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PROPERTIES OF PARTIALLY PURIFIED PHOTOSYNTHETIC REACTION CENTERS FROM SCENEDESMUS MUTANT 6E AND *ANABAENA VARIABILIS* GROWN IN THE PRESENCE OF DIPHENYLAMINE*

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SUMMARY

When membrane fragments of *Anabaena variabilis* grown in the presence of diphenylamine (designated diphenylamine-Anabaena) are treated with Triton X-100 and subjected to sucrose density gradient centrifugation, a bluish-green membrane fragment enirched in P700 is obtained. This high-P700 fragment, denoted HP700, contains three P700 molecules per 100 chlorophyll *a* molecules and reduces NADP at a rate that is approximately nine times higher than that of HP700 fragments prepared from normally cultured Anabaena by the use of Triton X-100 following extraction with organic solvents. An HP700 fragment has also been isolated from a carotenoidless Scenedesmus mutant 6E, by the use of Triton X-100 and sucrose density gradient centrifugation.

Both HP700 fragments show the characteristic rapid absorbance changes of P700 upon illumination. The fluorescence properties of the HP700 fragments at $-196°$ are different from those of the original membrane fragments. At $-196°$ the long wavelength fluorescence peak is located at a shorter wavelength (724 mμ) in the diphenylamine-Anabaena HP700 fragment and is lower in intensity than that observed with the membrane fragment. Long wavelength fluorescence at $-196°$ is low in the fluorescence spectra of the membrane fragments of Scenedesmus mutant 6E and is barely observable in the HP700 fragment. The fluorescence spectra of the HP700 fragments of both diphenylamine-Anabaena and Scenedesmus mutant 6E at $-196°$ show a shoulder or peak at 700 nm. The data on fluorescence properties of the HP700 fragments suggest that 730 nm fluorescence does not originate from P700.

INTRODUCTION

Fragments and pigment–protein complexes which correspond to Photosystems 1 and 2 have been isolated from spinach chloroplasts by the use of detergents[1–7]. The isolation and properties of such fragments obtained with Triton X-100 have been reported by this laboratory. Triton X-100 removes a small fragment which contains Photosystem 1, leaving a membrane residuum which contains Photosystem 2. The

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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two photosystems have also been isolated from *Anabaena variabilis* by the use of Triton X-100 (ref. 8). In the case of *A. variabilis*, Triton X-100 removes a small Photosystem 2 fragment, leaving behind a larger Photosystem 1 fragment. These Photosystem 1 fragments reduced $NADP^+$ at high rates and were enriched in reaction center chlorophyll, P700, and β-carotene.

Fragments enriched in P700, denoted "high P700" or HP700 fragment, have recently been isolated from four plant species in this laboratory[9-11]. The HP700 fragments are observed by electron microscopy to be smaller in shape than Photosystem 1 fragments. Since these HP700 fragments are simpler in pigment composition and contain relatively more P700 than Photosystem 1 fragments, they offer an opportunity to study the reaction center of Photosystem 1 in greater detail. However, $NADP^+$ reduction activity is greatly reduced in such preparations by the use of organic solvents during the preparation procedure.

In this paper we will describe a method for isolating an HP700 fragment which reduces $NADP^+$ at high rates. *Anabaena variabilis* cells grown in the presence of diphenylamine (diphenylamine-Anabaena) were used for this investigation. When grown under these conditions, this alga lacks the usual carotenoids except myxoxanthophyll[12]. Treatment of the photosynthetic membrane fragment of such algal cells with Triton X-100 produces an HP700 fragment which is highly active for $NADP^+$ reduction. This paper describes the photochemical and physical properties of the HP700 fragments prepared from diphenylamine-Anabaena and Scenedesmus mutant 6E in comparison with those prepared by the use of organic solvent and Triton X-100.

EXPERIMENTAL

Anabaena variabilis cells were grown in the modified Detmer's medium[13] in the absence or presence of diphenylamine (12 mg/l)[12]. After 9 days of culture, the cells were harvested. The cells grown in the absence and presence of diphenylamine will be described as normal-Anabaena cells and diphenylamine-Anabaena cells, respectively.

