Biochimica et Biophysica Acta, 1099 (1992) 74–80

BBABIO 43549

Presence of two chlorophyll *a'* molecules at the core of Photosystem I

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(Received 1 August 1991)

Key words: Chlorophyll *a'*; Photosystem I; Reaction center; HPLC

The pigment composition in cells and thylakoid membranes of a cyanobacterium, and in leaves and intact or P700-enriched chloroplasts of higher plants, has been reinvestigated by solvent extraction/HPLC. In acetone extracts, the amount of chlorophyll (Chl) *a'* relative to Chl *a* or P700 was nearly twice that in chloroform extracts: the latter gave a result in line with the stoichiometry of Chl *a'*/P700 = 1 found by chloroform extraction (Kobayashi, M. et al. (1988) Biochim. Biophys. Acta 936, 81–89). By two-step extraction, first with chloroform then with acetone, a roughly equal amount of Chl *a'* was extracted in each step, while more than 90% of Chl *a* was extracted already in the first step. For chloroplasts heavily enriched in P700, the residue of the first extraction exhibited a Chl *a'*/Chl *a* molar ratio outreaching the equilibrium value (0.33), as verified by the second extraction/HPLC. These results evidence that two Chl *a'* molecules exist at the Photosystem (PS) I core, and just one of them is at a site to which only such a hydrophilic molecule as acetone is accessible. A preliminary discussion is given on the possible role of the two Chl *a'* molecules in PS I.

Introduction

We previously detected chlorophyll (Chl) *a'*, the C13^2-epimer [1] of Chl *a*, at a sub-percent level against the major pigment Chl *a* in higher plant leaves by chloroform extraction/normal-phase HPLC [2]. By use of a binary solvent (hexane/2-propanol = 98.6/1.4, v/v) as HPLC eluent, subchloroplast particles with the Chl *a*/P700 molar ratio of 50–1000 exhibited an apparent Chl *a'*/P700 molar ratio of about 2, and the Photosystem (PS) II particles were devoid of Chl *a'*. These led us to speculate that P700 might be a Chl *a'* dimer [3].

Subsequently we noted that the 'Chl *a'* peak' in HPLC traces was contaminated with a somewhat variable but comparable amount of C20-chlorinated Chl *a*, formed in the course of extraction [4]. Modification of the eluent into a ternary system (hexane/2-propanol/methanol = 100:0.8:0.4) resulted in perfect resolution of the peak into two. We then reexamined the Chl *a'* content in fractionated PS I and PS II particles and ether-treated (P700-enriched) PS I particles with the Chl *a*/P700 molar ratio ranging from about 1000 down to 8, to find a stoichiometry of Chl *a'*/P700 ≃ 1 [5,6].

Throughout these measurements we used chloroform as an extraction solvent, in view of an extremely slow Chl *a*/*a'* epimerization (half-conversion period: about 600 h at 25°C) [7] and fairly good solubility of chlorophyllous pigments in this solvent. For instance, grinding of leaf tissues followed by 15-s sonication in chloroform liberated at least 75% of Chl *a* [2]. Since Chl *a'* is less polar than Chl *a*, as judged from the elution order in normal-phase HPLC, we had suspected that hydrophilic acetone would be less effective than chloroform to extract Chl *a'*. This point, however, remains to be examined in detail because the extraction efficiency depends not only on the in vitro solubility, but more importantly on the accessibility of solvent molecules to the target pigment sitting inside a polypeptide network.

In this work we hence compared chloroform and acetone with regard to their performance in extracting Chl *a* and Chl *a'* from a variety of plant samples. As a

Abbreviations: Chl, chlorophyll; Pheo, pheophytin; PS, Photosystem; RC, reaction center; HPLC, high-performance liquid chromatography.

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result, acetone was much better than chloroform in that it extracted both pigments almost completely, although a somewhat different observation had been made previously [2]. The Chl *a*/*a'* epimerization in acetone was negligible as long as the extraction/HPLC procedure was conducted in a short period. The most significant finding was that the amount of Chl *a'* relative to Chl *a* or P700 in acetone extracts was nearly twice that in chloroform extracts. This, as supported by other observations, shows the presence of two molecules of Chl *a'* at the core of PS I.

