FEBS Letters 356 (1994) 367–371

FEBS 14927

A novel peridinin–chlorophyll *a* protein (PCP) from the marine dinoflagellate *Alexandrium cohorticula*: a high pigment content and plural spectral forms of peridinin and chlorophyll *a*

Takehiko Ogata[a], Masaaki Kodama[a], Setsuzo Nomura[a], Masayuki Kobayashi[b], Tsunenori Nozawa[b], Tetzuya Katoh[c], Mamoru Mimuro[d,*]

[a]*School of Fisheries Sciences, Kitasato University, Sanriku-cho, Kesen-gun, Iwate 022-01, Japan*
[b]*Department of Biochemistry and Engineering, Faculty of Engineering, Tohoku University, Aoba, Sendai 980-77, Japan*
[c]*Department of Botany, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan*
[d]*National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan*

Received 19 October 1994; revised version received 16 November 1994

Abstract A new type of peridinin–chlorophyll *a* protein (PCP) was isolated from the marine dinoflagellate *Alexandrium cohorticula*. Unlike previous studies, PCP was obtained as a single component in the presence of a protease inhibitor. The monomer had a molecular mass of 37 kDa with 12 peridinin molecules associated with 2 chl *a* molecules. This pigment content was much higher than that reported previously. We observed a partial amino acid sequence of the N-terminus that is novel among photosynthetic pigment–protein complexes. Magnetic circular dichroism clearly indicated that chl *a* in PCP had monomeric features. Multiple spectral components were suggested for both chl *a* and peridinin. Based on the high pigment content, the optical properties were compared with those for a reported PCP containing 4 chl *a* and 1 peridinin.

Key words: Carotenoid; Dinoflagellate; Peridinin; Photosynthesis; Pigment–protein complex; *Alexandrium cohorticula*

1. Introduction

Dinoflagellates are unique in their photosynthetic pigment composition. In addition to chlorophyll (chl) *a* and chl *c*, almost all species contain peridinin as a major light harvesting pigment, while few species contain fucoxanthin. This difference is explained by the endosymbiosis of other organisms into dinoflagellates [1].

The photosynthetic pigment system of dinoflagellates consists of several kinds of membrane-bound pigment–protein complexes [2–5], similar to those in higher plants and algae [6]. Peridinin–chl *a* protein (PCP) is a water-soluble protein, light harvesting pigment–protein complex found in dinoflagellates [7]. The energy transfer efficiency from peridinin to chl *a* is known to be almost unity in this complex [8]. While PCP from *Amphidinium carterae* has been isolated and crystallized [9], its molecular structure remains unknown.

It is known that PCP exhibits multiple forms having different iso-electric points [10,11]. Several fractions of PCP have been isolated using ion-exchange column chromatography, and all of these have shown a similar absorption spectrum and a molecular mass of the monomer ranging from 32 to 39 kDa, depending on the species [7–10,12]. In some species, a subunit with a molecular mass of 15–17 kDa has been reported [11]. This water-soluble monomer form is an unusual functional form for a light harvesting complex, since efficient energy transfer to pigments in thylakoid membranes normally involves an associated form of pigment–protein complexes, as typically seen in phycobilisomes [13].

Since PCP is stable, it is an excellent candidate for the analysis of the optical properties of and energy transfer mechanism in carotenoid-containing pigment–protein complexes. Thus, we isolated PCP from the marine dinoflagellate *Alexandrium cohorticula*. We found a high pigment content (12 peridinin and 2 chl *a* molecules) per monomer and compared the optical properties with those reported previously, since the interaction between pigments has traditionally been discussed in terms of a low pigment content (4 peridinin and 1 chl *a* molecules per monomer) [8,11,12]. We also made novel use of magnetic circular dichroism (MCD) which provided good data on the interaction between the chl *a* molecules [14,15]. Other features of the protein chemistry are also discussed.

