Redox potential of chlorophyll *d in vitro*

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Abstract

Chlorophyll (Chl) *d* is a major chlorophyll in a novel oxygenic prokaryote *Acaryochloris marina*. Here we first report the redox potential of Chl *d in vitro*. The oxidation potential of Chl *d* was +0.88 V vs. SHE in acetonitrile; the value was higher than that of Chl *a* (+0.81 V) and lower than that of Chl *b* (+0.93 V). The oxidation potential order, Chl *b* > Chl *d* > Chl *a*, can be explained by inductive effect of substituent groups on the conjugated π-electron system on the macrocycle. Corresponding pheophytins showed the same order; Phe *b* (+ 1.25 V) > Phe *d* (+1.21 V) > Phe *a* (+1.14 V), but the values were significantly higher than those of Chls, which are rationalized in terms of an electron density decrease in the π-system by the replacement of magnesium with more electronegative hydrogen. Consequently, oxidation potential of Chl *a* was found to be the lowest among Chls and Phes. The results will help us to broaden our views on photosystems in *A. marina*.

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Abbreviations: Chl, chlorophyll; CV, cyclic voltammetry; DMF, dimethyl formamide; Phe, pheophytin; PS, photosystem; RC, reaction center; SWV, square wave voltammetry (SWV)

1. Introduction

In oxygenic photosynthetic organisms, chlorophyll (Chl) *a* (Fig. 1) is the major pigment that plays the key role in the electron transfer in both photosystem (PS) I and PS II reaction centers (RCs). In 1996, however, a Chl *d*-dominated cyanobacteria *Acaryochloris marina* was discovered [1], and much research on the pigment composition of this unique organism has been performed. In both PS I and PS II the surrounding antenna pigment is Chl *d* (Fig. 1).

In the case of PS I of *A. marina*, Chl *d*-type pigments also function as component(s) of the primary electron donor P740. P740 was initially proposed to be a homodimer of Chl *d* [2], later a homodimer of Chl *d'* [3] and finally a Chl *d/d'*

heterodimer (Fig. 2A) [4-6], just like the Chl *a/a'* for P700 in cyanobacteria and higher plants (Fig. 2B) [7,8]. The primary electron acceptor, A_0, in PSI of *A. marina* is not Chl *d* but was found to be Chl *a* (Fig. 2A) [9]. The midpoint potential, E_m, for P740 was reported to be +335 mV [2], which is significantly negative of ca. + 470 mV for P700 in other cyanobacteria [10-14]. Because of this, Chl *d* has been supposed to possess an oxidation potential lower than that of Chl *a*. The wavelength of the Chl *d* Q_Y-band, longer than that of Chl *a*, appears also to support the view that Chl *d* is oxidized more easily than Chl *a*. Such a view, however, still remains speculative. To elucidate the *in vivo* role of Chl *d*, it is of much importance to clarify its redox potential *in vitro* by electrochemical measurements.

In the case of PS II of *A. marina* whether Chl *d* acts as the primary electron donor in PS II is a matter of controversy; it has been suggested that the PS II primary donor is a Chl *d* dimer [13,15,16], a Chl *a* dimer [3-5,17-21], or a Chl *a/d* heterodimer (Fig. 2A) [6], while the identity of the primary electron acceptor of PS II in *A. marina* has been well defined as not Phe *d* but Phe *a* (Fig. 2A) [3-6,19,22], like other cyanobacteria (Fig. 2B). Our heterodimer model of Chl *a/d* was quite recently supported in part by the difference spectra of the PS II RC of *A. marina* in the blue light region (A. Telfer, personal communication).

It is well known that six molecules of Chl *a* are present as well as two Phe *a* molecules in the D1/D2/cyt b_{559} complex of Chl *a*-based organisms [23,24]: two corresponding to P680, two corresponding to the accessory, and two peripheral Chls *a* designated Chl a_Z (Fig. 2B). On the basis of pigment analyses, the accessory Chl *a* and Chl a_Z in Chl *a*-type oxygenic organisms are all replaced with Chl *d* in *A. marina* (Fig. 2A) [4-6,19].

Recently, it has been shown that the primary charge separation in PS II is

initiated from the excitation of accessory Chl *a*, AccChl *a*, of D1-branch in Chl *a*-type oxygenic organism: P-Acc*-Phe → P-Acc^{+}-Phe^{-}→P^{+}-Acc-Phe^{-} [25,26]. Therefore, the replacement of AccChl *a* with AccChl *d* is fundamentally necessary in PS II of *A. marina* (Fig. 2A) [6], because, if Acc was Chl *a*, energy transfer from antenna Chl *d* to AccChl *a* would be difficult because of the extremely uphill process. The primary charge separation initiated from AccChl *d* is hence most likely in the PS II RC of *A. marina* also, after energy transfer from antenna Chl *d* to AccChl *d* (Fig. 2A).

The chemical identity of the primary electron donor of the PS II RC in *A. marina* still remains to be resolved, as mentioned above. This uncertainty is mainly due to the difficulties associated with preparing the photoactive PS II core complexes, also due to the absence of experimental information about the oxidation potential of Chl *d* that is needed to be compared with that of Chl *a*, probably because Chl *d* had not been regarded as being present in any photosynthetic organisms until 1996.

Here we present the redox potentials of Chl *d* in acetonitrile and dimethyl formamide (DMF), comparing them with those of Chls *a*, *b*, Phes *a*, *b*, and *d*. In acetonitrile, the first oxidation potential, E^{1}_{ox}, of Chl *d* (+0.88 V vs. SHE) was more positive than that of Chl *a* (+0.81 V) and more negative than that of Chl *b* (+0.93 V). Corresponding pheophytins showed much higher values; Phe *a* (+1.14 V), Phe *b* (+ 1.25 V) and Phe *d* (+1.21 V). The E^{1}_{ox} value of Chl *a* was hence found to be the lowest among Chls and Phes. Note that oxygenic photosynthesis uses Chl *a* for P680 (Fig. 2B), although significantly high oxidation power is needed for water oxidation. The results obtained here will enlarge ones views on photosynthetic mechanisms of *A. marina*.

