Biochimica et Biophysica Acta, 1018 (1990) 67–71
Elsevier

BBABIO 43240

Chlorophyll *a'* and pheophytin *a*, as determined by HPLC, in photosynthesis mutants and double mutants of *Chlamydomonas reinhardtii*

Jeannine Maroc and Antoine Tremolieres

Laboratoire de Biochimie Fonctionnelle des Membranes Végétales (U.P.R. 54), C.N.R.S., Gif sur Yvette (France)

(Received 20 November 1989)
(Revised manuscript received 8 March 1990)

Key words: Chlorophyll *a'*; Pheophytin *a*; Photosynthesis mutant; (*C. reinhardtii*)

HPLC analysis of chlorophyll (Chl) *a'* and pheophytin (Pheo) *a* content was run for wild type *Chlamydomonas reinhardtii* and seven mutants with impaired photosynthetic functions. Four of them had no or little Photosystem II (PS II) activity. Two had no Photosystem I (PS I) activity and no P-700. In the seventh mutant, both photosystems were present but the main light-harvesting antenna (the chlorophyll-protein complex corresponding to PS II: CP II) was totally lacking because of the absence of Chl *b*. In mutants impaired in PS II activity, Pheo *a* is either lacking or found in only low amounts in direct relation with the loss in PS II activity. These results are in agreement with the well-known role of Pheo *a* as a primary electron acceptor in PS II. In mutants impaired in PS II activity the Chl *a'* level is normal. In mutants impaired in PS I activity (P-700 less mutants), only half of the normal content of Chl *a'* is present. So Chl *a'* is associated exclusively with PS I as demonstrated before by Watanabe (Watanabe, T., Kobayashi, M., Hongu, A., Nakazato, M., Hiyama, T. and Murata, N. (1985) FEBS Lett. 191, 252–256). But only half the Chl *a'* content could be P-700. This last result contradicts Watanabe's conclusion that the whole Chl *a'* constitutes P-700. On the other hand, there is no relation between the presence of Chl *a'* and the second Chl *a*-protein complex of PS I: CP 0a. The possible function of Chl *a'* related to PS I activity is discussed.

Introduction

The Chl *a'*, C10-epichlorophyll *a* was discovered in 1942 by Strain and Manning [1] in plant extracts. For a long time, Chl *a'* was considered as an artefact produced during preparation of the plant extracts. Recently, Chl *a'* has been detected in higher plants and cyanobacteria by Watanabe and co-workers [2,3]. Their results argue for the presence of Chl *a'* in vivo. Their subsequent studies of PS I and PS II particles revealed that Chl *a'* was associated exclusively with PS I at a Chl *a'*/P-700 molar ratio around 1. This suggested that Chl *a'* could be an integral part of P-700 [4–6].

The involvement of Pheo *a* as a primary electron acceptor in the PS II reaction center has been demonstrated by Klimov et al. [7] and largely strengthened by numerous biophysical measures. Omata et al. [8] have shown that two Pheo *a* molecules are present per PS II complex in spinach chloroplasts.

These two chlorophyllous pigments are present in small amounts in photosynthetic organisms, but it is now possible to identify and to measure them by HPLC according to Watanabe et al. [9,2,3].

The aim of this work was to investigate the Chl *a'* and Pheo *a* content in *Chlamydomonas reinhardtii* mutants, which have no PS II or no PS I activities or which lack the main light-harvesting antenna, in order to determine whether there is a correlation between the presence of these two pigments and the activity of one or the other of the two photosystems.

Materials and Methods

The characteristics of wild-type *C. reinhardtii* and of the mutants *Pg* 27, *Fl* 5, *Fl* 39, *Fl* 50 and the double mutants *Fl* 5 *Pg* 27, *Fl* 39 *Pg* 28 and *Fl* 50 *Pg* 27

Abbreviations: Chl, chlorophyll, CP, chlorophyll-protein complex; CP I, complex which corresponds to the PS I reaction center and its core antenna; HPLC, high-performance liquid chromatography; P-700, Chl *a* holochrome, active pigment in PS I; PAGE, polyacrylamide gel electrophoresis; Pheo, pheophytin; PS I, Photosystem I; PS II, Photosystem II.