2. Materials and methods

2.1. Algal culture and isolation of PCP

PCP was isolated from the marine dinoflagellate *Alexandrium cohorticula* originally collected from the Gulf of Thailand [16]. Cells were grown under autotrophic conditions in a T1 medium [17] at 25°C with light from a fluorescent lamp (white color) at an intensity of 51 μmol photon$\cdot m^{-2}\cdot s^{-1}$ in a 16:8 h cycle of light:dark. Cells at a late exponential phase were harvested and suspended in 50 mM Tris-HCl buffer (pH 7.5) containing a protease inhibitor PMSF (1 mM), and were disrupted by sonication three times (30-s sonication with 1-min interval) using an Ultrasonic Disrupter, UR-200P (Tomy Seiko, Japan). To reduce denaturation by heat, the temperature during disruption was kept at 5°C. After removing cell debris by centrifugation (10,000 × *g*, 30 min), a concentrated supernatant was applied to a Sephadex G-100 column (2.5 × 90 cm) equilibrated with the same buffer with PMSF. Further purification was carried out by Q-Sepharose column chromatography (2 × 12 cm), equilibrated with 1 mM Tris-HCl buffer (pH 8.0) and eluted with a gradient of NaCl from 0 to 0.2 M in the same buffer. SDS-PAGE analysis was performed according to the method of Laemmli [18] with a minor modification: 7.5 M urea was added to 16% polyacrylamide.

*Corresponding author. Fax: (81) (564) 53-7400.
E-mail: mimuro@sesame.nibb.ac.jp

Abbreviations: CD, circular dichroism; chl, chlorophyll; kDa, kilodaltons; MCD, magnetic circular dichroism; PCP, peridinin–chlorophyll *a* protein; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-poly acrylamide gel electrophoresis.

SSDI 0014-5793(94)01314-4

2.2. Spectroscopic analyses

Absorption and fluorescence spectra were measured as described previously [19]. The spectral sensitivity of the fluorometer was numerically corrected by using the radiation profile of a sub-standard lamp [19]. Circular dichroism (CD) [20] and magnetic circular dichroism (MCD) [14,15] were measured with a JASCO J-500 spectropolarimeter; an electromagnet with a field strength of 13.5 T was used to measure MCD.

2.3. Amino acid composition and sequence analysis

The amino acid composition was determined using a Hitachi amino acid analyzer (model 835) after hydrolysis by HCl for 24 h. A partial N-terminus sequence was determined using an automatic gas-phase sequencer (model 470A, Applied Biosystems).

2.4. Determination of pigments by HPLC

Pigments were extracted using an acetone/methanol mixture (1:1), and were identified using a combination of HPLC with a reverse-phase column (Wakosil 5C18, 0.46 × 25 cm; Wako Pure Chemicals, Japan) and a multi-channel photodiode-array detector (MULTI-340; JASCO, Japan). The extinction coefficients of chl *a* [21] and peridinin [22] in methanol at given wavelengths were obtained from previous studies.

3. Results

3.1. Single form of PCP

PCP accounted for more than 80% of the protein of the initial supernatant. There were two PCP fractions according to Sephadex G-100 column chromatography: one was eluted close to the void volume at an unknown molecular mass; and the other had a molecular mass of about 36 kDa. β-Mercaptoethanol treatment (1 mM) of the latter fraction revealed no small peptides, indicating that this fraction was a monomer. After further using Q-Sepharose ion-exchange column chromatography, elution patterns of this fraction varied depending on the presence of the protease inhibitor, PMSF. Multiple fractions were obtained in the absence of the inhibitor (Fig. 1A), each of which showed very similar molecular weights according to SDS-PAGE and their absorption spectra (data not shown). By contrast, only one fraction was obtained in the presence of PMSF (Fig. 1B) and its molecular weight was estimated to be 30 kDa using SDS-PAGE with 7.5 M urea (Fig. 2). In previous observations, several forms were consistently detected in purification using iso-electric focusing [10,11]. In previous reports [7–10,12], a protease inhibitor was not always added, which may have caused a partial degradation of polypeptides resulting in multiple fractions (see section 4). The same general elution pattern was also observed for *Alexandrium tamarense*, *Alexandrium catenella*, and *Gymnodinium catenatum* [23], suggesting that PCP is the single form in vivo.

