ISOLATION AND PARTIAL CHARACTERIZATION OF A NEW CHLOROPHYLL ASSOCIATED WITH THE REACTION CENTRE OF PHOTOSYSTEM I OF *SCENEDESMUS*

Dieter DÖRNEMANN and Horst SENGER
Fachbereich Biologie/Botanik, Philipps-Universität, Lahnberge, 3550 Marburg, FRG

Received 27 February 1981

1. Introduction

Kok discovered a chlorophyll protein complex which absorbed around 700 nm and showed a light-inducible redox change [1–3]. This complex, which is believed to contain chl *a*, was named P700 and identified as the reaction centre of PS I: such pigments have also been characterized in brown algae [4–6]. The chlorophyll of P700 is generally believed to be chl *a* attached to a specific protein [4–10]. It has also been suggested that the chlorophyll of P700 is a chl *a* dimer [11]. Although sub-chloroplast particle fractions have been highly enriched in P700 [7,9,12,13] the chromophore has not been investigated in detail and not the idea that the tetrapyrrolic chromophore is chl *a* has not been challenged.

A new chlorophyll has been extracted from a chl *b*-deficient pigment mutant of *Scenedesmus obliquus* which is enriched in P700. The new chlorophyll, purified by TLC, has absorption maxima at 672 and 433 nm in acetone, and its fluorescence emission peak like the absorption maxima, is also shifted ~9 nm towards longer wavelengths than the emission peak of chl *a*. The phaeophytin derived from the new chlorophyll likewise differs from phaeophytin *a* since its absorption maxima and fluorescence emission maximum are also shifted towards longer wavelengths than those of phaeophytin *a*. The molar ratio of the new chlorophyll obtained in solution relative to that of chl *a* parallels that of P700 to chl *a* observed in vivo.

The new chlorophyll prosthetic group was also obtained from other pigment mutants and the wild type of *Scenedesmus*. It could not be found in cells in which the formation of the photosynthetic centre of PS I was suppressed by chloramphenicol.

This new chlorophyll may derive from the chromophore of the reaction centre P700 pigment or of another pigment of the same reaction centre associated in a 1:1 molar ratio with P700: the name chl RCI is proposed.

Abbreviations: chl *a* and *b*, chlorophylls *a* and *b*; chl RCI, a newly discovered chlorophyll described in the text; DCPIP, 2,6-dichlorophenol-indophenol; PS I, II, photosystems I, II; TLC, thin-layer chromatography

Some data shown in this paper were presented at the 5th International Congress on Photosynthesis, September 1980, Halkidiki, Greece

2. Methods

Cells of the X-ray induced [14] pigment mutants C-6E, C-6D and C-2A′ [15] and of the wild-type *Scenedesmus obliquus* were grown heterotrophically in the dark at 30°C as in [16]. Cells were harvested by centrifugation from 3-day-old cultures at the end of the logarithmic growth phase. A small amount of magnesium hydroxide-carbonate (Merck, catalogue no. 5827) was added to the pellet which was exhaustively extracted with hot methanol. The methanol was evaporated in vacuo at 60°C and the pigment residue dissolved in acetone. TLC was carried out on preparative Merck Kieselgel plates (20 × 20 cm; 2.0 mm). The plates were developed in petroleum ether:propan-2-ol:H_2O (100:10:0.25, by vol.), dried under N_2 and developed again in the same solvent system. Moving at the front of the main chl *a* band was the new pigment chl RCI. The front of this main band was eluted with acetone and subjected a further 3 times to TLC using analytical Merck Kieselgel 60

plates (20 × 20 cm; 0.2 mm) and the above-mentioned solvent system: for each of the 3 runs the plates were dried under N_2 and developed again in the same solvent system. Residual traces of phaeophytin were removed from the new chlorophyll fraction by TLC on Kieselgel plates containing ascorbate (10^{-2} M). The whole procedure was carried out under dim green light. The molar ratio of chl *a*:chl RCI was calculated from their absorption maxima using the molar extinction coefficient of chl *a* [13].

