Biochimica et Biophysica Acta, 936 (1988) 81–89
Elsevier

BBA 42865

Chlorophyll *a*′/P-700 and pheophytin *a*/P-680 stoichiometries in higher plants and cyanobacteria determined by HPLC analysis

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(Received 21 March 1988)

Key words: Chlorophyll *a*′; Pheophytin *a*; Reaction center; Photosynthetic apparatus; HPLC

The chlorophyll (Chl) *a*′ and pheophytin (Pheo) *a* contents in photosynthetic organs have been reinvestigated by means of silica HPLC. Previously reported data (Watanabe, T. et al. (1985) BBA 807, 110–117 and FEBS Lett. 191, 252–256) were found to contain errors from two sources: slight epimerization and pheophytinization of Chl *a* during pigment extraction and PS I particle preparation, and an accidental overlap of a Chl *a* alteration product (*meso*-chlorinated Chl *a*) with Chl *a*′ on the HPLC trace. The pigment molar ratios determined with the improved extraction/HPLC procedure were Chl *a*/Chl *a*′ ≒ 460 and Chl *a*/Pheo *a* ≒ 120 in more than 100 samples from 13 different higher plants, and Chl *a*/Chl *a*′ ≒ 125 and Chl *a*/Pheo *a* ≒ 100 in three cyanobacteria. These values, when combined with the PS I particle composition (Chl *a*′/P-700 = 1) and the widely accepted molar ratio Pheo *a*/P-680 of 2, yield P-680/P-700 = 1.9 ± 0.5 and 0.65 ± 0.20 as reaction center stoichiometries in higher plants and cyanobacteria, respectively. The present results correspond well with the recently published Chl *a*/reaction center stoichiometry data acquired by (photo-)redox titrations of key components.

Introduction

The presence of metal-free chlorophyll (Chl) *a*, or pheophytin (Pheo) *a*, in photosynthetic apparatus had been questioned for a long time, until Klimov et al. [1,2] demonstrated indirectly that it might function as the primary acceptor in photosystem (PS) II reaction center. Chl *a*′, or the C-10 epimer of Chl *a* (see Ref. 3 for carbon numbering), was first described in 1942 [4], but since then most workers have regarded it as merely an extraction artifact. Only a few investigators [5,6] have claimed that it may be possible that Chl *a*′ is an ingredient in vivo.

We have attempted to quantitate minor pigments in photosynthetic organs by means of HPLC. Previous work [7] suggested the presence of one molecule of Chl *a*′ and one molecule of Pheo *a* per approx. 300 and 60 molecules of Chl *a*, respectively, in higher plant leaves. Subsequent analysis of a series of PS I particles under identical HPLC conditions showed that roughly two Chl *a*′ molecules are associated with P-700 [8]. More

Abbreviations: Chl, chlorophyll; Pheo, pheophytin, PS, Photosystem; HPLC, high-performance liquid chromatography; Q, quinone acceptor.

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recently interaction of Chl a' with a 65 kDa subunit protein of PS I reaction center was demonstrated [9].

After further accumulation of analytical data, however, we noted that the contents of Pheo a and Chl a' were variable, albeit slightly , from one HPLC measurement to another. The present work was undertaken to unravel the causes for such a scatter of analytical data and, on this basis, to obtain more reliable values for the Chl a' and Pheo a contents in higher plants, PS I particles and cyanobacteria.

Briefly, two matters have been identified as the principal sources of the data scatter: alteration of Chl a through contact with tissue-derived substances on disruption of cells and chloroplasts, and an accidental overlap of one of the Chl a alteration products with Chl a' on the HPLC trace. By carefully examining and improving the extraction/HPLC conditions, we were able to suppress these artifact-producing phenomena satisfactorily. The newly established analytical data are in line with the stoichiometries Chl a'/P-700 = 1 and Pheo a/P-680 = 2 for every plant species examined here, and the P-680/P-700 molar ratios of about 1.9 in higher plants and about 0.65 in cyanobacteria.