Materials and methods

2.1 Pigment preparation

Chls *a*, *b* and *d* were extracted and purified as described elsewhere [3,6,27,28]. Briefly, Chls *a* and *b* were extracted from parsley (*Petroselinum crispum*) and Chl *d* from *Acaryochloris marina* MBIC11017, which were then purified by normal-phase HPLC. Phes *a*, *b* and *d* were prepared by pheophytinization of Chls *a*, *b* and *d* respectively, as described before [27].

2.2 Materials Purification

Acetonitrile and dimethyl formamide (DMF) (both from Aldrich, anhydrous grade: water < 50 ppm) were deoxidized and dried before use. The solvent was subjected to freeze-pump-thaw cycles at least three times under about 10^{-5} torr. Under nitrogen atmosphere, the deoxidized solvent was then dried for 24 h with the activated molecular sieves (4A 1/16, Wako), pretreated in vacuo at 473 K over 24 h. Tetra-*n*-butylammonium perchlorate (Bu_4NClO_4, TBAP) (Aldrich, Electrochemical grade: > 99.0 %), was used as the supporting electrolyte, which was recrystallized from methanol solution and was then dried in vacuo at 333 K over 24 h.

2.3 Electrochemical measurements

The redox potentials of chlorophylls were measured by both cyclic voltammetry (CV) and square wave voltammetry (SWV). Signal-to-noise ratio of SWV is generally better than that of CV, especially for measuring redox couples at such low concentration (ca. 0.5 mM) as the present case [29,30]. Both measurements were performed with an ALS model 620A electrochemical analyzer. Scan speed for CV was 0.1 V/s. Parameters for SWV were V_{step} = 5.0 mV, AC signal (V_{pulse}) = 25 mV, and p-p at 8 Hz. The measurements were carried out in an air-tight electrochemical cell containing small compartment for a sample solution equipped

with a glass filter that can be degassed and filled with dry N_2. A platinum disk electrode with 1.6 mm in diameter (outer diameter: 3 mm) was used as the working electrode, and a platinum black wire fabricated in the small compartment (internal diameter: 8.9 mm) as the counter electrode. An Ag/AgCL electrode, chosen for good reproducibility despite possibility of junction potential, was connected through a salt bridge to the outer electrolytic solution of the small components.

The ferrocene-ferrocinium redox couple was used to estimate junction potential changes upon changing solvents. After each measurement, the redox potentials of the ferrocene-ferrocinium were measured as +0.45 V and +0.53 V vs. Ag/AgCL in acetonitrile and DMF, respectively.

3. Results

Typical cyclic voltammogram (CV) and square wave voltammogram (SWV) for Chl *d* in acetonitrile are illustrated in Fig. 3. Four reversible redox reactions were clearly resolved. Similar trends were observed for Chl *a*, Chl *b*, Phe *a*, Phe *b* and Phe *d* (see Fig. 4 for Chls *a*, *b* and *d* in acetonitrile and DMF).

The anodic sweep of CV for Chl *d* in acetonitrile showed that E^2_{red} = -1.20, E^1_{red} = -0.88, E^1_{ox} = +0.93, E^2_{ox} = +1.12 V vs. SHE, and the cathodic sweep showed that E^2_{red} = -1.32, E^1_{red} = -0.94, E^1_{ox} = +0.84 and E^2_{ox} = +1.06 V vs. SHE (Fig. 3A), resulting in E^2_{red} = -1.26, E^1_{red} = -0.91, E^1_{ox} = +0.88 and E^2_{ox} = +0.89 V vs. SHE. The values agreed well with the redox potentials obtained from the SWV: E^2_{red} = -1.26, E^1_{red} = -0.91, E^1_{ox} = +0.88, and E^2_{ox} = +0.89 V vs. SHE (Fig. 3B)

In Table 1, we summarize the values of redox potentials for Chls *a*, *b*, *d*, Phes *a*, *b* and *d* examined here. Chl *d* showed higher oxidation potentials (+0.88 and +0.92 V vs. SHE in acetonitrile and DMF, respectively) than Chl *a* (+0.81 and +0.86 V), lower than Chl *b* (+0.94 and +0.96 V), and much lower than Phe *a* (+1.14 and +1.23

V), Phe *b* (+1.25 V in acetonitrile) and Phe *d* (+1.21 V in acetonitrile).

4. Discussion

4.1 Oxidation potentials of Chls *a*, *b* and *d*

Chl *d* had been thought to have a lower oxidation potential than Chl *a* even though no experimental evidence had been present, mainly because the midpoint potential, E_m, for P740 in *A. marina* was shown to be +335 mV [2], significantly more negative than that for P700 in other cyanobacteria (around +470 mV) (Fig. 5) [10-14], where P740 is a heterodimer of Chl *d/d'* [4,5] and P700 is a heterodimer of Chl *a/a'* [7,8] (Fig. 2). The fact that the Q_Y-band of Chl *d* is at the longest wavelength compared with Chls *a* and *b* (Fig. 5) seems to have led to some misapprehensions concerning the oxidation potential of Chl *d*; one estimated that Chl *d* had the lowest oxidation potential of all Chls. Consequently, experiments were done.