Correspondence: J. Maroc, Laboratoire de Biochimie Fonctionnelle des Membranes Végétales (UPR 54), CNRS, Bâtiment 9, F-91198 Gif sur Yvette, Cedex, France.

isolated in our laboratory have been described in preceding papers [10–15]. Some of them are presented in Table I. Algae were grown in light, in Tris-acetate medium [16] as previously reported [10].

For pigment extraction, the algae collected by centrifugation were washed in a 0.01 M potassium phosphate buffer (pH 7.5). Then the pellet was extracted by homogenization, at 4°C, in 30 ml acetone/0.01 M phosphate buffer (pH 7.5) (80 : 20, v/v). The extract was filtered on frittered glass. The chlorophylls and the carotenoids in the 80% acetone extract were rapidly transferred to hexane. Emulsion must be avoided and the total procedure must be rapid, in order to minimize (< 10 min) contact with acetone. The concentrated hexane extract is immediately analysed by HPLC.

The HPLC procedure described by Watanabe et al. [9,2] was used with some modifications. In our study, for analytical HPLC, the silica column was a 250 mm length, 4 mm diameter, Hibar pre-packed column RT, Lichrosorb Si 60 5 μm (Merck), cooled to approx. 4°C in an ice-water bath. The sample was eluted isocratically with hexane/2-propanol (98.5 : 1.5, v/v) at a flow rate of 0.7 ml/min. The loop size was 20 μl and the detection wavelength was 430 nm. Sometimes, to characterize unambiguously the pheophytin *a*, detection was made at 408 nm: Pheo *a*, $A_{408}/A_{430} = 4$; Chl *a'* (and *a*), $A_{408}/A_{430} = 0.88$.

The HPLC procedure was checked with standard samples of Chl *a*, Chl *a'* and Pheo *a*. Chl *a* was extracted from spinach chloroplasts and purified by preparative scale HPLC. A fraction of Chl *a* in diethyl ether was pheophytinized by shaking with 1 ml of cold HCl (25%, v/v). The acid phase was neutralized with solid sodium acetate and Pheo *a* in diethyl ether was transferred to hexane [17]. Chl *a'* was obtained by treating Chl *a* with 0.1 M imidazole in diethyl ether for one night at 21°C in the dark [18]. Subsequently, Chl *a* and Chl *a'* were separated by preparative HPLC.

The chlorophyll and pheophytin concentrations were measured in 80% acetone according to MacKinney [19] and Arnon [20], or in pure acetone according to Watanabe et al. [9].

With the column used by Watanabe (Senshupack 1151-N, 150 mm length), Chl *a'* eluted before Pheo *a*. But, if the authors observed some overlap between Chl *a'* with the solvent, hexane/2-propanol (98.5 : 1.5, v/v), they finally used [3] a ternary solvent (hexane/2-propanol/methanol, 100 : 0.8 : 0.4, v/v) to overcome this problem and to obtain a clear separation between Pheo *a*, Chl *a'*, Chl RC1 [3,5,21–24] and Chl *a*.

As seen in Fig. 1, with the column used in this work and the binary solvent (hexane/2-propanol, 98.5 : 1.5, v/v), the Pheo *a* eluted before Chl *a'*. The identification of the retention times of the two components was confirmed by comparison of the absorption at 430 nm (Fig. 1A) and at 408 nm (Fig. 1B). At 408 nm, the first peak is four times higher than at 430 nm, showing this peak is the Pheo *a*. In this system, Chl *a'* eluted only 1.5–2 min later than Pheo *a* and two other minor peaks are observed to elute a short time after Chl *a'*. The identities of these two minor peaks remains unknown, but one of them could be the Chl RC1. The two peaks are clearly visible in Fig. 1, but appear only as traces in Fig. 2, because the concentration of chlorophylls in the column is much higher for Chl *b* containing strain analysis (80 μg Chl *a*; Fig. 1) than for Chl *b*-less mutant analysis (11–26 μg Chl *a*; Fig. 2). Using the ternary solvent of Watanabe with our column, the best separation of Pheo and Chl *a'* was achieved (3–4 min), but in this system the two minor peaks overlap with the Chl *a'*. For this reason we used the binary solvent system.