Materials and Methods

Plant materials. Young leaves, apparently healthy and active in photosynthesis, were harvested from a total of 13 higher plants (*Hydrangea macrophylla, Spinacia oleracea, Chenopodium album, Chrysanthemum maximum, Erigeron canadensis, Fragaria chiloensis, Angelica keiskei, Brassica rapa, Cryptotaenia canadensis, Oenanthe stolonifera, Salpichroa rhomboidea, Lycopersicon esculentum* and *Rhododendron pulchrum*) grown under sunlight. Three species of cyanobacteria, *Synechococcus* sp. (ATCC 27144), *Anabaena* sp. (ATCC 29151), and *Spirulina platensis* (M-135) were used as materials. The former two were grown in BG-11 medium [10] without NaCl and the latter in SOT [11], all under white fluorescent tubes (approx. 40 $\mu E \cdot m^{-2} \cdot s^{-1}$) at $30 \pm 1°C$ for 7–8 days.

PS I particles. P-700-enriched particles were prepared by digitonin solubilization of spinach chloroplasts followed either by centrifugation [12] or by a diethyl ether wash [13,14]. The P-700 concentration was assayed via the flash-induced absorption change for the former particles and the ferricyanide-oxidized minus ascorbate-reduced difference spectrum for the latter particles, based on a common absorption coefficient of 64 $mM^{-1} \cdot cm^{-1}$ for P-700 [15]. The Chl a/P-700 molar ratio in these PS I particles ranged from about 300 down to about 8.

Pigment extraction. The leaf tissue of a higher plant was ground in a glass mortar for 1 min with approx. 60 g of anhydrous Na_2HPO_4 (−20 °C) as a desiccant. The leaf tissue/desiccant weight ratio proved to be one of the crucial factors in ensuring the molecular integrity of Chl-type pigments in the course of extraction, as will be detailed later. The ground material was transferred into a glass beaker, to which 30 ml of chloroform (−20 °C) was added, and the mixture was sonicated for 30 s. It was then filtered through a glass filter (Whatman GF/C, prewashed with chloroform) under suction, and the residue was washed twice with 5 ml of cooled chloroform for exhaustive extraction. The filtrate was immediately dried in a rotary evaporator under 0.01 atm.

The same practice was followed in pigment extraction from cyanobacteria (after separating the culture medium by centrifugation at $10000 \times g$ at −10 °C for 1 min) and PS I particles.

HPLC analysis. The green solid material obtained by the above procedure was immediately redissolved in approx. 10 μl of chloroform, and an aliquot of 0.4–3.0 μl was injected to a silica HPLC column (Senshupak 1151-N, 4.6 mm (diameter) × 150 mm, cooled to approx. 4 °C in an ice-water bath). The sample was eluted isocratically with hexane/2-propanol/methanol (100 : 0.8 : 0.4, v/v) at a flow rate of 0.5–0.8 ml/min. Pigment components were monitored by means of an Oyo-Bunko-Kiki visible absorbance detector UVI-LOG-7 (425 nm) and a JASCO fluorescence detector FP-110 (λ_{ex} 365 nm, λ_{em} 670 nm) placed in series.

Sufficiently pure Chl a (epimeric purity 99.97%, see below), Chl a' (99.9% or higher), Pheo a (99.9%) and Chl b (99.9%) were prepared by means of preparative-scale HPLC [3], and were used as internal or external standards in pigment identification and quantitation.

Results

Improvement in HPLC resolution

Under previously employed HPLC conditions in which the eluent had been a binary mixed solvent (hexane/2-propanol, 100 : 0.8–1.5) [7,8], we later noticed that the Chl *a′* peak was sometimes contaminated with a weakly fluorescent component, by comparing the absorbance/fluorescence intensity ratio on the HPLC trace between the plant extract and the standard Chl *a′* sample. In addition, the Chl *a′* peak occasionally appeared to be a closely spaced doublet (Chl *a′* and X). In view of this, we first looked for a better eluent capable of separating this doublet into two isolated peaks, to reach a hexane/2-propanol/methanol (100 : 0.8 : 0.4) ternary solvent system.