For the in vivo determination of P700 [17,18] cells were disrupted in a Vibrogen cell mill [19] and the P700 in the cellfree preparation was reduced by ascorbate (10 mM) and measured against an aliquot oxidized by blue light (444 nm, 12 W/m^2) using an Aminco DW2 spectrophotometer with a filter system to screen the actinic light from the photomultiplier.

To obtain cells free of the reaction centre pigment (P700) of PS I, mutant C-2A′ of *Scenedesmus* was greened for 7 h in light (20 W/m^2) in the presence of chloramphenicol (5 μg/ml of cell suspension) to suppress the transcription in the plastids.

Low- and room-temperature absorption spectra were obtained with a Shimadzu MPS-5000 spectrophotometer and fluorescence spectroscopy was performed in a Shimadzu RF-502 spectrofluorimeter automatically corrected against a rhodamine *b* standard.

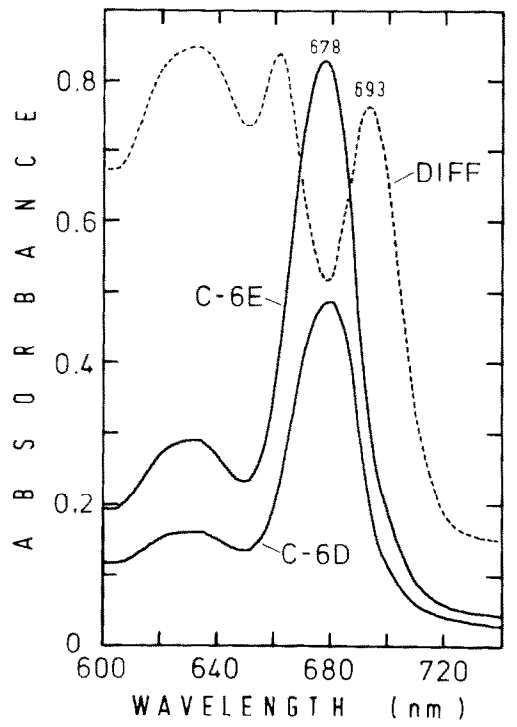

Fig.1. In vivo absorption spectra (C-6E and C-6D) and difference spectrum (C-6E minus C-6D) of the *Scenedesmus* mutants C-6D and C-6E. Spectra were recorded in a Shimadzu MPS 5000 spectrophotometer at 77 K, lightpath 1 mm. The samples had a density of 150 μl packed cell vol./ml nutrient solution.

3. Results and discussion

The pigment mutant C-6E of *Scenedesmus obliquus* lacks all carotenoids and chl *b*, but has chl *a* and a relatively greater amount of P700 than found in the wild type: the ratio of chl *a*: P700 has been reported to be 91:1 [18] and was found to be 80:1 here. PS I activity in cell-free preparations of C-6E was measured by recording oxygen uptake after adding methylviologen reduced with ascorbate-DCPIP as electron donor system: activity was as high as 90 μmol O_2. mg chl^{-1}. h^{-1}. No PS II activity should be detected.

An absorption difference spectrum at low temperature of the dark-grown mutant C-6E minus the mutant C-6D, which produces under the same conditions only chl *a* and a smaller amount of P700, is shown in fig.1. The samples were brought to approximately the same density but stray light effects caused peak heights to vary considerably; nevertheless, a peak at 693 nm and a trough at 678 nm were observed and we suggest that they are attributable to P700 [2] and chl *a* [20] (both in vivo), respectively.