Results

The photochemical characteristics of the strains are summarized in Tables I and II. Results are presented in Tables II and Figs. 1 and 2.

Wild type

In the wild strain, the ratios of Chl *a*/Chl *a'* and Chl *a*/Pheo *a* are not different from the values found by Kobayashi [3] in higher plants. These authors found 460 ± 90 for the Chl *a*/Chl *a'* ratio and we found

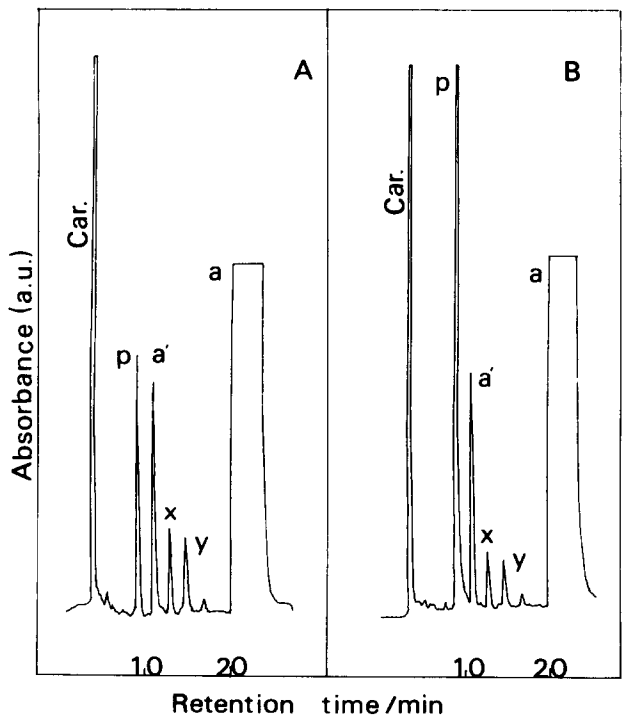

Fig. 1. HPLC traces of the wild strain of *C. reinhardtii*. Column load: 80 μg of Chl *a* + Chl *b*; Car., carotenoid; P, pheophytin *a*; *a'*, Chl *a'*; a, Chl *a*. Detection wavelength, 430 nm (A) or 408 nm (B). Eluent, hexane/2-propanol (197 : 3, v/v), a.u., arbitrary unit.

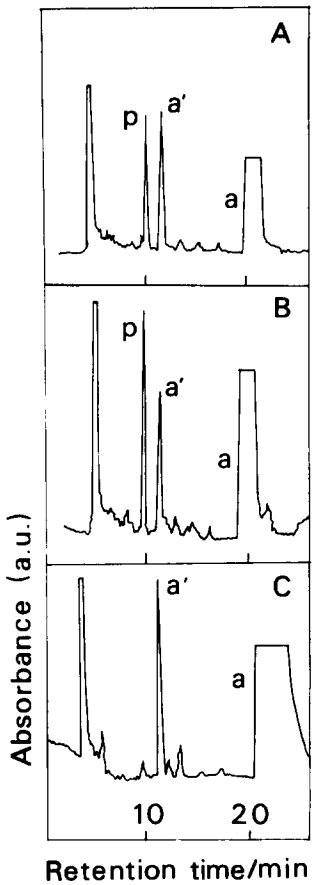

Fig. 2. HPLC traces of three Chl *b*-less strains of *C. reinhardtii*. (A) *Pg* 27, control, 11 μg of Chl *a*; (B) *Fl* 5 *Pg* 27, P-700-less strains, 26 μg of Chl *a*; (C) *Fl* 39 *Pg* 28, PS II-deficient strain, 12 μg of Chl *a*. Detection wavelength, 430 nm. Eluent, hexane/2-propanol (197:3, v/v).

500 ± 60. Kobayashi et al. found a Chl *a*/Pheo *a* ratio of 120 ± 20; we found 100 ± 10 for *C. reinhardtii*.