Materials and Methods

Cyanobacterium: cells and thylakoid membranes

Synechocystis PCC 6714 was cultured at 26°C in the medium of Herdman et al. [8] as modified by Astier et al. [9]. Cells grown under four different light conditions were subjected to analysis: cells were grown initially (sample 1) under PS II light, which selectively excites the PS II antenna ($\lambda_{max} = 625$ nm). The light was then replaced with PS I light ($\lambda > 650$ nm), and cells were harvested at 7.5 h (sample 2), 15 h (sample 3) and 30 h (sample 4) thereafter. The reaction center (RC) molar ratio, RC I/RC II, was evaluated to be 2.2, 1.4, 1.2 and 1.0 for sample 1, 2, 3 and 4, respectively (unpublished results).

For the preparation of thylakoid membranes [10], cells were suspended in a Tricine-NaOH buffer (50 mM, pH 7.5), disrupted with a French press, and then treated with intermittent sonication (20 kHz) at ice-water temperature. After removal of unbroken cells, the homogenates were centrifuged at $140\,000 \times g$ for 1 h. The precipitated membranes were washed three times with the Tricine-NaOH buffer.

Higher plants: leaf tissues and chloroplasts

Fresh spinach and hydrangea were employed as the sources for leaf tissue specimen. Chloroplasts were prepared from spinach as follows. Leaves were homogenated with a mechanical mixer in a 50 mM phosphate buffer (pH 7.8) containing 10 mM NaCl. Removal of the debris by filtration was followed by centrifugation at $300 \times g$ for 5 min, and the supernatant was then centrifuged at $2000 \times g$ for 8 min to precipitate chloroplasts, which were finally lyophilized.

P700-enriched chloroplasts

Spinach chloroplasts, exhibiting the Chl *a*/P700 molar ratio of ca. 500, were enriched in P700 by treatment with diethyl ether [11] at various degrees of water saturation. Chloroplasts with the Chl *a*/P700 molar ratios of about 162, 100, 48, 22, 15 and 12 were thus obtained. Whole PS I particles (Chl *a*/P700 ≃ 200) were separately prepared and also submitted to the analysis. The absorption coefficient of 64 mM^{-1} cm^{-1} [12] was used to quantitate P700 spectrophotometrically.

Pigment extraction

Chloroform extraction was performed as in the previous work [6]. Briefly, a sample was ground with a large amount of Na_2HPO_4, and then sonicated for about 1 min in reagent grade chloroform (Wako Pure Chemicals). The extraction efficiency of Chl *a* was generally 90–95% and occasionally 97–99%, depending on the nature of sample.

The method of acetone extraction was varied according to whether the sample was a solid (leaf tissue and lyophilized chloroplasts) or a suspension (ether-treated chloroplasts, *Synechocystis* cells and thylakoids). In the former case, less than 120 mg of sample was ground with 60 g of Na_2HPO_4 and then sonicated (20 kHz, 50 W) for 1 min in 10 ml of reagent grade acetone (Wako Pure Chemicals) at 20–25°C. In the latter case, about 10 μl of sample was mixed with 10 ml of acetone and sonicated for 1 min as above. The acetone solution thus obtained was filtered through a Tosoh Teflon filter H-25-5 (0.45 μm). The residue on the filter was further washed twice, each time with 10 ml of acetone. No pigments were extracted by the second wash, and the overall extraction efficiency of Chl *a* was estimated to be higher than 99.9%. The whole procedure of acetone extraction was completed within 5 min.

A two-step extraction, first with chloroform and then with acetone, was also conducted to examine the extraction efficiency of Chl *a'*.