The in vivo absorption peaks of chloroplasts and unicellular algae between 695 and 700 nm has been attributed to P700, which has been assumed to consist of chl *a* [7–10] or a chl *a* dimer [11] associated with a specific protein. To test this assumption cells of mutant C-6E were extracted with hot methanol, the extract vacuum dried, transferred into acetone and its absorption recorded against a sample of purified chl *a* as a difference spectrum (fig.2). This difference spectrum shows a trough at 661 nm attributable to chl *a* and a peak at 673 nm which cannot be attributed to chl *a*.

Repeated TLC of the total extract of C-6E (see section 2) yielded a new chlorophyll separated quite distinctly from chl *a*. The absorption spectrum of the new chlorophyll in acetone is shown in fig.3 and its absorption characteristics are compared with those of chl *a* in table 1.

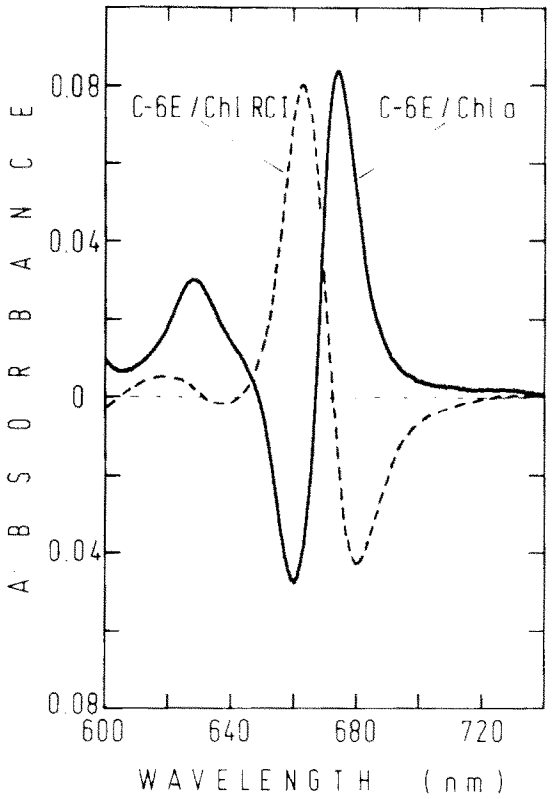

Fig.2. Difference spectra of a total acetone extract of mutant C-6E minus a purified chl *a* solution (——) and of a C-6E total acetone extract minus a purified chl RCI solution (– – –); chl *a* and chl RCI were also dissolved in acetone. Spectra were recorded at room temperature in a Shimadzu MPS 5000 spectrophotometer, lightpath 1 cm.

The fluorescence emission spectrum at room temperature (see table 1) shows a peak (675 nm) which is 8 nm higher than that of chl *a* (667 nm). From the new chlorophyll chl RCI a phaeophytin (Phaeo RCI) was prepared which clearly emits higher wavelength fluorescence than that of the phaeophytin of chl *a* (table 1). When the absorption of the total extract of mutant C-6E was recorded against that of the purified new chlorophyll the resulting difference spectrum showed the absorption peak of chl *a* and a trough attributable to the absorption of chl RCI (fig.2).

To clarify the correlation between the reaction centre P700 in vivo and the new chl RCI we determined the molar ratio of chl *a* and P700 in vivo and compared it with the ratio of chl *a* to chl RCI in acetone extracts of several cell preparations (table 2). To calculate the concentration of the new chl RCI we