As a typical example, the HPLC trace for a chloroform extract of *Spinacia oleracea* leaf tissue, recorded using the novel eluent, is shown in Fig. 1. The Chl *a′* and component X peaks are now completely separated. The relative height of the component X peak showed a significant scatter from one measurement to another, even in samples from a common plant species. (The HPLC trace in Fig. 1 represents a case in which the relative amount of X is exceptionally high, being comparable to that of Chl *a′*. In many runs, under an improved extraction procedure described later, the content of X was much lower, frequently below the detection limit.) Based on this and other pieces of evidence, we were able to verify [16] that component X is an alteration product of Chl *a*, in which the δ-methine carbon has undergone chlorination [17,18] possibly by an attack of electrophilic chlorine species ($Cl\cdot$, Cl^+ and/or Cl_2) [19,20] formed from cytoplasm- or vacuole-derived chloride ion during tissue grinding. The accidental overlap of the Chl *a′* peak with the δ-chlorinated Chl *a* peak was one of the factors causing observation of higher Chl *a′* contents in our previous measurements [7,8].

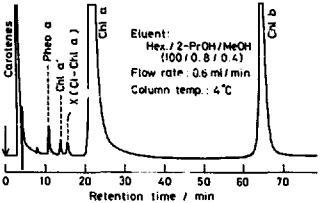

Fig. 1. HPLC trace for a chloroform extract of *Spinacia oleracea* leaf tissue. Detection wavelength, 425 nm.

Improvement in extraction procedure

Even with the use of the novel eluent, the amounts of Chl *a′* and Pheo *a* relative to Chl *a*, evaluated from HPLC peak areas, still exhibited a scatter, in the ranges 0.19–0.5% and 0.75–1.5%, respectively, in higher plants. This suggested that Chl *a* molecules had undergone alterations (epimerization and/or pheophytinization) at some stage(s) of extraction.

The plant cytoplasm and vacuole contain a variety of acids, bases and inorganic ions. Chl *a* is pheophytinized by acids [21], epimerized by bases [22], and chlorinated by an electrophilic attack of chlorine species [16,19,20]. On grinding the leaf tissue, Chl *a* molecules liberated from structural proteins come into contact with cytoplasm- or vacuole-derived substances. Such substances should be active only in an aqueous or wet environment. Rapid desiccation of plant tissues would then be an effective means for suppressing pigment alterations. The use of a fairly large amount of desiccant (anhydrous Na_2HPO_4) in our tissue grinding practice, is based on these considerations.

The efficiency of tissue desiccation, or inhibition of pigment alteration, should be a function of the leaf tissue/desiccant weight ratio during grinding. A higher weight ratio would lead to a higher degree of pigment alteration, since Chl *a* molecules should locally encounter the wet environment more frequently. By choosing a common weight (60 g) of anhydrous Na_2HPO_4, we examined how the weight of co-ground *H. macrophylla* leaf tissue affects the apparent contents of Chl *a′* and Pheo *a* relative to Chl *a* in extracts. The results are summarized in Fig. 2. As expected, the apparent Chl *a′* and Pheo *a* contents increased with increasing leaf tissue weight. The analytical concentrations of Chl *a′* and Pheo *a*, however, appear to intersect with the ordinate at characteristic non-zero values, 0.21 (±0.01)% and

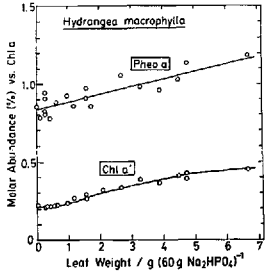

Fig. 2. Apparent Chl a' and Pheo a contents relative to Chl a in chloroform extracts prepared after grinding *H. macrophylla* leaf tissue of various weights with 60 g of anhydrous Na_2HPO_4 (−20 °C).

0.83 (±0.05)% of Chl a, respectively, when the weight of leaf tissue kneaded with 60 g desiccant is small enough, i.e., below approx. 600 mg. These values could thus be regarded as reflecting the pigment composition in vivo.