HPLC analysis

The chloroform or acetone extract was dried in a rotary evaporator, then redissolved in 10–20 μl of chloroform, and finally a 2-μl aliquot was injected into a silica HPLC column (Neopack N-9122, 250 mm × 4.6 mm i.d.) cooled to 4°C. Pigments were eluted isocratically with hexane/2-propanol/methanol (100/1.0/0.5) at a flow rate of 1.2 ml/min with a Shimadzu absorbance detector Model SPD-10A adjusted to 425 nm.

All the operations described above were conducted under dim light.

Results and Discussion

Chl a' content in Synechocystis

Typical HPLC traces for chloroform and acetone extracts of *Synechocystis* cells (sample 1, see above) are shown in Fig. 1A and B, respectively. β-Carotene (β-Car), Pheo *a*, Chl *a'*, and Chl *a* are clearly detected in both extracts. When the Chl *a* peak intensity was arbitrarily scaled to a common value, the relative amounts of the three pigments, β-Car, Pheo *a* and Chl *a*, remained unchanged by the change of extraction solvent. Of significance is the roughly 2-fold increase in

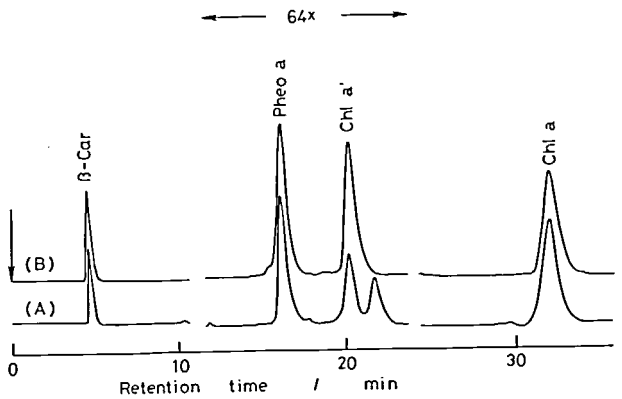

Fig. 1. HPLC traces for a chloroform extract (A) and acetone extract (B) of *Synechocystis* PCC 6714 cells.

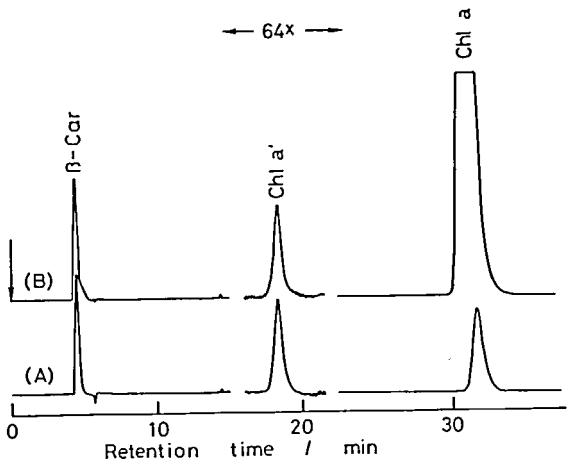

Fig. 2. HPLC traces for acetone extracts of *Synechocystis* cells (A) and of *Synechocystis* cells plus 99.97% pure Chl *a* (B).

the relative amount of Chl *a'* in going from chloroform to acetone; 0.74% in chloroform extract to 1.41% in acetone extract with respect to Chl *a*, for this particular example. On the contrary, both chloroform and acetone can liberate another minor pigment, Pheo *a*, to nearly the same extent.

The peak at 21–22 min in trace A reflects the formation of C20-chlorinated Chl *a* from the major pigment Chl *a* (but not from Chl *a'*) in the course of extraction [4]. We qualitatively noted that the degree of this alteration was higher when the plant tissue was not sufficiently dehydrated or when the tissue was exposed to excessive light during chloroform extraction. In sharp contrast, use of acetone well suppressed the C20-chlorination of Chl *a*. This suggests that the C20-chlorination occurs by a reaction of Chl *a* with a chlorine species liberated mainly from decomposition of chloroform. Details of this alteration are however beyond the scope of the present paper. In any event the amount of C20-chlorinated Chl *a* was too small to affect the discussion that follows.