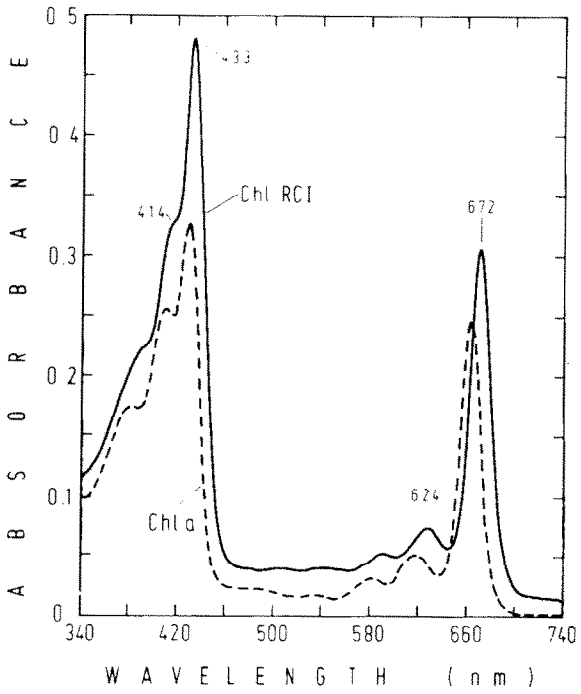

Fig.3. Absorption spectra of purified chl *a* and chl RCI, in acetone. Spectra were recorded in a Shimadzu MPS 5000 spectrophotometer at room temperature, light path 1 cm.

assumed, as a first approximation, that chl RCI and chl *a* had identical molar extinctions coeffcients and we used the spectral data for chl *a* of MacKinney [21]. The concentration, so calculated, of the new chl RCI and of P700 was almost identical in the total cells of C-6E and in a PS I-particle-enriched sucrose

Table 1
Absorption- and fluorescence-emission maxima of chl *a*, chl RCI and their phaeophytins in acetone

Pigment	Absorption maxima (nm) in acetone					Fluorescence emission maxima (nm)
Chl *a*	410	430		618	663	667
Phaeo *a*	411	505	533	611	668	673
Chl RCI	414	433		624	672	675
Phaeo RCI	415	515	545	620	678	682

Absorption spectra were recorded on a Shimadzu MPS 5000 spectrophotometer with 1 cm light path, fluorescence emission spectra on an automatically corrected Shimadzu RF 502 fluorescence spectrophotometer. Fluorescence excitation band width was 15 nm and emission band width 3 nm. All spectra were recorded at room temperature.

Table 2
Molar ratios of chl *a*/P700 (in vivo) and of chl *a*/chl RCI (in acetone)

Material		Pigments	Ratio
Mutant C-6E		chl *a*/chl RCI (extract)	89
		chl *a*/P700 (in vivo)	80
Sucrose density gradient PSI particles (C-6E)		chl *a*/chl RCI (extract)	28
		chl *a*/P700 (in vivo)	28
Mutant C-2A′ after 7 h greening	−CAP	chl *a*/chl RCI (extract)	400
		chl *a*/P700 (in vivo)	300–500
	+CAP	chl *a*/chl RCI (extract)	∞
		chl *a*/P700 (in vivo)	∞

Corresponding data were taken from aliquots of the same samples. The material was prepared as in section 2. P700 was determined as redox difference [9]. For the calculation of the chlorophyll concentrations the molar extinction coefficient of chl RCI was assumed to be the same as chl *a*

density fraction. In the greening mutant C-2A′ the concentration of the new chl RCI is lower and the determination less accurate. The new chlorophyll could be demonstrated in extracts of the wild-type cells of *Scenedesmus obliquus*, but its concentration was too low to allow a quantitative measurement. When mutant C-2A′ was greened in the presence of chloramphenicol (see section 2) no reaction centres were formed and only antenna pigments appeared: this culture had no PS I activity and no P700 could be detected in vivo and likewise no new chl RCI could be detected in solvent extracts.

We conclude from the above results that the new chl RCI derives from the reaction centre of PS I and is either identical with the chlorophyll prosthetic group of the in vivo P700 pigment or of another reaction centre I pigment associated in a 1:1 molar ratio with P700. We suggest that chl RCI is an allomer of the corresponding naturally-occurring pigment: whereas the pigment of the extraction with hot methanol or cold acetone gave a positive Molisch phase test, the test was no longer positive after subjecting the pigment to TLC. The difference spectrum of the pigments was identical before and after the loss of the positive Molisch test. Whether one of the 8 species of chl *a* and chl *b* [22] is identical with chl RCI cannot be decided since their absorption characteristics are given only for the blue region of the spectrum.