The Scenedesmus mutant 6E culture was a gift of Professor N. I. Bishop. These cells were cultured heterotrophically in the dark for 5 days on nitrate medium supplemented with 0.5% glucose and 0.25% yeast extract[14].

Membrane fragments containing the photosynthetic apparatus were prepared from Anabaena cells by the sonication procedure described earlier[8]. The same procedure was used for the preparation of membrane fragments from Scenedesmus mutant 6E, but 1 mM ascorbate was also present, since without ascorbate most of the chlorophyll in the mutant cells was destroyed during the preparation of the membrane fragments.

For the isolation of HP700 fragments from the membrane fragments of diphenylamine-Anabaena and Scenedesmus mutant 6E, all steps were carried out at 0° in the presence of 0.01 M Tris–HCl buffer (pH 7.5) (also containing 1 mM ascorbate for Scenedesmus mutant 6E). A solution of 10% Triton X-100 in buffer was added to an equal volume of the membrane fragment suspension (0.3 mM for diphenylamine-Anabaena and 0.2 mM for Scenedesmus mutant 6E in terms of chlorophyll concentrations). A portion of the suspension (3 ml) was layered on a discontinuous sucrose gradient (2–20% in 2% steps) and centrifuged at 131 000 $\times g$ for 20 h in

a Spinco Model L2-65 ultracentrifuge with SW27 rotor. The HP700 fragments of both diphenylamine-Anabaena and the Scenedesmus mutant 6E were located in the 8% layer. The HP700 and Photosystem I fragments from normal-Anabaena cells were prepared by the method previously reported[8,9].

A Cary 14 recording spectrophotometer was used to measure absorption spectra. Difference spectra (ferricyanide oxidized-*minus*-dithionite reduced, light-*minus*-dark) as well as the kinetic curves of the light-induced bleaching of P700 were measured with a Phoenix dual-wavelength scanning spectrophotometer. Fluorescence measurements were made according to the procedure described previously[8]. All fluorescence spectra shown in this paper are tracings of the original recordings and were not corrected for the efficiency of the monochromator or for the sensitivity of the photomultiplier.

For the preparation of crude enzymes used in the assay for $NADP^+$ reduction, a sonicated Anabaena cell suspension in distilled water was brought to 60% satn. with $(NH_4)_2SO_4$ by adding the solid salt. The supernatant obtained after centrifugation at 20 000 × *g* for 15 min was filtered through glass-wool and was brought to saturation with $(NH_4)_2SO_4$. A precipitate was collected after 2 days by centrifugation at 20 000 × *g* for 30 min, dialyzed against 1 mM Tris–HCl buffer (pH 7.5) and used as the crude enzyme preparation.

Chlorophyll *a* was determined after extraction of the material with 80% acetone, using an extinction coefficient of $7.54 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ at 663 nm (ref. 15). Protein was determined by the method of LOWRY *et al.*[16].

RESULTS

HP700 fragments from diphenylamine-Anabaena and Scenedesmus mutant 6E

Treatment of diphenylamine-Anabaena membrane fragments with 5% Triton X-100 yielded bluish green HP700 fragments which are separated by sucrose density gradient centrifugation. Fig. 1A shows the appearance of the centrifuge tube after