Intactness of Chl a during acetone extraction

Before examining the quantitative aspect of Chl *a'* extraction, it was necessary to critically ensure the absence of Chl $a \rightarrow a'$ epimerization during acetone extraction. For this, as in chloroform extraction [6], we added 99.97% pure Chl *a* to a plant specimen, and subjected the mixture to the same procedure as the acetone extraction/HPLC mentioned above. Such experiments were repeated for all the specimens employed in this work, but only the results for *Synechocystis* cells (sample 1) are presented here.

Fig. 2 shows the HPLC traces before (A) and after (B) admixing of 99.97% pure Chl *a*, to a level roughly 10-times that in the original cells. The Chl *a'* to β-Car molar ratio did not change by the admixing, and the sole consequence is an increase in the relative amount of Chl *a* in trace B.

The results of such measurements are plotted in Fig. 3 in the form of the Chl *a'*/β-Car molar ratio against the Chl *a*/β-Car molar ratio, the latter reflecting the degree of Chl *a* admixing to the original cells. Since the Chl *a'*/β-Car molar ratio remains practically constant by this admixing, we conclude that acetone is sufficiently inert in promoting the Chl $a \rightarrow a'$ epimerization under the present conditions. Essentially identical results were obtained for other specimens (samples 2–4 of *Synechocystis* cells, thylakoid membranes, higher plant leaves, intact and P700-enriched chloroplasts). In a recent work we studied the pigment composition in the PS II reaction center (D1/D2/cytochrome *b*-559) complex by acetone extraction/HPLC, and detected no Chl *a'* in the extract [13]. This is in line with the present finding, and indicates the superiority of acetone as extraction solvent for chlorophyllous pigments.

Comparison of chloroform and acetone in extracting Chl a' from the cyanobacterium

A series of measurements such as the one shown in Fig. 1 were carried out on samples 1–4 (cells) and

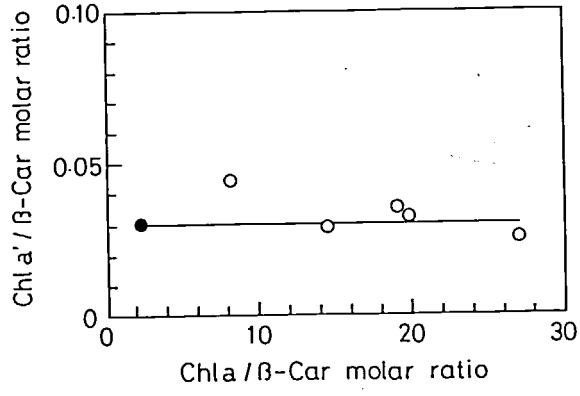

Fig. 3. Dependence of the Chl *a'*/β-Car molar ratio on the Chl *a*/β-Car molar ratio in acetone extracts of [*Synechocystis* cells plus varying amounts of 99.97% pure Chl *a*]. The solid circle (•) represents the original cells.

TABLE I

Chl a'/Chl a molar ratios in chloroform and acetone extracts of Synechocystis PCC 6714 cells

Sample [a]	[Chl a'/Chl a]·1000 (mean ± S.D.) in		B/A [b]
	Chloroform extract (A)	Acetone extract (B)	
1	7.42 ± 0.26 ($n = 4$)	14.4 ± 0.98 ($n = 3$)	1.94
2	5.72 ± 0.38 ($n = 4$)	11.6 ± 0.81 ($n = 3$)	2.02
3	5.36 ± 0.29 ($n = 4$)	12.1 ± 0.54 ($n = 3$)	2.25
4	6.74 ± 1.34 ($n = 4$)	12.4 ± 1.10 ($n = 3$)	1.84

[a] See text for the nature of samples 1–4.
[b] Ratio of the mean values.

samples 1′–4′ (thylakoid membranes). The results are summarized in Tables I and II. It is seen that the amount of Chl *a'* relative to Chl *a* in acetone extracts is nearly twice that in chloroform extracts. The Chl *a'*/Chl *a* molar ratios in the cell samples 1, 3 and 4 are in good agreement with those in the corresponding thylakoid membrane samples 1′, 3′ and 4′. Exceptionally, the values for sample 2 are somewhat lower than those of the corresponding sample 2′, for an unknown reason. The data scatter is neatly smaller for membranes than for their starting materials (cells).