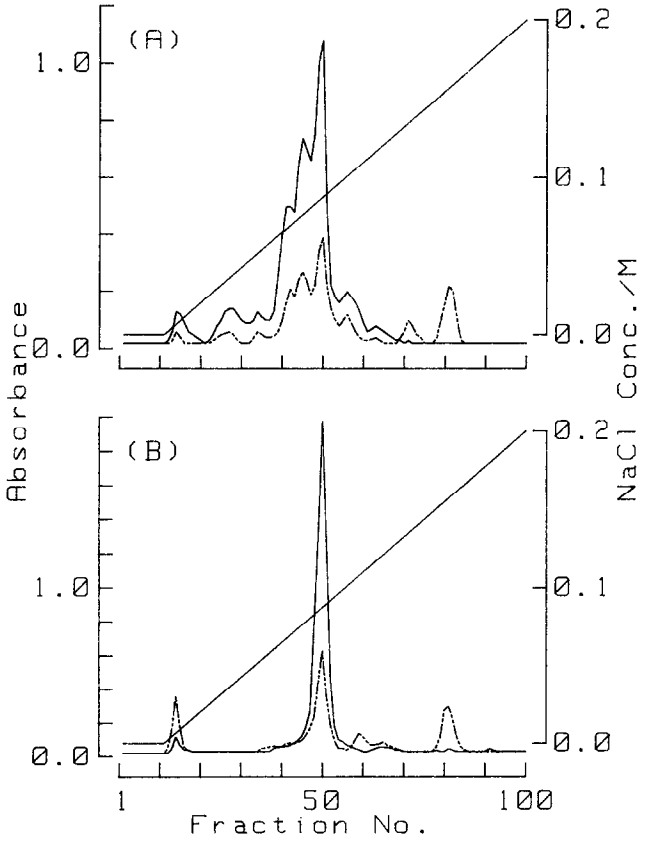

Fig. 1. Elution patterns of PCP from Q-Sepharose column chromatography. (A) Pattern in the absence of PMSF, and (B) pattern in the presence of PMSF (1 mM). A PCP fraction purified by Sephadex G-100 column chromatography was applied and eluted with an NaCl gradient from 0 to 0.2 M. Solid lines, absorbance monitored at 470 nm; broken lines, absorbance monitored at 280 nm.

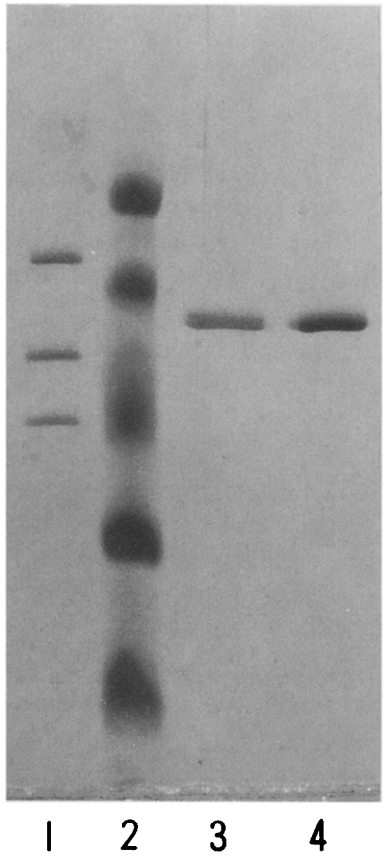

Fig. 2. SDS-PAGE pattern of purified PCP from *A. cohorticula*. Lane 1, three peripheral proteins of the electron donor side of photosystem II isolated from spinach having molecular weights of 33, 24, and 17 kDa; lane 2, pre-stained molecular weight marker polypeptides having molecular weights of 42.4, 27.5, 19, 14, and 6 kDa; lane 3, PCP, 20 μg; and lane 4, PCP, 40 μg.

