [22] that showed proper orientation of reconstituted quinones at the A_1 site and also suggested different orientation of benzoquinone compounds [92]. Zech et al. [23] estimated almost the same distance and orientation of some quinones reconstituted into PS I. The A_1 location is almost consistent with that estimated by the X-ray crystal structure in which the two positions of the phyloquinone molecules were proposed in the PS I reaction center [5]. The results confirm that ether extraction preserves the geometry of the $P700$ - A_0 - A_1 arrangement. An ESEEM study of PS I in oriented films by Yoshii et al. [98] estimated the

angle of the $P700^+ - A_1^-$ axis to be 24° tilted with respect to the membrane normal and suggested the $P700 - C'_3$ side rather than the $P700 - C_3$ side seen in the X-ray structure (see Fig. 1) to be the active electron transfer branch inside PS I. This result is a little different from that estimated in the crystal of PS I by Bittle et al. [99].

4.8. Reaction of Fe-S centers in the absence of phylloquinone

Even after the complete extraction of phylloquinone by ether, continuous or repeated flash illuminations at 10 K reduced F_A/F_B to some extent [32]. The g_x value of the F_X EPR signal was shifted to the lower magnetic field, presumably due to the structural change of PS I RC due to the loss of Chls, since the readdition of Chl *a* partially recovered the shift [29]. However, the reaction of F_X did not seem to change significantly. The reaction time between A_0 and F_X in the absence of phylloquinone at room temperature seems to be 300–400 ns, judging from the 8–10% flash yield of reduced F_A/F_B that was obtained in the competition against the charge recombination reaction between $P700^+$ and A_0^- that occurs with a 30 ns time constant [32,91]. The yield increased to 25% at the cryogenic temperature [32]. This high yield in the absence of phylloquinone may reflect the heterogeneity of PS I in which some RCs are functional even without phylloquinone [32]. Another plausible idea is to assume the A_0^- to F_X reaction time to be 400 ns also at 77 K, then the high yield can be interpreted by the slow-down of the competing reaction between A_0^- and $P700^+$, which is known to occur with a slower 100 ns time constant at 77 K [1,2]. In either case, phylloquinone is an essential component for the efficient electron transfer in PS I.

The latter consideration might also be adapted to the results in the RC of heliobacteria, in which the extraction of menaquinone did not significantly affect the electron transfer rate from A_0 to F_X as reported by Kleinherenbrink et al. [85]. If the reaction time between A_0 and F_X in the heliobacterial RC is 10 times faster (i.e. around 40 ns) due to the somewhat shorter distance or the different energy gap compared to that in PS I, an almost sufficient yield of A_0 to F_X electron transfer will be achieved even after the ex-

traction of menaquinone. Accurate quantitative measurements of the electron transfer rate will solve the mystery of homodimer RCs.

5. Natural exchange of chlorophylls: PS I reaction center with chlorophyll *d* and P740

The new type of oxygenic prokaryote *A. marina* contains chlorophyll *d* as a major pigment with a few percent of chlorophyll *a* as reported by Miyashita et al. [100]. Chlorophyll *d* has a formyl group on ring IV of the chlorine ring in its chemical structure (Fig. 7) and its function in photosynthesis has not been known until the discovery of *A. marina*. It was assumed that the new pigment chlorophyll *d* may function simply as antenna as is the case in all the other oxygenic plants and cyanobacteria. However, Hu et al. [24] showed that the purified PS I RC complex of this organism has P740 (named after its Qy band at 740 nm) as the primary donor, which is a special pair of chlorophyll *d* instead of chlorophyll *a* dimer P700 that has been ubiquitously found in PS I. Fig. 8 represents the absorption spectrum of the purified PS I RC complex and the light-induced difference absorption spectrum of P740. The complex contains 145 Chl *d*, 0.8 Chl *a*/P740 and shows an absorption peak at 705 nm. The kinetics of P740 and the iron-sulfur center are similar to those known with P700 and F_A/F_B in the ever known PS I. The midpoint redox potential of P740 was determined to be 335 mV. The E_m value is more negative than that of P700 by 100 mV and seems to produce sufficient reducing power to compensate the 90 meV lower energy of the 740 nm photon compared to that at 700 nm.