Chl a' content in higher plants

Spinach and hydrangea leaf tissues and spinach-derived chloroplasts gave essentially the same results as to the difference in the Chl *a'* extractability between chloroform and acetone. As a typical example, HPLC traces for chloroform and acetone extracts of spinach leaf tissues are displayed in Fig. 4A and B, respectively. A significant increase in the relative amount of Chl *a'* in going from chloroform to acetone is again evident.

In a previous work [6] we noted that initial dehydration is a crucial factor for ensuring the pigment integrity in extracting chlorophyllous pigments from leaves. In chloroform extraction, we used 60 g of the desiccant, Na_2HPO_4, and found that the pigment integrity was maintained when the leaf weight was within 0.5 g [6]. In the present work a similar examination was done by keeping the latter within 0.12 g, for both chloroform and acetone extractions. The results shown in Fig. 5 demonstrate that the relative abundance of Chl *a'* is fairly constant under these conditions.

TABLE II

Chl a'/Chl a molar ratios in chloroform and acetone extracts of Synechocystis PCC 6714 thylakoid membranes

Sample [a]	[Chl a'/Chl a]·1000 (mean ± S.D.) in		B/A [b]
	Chloroform extract (A)	Acetone extract (B)	
1′	7.28 ± 0.20 ($n = 4$)	13.9 ± 0.66 ($n = 3$)	1.91
2′	6.94 ± 0.38 ($n = 4$)	13.7 ± 0.17 ($n = 4$)	1.97
3′	6.13 ± 0.39 ($n = 4$)	12.3 ± 0.35 ($n = 4$)	2.01
4′	6.92 ± 0.31 ($n = 4$)	12.9 ± 0.60 ($n = 3$)	1.86

[a] Samples 1′–4′ were prepared, respectively, from samples 1–4 in Table I.
[b] Ratio of the mean values.

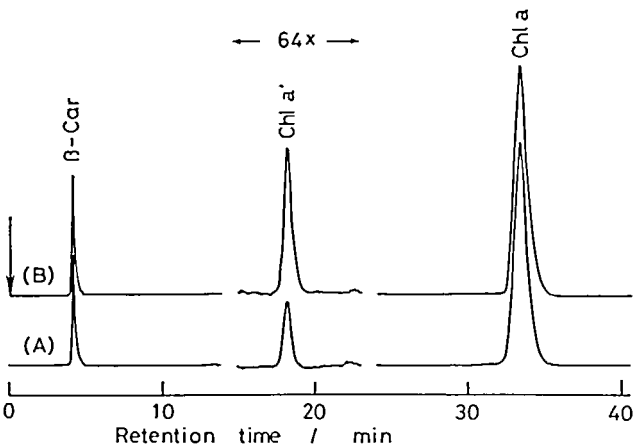

Fig. 4. HPLC traces for a chloroform extract (A) and acetone extract (B) of spinach leaf tissue.

The data in Fig. 5 are summarized, in the form of mean ± S.D., as follows.

Acetone extraction:

$$\text{Chl } a'/\text{Chl } a = (0.47 \pm 0.03) \cdot 10^{-2} \; (n = 15)$$

Chloroform extraction:

$$\text{Chl } a'/\text{Chl } a = (0.24 \pm 0.08) \cdot 10^{-2} \; (n = 4)$$

The latter value is within the error limit of the previous result (0.22 ± 0.04%) for 13 different higher plants [6]. Of more significance is the fact that, as for the *Synechocystis* samples described above, the relative amount of Chl *a'* in acetone extracts is approximately twice that in chloroform extracts.