The E^1_{ox} value for Chl *d* obtained in this study is higher than that of Chl *a*. This result can be explained by inductive effect of substituent groups on the macrocycle, as follows. The redox potential of a π-conjugated molecule, like chlorophyll, is affected by the nature of substituent groups on the π-electron system [31,32]. The -CHO substituent is an electron-withdrawing group (→CHO), and reduces the electronic density in the π-system of chlorophyll. The replacement of $-CH=CH_2$ at C3 of Chl *a* by →CHO to yield Chl *d* causes the macrocycle to be electron poor, thus rendering the molecule less oxidizable (E^1_{ox}: Chl *d* > Chl *a*). Similarly, replacement of $-CH_3$ at C7 of Chl *a* to yield Chl *b* makes E^1_{ox} more positive than that of Chl *a*. Therefore, the E^1_{ox} order becomes Chls *b*, *d* > Chl *a*. When one pays attention to the group of $-CH_3$ at C7 of Chl *d* and the group of $-CH=CH_2$ at C3 of Chl *b*, the $-CH_3$ group is more electron-donating ($\leftarrow CH_3$), thus

making the macrocycle of Chl *d* more electron rich, and hence the oxidation potential less positive (Chl *b* > *d*); consequently the E^1_{ox} order results in Chl *b* > Chl *d* > Chl *a*.

We should note that inductive effects on the absorption wavelengths and intensities of Q_Y-bands of chlorophylls strongly depend on the substitution position, due to the presence of two different electronic transitions polarized in the x and y directions (see Fig.1) [33-35]. Replacement of the electron-donating group, $-CH_3$, at ring II of Chl *a* by the electron-withdrawing group, -CHO, yielding Chl *b*, causes the blue-shift and significant intensity reduction of Q_Y-band (Fig. 6A). In contrast, replacement of $-CH=CH_2$ at ring I of Chl *a* by -CHO, yielding to Chl *d*, causes the red-shift and intensity increase of Q_Y-band (Fig. 6A). Similar phenomena are clearly seen in BChls *b* and *g*; both pigments have the same macrocycle, but the substituents at ring I are $-COCH_3$ for BChl *b* and $-CH=CH_2$ for BChl *g*, respectively (electron-withdrawing effect: $-COCH_3$ > $-CH=CH_2$), resulting in the red-shift and intensity increase of Q_Y-band in BChl *b* as compared with BChl *g* (Fig. 6B). The oxidation potential of BChl *b* can be hence expected to be higher than that of BChl *g* as mentioned above, and such measurements are now under way.

4.2 Comparison of oxidation potentials of P700 and P740 with Chls *a* and *d*

As mentioned above, E_m for P740 in *A. marina* was reported to be lower than that for P700 by 100 mV or more, while in acetonitrile E^1_{ox} for Chl *d* was higher than Chl *a* by 70 mV, indicating that negative shift of potential caused by dimerization of chlorophyll to constitute P740 and P700 in PS I is larger for Chl *d* in *A. marina* than Chl *a* in other oxygenic organisms (see Fig. 5). Excitation energy of 1.68 eV for P740 is smaller than that for P700 (1.77 eV) by 90 mV, and the primary electron acceptor, A_0, in PSI is the same molecule, namely, Chl *a* [9] (see Fig. 2). If the redox potential of A_0 in *A. marina* is assumed to be the same as that of A_0 in other PS

I (ca. -1.05 V), the differences between P^*/P^+ and A_0/A_0^- are calculated to be 300 mV for *A. marina* and 250 mV for other PS I, respectively: the former is greater than the latter by 50 mV.

Compared with E^1_{red} of Chl *a* (-1.12 V in acetonitrile), E^1_{red} of Chl *d* (-0.91 V) is less negative (Fig. 5). The E_m (F_x/F_x^-) value for P700 is -0.67 V [36], which is positive enough for the electron transfer from both Chl a^- and Chl d^- to F_x. Further, if A_0 were Chl *d* in *A. marina*, E_m (A_0/A_0^-) would be expected to be less negative. In this case, more positive values for both $P740^*/P740^+$ and $P740/P740^+$ couples are enough. The reason why *A. marina* uses Chl *a* as A_0 is still an open question.

If the reported value of E_m for P740 shown in Fig. 5 is right, interaction between the special pair chlorophylls, Chl *d'* and Chl *d*, must be much stronger than that between Chl *a'* and *a* for P700, because the negative shift of E_m on formation of P740 was significantly greater. Further fundamental measurements of E_m for P740 and A_0 in PS I of *A. marina* are needed to clarify the mechanisms of PS I in *A. marina*. Such measurements are under way, and more details will be discussed elsewhere.

4.3 Higher oxidation potentials of Phe *a*, Phe *b* and Phe *d*

For water oxidation, higher oxidation potential is believed to be favorable, and if so Chl *b* might be most preferable for P680 due to its highest oxidation potential of all Chls (Fig. 4, Table 1). In that context, Chl *a* might be most unsuitable, and Chl *d* rather than Chl *a* looks slightly preferable. We should note, however, that Chl *b* has not yet been used in P680 in oxygenic photosynthesis.

Considered solely from the view point of water oxidation, Phes are much more favorable than Chls due to their amazingly high oxidation potentials: Phe *a* (+1.14 V), Phe *b* (+1.25V), Phe *d* (+1.21V) in acetonitrile (Table 1); their very high potentials are rationalized in terms of an electron density decrease in the π-system by the

replacement of Mg with more electronegative H [32,37,38]. Phes, however, have not yet been found to function as the primary electron donor of PS II in natural oxygenic organisms. One reason might be that Phe itself (and proteins around it) could not withstand chemical modification due to the high oxidation potential.

We cannot conclude at present which model is right for the special pair in PS II of *A. marina*; a Chl *a* dimer, a Chl *d* dimer or a Chl *a/d* heterodimer. Our results, however, will help us to understand the photosynthetic mechanisms of *A. marina*. Further, the *in vivo* direct measurement of the redox potential of special pair in PS II of *A. marina* is fundamentally needed and such a measurement is now under way. More details will be discussed elsewhere.

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Figure legends

Fig. 1 Molecular structure and carbon numbering of chlorophylls, according to the IUPAC numbering system.