Pg 27

In *Pg* 27, the two photosystems were functional. However, *Pg* 27 does not contain Chl *b* and, therefore, the main light-harvesting antenna (CP II) and the peripheral PS I antenna (CP 0) are absent. In this mutant, the ratios Chl *a*/Chl *a'* and Chl *a*/Pheo *a* were 50% lower than those obtained in the wild strain. But the Chl *a* content of *Pg* 27 is half that of the wild strain, because of the lack of the main light-harvesting antenna in this mutant. If we consider that all the Chl *a* is then associated with the reaction centers, we can conclude that the *Pg* 27 content is normal in Pheo *a* and in Chl *a'*.

Fl 5

Fl 5 is a mutant lacking P-700 and therefore showing no PS I activity. As shown in Table II, this mutant has a normal Pheo *a* content, but its Chl *a'* content is approximately half that of the wild type.

Fl 5 Pg 27

The double mutant *Fl* 5 *Pg* 27 results from a cross between the mutant *Fl* 5 which lacks P-700 and the mutant *Pg* 27 which lacks Chl *b* (Table I) and the two light-harvesting antennae. If we compare this mutant with the parent *Pg* 27, it has the same pheophytin content but contains only half the amount Chl *a'*. This mutant shows the same Chl *a'* deficiency as its other parent *Fl* 5.

Fl 39

The mutant *Fl* 39 has no PS II activity and does not possess the CP of this photosystem. The ratio Chl *a*/Chl *a'*, as determined by HPLC, is normal, but we did not find any pheophytin in this mutant.

Fl 39 Pg 28

The double mutant *Fl* 39 *Pg* 28 was obtained by irradiation of the parent *Fl* 39, then screening of the Chl *b*-less mutants. This Chl *b*-less mutant has no PS II activity, no PS II linked CP and no light-harvesting antennae. So it is comparable to PS I-enriched particles, with the difference that here no Triton X-100 or electro-

TABLE I

Photochemical characteristics of the wild type and of seven mutants of C. reinhardtii

Strains	PS I activity	PS II activity	PS I complexes			PS II complexes	CP II (chl *b*)
			P-700	CP 0	CP 0a		
Wild type	+	+	+	+	+	+	+
Fl 5	−	+	−	+	+	+	+
Fl 39	+	−	+	+	+	−	+
Fl 50	+	tr.	+	+	−	tr.	+
Pg 27	+	+	+	−	+	+	−
Fl 5 *Pg* 27	−	+	−	−	+	+	−
Fl 39 *Pg* 28	+	−	+	−	n.d.	−	−
Fl 50 *Pg* 27	+	tr.	+	−	n.d.	tr.	−

n.d., not determined; tr., traces.

TABLE II

Ratios Chl a/Chl a', Chl a/Pheo a, Chl a and Chl b contents of wild type and of seven mutants of C. reinhardtii

Strains	$\frac{\text{Chl } a}{\text{Chl } a'}$	$\frac{\text{Chl } a}{\text{Pheo } a}$	Chl *a* [a]	Chl *b* [a]
Wild type	500± 60	100±10	32.8	13.7
Fl 5	870±120	115±10	27.5	15.2
Fl 39	475± 25	+∞ [b]	30.3	11.7
Fl 50	500± 30	430±30	14.1	7.4
Pg 27	270± 30	70±10	14.7	0.0
Fl 5 *Pg* 27	535± 25	70±15	9.5	0.0
Fl 39 *Pg* 28	130± 10	+∞ [b]	21.8	tr.
Fl 50 *Pg* 27	200± 20	190±40	13.8	0.0

[a] μg chlorophyll per mg dry matter.
[b] Pheophytin content = 0.
tr., traces.

phoretic treatment were present that could result in pheophytinization or epimerization of the pigments. The Chl *a*/Chl *a'* ratio in this mutant is lower than in the control-type *Pg* 27. But in *Fl* 39 *Pg* 28, all the Chl *a* containing CP of the PS II are absent. So we can consider that this ratio is normal. No Pheo *a* was isolated from this mutant.

Fl 50 and Fl 50 Pg 27

The double mutant *Fl* 50 *Pg* 27 is the product of a cross between the mutant *Fl* 50 and the mutant *Pg* 27.

The two mutants are partially deficient in PS II content and contain only traces of the PS II-linked CP and of their apoproteins, as shown by PAGE [13]. Using HPLC, we have detected only traces of Pheo *a* (25–30% of wild type). For the strain *Fl* 50, this result confirms an earlier study, of fluorescence induction in *Fl* 50, by Briantais [14]. This study indicated that probably only 20% of the PS II reaction centers were functional. In these two mutants, partially depleted in the polypeptides of the PS II reaction center, the Pheo *a* is markedly lowered.