3.2. Amino acid composition and partial N-terminal sequence of PCP

Table 1 shows the amino acid composition of the PCP monomer. The molecular mass was calculated to be about 37 kDa.

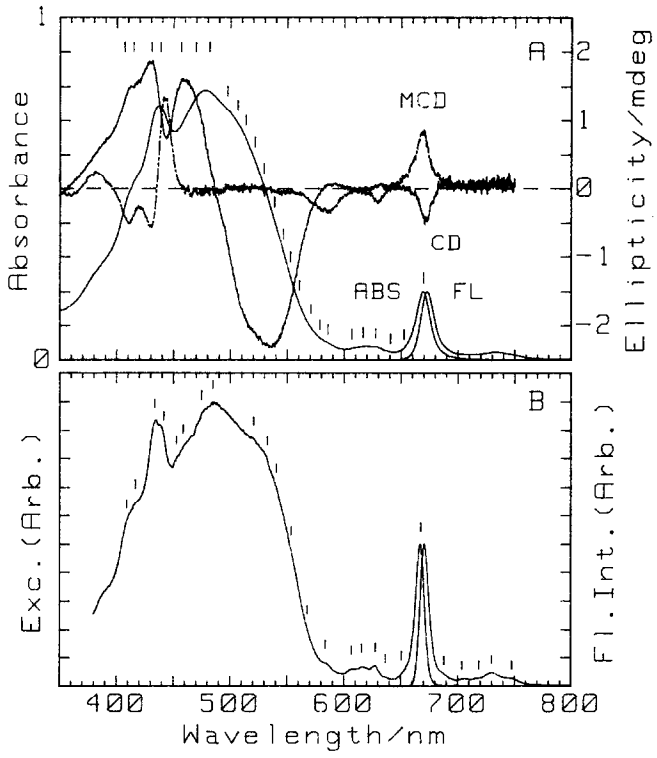

Fig. 3. Optical properties of PCP. (A) Absorption, fluorescence, CD (solid line), and MCD (broken line) spectra of PCP at 15°C; and (B) fluorescence emission and excitation spectra of PCP at −196°C. Small bars along the absorption (A) and excitation (B) spectra indicate the location of maxima estimated by the second derivative spectrum. The maximum intensity of the emission spectra were normalized to that of the absorption spectra. For the emission spectra, the excitation wavelength was 480 nm (bandwidth, 3 nm) and fluorescence was monitored with a bandwidth of 1 nm. For the excitation spectra, fluorescence was monitored at 740 nm with a bandwidth of 3 nm and excitation was monitored with a bandwidth of 3 nm, for both temperature conditions. Samples were suspended in 20 mM phosphate buffer (pH 7.0). For the low temperature spectrum, poly(ethylene glycol) 4000 was added to a sample solution (final concentration, 15%) to obtain homogeneous ice.

This value was significantly larger than that estimated by SDS-PAGE with urea, but in the absence of urea, the molecular mass did correspond to the SDS-PAGE pattern. Since PCP is water-soluble, we observed significant amounts of glutamic acid, aspartic acid, and basic amino acids, such as lysine and serine. There were four histidine residues, which was sufficient to account for a ligand to chl *a*. A partial N-terminal sequence of PCP was accomplished using an automatic gas-phase sequencer.