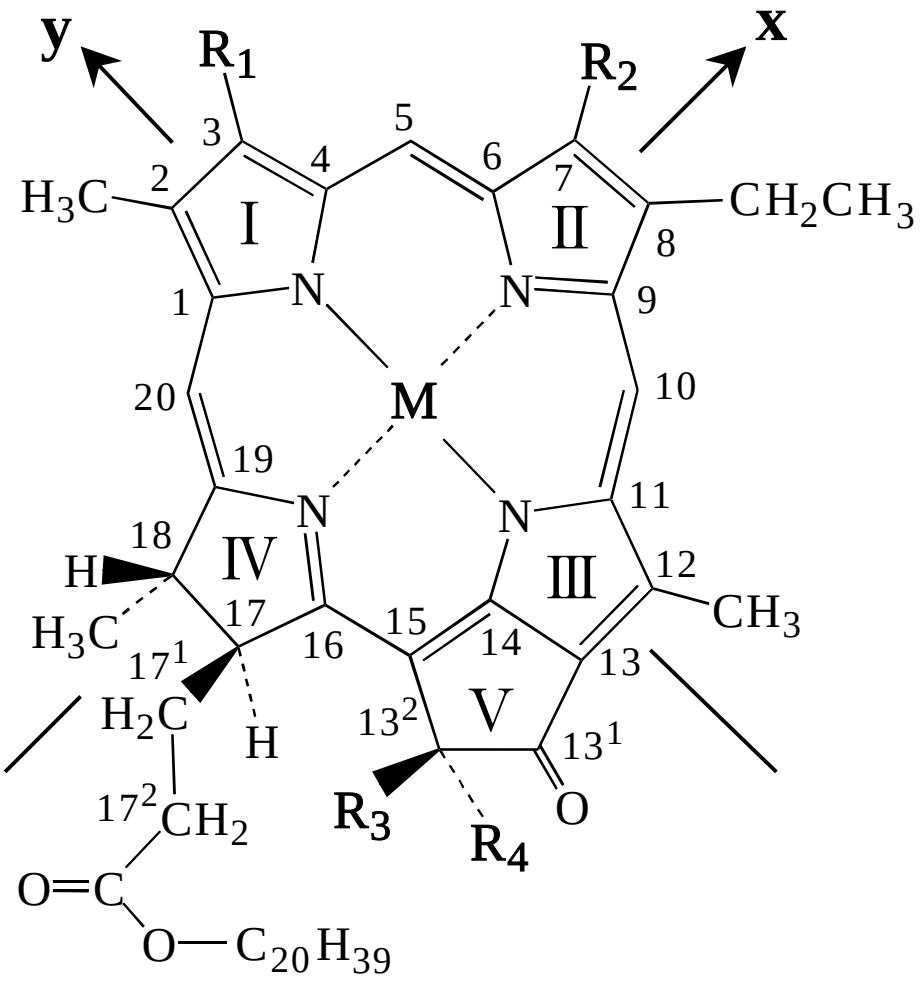
Fig. 2 Models for pigment arrangements in photosystems of (A) *A. marina* and (B) cyanobacteria. In model (A), the primary electron donor of PS I is a heterodimer of Chl *d/d'*, the active A_0 in PS I is Chl *a*, the primary electron donor of PS II is a heterodimer of Chl *a/d*, both accessory and Chlz are Chl *d*, and the primary electron acceptor in PS II is Phe *a*.

Fig. 3 Typical (A) cyclic and (B) square wave voltammograms of Chl *d* (0.5 mM) in acetonitrile with 0.1 M TBAP. Scan speed for CV was 0.1 V/s. In SWC, step was 5 mV, amplitude was 25 mV, and frequency was 8 Hz.

Fig. 4 Square wave voltammograms of Chls *a*, *b* and *d* in (A) acetonitrile and (B) DMF.

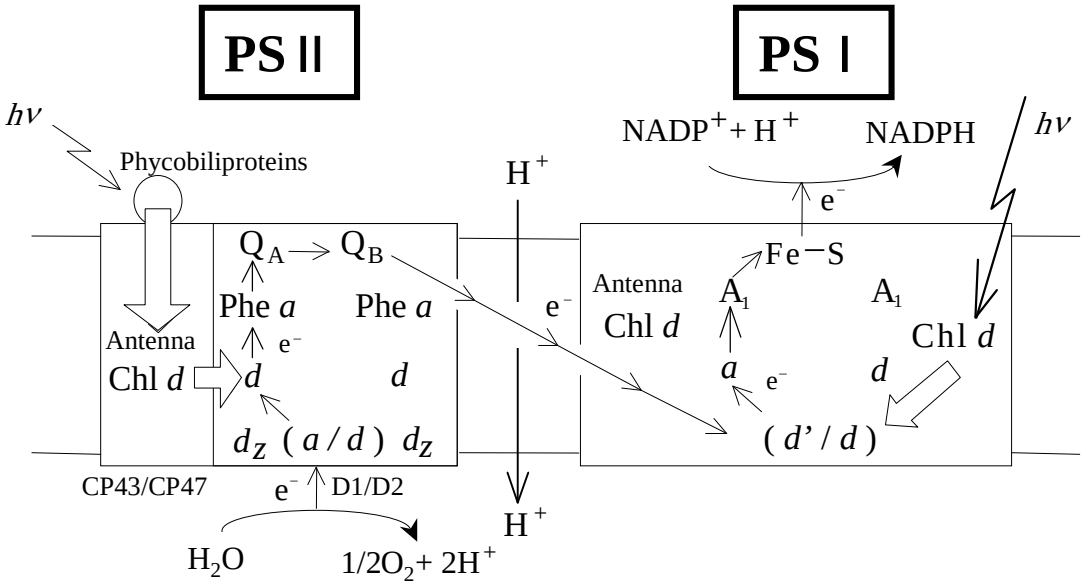
Fig. 5 Schematic comparison of redox potentials of Chl *a* and Chl *d* with P700, P740 and A_0. Redox potentials of Chl *a* and Chl *d* in vitro are in acetonitrile. The primary electron acceptor, A_0, is Chl *a* for both P700 and P740, but the midpoint potential of A_0/A_0^- shown in this illustration is for P700, the value for P740 has not been determined yet, See text for more detail.