In light of these findings, it is now clear why we observed apparently higher Chl a' and Pheo a contents, approx. 0.33% and 1.7% of Chl a, respectively, in previous measurements [7]. In those experiments we ground 1–5 g leaf tissue with 10–30 g anhydrous Na_2SO_4, or at least 2 g of the former per 60 g of the latter. Referring to Fig. 2, under such ill-controlled conditions the apparent Chl a' content easily exceeds 0.3%, as long as the desiccating power is similar for Na_2HPO_4 and Na_2SO_4. The previously observed Pheo a content (1.7%) is, however, slightly higher than the maximum value (approx. 1.2%) noted in Fig. 2: this suggests the occurrence of faster pheophytinization in crushed tissues in contact with Na_2SO_4, probably because of its lower activity as compared to Na_2HPO_4 in scavenging tissue-derived acidic substances.

When leaf tissues were ground in the absence of a desiccant and then extracted with chloroform, the relative contents of Chl a', Pheo a and δ-chlorinated Chl a assayed from HPLC traces showed a significant scatter and were generally higher, in the ranges 0.3–0.5%, 1.5–3.0%, and 0.5–1.5% of Chl a, respectively. Based on the observations mentioned above, these values evidently contain contribution from Chl a alterations in the course of grinding/extraction.

Pigment integrity in the entire course of extraction/HPLC

In order to examine whether the 0.2% level of Chl a' and the 0.8% level of Pheo a (Fig. 2) derive from in vivo or are Chl a alteration products formed during extraction/HPLC, the following experiments were conducted. First an extra pure Chl a sample, containing 0.03% Chl a' as the sole impurity (Fig. 3), was prepared. 99.97% pure Chl a of various weights was then kneaded with *H. macrophyllc* leaf tissue, and the pigments were extracted from the mixture and analyzed by HPLC as described above. If the molecular integrity of the 99.97% pure Chl a is preserved throughout the entire course of extraction/HPLC, both the Chl a'/Chl a and Pheo a/Chl a molar ratios in the resulting extracts would decrease as the amount of admixed pure Chl a increases. In this experiment, the Chl a/Chl b molar ratio in the extract is a measure for the degree of pure Chl a admixing.

In Fig. 4 the typical HPLC traces for *H. macrophylla* leaf tissue alone (A, Chl a/Chl $b = 2.1 \pm 0.1$) and for the leaf tissues with higher Chl a/Chl b molar ratios due to admixing of the pure Chl a (B, C) can be seen. As is seen, the relative contents of Pheo a and Chl a' are lowered systematically by the admixing. Fig. 5 summarizes quantitatively

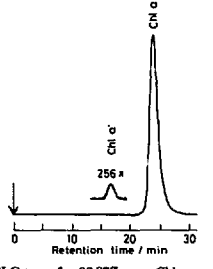

Fig. 3. HPLC trace for 99.97% pure Chl a, containing Chl a' as the sole impurity.

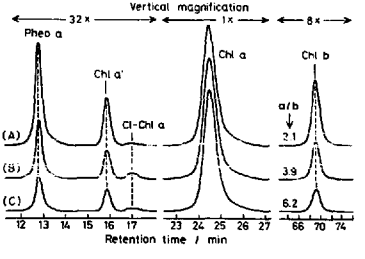

Fig. 4. HPLC traces for chloroform extracts of *H. macrophylla* leaf tissue alone (A) and of (99.97% Chl *a* plus *H. macrophylla* leaf tissue) mixtures (B, C). The Chl *a* peaks are normalized to a common intensity.

the courses of Chl *a'* and Pheo *a* molar abundance lowering with the progress of pure Chl *a* admixing. Most of the data points fall on the working curves (b) and (d), which denote the cases in which the extraneous 99.97% Chl *a* remains intact during extraction/HPLC. Hence, if both endogenous and extraneous pigments have experienced a similar chemical environment, the low levels of Pheo *a* and Chl *a'* found in extracts are not artifacts, but were present in plant tissues just before extraction. A further discussion on these results will be given later.