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1                    11
D E I G D AA K KLGDASYAFA K E
21                   31
V D (C) N N GI F LQAPGKFQPL (E) A
41                   49
L K ( ) I ( ) KH ( ) V
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A homology search of this partial sequence was done on other pigment–protein complexes isolated from higher plants and algae, but no significant indication of homology with PCP was observed. In this sense, PCP is a unique polypeptide in the photosynthetic apparatus, among antenna polypeptides. The amino acid sequence of PCP from *Heterocapsa pygmaea* deduced from cDNA was very different [24] due, perhaps, to the subunit structure of PCP in this species.

3.3. Stoichiometry of pigments in PCP

In previous reports, two stoichiometric types were identified for the PCP pigments: one had a ratio of peridinin-to-chl *a* of 4 [8,11,12], and the other a ratio of 4.5 (9 peridinin and 2 chl *a* molecules in one polypeptide) [10]. The PCP isolated from *A. cohorticula*, however, contained a larger number of pigments: the ratio of peridinin-to-chl *a* was estimated to be 6 according to HPLC analysis. Two forms of peridinin were detected by HPLC; one was the *trans* form (more than 90%) and the other was the *cis* form. The *cis* form is usually not found in antenna complexes [25], and so it may be formed during the isolation. The actual number of pigments on the monomer base was 12 peridinin and 2 chl *a*. This represents the largest reported number of pigments per unit polypeptide of PCP at a molecular weight of 37 kDa. The same pigment stoichiometry has been found in PCP isolated from *A. catenella* [23].

3.4. Spectroscopic characteristics of PCP

Fig. 3 shows the optical properties of PCP. A broad band was found in the absorption spectrum at 15°C (Fig. 3A), which originated from peridinin at around 475 nm. Two peaks, at 436 and 669 nm, were assigned to chl *a*. The second derivative spectrum showed several bands both in the Soret region of chl *a* and in the main absorption of peridinin, which appeared as small bars on the absorption spectrum. This suggests spectral heterogeneity of peridinin (see section 4). Upon preferential excitation of peridinin at 480 nm, fluorescence from chl *a* was observed with a peak at 672 nm (Fig. 3A), indicating efficient energy transfer from peridinin to chl *a*. The excitation spectrum monitored at 740 nm was essentially identical to the absorption spectrum (data not shown).

In the CD spectrum (Fig. 3A), a small negative peak at 673 nm and a trough at 442 nm corresponded to chl *a*. These bands were slightly red-shifted compared with the absorption spectrum, suggesting plural forms of chl *a*. A large negative band

Table 1
Amino acid composition of PCP isolated from *A. cohorticula*

Amino acid	Mol %	Deduced no.
Asp	12.5	42
Thr	2.6	9
Ser	7.5	25
Glu	6.2	21
Pro	4.9	16
Gly	7.5	25
Ala	18.4	61
Val	8.5	28
Met	2.6	9
Ile	4.9	16
Leu	7.2	24
Tyr	3.0	10
Phe	3.3	11
Lys	8.2	27
His	1.3	4
Arg	0.3	1
Total	100.0	329

Tryptophan and cystein were not determined.

at 535 nm, a positive band around 455 nm, and a shoulder around 510 nm were attributed to peridinin (Fig. 3A). The MCD spectrum of PCP (Fig. 3A) in the Q_y and Q_x region of chl *a* was similar to the CD spectrum, but with an opposite sign and slightly blue-shifted peaks. One positive peak (440 nm) and two negative peaks (411 and 430 nm) were found in the Soret region. This MCD spectrum was very similar to that of monomeric chl *a* [15,26], which clearly indicates that chl *a* in PCP maintains its monomeric features. No MCD signal for peridinin was detected. Since the 411 and 430 nm MCD bands corresponded to those in the CD bands, the 411 and 430 nm CD bands were assigned to chl *a*, and thus the CD band in the wavelength region from 445 to 565 nm came exclusively from peridinin.