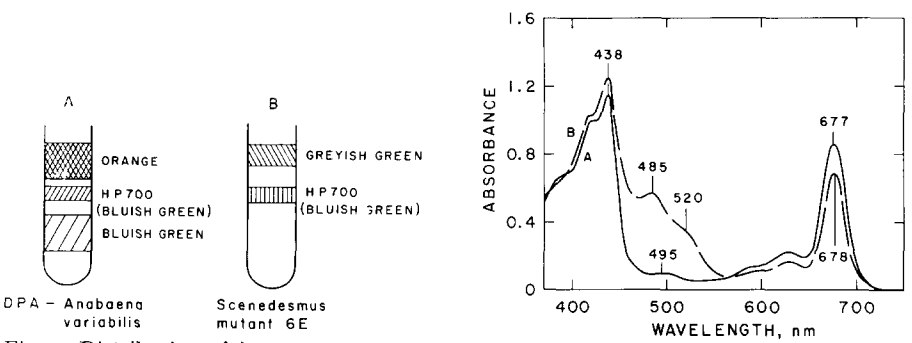


Fig. 1. Distribution of fragments produced through the action of Triton X-100 on the original membrane fragment of *A. variabilis* grown in the presence of diphenylamine (DPA-*Anabaena variabilis*) (A) and Scenedesmus mutant 6E (B) after centrifugation for 20 h at 131 000 × *g* through a discontinuous gradient of sucrose (2–20%). HP700 designates the HP700 fragment which is enriched in P700.

Fig. 2. Absorption spectra of the HP700 (Curve A) and membrane (Curve B) fragments prepared from diphenylamine-Anabaena cells. The fragments were suspended in 0.01 M Tris–HCl buffer (pH 7.5).

the separation was complete, with the HP700 fragment concentrated as a well defined band at 8% sucrose. Approx. 20% of the total chlorophyll was found in this band. A diffuse bluish-green band which was observed around 14% sucrose contained partially fragmented membranes. Most of the carotenoids and approx. 65% of the total chlorophyll were contained in the orange band at the top of the tube. This band was highly fluorescent, which indicates the presence of solubilized chlorophyll and carotenoids.

The absorption spectra of the HP700 and membrane fragments prepared from diphenylamine-Anabaena cells are shown by Curve A and B in Fig. 2, respectively. The absorption maxima of the red band of chlorophyll *a* in the HP700 and membrane fragments were located at 677 and 678 nm, respectively, and both Soret bands were maximal at 438 nm. As shown by a peak at 485 nm and shoulder around 520 nm in Curve B, the membrane fragment contains carotenoids, most of which is myxoxanthophyll[12]. Most of these carotenoids were removed from the membrane fragment by Triton treatment and only a trace of carotenoid was observed in the HP700 fragments, as shown by a slight shoulder around 495 nm in Curve A.

Fig. 1B shows the appearance of the centrifuge tube after sucrose density gradient centrifugation of the membrane fragments of Scenedesmus mutant 6E treated with 5% Triton X-100. The bluish-green HP700 fragment is concentrated at 8% sucrose. Another band located at the top of the tube contained solubilized chlorophyll *a*, some of which had been converted to pheophytin *a* during centrifugation.

Curves A and B in Fig. 3 show the absorption spectra of the HP700 and membrane fragments of Scenedesmus mutant 6E, respectively. The red and Soret bands of chlorophyll *a* in both fragments were located at 674 and 437 nm, respectively. A peak at 413 nm, due to pheophytin *a*, was observed in both fragments. When the membrane fragments were kept at 0° for 1 or 2 days, the peak at 413 nm became

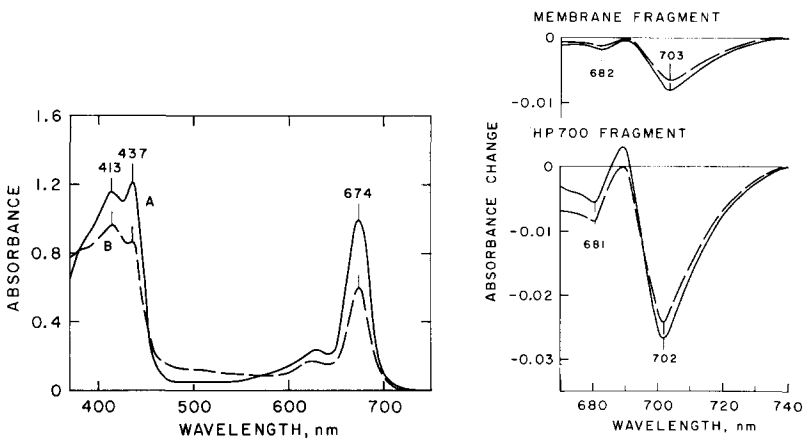


Fig. 3. Absorption spectra of the HP700 (Curve A) and membrane (Curve B) fragments of Scenedesmus mutant 6E. The fragments were suspended in 0.01 M Tris–HCl buffer (pH 7.5).