This organism contains a small undeveloped phycobilisome [101] and a PS II reaction center that also uses Chl *d* as antenna [102]. As for the chemical nature of the primary donor chlorophyll of PS II, Mimuro et al. suggested it to be a Chl *a* dimer (P680) based on the observation of long-lived fluorescence at 680 nm [103] and uphill energy transfer from Chl *d* to this fluorescence band [104]. A preliminary spectroscopic study, however, detected almost no absorbance changes around 680 nm and detected changes only around 720 nm (Iwaki et al., unpublished results), so that the chemical identity of

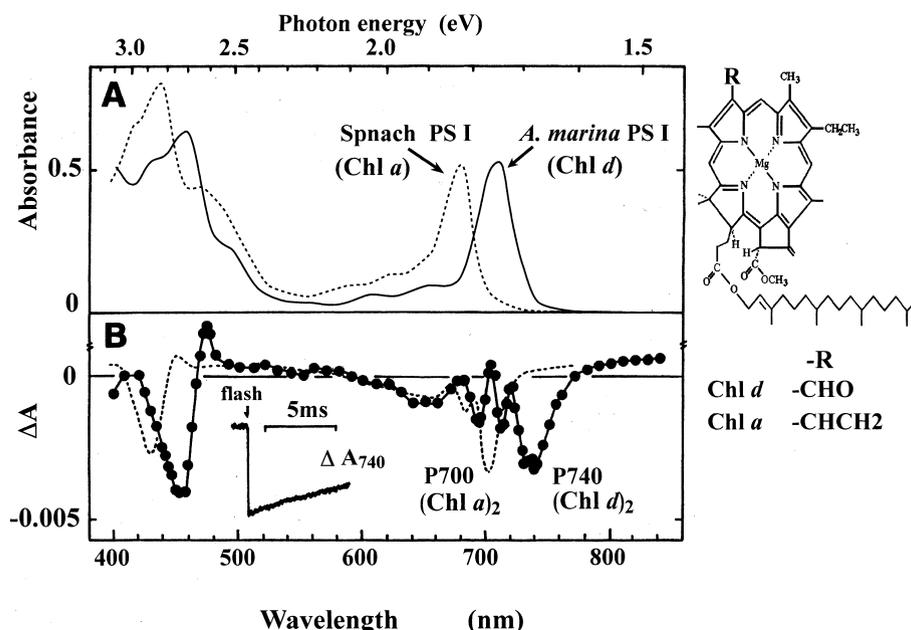


Fig. 7. (A) Comparison of absorption spectrum of chlorophyll *d* containing PS I RC preparation isolated from *A. marina* and chlorophyll *a* containing PS I of spinach. —, *A. marina*; ---, spinach PS I. (B) Laser flash-induced difference absorption spectra of P740 measured in *A. marina* PS I. ---, difference absorption spectrum of P700 in spinach PS I particles. The molecular formulae of chlorophyll *d* and *a* are also shown. The PS I preparation of *A. marina* contains 140 Chl *d*:1 Chl *a*:1 P740 [24]. Figures are drawn after [24].

the electron donor chlorophyll in PS II of *A. marina* still remains to be studied.

The extraction of PS I RC of this organism with dry ether extracted two molecules of phylloquinone/P740 and increased P740 triplet state formed by the charge recombination. Reconstitution of phylloquinone or artificial dimethylnaphthoquinone recovered the electron transfer to the iron-sulfur centers. The ether, however, did not significantly extract the antenna chlorophyll *d* (Iwaki et al., unpublished data).

The organism was isolated from a sea ascidian that also contains prochlorophyte species as a cohabitant, which is a cyanobacterium-type organism that uses chlorophyll *b* and *a* as the antenna and the RC chlorophyll, respectively. *A. marina*, which has chlorophyll *d* both for antenna and P740, seems to have the advantage of capturing longer wavelength light, which is not used by the prochlorophyte species. This organism, as well as prochlorophyte species, is positioned amid cyanobacterial varieties in a molecular phylogenetic tree based on rRNA [105] or amino acid sequences of PS I and II reaction center polypeptides (Miyashita et al., personal communication).

Another example of natural chlorophyll exchange

was an aerobic purple photosynthetic bacterium, *Acidiphilium rubrum*, which was isolated from acidic mine waste drainage [106]. Natural photosynthesis has long been thought to use only Mg-chlorophylls

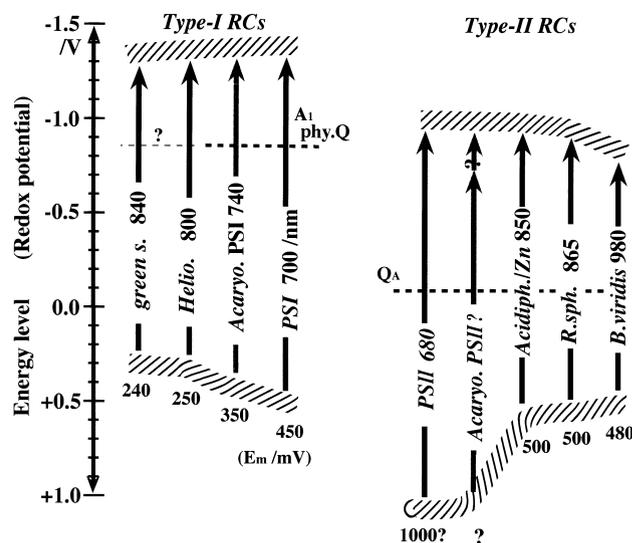


Fig. 8. Redox properties of reaction center chlorophylls in type I and type II reaction centers of various photosynthetic organisms. Values of E_m and the peak wavelengths of the special pair of each RC are shown. See text for details.