Fig. 6 Comparison of the absorption spectra of (A) Chls *a*, *b* and *d*, and (B) BChls *b* and *g* in diethyl ether. Spectra were normalized by Soret-band.



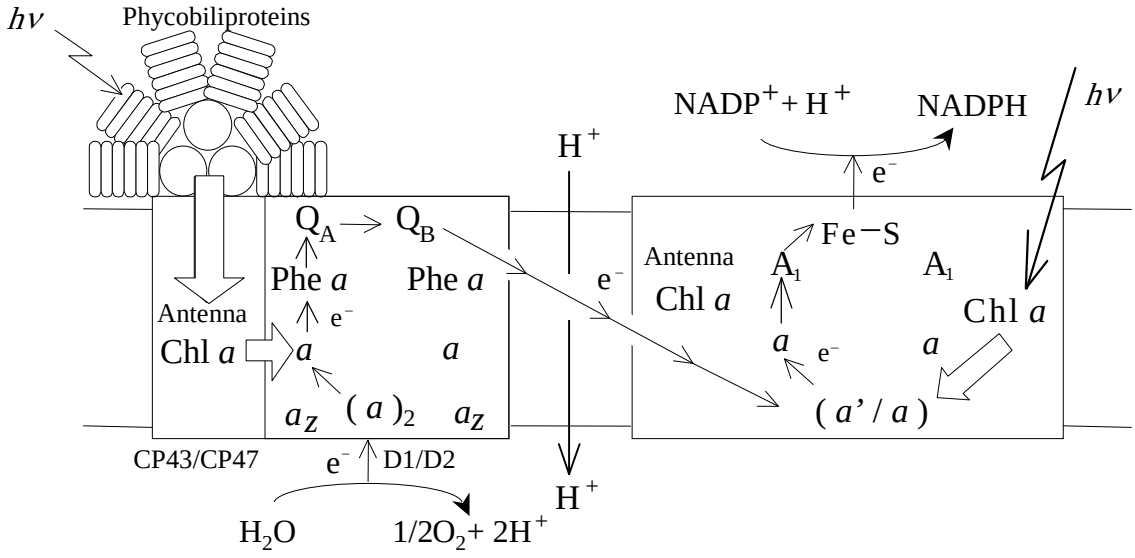
	M	R_1	R_2	R_3	R_4
Chl *a*	Mg	$CH=CH_2$	CH_3	H	$COOCH_3$
Chl *a'*	Mg	$CH=CH_2$	CH_3	$COOCH_3$	H
Phe *a*	2H	$CH=CH_2$	CH_3	H	$COOCH_3$
Chl *b*	Mg	$CH=CH_2$	CHO	H	$COOCH_3$
Phe *b*	2H	$CH=CH_2$	CHO	H	$COOCH_3$
Chl *d*	Mg	CHO	CH_3	H	$COOCH_3$
Chl *d'*	Mg	CHO	CH_3	$COOCH_3$	H
Phe *d*	2H	CHO	CH_3	H	$COOCH_3$

(A)