Fig. 4. Difference spectra of ferricyanide oxidized-*minus*-dithionite reduced samples (———) and light-*minus*-dark difference spectra measured in the presence of 1 mM ascorbate (– – – –) for the membrane and HP700 fragments prepared from diphenylamine-Anabaena cells. Each fragment was suspended in 0.01 M Tris–HCl buffer (pH 7.5) and had a chlorophyll content of 16.5 μg chlorophyll *a*/ml and an absorbance of 1.0 at the absorption maximum of the red band.

much higher and the Soret band of chlorophyll *a* was observed only as a shoulder. However, most of the pheophytin *a* was removed by Triton treatment and the HP700 fragments from such membrane fragment showed the same absorption spectrum as Curve B in Fig. 3. As seen from Curves A and B in Fig. 3, which have no peak or shoulder around 480 nm, these fragments lack chlorophyll *b*.

P 700 and protein content

The HP700 and membrane fragments from both algae show an absorbance change in the 700 nm region when illuminated or treated with ferricyanide. Solid curves in Fig. 4 show the difference spectrum between ferricyanide-oxidized and dithionite-reduced samples for the HP700 and membrane fragments from diphenylamine-Anabaena cells, showing a good coincidence with the light-*minus*-dark spectrum measured in the presence of 1 mM ascorbate (dotted curves). The HP700 and membrane fragments show the maximal absorbance changes at 702 and 703 nm, respectively, accompanied by another change around 680 nm.

Curves A and B in Fig. 5 show the light-*minus*-dark spectra of the HP700 and membrane fragments prepared from Scenedesmus mutant 6E, respectively. Both spectra show a maximal absorbance change at 694 nm accompanied by another change at 678 nm.

The HP700 fragments prepared from diphenylamine-Anabaena and Scenedesmus mutant 6E by treatment with Triton X-100 alone, contain one P700 per approx. 30 chlorophylls; the ratio is almost the same as that of the HP700 fragments prepared from normal-Anabaena cells and spinach chloroplasts by the use of organic solvents and Triton X-100. These data are summarized in Table I. As seen from this table,

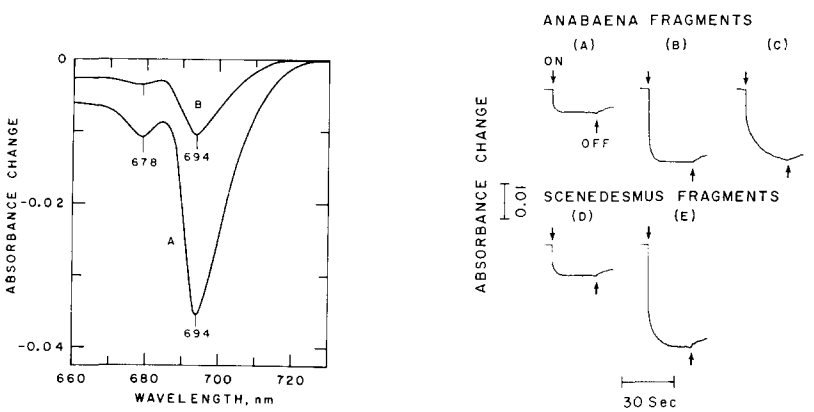


Fig. 5. Light-*minus*-dark difference spectra measured in the presence of 1 mM ascorbate for the HP700 (Curve A) and membrane (Curve B) fragments of Scenedesmus mutant 6E. Each fragment was suspended in 0.01 M Tris–HCl buffer (pH 7.5) and had a chlorophyll content of 17.0 μg chlorophyll *a*/ml and an absorbance of 1.0 at the absorption maximum of the red band.