Chl a' and Pheo a contents in higher plants

The analytical values of Chl *a'* and Pheo *a* contents, under experimental conditions where molecular alterations were negligible, were significantly uniform among more than 100 leaf tissue samples from the 13 different higher plants listed above. When expressed as the number of Chl *a* molecules per molecule of the minor pigments, the results are summarized as follows:

$$\text{Chl } a/\text{Chl } a' = 460 \pm 90 \quad (1)$$

$$\text{Chl } a/\text{Pheo } a = 120 \pm 20 \quad (2)$$

Essentially the same values were obtained for chloroplasts.

Chl a' and Pheo a contents in cyanobacteria

The Chl *a'* and Pheo *a* contents in cyanobacteria, averaged over the three species mentioned above, were found to be slightly different from

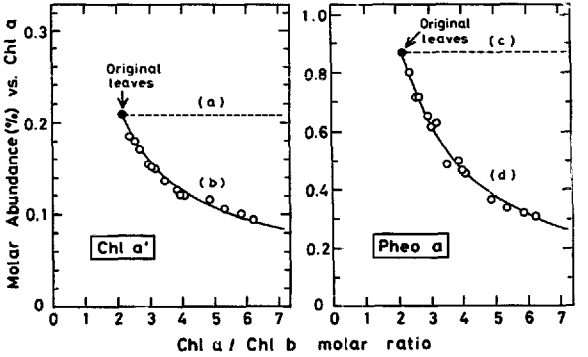

Fig. 5. Decrease in the relative contents in chloroform extracts, of Chl *a'* and Pheo *a* by the admixing of increasing amounts of 99.97% pure Chl *a* to *H. macrophylla* leaf tissue before extraction. The solid curves (b) and (d) represent pure Chl *a* remaining intact during the entire course of extraction/HPLC.

TABLE I

COMPARISON OF Chl *a*/P-700, Chl *a*/P-680 AND P-680/P-700 STOICHIOMETRIES IN HIGHER PLANTS AND CYANOBACTERIA DETERMINED BY HPLC ANALYSES AND OTHER METHODS

Index for P-680 content: Q_A, light-induced absorbance change at 325 nm in the presence of ferricyanide (+FeCy) or in its absence (−FeCy); O_2 or H^+, oxygen or proton yield per flash; atrazine, amount of atrazine bound.

Species	Chl *a* / P-700	Chl *a* / P-680	P-680 / P700	Index for P-680 content	Ref.
Higher plants, 13 species (see Materials and Methods)	460 (±90)	240 (±40)	1.90 (±0.50)	Pheo *a*	Present study
Spinacia oleracea	478	272	1.76	Q_A (+FeCy)	25
	433 [a] (±30)	209 [a]	2.07 (±0.13)	Q_A (+FeCy)	26
	–	220 (±14)	–	Pheo *a*	23
	435	405	1.07	Q_A (−FeCy)	27
	397 [a] (±10)	387 [a]	1.02 (±0.10)	O_2, H^+	28
Pisum sativum	380 (±20)	220 (±60)	1.70 (±0.50)	atrazine	29
	405 (±10)	–	–	–	30
	600 [a]	265 [a]	2.25	atrazine	31
	600 [a]	256 [a]	2.32	Q_A (+FeCy)	31
	600 [a]	431 [a]	1.38	Q_A (−FeCy)	31
	600 [a]	470 [a]	1.27	O_2	31
Nicotiana tabacum	390	230	1.70	Q_A (+FeCy)	32
Glycine max	500	270	1.85	Q_A (+FeCy)	33
Hordeum vulgare	490	260	1.88	Q_A (+FeCy)	33
Lycopersicon esculentum	370	448 [a]	0.83	H^+	30, 34
Cyanobacteria					
Synechococcus sp. (*Anacystis nidulans*)	136 (±4)	218 (±3)	0.62 (±0.02)	Pheo *a*	Present study
	109	–	–	–	35
	–	220 (±18)	–	Pheo *a*	23
	–	211 (±15)	–	O_2	36
	158 (±9)	370 (±34)	0.42 (±0.04)	O_2	37
Anabaena sp.	127 (±7)	208 (±30)	0.61 (±0.07)	Pheo *a*	Present study
	147 (±11)	285 (±13)	0.49 (±0.04)	O_2	37
	128	–	–	–	35
	130	–	–	–	38
Spirulina platensis	113 (±3)	154 (±6)	0.73 (±0.05)	Pheo *a*	Present study

[a] Recalculated from the published data assuming Chl *a*/Chl *b* = 2.6.

those in higher plants, and are summarized as follows:

$$\text{Chl } a/\text{Chl } a' = 125 \pm 15 \quad (3)$$

$$\text{Chl } a/\text{Pheo } a = 100 \pm 25 \quad (4)$$

These values, however, appear to be somewhat characteristic of the species, as will be detailed later.