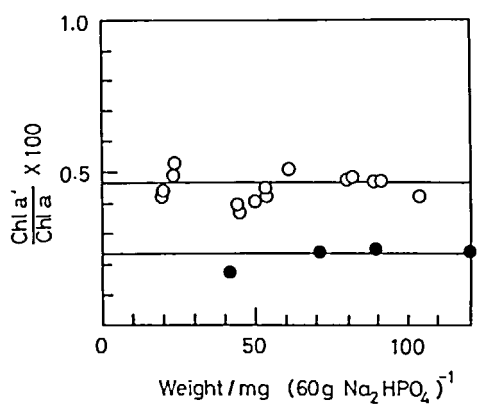

Fig. 5. Relative abundances of Chl *a'* by chloroform extraction (●) and by acetone extraction (○) in spinach leaf tissue, for a series of runs where the weight of leaf tissue, ground with 60 g of desiccant, was varied.

Chloroform / acetone two-step extraction

The experimental observations up to this stage strongly suggested that chloroform could liberate only half the amount of Chl *a'* present in vivo, and the remaining half could be extracted with acetone. In order to verify this, a two-step extraction was carried out on a variety of plant specimens.

A result for spinach leaf tissue is displayed in the form of HPLC traces in Fig. 6. Trace A represents the initial chloroform extract, where the amount of Chl *a'* is about 0.20% against Chl *a*. The residue was then extracted with acetone, yielding trace B which is magnified 32-fold with respect to trace A. In trace B, the amount of Chl *a'* is 7.7% against Chl *a*, namely the Chl *a'* in the leaf tissue has been 40-fold concentrated by the initial chloroform extraction. When the residue of the acetone extraction was re-extracted with acetone, trace C was obtained. No pigments were detected on trace C, indicating that pigment extraction was almost complete at the stage of trace B.

The absolute amounts of Chl *a'* and Chl *a* were calculated on the basis of traces A and B to give the following figures:

Trace A:	Chl *a'* = 13 pmol,	Chl *a* = 6600 pmol
Trace B:	Chl *a'* = 15 pmol,	Chl *a* = 190 pmol

This shows that the efficiency of the initial chloroform extraction was about 97% for Chl *a* but roughly half (46%) for Chl *a'* as expected.

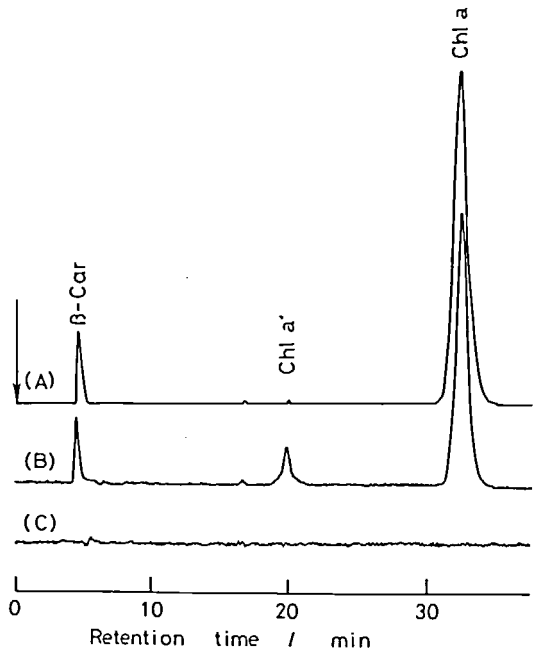

Fig. 6. HPLC traces for a two-step extraction, first with chloroform (A) then with acetone (B), of spinach leaf tissue. Re-extraction of the residue with acetone yielded trace C. Traces B and C are 32-fold magnified vertically with respect to trace A.