Fig. 6. Light-induced absorbance changes related to P700 measured in the presence of 1 mM ascorbate. A, diphenylamine-Anabaena membrane fragment; B, diphenylamine-Anabaena HP 700 fragment; C, normal-Anabaena HP700 fragment; D, membrane fragment of Scenedesmus mutant 6E; E, HP700 fragment of Scenedesmus mutant 6E. The absorbance changes were followed at 703, 702 and 701 nm for A, B and C, respectively and at 694 nm for D and E. The chlorophyll content of each sample was 16.5 μg/ml. The actinic light was from a tungsten lamp and reached the sample after passing through a blue filter (Corning CS-5-60). The intensity of the actinic light at the sample was $2.0 \cdot 10^4$ erg·cm^{-2}·sec^{-1}.

diphenylamine-Anabaena membrane fragment contains almost the same amount of P700 as does the normal-Anabaena membrane fragment. The P700 content of the membrane fragment of the Scenedesmus mutant 6E is close to that of the Photosystem I fragments of spinach and Anabaena[8,17].

The last column of Table I shows the protein content of each fragment. The HP700 fragment in each case contained less protein than the original membrane fragments, showing that considerable protein was removed from the membrane fragment during the isolation of the HP700 fragments. Also notable is the markedly lower protein in the Anabaena fragments, by comparison with both Scenedesmus and spinach.

TABLE I

P700 AND PROTEIN CONTENTS OF MEMBRANE AND HP700 FRAGMENTS

	*P700**	*Protein***
P700 fragments		
Prepared by treatment with Triton X-100 alone from:		
Diphenylamine-Anabaena	2.8	0.8
Scenedesmus mutant 6E	3.6	4.1
Prepared by the use of organic solvents and Triton X-100 from:		
Normal-Anabaena (ref. 9)	3.1	0.3
Spinach chloroplast (ref. 11)	3.3	3.2
Membrane fragments		
from:		
Diphenylamine-Anabaena	0.8	1.3
Normal-Anabaena	0.8	0.6
Scenedesmus mutant 6E	1.1	8.6

* P700 in moles per 100 moles chlorophylls, assuming P700 has the same extinction coefficient as chlorophyll *a*. These values are the ratios of the oxidized-*minus*-reduced absorption change of P700 to the absorbance at the red peak × 100.

** Protein in mg per 100 nmoles chlorophylls.

TABLE II

$NADP^+$ PHOTOREDUCTION ACTIVITIES OF ANABAENA FRAGMENTS

The reaction mixture contained: 0.2 mM DCIP, 2 mM sodium ascorbate, 0.4 mM $NADP^+$, 6 mM $MgCl_2$, 15 μM chlorophyll, 0.1 % Triton X-100 and saturating amount of crude enzymes prepared from *A. variabilis*. Reactions were run anaerobically under illumination with red light (Corning filter CS 2-58) at $1.5 \cdot 10^5$ erg·cm^{-2}·sec^{-1}.

Fragment	*NADPH (μmoles/mg chlorophyll per h)*
Fragments from diphenylamine-Anabaena cells	
Membrane fragment	169
HP700 fragment	185
Fragments from normal-Anabaena cells	
Membrane fragment	164
Photosystem I fragment	202
HP700 fragment	20

$NADP^+$ photoreduction activities

The $NADP^+$ photoreduction activities of the various Anabaena fragments are described in Table II. As seen in this table, the HP700 fragment prepared from diphenylamine-Anabaena cells reduces $NADP^+$ at high rates, while only small activity was found in the HP700 fragment prepared from normal-Anabaena cells by the use of organic solvents and Triton X-100. The activity of the diphenylamine-Anabaena HP700 fragment was slightly higher than that of the original membrane fragment on the chlorophyll basis, however, the activity was lower (about 1/3) on the P700 basis. The Photosystem 1 fragment showed about 20% higher activity than the original membrane fragment. These fragments reduced $NADP^+$ at very low rates or did not show any activity when supplemented with the emzymes prepared from spinach.

The HP700 and membrane fragments prepared from Scenedesmus mutant 6E did not show any $NADP^+$ reduction activity upon illumination in the presence of appropriate enzymes from either spinach or *A. variabilis*.