Chl a' contents in PS I particles

In view of the possibility that we had overestimated the Chl *a'* content in a previous study [7], the pigment composition of PS I particles was re-investigated by the improved extraction/HPLC procedure. The result given in Fig. 6 corresponds well with the stoichiometry

$$\text{Chl } a'/\text{P-700} = 1 \quad (5)$$

for a wide range of PS I particles with Chl *a*/P-700 molar ratios from 1000 down to about 8. (The particle of Chl *a*/P-700 = 1000 alone is actually a PS II particle.) This indicates that Chl *a'* is one of the eight Chl molecules constituting the PS I reaction center, including P-700, though we are not yet sure about the exact role or function of Chl *a'* in driving PS I photochemistry. In PS I particles, Pheo *a* and Chl *b* were detected in trace amounts only, so that these pigments cannot be essential components of PS I.

In our previous work [8], where the Chl *a'*/P-700 molar ratio was evaluated to be around 2, the PS I particles had been prepared through heavier treatments of spinach chloroplasts; Triton X-100 fractionation, SDS-polyacrylamide gel electrophoresis, sucrose-gradient centrifugation, and DEAE-Sepharose column chromatography. Epimerization and/or δ-chlorination of Chl *a* during these treatments were evidently the causes for observation of higher Chl *a'*/P-700 molar ratios.

P-680/P-700 stoichiometry in photosynthetic apparatus

The presence of two Pheo *a* molecules as the primary electron acceptor of PS II reaction center (P-680) has been well established by recent studies [23,24]. Combining Pheo *a*/P-680 = 2 with Eqns. 1–5, one obtains the following pigment/reaction center stoichiometries

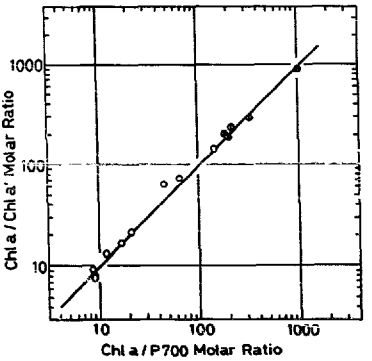

Fig. 6. Relationship between the Chl *a*/Chl *a'* molar ratio and the Chl *a*/P-700 molar ratio for a series of PS I-enriched particles. ①, digitonin-solubilized then centrifuged; ○, digitonin-solubilized and subsequently, ether-washed; ●, PS II particle.

$$\text{Chl } a/\text{P-700} = 460 \pm 90 \quad (6)$$

$$\text{Chl } a/\text{P-680} = 240 \pm 40 \quad (7)$$

$$\text{P-680/P-700} = 1.9 \pm 0.5 \quad (8)$$

for the 13 higher plants, and

$$\text{Chl } a/\text{P-700} = 125 \pm 15 \quad (9)$$

$$\text{Chl } a/\text{P-680} = 200 \pm 50 \quad (10)$$

$$\text{P-680/P-700} = 0.65 \pm 0.20 \quad (11)$$

for the three cyanobacteria examined in the present investigation. In Table I, these values, obtained by simple yet absolute HPLC determination of Chl-type pigments, are compared with recently published stoichiometry data accumulated through (photo-)redox titrations of reaction center key components.

Discussion

For a series of higher plants, a good agreement is seen in Table I among the Chl *a*/P-700 ratios

assayed by different workers. Obviously, this is a consequence of the use of a common method for quantitating P-700; oxidized-minus-reduced absorbance measurement with a P-700 absorption coefficient of 64 $mM^{-1} \cdot cm^{-1}$ [15].