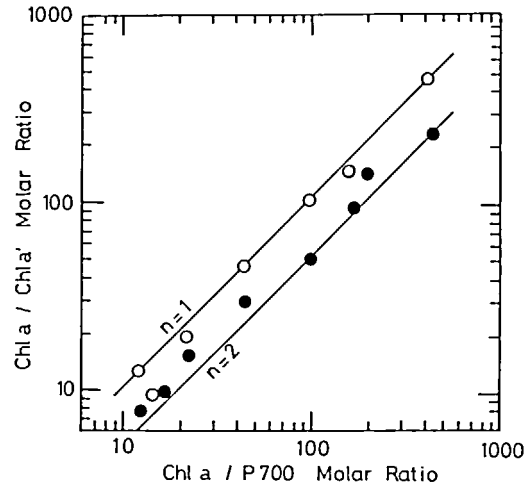

Fig. 7. Relationship between the Chl *a*/Chl *a'* molar ratio and the Chl *a*/P700 molar ratio for a series of P700-enriched chloroplasts by chloroform extraction (○) and by acetone extraction (●). The Chl *a'*/P700 molar ratio is denoted by *n*.

A typical result from a similar two-step extraction for spinach chloroplasts was as follows:

Chloroform extraction:	Chl *a'* = 27 pmol,	Chl *a* = 9800 pmol
Acetone extraction:	Chl *a'* = 26 pmol,	Chl *a* = 250 pmol

Here the efficiency of the initial chloroform extraction is 98% for Chl *a* and 51% for Chl *a'*. This is again in line with the expectation.

Chl a' content in P700-enriched chloroplasts

As in the previous work [6], spinach chloroplasts were treated with diethyl ether at various degrees of water saturation, and the contents in P700 and Chl *a'* were assayed by independent measurements. The results, together with those for intact chloroplasts (Chl *a*/P700 = 500) and PS I particles (Chl *a*/P700 = 200) are depicted in Fig. 7. The open and solid circles are, respectively, from chloroform and acetone extractions, and *n* denotes the Chl *a'*/P700 molar ratio. The open circles are well on the line corresponding to the stoichiometry of Chl *a'*/P700 = 1 as found before by chloroform extraction [6]. The data points for acetone extraction, however, are fairly close to the line representing the stoichiometry of Chl *a'*/P700 = 2.

The two-step extraction was applied also to the P700-enriched chloroplasts. The results were generally the same as for the leaf tissues presented above, i.e., roughly half the amount of Chl *a'* was extracted with chloroform and the remaining half was subsequently extracted with acetone. In addition, chloroplasts highly enriched in P700 gave a remarkable result. In a manner similar to that for Fig. 6 stated above, the result for the chloroplasts exhibiting a Chl *a*/P700 molar ratio of approx. 12 is illustrated in Fig. 8. When expressed by

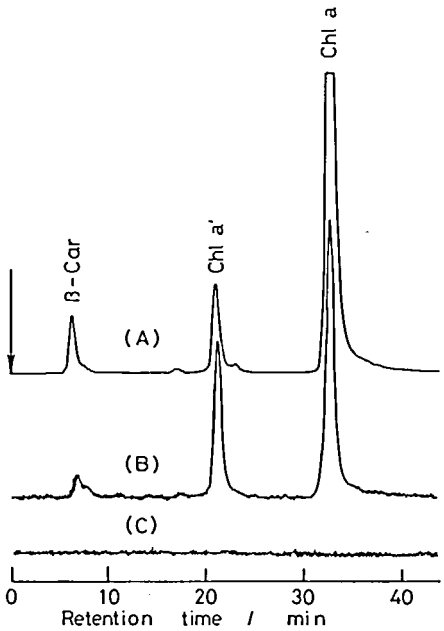

Fig. 8. HPLC traces for a two-step extraction, first with chloroform (A) then with acetone (B), of ether-washed chloroplasts having a Chl *a*/P700 molar ratio of about 12. Re-extraction of the residue with acetone yielded trace C. Traces B and C are 2 fold magnified vertically with respect to trace A.

the absolute amounts of Chl *a'* and Chl *a*, the following figures are derived from the HPLC traces:

Trace A:	Chl *a'* = 85 pmol,	Chl *a* = 1100 pmol
Trace B:	Chl *a'* = 74 pmol,	Chl *a* = 160 pmol

The figures for trace B correspond to the Chl *a'*/Chl *a* molar ratio of 0.46. In a separate measurement with the same sample, we observed a value of 0.48 for this molar ratio. These are neatly higher than the thermodynamic equilibrium value of 0.33 [14], and provide unequivocal evidence that the Chl *a'* was indeed present in vivo and was not a product of Chl *a* → *a'* epimerization during extraction. A similar observation was made recently by acetone extraction/HPLC on ether-treated *Heliobacterium chlorum* membranes [15], though the pigment in question then was not Chl *a'* but bacteriochlorophyll (BChl) *g'*.