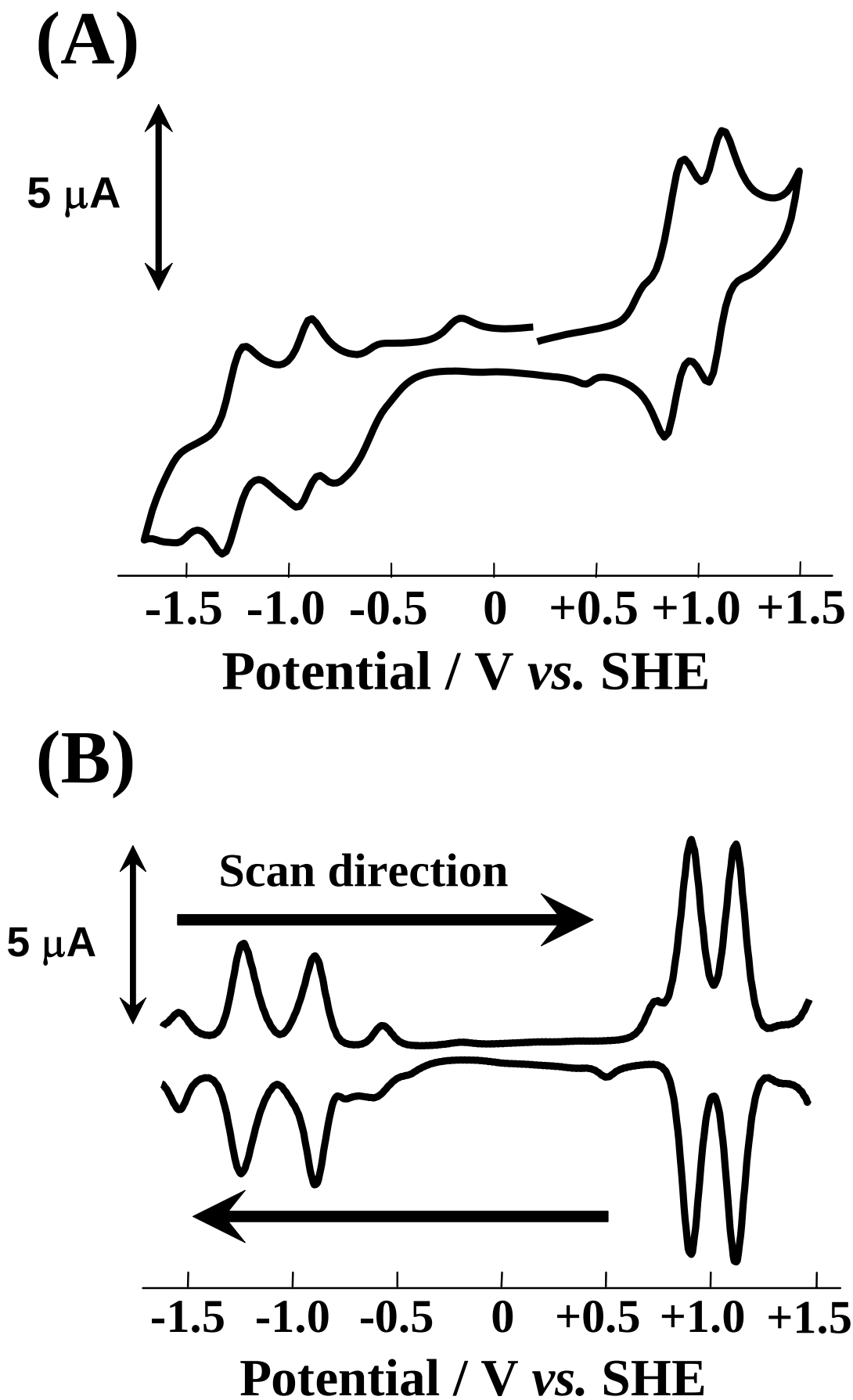


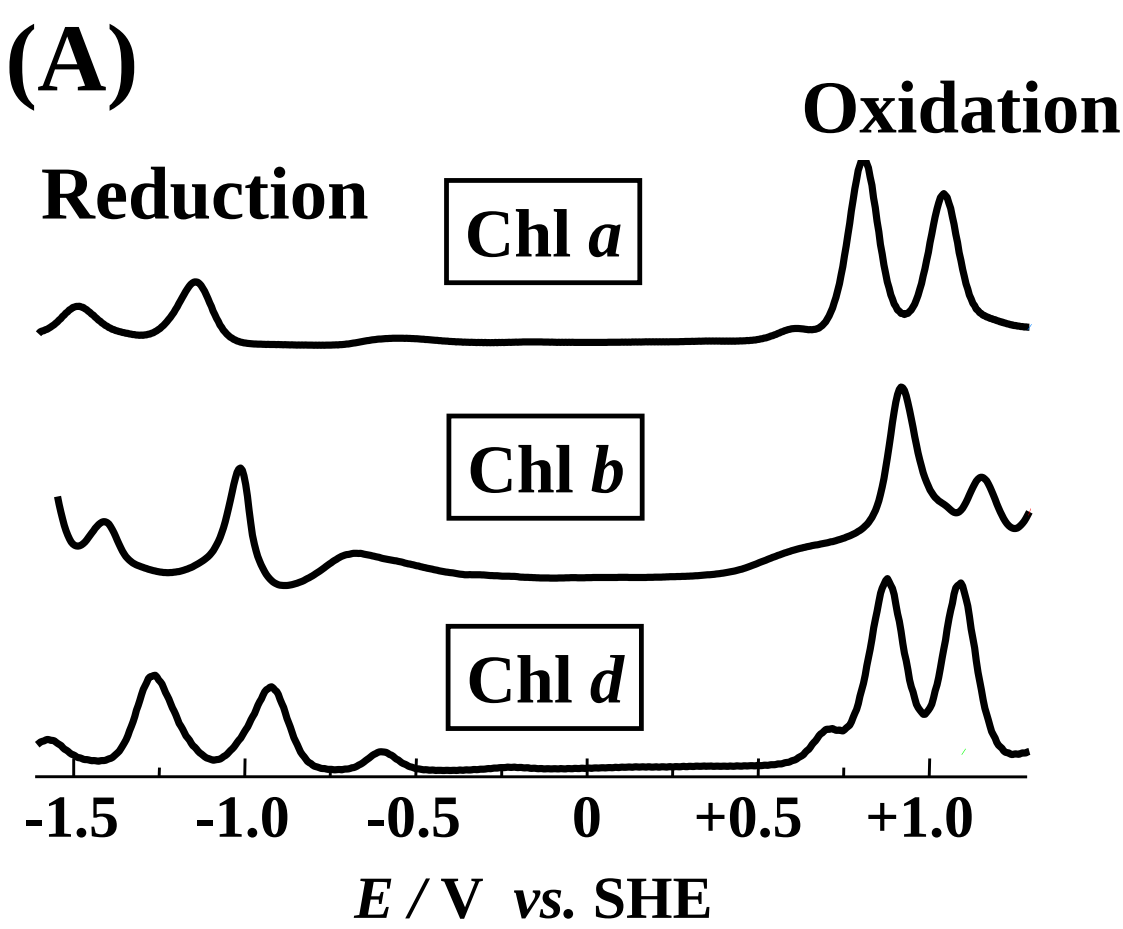
A. marina

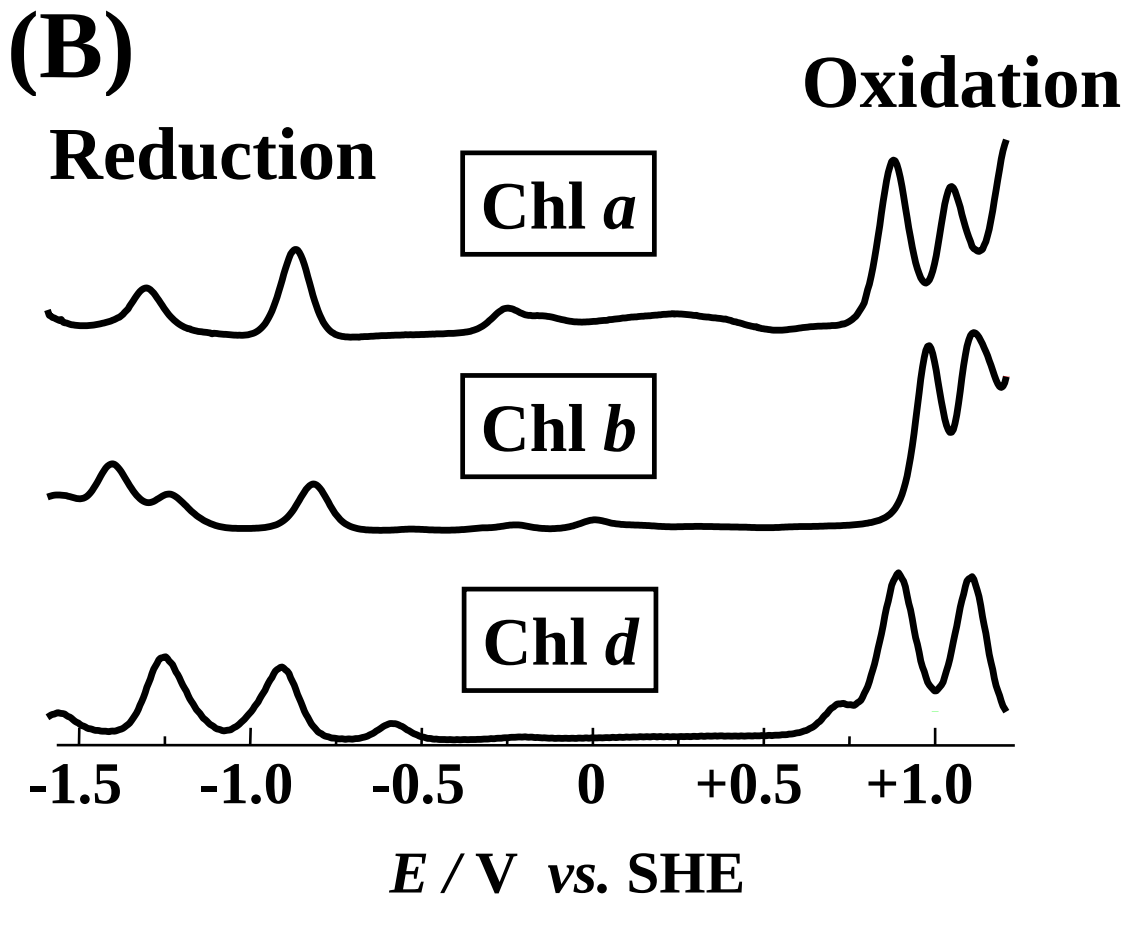
(B)

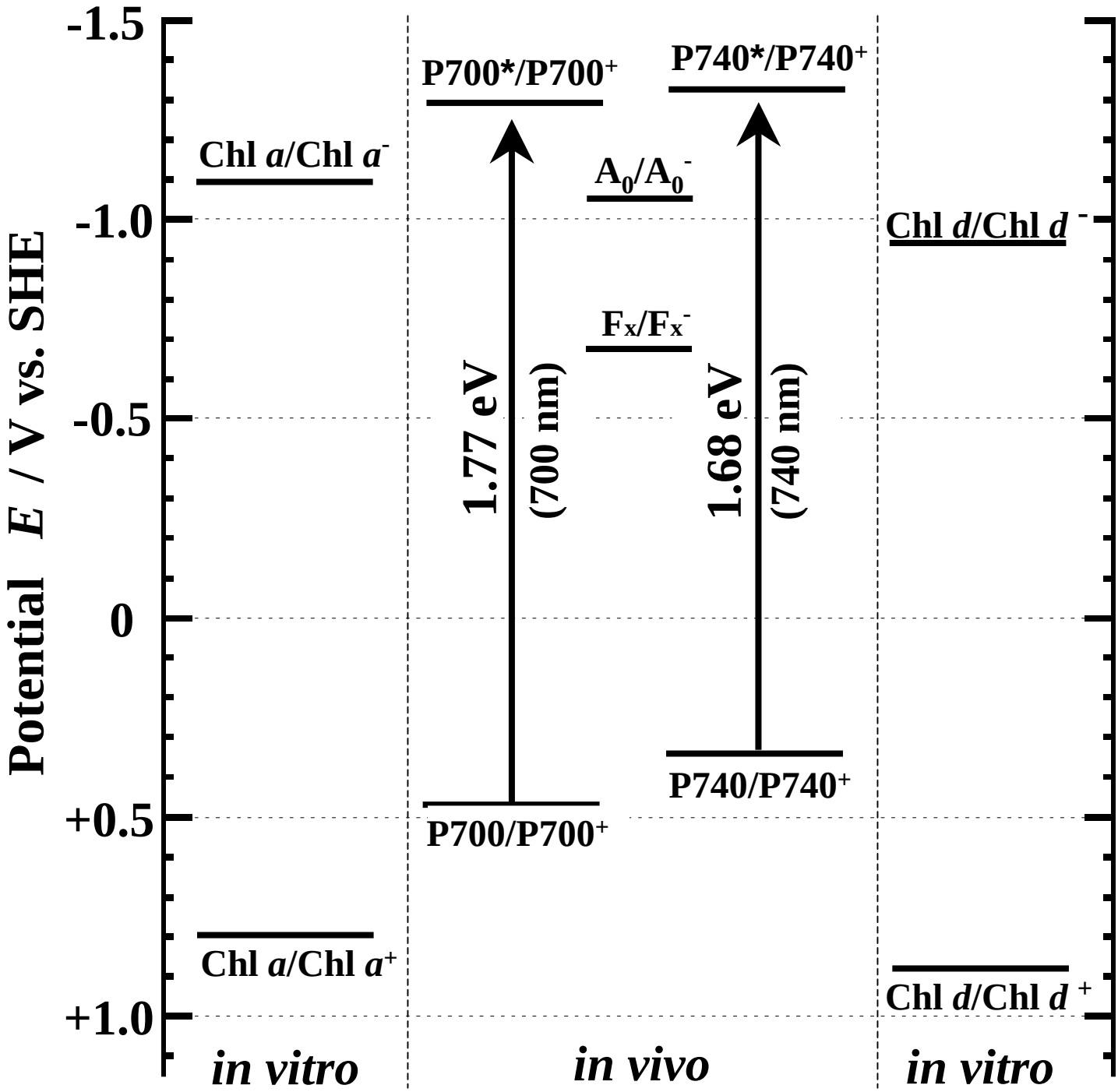


Cyanobacteria


(A)
5 μA
-1.5 -1.0 -0.5 0 +0.5 +1.0 +1.5
Potential / V vs. SHE
(B)
Scan direction
5 μA
-1.5 -1.0 -0.5 0 +0.5 +1.0 +1.5
Potential / V vs. SHE