(‘chlorophyll’ has been the name given to Mg-protoporphyrins), although Zn containing porphyrins have been used in a wide variety of artificial photosynthetic systems. Several species belonging to the genus *Acidiphilium* are now found to contain Zn instead of Mg as the central metal of BChl *a* in the light harvesting (LHI) and RC complexes and to perform efficient photosynthesis [106–108]. The natural exchange of chlorophyll in RC complexes, therefore, might be an event occurring in the course of evolution more frequently than ever expected.

6. Design of reaction center

The discovery of P740 in *A. marina* suggested the flexibility of the PS I reaction center for the usage of chlorophylls. On the other hand, quinone reconstitution studies indicated the highly specific optimization mechanism of the reaction between A_0 and A_1 through the fine matching of ΔG^0 and λ . Fig. 8 summarizes the energy diagrams of electron donor chlorophylls of various type I RCs. P740 absorbs 740 nm light quantum that gives an energy smaller than that absorbed by P700. The energy deficiency, however, seems to be compensated by the negative shift of E_m of P740 so that the estimated reducing power of P740* is comparable to that of P700* [24]. A similar situation seems to hold for the BChl *g* dimer P798 of heliobacteria or the BChl *a* dimer P840 of green sulfur bacteria. In all the type I RCs, the reducing power of the excited special pair chlorophylls are adjusted to be almost equal through the matching of E_m to fill the change in photon energy.

Fig. 8 also shows the reducing power of the wide variety of type II RCs including the Zn-BChl *a*-based P850 of *A. rubrum*. The photon energies used by the special pairs of type II RCs cover a range of 1.4–1.8 eV that is wider than that of type I RCs. Including the PS II RC that shows the extremely positive E_m of P680, the reducing powers of all the type II RCs are in a narrow range. Type II RCs thus seem to have maintained their reducing power at an almost constant level, sufficient to produce quinols that feed reducing power to cytochrome *b-c* (*b_{6-f}*) complexes, although the chemical identity as well as the redox property of special pairs of PS II in *A. marina* still remain to be determined.

Type I and II RCs produce constant but significantly different reducing powers. The reducing power seems to be maintained by the tuning of E_m of special pairs. The tuning seems to adapt easily to the fine matching of $-\Delta G^0$ and λ , which is critical for efficient electron transfer in the reducing side. Both types of readjustment must have been operated during molecular evolution. However, the E_m tuning would have been efficient only in combination with the matching of the extra RC electron donor molecules, as suggested from a wide variety of soluble and membrane-bound electron donors to type I and II RCs [1,2,6]. A new type of coupling mechanism of RC and cytochrome *b-c* complex as shown for the membrane-bound cytochrome *c_z* in green sulfur bacteria [109] may give us a clue to understand the chlorophyll exchanging history in a series of type I RC. A change in the extra RC components such as cytochrome *b-c* complex vice versa would also have led to an alteration of RC pigment, although it might be more difficult to be assumed.

7. Conclusion

The ether-extracted PS I RC has been an experimental system suitable for the study of primary electron and energy transfer processes in the core moiety of PS I. The activity of the 9 or 11Chl/P700 particles clearly indicates that the Chls in the core moiety of PS I that are essential for electron transfer can selectively remain after the extraction. The quinone exchange also revealed features of electron transfer in PS I RC. It suggests each step of forward electron transfer from A_0 to A_1 , F_X and to $F_A F_B$ to be a downhill energy reaction, and the backward electron transfer from $F_A F_B$ or F_X to P700 to be mediated by A_1 through the uphill energy electron transfer step from F_X to A_1 . Modification of the A_1 redox potential by the reconstitution of different quinones thus affects both the rate of electron transfer from A_0 to A_1 and from F_X or $F_A F_B$ to P700. The small energy difference between A_1 -phyloquinone and F_X as well as the temperature-independent reaction of A_1 with P700 seems to result in the low efficiency of FeS reduction at low temperature. Intact PS I thus seems to be a highly optimized system that has developed to produce the strong reducing power required for

efficient CO₂ fixation at room temperature. The system presumably has evolved from an origin that is common with type II RCs. A unique idea of Ikegami to deplete Chls from PS I RC by using an organic solvent led us to the wide trials of RC modification through the exchange of chlorophylls and quinones.

New types of RCs found in *A. marina* and *A. rubrum* as well as a wide variety of ever-known type I and II RCs suggest that the highly optimized modern photosynthesis has evolved through vast trials of natural exchanges of pigments, cofactors, metals and polypeptides. The exchange methods as well as mutagenesis will be useful to explore this history and possibilities hidden in the RC structures, in combination with the structural information obtained by X-ray crystallography. Artificial exchanges of chlorophylls, carotenoids, and lipids, available on the basis of the ether extraction method, will lead to a new understanding of the relationship between the structure and function of photosynthetic RCs found in a wide variety of organisms.

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