Possible role of Chl a' in vivo

From the present findings it is now obvious that two out of a dozen chlorophyllous molecules at the core of PS I are Chl *a'*. The result of two-step extraction experiments suggests that one of them resides deep in the structural protein, that is, at a site which is accessible by such a hydrophilic molecule as acetone but inaccessible by hydrophobic chloroform (in constrast, both chloroform and acetone can apparently access the site where Pheo *a* molecules are situated, as indicated by the result in Fig. 1).

In conjunction with the long-standing view that P700 might be a dimer of a Chl *a*-type pigment [16], the present results incite us to suppose that P700 be a Chl *a'* dimer. We once stated such a speculation (albeit at a stage where Chl *a'* and C20-chlorinated Chl *a* were not separated in our HPLC) based on a similarity of the oxidized-minus-reduced difference spectrum between $(\text{Chl } a')_2$ and P700 [3] and the apparent recovery of the P700 activity by addition of Chl *a'* to the PS I core apoprotein [17]. The present results would justify the renaissance of this speculation. Of much relevance is the detection of two molecules of BChl *g'*, the $C13^2$-epimer of the major pigment (BChl *g*), in the reaction center of heliobacteria by acetone extraction/HPLC [18]. Since the reaction center of heliobacteria is of the PS I type [19], it is possible that a dimer of the stereoisomer of the major pigment constitutes the reaction center in both organisms. Chl *a* and Chl *a'* exhibit exactly the same UV-visible absorption [20] and redox properties (unpublished results) in the monomeric state, but behave very distinctly once they form dimers or aggregates (unpublished results). The choice, if any, of the dimer of Chl *a'* or BChl *g'* as the reaction center may therefore be due to their particular spectroscopic and redox properties which are not produced with Chl *a* or BChl *g* as building blocks. Efforts are currently under way to reinvestigate closely the physicochemical properties of $(\text{Chl } a')_2$ in comparison with P700.

In the current discussion of PS I photochemistry, the functioning of a Chl *a*-type pigment is envisaged as the primary acceptor A_0 [21]. If there exists, as in bacterial RCs, two electron relay pathways of which only one is active within RC I, there is another possibility that the two Chl *a'* molecules are the active and silent A_0. The choice of Chl *a'* as A_0, however, is hard to imagine because the Chl *a'* molecule should function there as monomer; monomeric Chls *a* and *a'* are identical to each other as to spectroscopic and redox behaviors (vide supra).

Recently Maroc and Tremolieres [22] studied the pigment composition in *Chlamydomonas reinhardtii* by acetone extraction/normal-phase HPLC. In P700-lacking mutants the relative amount of Chl *a'* was about half that in normal strains. This observation is hardly reconciled with the stoichiometry Chl *a'*/P700 = 1 [6], because we then have to assume the presence of a 'half-molecule' of Chl *a'* in P700-less mutants. In the light of our renewed hypothesis that P700 be a Chl *a'* dimer, however, their finding is rationalized by assuming that the P700 activity is readily impaired by a loss of just one, and not necessarily both, of the two Chl *a'* molecules in the PS I core.

Acknowledgments

We are grateful to Prof. Y. Fujita and Mr. A. Murakami, National Institute for Basic Biology, for the supply of the *Synechocystis* strain and generous guidance in preparing thylakoid membranes with their facilities. Thanks are extended to Prof. V.V. Klimov, Institute of Soil Science and Photosynthesis, USSR, for a stimulating discussion. We thank Mr. J. Suzuki for his help with the experiments.

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