(A)
Oxidation
Reduction
Chl a
Chl b
Chl d
-1.5
-1.0
-0.5
0
+0.5
+1.0
E / V vs. SHE


(B)
Oxidation
Reduction
Chl a
Chl b
Chl d
-1.5
-1.0
-0.5
0
+0.5
+1.0
E / V vs. SHE


-1.5
-1.0
-0.5
0
+0.5
+1.0
Potential E / V vs. SHE
Chl a/Chl a-
Chl a/Chl a+
P700*/P700+
P700/P700+
1.77 eV
(700 nm)
A0/A0-
Fx/Fx-
P740*/P740+
P740/P740+
1.68 eV
(740 nm)
Chl d/Chl d -
Chl d/Chl d +
in vitro
in vivo
in vitro

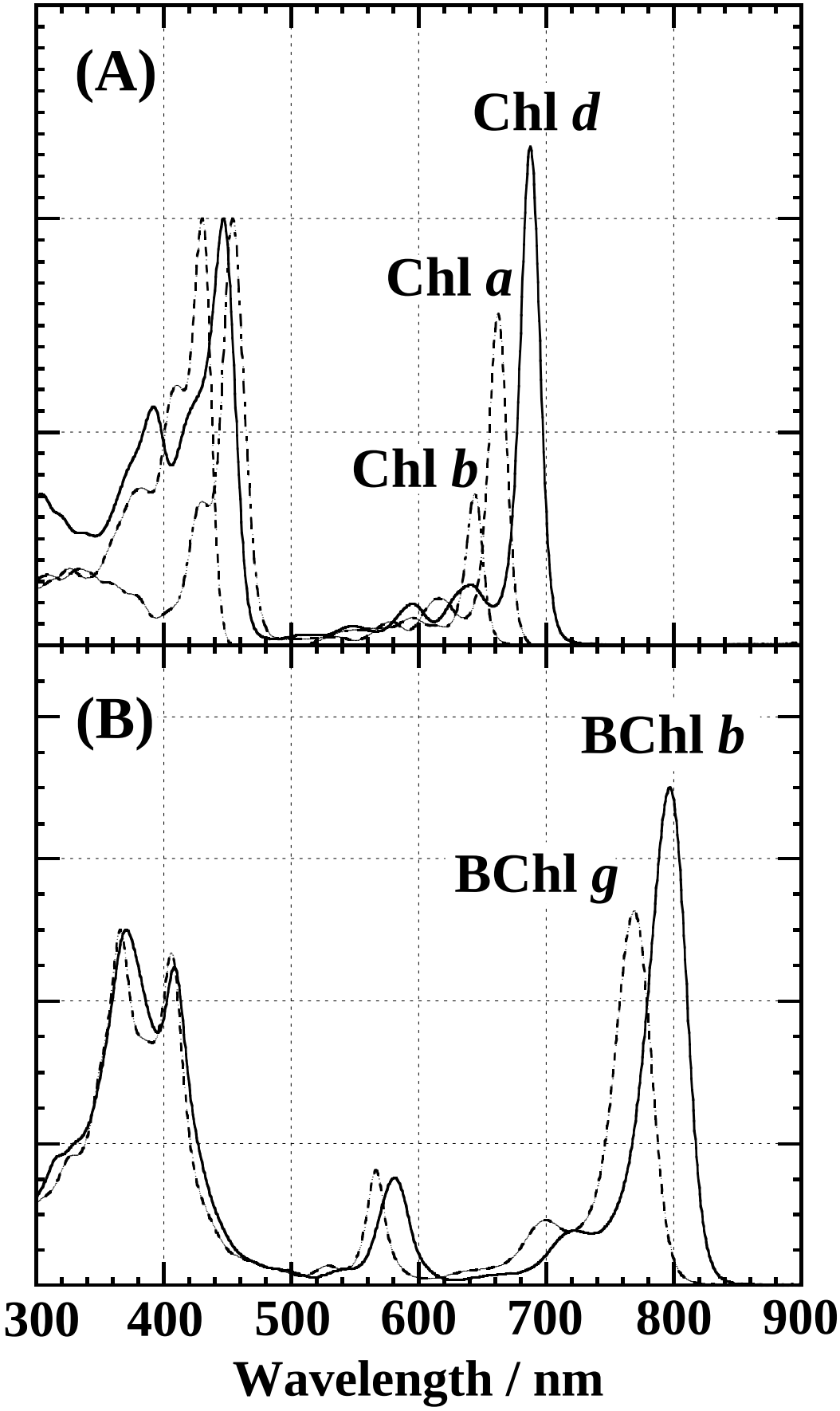

(A)
Chl *d*
Chl *a*
Chl *b*
(B)
BChl *b*
BChl *g*
300
400
500
600
700
800
900
Wavelength / nm

Table 1 Redox potentials of Chls *a* , *b* , *d* and Phes *a, b, d* in acetonitrile and dimethylformamide (DMF)

	E^{2}_{red}	E^{1}_{red}	E^{1}_{ox}	E^{2}_{ox}	Solvent
	V vs. SHE				
Chl *a*	-1.46	-1.12	0.81	1.04	acetonitril
Chl *b*	-1.41	-1.02	0.94	1.15	ibid.
Chl *d*	-1.27	-0.91	0.88	1.09	ibid.
Phe *a*	-1.00	-0.75	1.14	1.49	ibid.
Phe *b*	-1.05	-0.64	1.25	1.58	ibid.
Phe *d*	-0.87	-0.63	1.21	1.50	ibid.
Chl *a*	-1.32	-0.88	0.86	1.04	DMF
Chl *b*	-1.25	-0.83	0.96	1.11	ibid.
Chl *d*	-1.10	-0.70	0.92	1.05	ibid.
Phe *a*	-0.99	-0.66	1.23	1.36	ibid.