The fluorescence properties changed significantly at −196°C (Fig. 3B). In the emission spectrum, the main band at 671 nm became sharp and several minor bands were also detected at 689, 704, 720, 730, and 747 nm (small bars). In the excitation spectrum, several bands were resolved; in the Soret region of chl *a*, the band split into two bands, appearing at 434 and 441 nm (small bars). Multiple bands were also resolved by the second derivative spectrum (small bars on the spectrum, Fig. 3B). Compared with the band locations at 15°C (Fig. 3A), almost all the bands were consistent, except that some bands were missing in the excitation spectrum at −196°C. This was mainly due to a poor S/N ratio in the excitation spectrum. The presence of several bands indicated plural spectral forms of peridinin. The main excitation band of chl *a* at −196°C was located at 667 nm, with a narrower bandwidth than that observed at 15°C. Several minor bands were observed at 605, 616, 628, 638, and 654 nm, as reported previously [11,27]. According to the wave number scale, these minor bands correspond to the mirror image of the fluorescence spectrum, which indicates that they originated from vibrational bands of chl *a*. This vibrational structure was similar to that of free chl *a*, as reported by Kwa et al. [28]. Therefore, chl *a* in *A. cohorticula* PCP appears to maintain its monomeric features, even when two chl *a* molecules are confined to a single peptide. This is consistent with the results deduced from the MCD spectrum of PCP (Fig. 3A).

4. Discussion

Under the presence of a protease inhibitor, PMSF, we obtained only one form of PCP; multiple forms in previous studies [7–10,12] might arise from partial degradation with a cleaved fragment in a small size. A similar observation was reported by Haxo [10].

PCP isolated from *A. cohorticula* contains 12 peridinins and 2 chls *a* per 37-kDa monomer. The number of peridinin molecules per PCP monomer has been reported to be 8 to 9 [8,10–12], thus the peridinin content in *A. cohorticula* is considered to be high. This high content has also been observed in *A. catenella* [23]. Similarly, the molar ratio of peridinin-to-chl *a* in *A. tamarense* and *G. catenatum* has been reported to be 10:2 [23]. These results suggest species specificity in pigment content. Thus, it is important to carefully determine the pigment content and apparent multiple forms of purified PCP. We found an associated form of PCP at the void volume following gel-filtration chromatography, as similarly reported by Prézelin et al. [11]. This suggests that the monomer PCP may be incorporated into an associated form, which corresponds to the in vivo functional form that interacts with pigments in thylakoid membranes.

The present report is the first to document inhomogeneity of the spectral components for chl *a* and peridinin in PCP. Since 12 peridinin molecules are associated with 1 polypeptide, it was reasonable to expect spectral heterogeneity, and several bands were observed in the second derivative spectrum (small bars in Fig. 3). It is known that the vibrational progression of the S_2 state of carotenoids has an energy gap of about 1400 cm^{-1} between each vibrational state [29]. When we applied the same magnitude to the absorption spectrum of peridinin, three possible sets of such a progression could be seen on the resolved components: 457–497–539 nm, 470–513–553 nm, and 482–522–561 nm. However these spectral forms were not yet confirmed. A strong interaction between peridinin molecules has been proposed based on the nearly equal rotatory strength of the dispersed type CD spectrum with a zero-crossing point at 486 nm [8]. Our assignment did not support this interpretation, however, since a positive band in the wavelength region shorter than 485 nm originated, in part, from chl *a* (Fig. 3A). At present, we do not have positive data confirming a strong interaction between peridinin molecules in PCP.

Acknowledgements: We would like to thank Prof. Y. Koyama of Kwansei Gakuin University for giving valuable advice, and Mr. H. Kojima and Ms. H. Kajiura of the National Institute for Basic Biology for their help with the analyses of amino acid composition and sequence. Thanks are also due to Mr. T. Tomo for assistance with the SDS-PAGE analysis. This work was financially supported, in part, by a Grant-in-Aid for Cooperative Research (02305014) from the Ministry of Education, Science and Culture, Japan. M.M. thanks the Ciba-Geigy Foundation (Japan) for the Promotion of Science for financial aid.

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