P700 bleaching kinetics

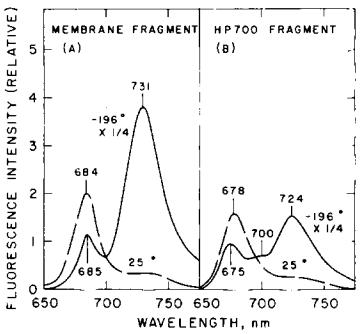
Fig. 6 shows the kinetics of the light induced absorbance changes of P700, obtained with diphenylamine-Anabaena membrane fragment (A), the HP700 fragment prepared from diphenylamine-Anabaena cells (B) and HP700 fragment from normal-Anabaena cells (C) in the presence of 1 mM ascorbate. The P700 absorbance changes were followed at the peak position of their oxidized-*minus*-reduced spectra; 703, 702 and 701 nm for (A), (B) and (C), respectively. The magnitude of the light induced absorbance changes of the HP700 fragments prepared from diphenylamine- and normal-Anabaena cells was approx. 3 times greater than that of the membrane fragment. The rate of the P700 bleaching in the diphenylamine-Anabaena HP700 fragment (B) was as fast as that of the membrane fragment (A), while the rate was much slower in the HP700 fragment prepared from normal-Anabaena cells by the use of organic solvents and Triton X-100 (C). The light intensity used in these experiments, which was the maximum intensity obtained from the tungsten lamp, was close to the saturating intensity. Though a slightly higher rate of P700 bleaching was obtained with higher light intensity, the rate was much slower in the normal-Anabaena HP700 fragment than in the diphenylamine-Anabaena membrane and HP700 fragments. Since the $NADP^+$ reduction activity of the diphenylamine-Anabaena HP700 fragment is much higher than that of the normal-Anabaena HP700 fragment, there might be some correlation between the slow rate of P700 bleaching and the low $NADP^+$ reduction activity of the normal-Anabaena HP700 fragment.

The kinetics of the light induced absorbance changes of P700, obtained with the membrane (D) and HP700 fragments (E) of Scenedesmus mutant 6E are also shown in Fig. 6. The P700 absorbance changes were followed at 694 nm in the presence of 1 mM ascorbate. The magnitude of bleaching of the HP700 fragment was approximately three times higher than that of the membrane fragment.

Fluorescence measurements

Fig. 7 shows the fluorescence spectra at room (25°) and liquid nitrogen temperature (−196°) for the HP700 and membrane fragments prepared from diphenylamine-Anabaena cells. At 25° the HP700 and membrane fragment showed a peak at 678 and 684 nm, respectively. The fluorescence yield was greatly enhanced at −196°.

The fluorescence spectrum of the HP700 fragment showed a peak located at 724 nm and a shoulder around 700 nm, while that of the membrane fragment showed peaks at 731 and 685 nm. The fluorescence spectrum of the HP700 fragment at −196° also showed a peak at 675 nm, indicating some contamination by solubilized chlorophyll.



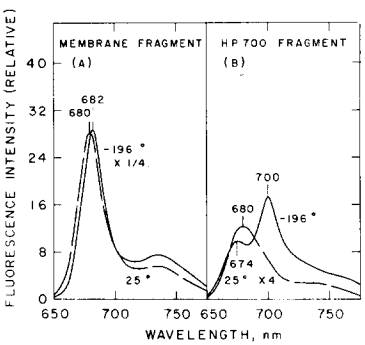


Fig. 7. Fluorescence spectra of the membrane (A) and HP700 (B) fragments prepared from diphenylamine-Anabaena cells at room (25°) and liquid N_2 temperature (−196°). Each sample had the same chlorophyll content.

Fig. 8. Fluorescence spectra of the membrane (A) and HP700 (B) fragments of Scenedesmus mutant 6E at room (25°) and liquid N_2 temperature (−196°). Each sample had the same chlorophyll content.