In *Fl* 50, the Chl *a*/Chl *a'* ratio is the same as in the wild type and in *Fl* 50 *Pg* 27 this ratio is almost the same as in *Pg* 27. The Chl *a'* content is normal in these two mutants. The strain *Fl* 50 has been studied here because it displays a second important mutation: *Fl* 50 is a CP 0a-lacking mutant. CP 0a is a new Chl *a*-protein complex related to PS I and was recently identified by us [14,15]. In the present study we noted that, despite the lack of CP 0a, the Chl *a'* content of *Fl* 50 is normal.

Discussion and Conclusion

We did not use Watanabe's extraction procedure with chloroform and anhydrous Na_2HPO_4. A rapid acetone extraction (1 min) seemed more appropriate for an algae wet pellet. Therefore, prolonged grinding and sonication were not required. Watanabe quoted that pheophytinization was considerably higher in acetone than in chloroform. We did not observe such a pheophytinization: we found two mutants (*Fl* 39; *Fl* 39 *Pg* 28) which contained no Pheo *a* (Fig. 2C). So, brief acetone extraction, followed by HPLC separation (< 15 min) at 4°C are not factors of pheophytinization with our biological material. With higher plants leaves, pheophytinization could perhaps be more easily induced by the presence of a high content of acidic components in some leaves. The two mutants which did not contain Pheo *a* are mutants totally depleted of PS II activity. Two other mutants (*Fl* 50, *Fl* 50 *Pg* 27) contained only 25–30% of Pheo *a* normal content. These two mutants are partially depleted of PS II activity and of the polypeptides of the PS II reaction center. Mutants such as *Fl* 5 and *Fl* 5 *Pg* 27 which have impaired PS I but active PS II, have a normal Pheo *a* content. These results agree well with the crucial role proposed for Pheo *a* in the PS II photochemistry.

Concerning Chl *a'* we cannot prove whether or not it is a natural product. If there is artificial epimerization in the course of our work, it must occur during the extraction or during the HPLC separation (12 min) at 4°C. We do not think that a silica column could be a good factor of epimerization, a base-catalysed reaction [18].

Here, we have approached the study of Chl *a'* with new biological material – photosynthesis mutants. With PS II- or PS I-lacking mutants, we could avoid the damaging Triton X-100 treatment which is useful for preparing PS II or PS I-enriched particles. With the Chl *b*-less double mutants, we easily obtained clearer HPLC charts than with the Chl *b* containing strains or the higher plant leaves.

The strains affected only in PS II (*Fl* 50, *Fl* 39, *Fl* 50 *Pg* 27, *Fl* 39 *Pg* 28) had normal Chl *a'* content as compared with the control strains. So there was no constant stoichiometry between PS II and Chl *a'*. We have confirmed the link between Chl *a'* and the PS I as first evidenced by Watanabe. But there is some discrepancy on the possible state of Chl *a'* in PS I. The two PS I-deficient mutants, which lacked P-700, also lacked Chl *a'*. But this deficiency is partial. The two mutants still contained half the normal Chl *a'* content.

This result is supported by the good reproducibility of the measures and the fact that half of the normal content of Chl *a'* found in *Fl* 5 is also found in the double mutant arising from this strain. Therefore, only half and not all the Chl *a'*, could be assumed to correspond to P-700, as suggested by Watanabe's work on subchloroplasts [4]. What could be the state in the PS I of the remaining Chl *a'*? The double mutant *Fl* 5 *Pg* 27 contains 50% of the Chl *a'* and, on electrophoretograms concerning this P-700-less strain, only one PS I-linked CP is visible: CP 0a [14,15]. This reduced pool of Chl *a'* could be entirely CP 0a. Such a hypothesis can be rejected: with the CP 0a-less mutant, *Fl* 50, there was no link between Chl *a'* and CP 0a. By curve analysis of low-temperature absorption spectra, Ikegami and Itoh [25] have detected ten different Chl *a* in P-700-enriched particles. Two of them are assumed to be P-700 (Chl *a* and Chl *a'* have similar absorption spectra). Is one of the eight other chlorophylls, the second half of Chl *a'*?