The absorption of chl RCI at 672 nm, which is 9 nm higher than that of chl *a* at 663 nm, might be consistent with the substitution of the ethyl group on ring II of chl *a* by a vinyl group; however, studies on the chemical structure of chl RCI are under way. The investigation will also be extended to other algae and higher plants.

Acknowledgements

We thank Dr G. Brinkmann for valuable suggestions, Ms Irmgard Koss for skilled technical assistance and Ms Ilse Krieger for typing the manuscript. We also thank Dr R. J. Porra for helpful discussion and correction of the English text.

References

[1] Kok, B. (1956) Biochim. Biophys. Acta 22, 399–401.
[2] Kok, B. (1961) Biochim. Biophys. Acta 48, 527–533.
[2a] Kok, B. and Hoch, G. (1961) in: Light and Life (McElroy, W. D. and Gloss, B. eds) pp. 397–416, John Hopkins Press, Baltimore MD.
[3] Witt, H. T. and Wolff, Ch. (1970) Z. Naturforsch. 256, 387–388.
[4] Anderson, J. M. and Barrett, J. (1979) in: Chlorophyll Organization and Energy Transfer in Photosynthesis, Ciba Found. Symp. 61, pp. 81–104, Excerpta Medica/Elsevier-North-Holland, Amsterdam, New York.
[5] Barrett, J. and Anderson, J. M. (1980) Biochim. Biophys. Acta 590, 309–323.
[6] Barrett, J. and Thorne, S. W. (1980) FEBS Lett. 120, 24–28.
[7] Butler, W. L. and Kitajima, M. (1975) Biochim. Biophys. Acta 396, 72–85.
[7a] Butler, W. L. (1978) Annu. Rev. Plant. Physiol. 29, 345–378.
[8] Sauer, K. (1975) in: Bioenergetics of Photosynthesis (Govindjee, ed) pp. 115–181, Academic Press, New York.
[9] Thornber, J. P. (1975) Annu. Rev. Plant. Physiol. 26, 127–158.
[10] Witt, H. T. (1979) Biochim. Biophys. Acta 505, 355–427.
[11] Shipman, L. L., Cotton, T. M., Norris, J. R. and Katz, J. J. Proc. Natl. Acad. Sci. USA 73, 1791–1794.
[12] Sane, P. V. and Park, R. B. (1970) Biochem. Biophys. Res. Commun. 41, 206–210.
[13] Thornber, J. P. and Olson, J. M. (1971) Photochem. Photobiol. 14, 329–341.
[14] Bishop, N. I. (1971) in: Methods in Enzymology, vol. 23, pt A (San Pietro, A. ed) pp. 130–143, Academic Press, London.

[15] Wellburn, F. A. M., Wellburn, A. R. and Senger, H. (1980) Protoplasma 103, 35–54.
[16] Senger, H. and Bishop, N. I. (1972) Plant Cell Physiol. 13, 633–649.
[17] Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160–171.
[18] Ogawa, T. and Vernon, L. P. (1970) Biochim. Biophys. Acta 197, 292–301.
[18a] Vernon, L. P., Shaw, E. R., Ogawa, T. and Raveed, D. (1971) Photochem. Photobiol. 14, 343–357.
[19] Senger, H. and Mell, V. (1977) in: Methods in Cell Biology (Prescott, D. M. ed) vol. 25, pp. 201–219, Academic Press, London, New York.
[20] French, C. S. and Prager, L. (1969) in: Progress in Photosynthesis Research (Metzner, H. ed) pp. 555–564.
[21] MacKinney, G. (1941) J. Biol. Chem. 140, 315–322.
[22] Freyssinet, G., Rebeiz, C. A., Fenton, J. A., Khanna, R. and Govindjee (1980) Photochem. Photobiophys. 1, 203–212.