Based on these findings, the result of the dilution experiments (Fig. 5) could be regarded as strong evidence that the low level of Chl *a'* found in extracts of 'undiluted' plant tissues is not an experimental artifact. An argument against this would be to invoke a possibility that a single Chl *a* molecule or its derivative, present in a special environment very close to P-700, has been selectively epimerized to Chl *a'* on disintegration of the protein network by tissue crushing. However, since Chl *a'* is higher in Gibbs free energy than Chl *a* by about 2.7 kJ/mol [22], and as long as the epimerization proceeds as a normal chemical reaction, the maximum fraction of Chl *a'* in the resulting epimer mixture should be 25%, irrespective of the nature of the starting species (Chl *a*, Chl *a'*, or an enol). Therefore, even when epimerization is completed during our 1-min tissue crushing period, we have to assume the presence of four special Chl *a*-type pigments in the vicinity of P-700. If the epimerization has proceeded only slightly within 1 min (and this is perhaps more probable) much more special Chl *a* molecules (out of only eight Chl *a* molecules, see Fig. 6) are needed to yield one molecule of Chl *a'* during crushing/extraction.

The sole argument against endogenous pigments being intact during crushing may be to suppose the presence of a special enzyme which catalyzes the energetically uphill process Chl *a* → Chl *a'* or the enol → Chl *a'* conversion, which are both reversible reactions and can normally yield at most 25% Chl *a'*, with 100% efficiency. Whether such a process takes place is, however, beyond the scope of the present work.

The Chl *a*/P-680 molar ratio determined here by simple HPLC quantitation of Chl *a* and Pheo *a* (240 ± 40) more than adequately covers the range of literature value obtained by quantitating Pheo *a*, atrazine bound, or Q_A in the presence of ferricyanide (200–270). We note, however, that the Chl *a*/P-680 ratio evaluated by quantitating Q_A in the absence of ferricyanide or by measurement of oxygen or proton yield by flash excitation (387–480) is roughly twice the value mentioned above. This may reflect the presence of two different electron acceptors, Q_A and Q_{400} [31,39–41], of which only Q_A can be detected in the latter mode of measurement. Q_{400}^{+} was recently suggested to be Fe(III), the oxidized form of Fe(II) associated with quinone acceptors Q_A and Q_B [42,43]. Since Q_{400} can accept electrons only in its oxidized state, both Q_A and Q_{400} are detectable in the presence of ferricyanide.

Based on our HPLC analyses with the sole assumption that Pheo *a*/P-680 = 2, the P-680/P-700 molar ratio is evaluated to be 1.9 ± 0.5 for the 13 higher plants examined. This value is again in the range of many recently reported values (1.70–2.30) listed in Table I. The P-680/P-700 stoichiometry assayed from Q_A quantitation in the absence of ferricyanide or from oxygen or proton yield measurements is, for reasons mentioned above, significantly lower than this (0.83–1.4).

The Chl *a*/P-700, Chl *a*/P-680 and P-680/P-700 stoichiometries evaluated from HPLC analyses of pigment composition in cyanobacteria are also fairly close to those obtained by other methods [23,35–38,44,45]. Thus, the body of Table I provides strong evidence that simple HPLC quantitation of Chl-type pigments is, as long as it is carried out under conditions where even minute alterations of these labile pigments are negligible, an effective means for evaluating reaction center concentrations in photosynthetic apparatus.

Currently, a controversy prevails as to the molar ratio of a PS II subunit cytochrome *b*-559 to P-680, namely between 1 [46] and 2 [44,47,48]. The former value is in line with our analytical results, since the Chl *a*/cytochrome *b*-559 molar ratio in higher plants has been established at about 200 [47,49]. In view of such uncertainty, a reliable technique to quantitate P-680 should be devised in future investigations.

Acknowledgments

The authors are grateful to professor H. Scheer, Botanical Institute, University of Munich, for valuable discussions. This work was supported partly by Iwatani Naoji Foundation and a Grant-in-Aid (No. 62602508) from the Ministry of Education, Science and Culture to T.W.

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