There is another possibility. It is possible that all the Chl *a'* could be linked to P-700. In the two P-700-less mutants, there is no formation of P-700 apoproteins. If the two mutants are still able to synthesize the Chl *a'*, in absence of the P-700 apoproteins, P-700 cannot be formed and therefore free Chl *a'* could partially exist in the algae cells.

New mild procedures and new mutants are necessary to investigate throughly the problems of the existence and of the role of Chl *a'* in PS I.

Acknowledgments

The authors are grateful to Mrs. D. Guyon and Mrs. A. Trouabal for supplying us with the algae. Thanks are also due to Dr. S. Brown for reading the manuscript, to Mr. R. Boyer and to Mrs. M. Montaggioni.

References

1 Strain, H.H. and Manning, W.M. (1942) J. Biol. Chem. 146, 275–276.
2 Watanabe, T., Nakazato, M., Mazaki, H., Hongu, A., Konno, M., Saitoh, S. and Honda, K. (1985) Biochim. Biophys. Acta 807, 110–117.
3 Kobayashi, M., Watanabe, T., Nakazato, M., Ikegami, I., Hiyama, T., Matsunaga, T. and Murata, N. (1988) Biochim. Biophys. Acta 936, 81–89.
4 Watanabe, T., Kobayashi, M., Hongu, A., Nakazato, M., Hiyama, T. and Murata, N. (1985) FEBS Lett. 191, 252–256.
5 Watanabe, T., Kobayashi, M., Nakazato, M., Ikegami, I. and Hiyama, T. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. 1, pp. 303–306, Martinus Nijhoff, Dordrecht.
6 Hiyama, P., Watanabe, T., Kobayashi, M. and Nakazato, M. (1987) FEBS Lett. 214, 97–100.
7 Klimov, V.V., Dolan, E., Shaw, E.R. and Ke, B. (1980) Proc. Natl. Acad. Sci. USA 77, 7227–7231.
8 Omata, T., Murata, N. and Satoh, K. (1984) Biochim. Biophys. Acta 765, 403–405.
9 Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M. and Saitoh, S. (1984) Anal. Chem. 56, 251–256.
10 Garnier, J. and Maroc, J. (1972) Biochim. Biophys. Acta 283, 100–114.
11 Maroc, J. and Garnier, J. (1973) Biochim. Biophys. Acta 292, 477–490.
12 Picaud, A., Dubertret, G., Guyon, D. and Hervo, G. (1981) in Photosynthesis (Akoyunoglu, G., ed.), Vol. III, pp. 405–415, Balaban International Science Services, Philadelphia.
13 Maroc, J., Guyon, D. and Garnier, J. (1983) Plant Cell Physiol. 24, 1217–1230.
14 Garnier, J., Maroc, J. and Guyon, D. (1986) Biochim. Biophys. Acta 851, 395–406.
15 Maroc, J., Garnier, J. and Guyon, D. (1989) J. Photochem. Photobiol. B. Biol. 4, 97–109.
16 Gorman, D.S. and Levine, R.P. (1965) Proc. Natl. Acad. Sci. USA 54, 1665–1669.
17 Rebeiz, C.A. and Castelfranco (1971) Plant Physiol. 47, 33–37.
18 Watanabe, T., Masaki, H. and Nakazato, M. (1987) Biochim. Biophys. Acta 892, 197–206.
19 MacKinney, G. (1941) J. Biol. Chem. 140, 315–322.
20 Arnon, D.E. (1949) Plant Physiol. 24, 1–15.
21 Dörnemann, D. and Senger, H. (1981) FEBS Lett. 126, 323–327.
22 Dörnemann, D. and Senger, H. (1982) Photochem. Photobiol. 35, 821–826.
23 Senge, M., Dörnemann, D. and Senger, H. (1988) FEBS Lett. 234, 215–217.
24 Senge, M. and Senger, H. (1989) Biochim. Biophys. Acta 977, 177–186.
25 Ikegami, I. and Itoh, S. (1988) Biochim. Biophys. Acta 934, 39–46.