The 730 nm fluorescence peak at −196° is characteristic of Photosystem I preparations[18,19] and has been attributed to the chlorophyll molecules (or C_{705}) serving as energy collector[20]. The fluorescence data described in this paper suggest that the chlorophyll molecule, C_{705}, is different from P700 since the fluorescence intensity of the HP700 fragment at 730 nm is much less than that of the membrane fragment, in spite of the 3 times higher P700 content in the HP700 fragment. Most of the chlorophyll molecules, C_{705}, might be removed from the membrane fragment by treatment with Triton X-100 during the isolation of the HP700 fragment.

The fluorescence spectra of the HP700 and membrane fragments of Scenedesmus mutant 6E at 25° and −196° are shown in Fig. 8. At 25° both fragments have a peak at 680 nm, and the fluorescence intensity of the membrane fragment at 680 nm was 9 times higher than that of the HP700 fragment. The fluorescence spectrum of the membrane fragment at −196° was similar in shape to the fluorescence spectrum at 25°, but the fluorescence intensity at −196° was higher and a peak was shifted to 682 nm. A marked increase in fluorescence intensity at 730 nm was not observed at the low temperature.

The low temperature fluorescence spectrum of the HP700 fragment from the mutant showed peaks at 674 nm, due to solubilized chlorophyll, and at 700 nm; no peak was observed around 730 nm. The fluorescence peak at 700 nm might be attributed to the chlorophyll molecule closely related to P700, since 700 nm fluorescence was also observed in the HP700 fragment preparations from diphenylamine-, normal-Anabaena cells and spinach chloroplasts[9,11].

The fluorescence data of the Scenedesmus mutant 6E again suggest that the

chlorophyll molecule, C_{705}, differs from P700, and that the chlorophyll molecule, C_{705}, is missing (or the content is very small) in the fragments of Scenedesmus mutant 6E.

DISCUSSION

It has been demonstrated in the previous studies that the prior removal of carotenoids from photosynthetic membrane by extraction with organic solvents allows for the subsequent removal of bulk chlorophyll and solubilization of HP700 fragment by Triton X-100 (refs. 9–11). This is also demonstrated in the present study, where the carotenoidless Scenedesmus mutant 6E and diphenylamine-Anabaena were used and the HP700 fragments were prepared without using organic solvents. In the case of diphenylamine-Anabaena, the membrane fragments from this alga contain high amounts of myxoxanthophyll[12]. However, myxoxanthophyll did not interfere to the action of Triton X-100, which might be due to the fact that most of the myxoxanthophyll is distributed in Photosystem 2 fragment[8].

Although the diphenylamine-Anabaena HP700 fragment has much higher $NADP^+$ photoreduction activity than the normal-Anabaena HP700 fragment, the activity is lower (about 1/3) than that of the original membrane fragment on the basis of P700 content. This might be due to the loss of some factor (primary electron acceptor?) or some alteration of the structure, making the HP700 fragment less accessible to the necessary enzymes (ferredoxin *etc.*).

All the fluorescence data described in this paper suggest that the chlorophyll molecule, C_{705}, which has a fluorescence maximum at 730 nm at $-196°$ (ref. 20) differs from P700. This is consistent with the observation made by KOK[21] that a Scenedesmus mutant lacking P700 exhibits 730 nm fluorescence at $-196°$. The low intensity of long wavelength fluorescence of the diphenylamine-Anabaena HP700 fragment at $-196°$ suggests that the content of the chlorophyll molecule, C_{705}, is much lower in the HP700 fragment than in the original membrane fragment. The chlorophyll molecule, C_{705}, might be missing (or the content is very small) in the HP700 fragment of Scenedesmus mutant 6E, since the long wavelength fluorescence at $-196°$ is barely observable in this HP700 fragment.

Fluorescence at 700 nm appears at $-196°$ in the HP700 fragments of both diphenylamine-Anabaena and Scenedesmus mutant 6E. The 700 nm fluorescence at $-196°$ could originate from either P700 or a chlorophyll molecule closely related to P700. In the case of diphenylamine-Anabaena HP700 fragment, it is difficult to relate the 700-nm fluorescence with P700 since the reduced-*minus*-oxidized spectra of the diphenylamine-Anabaena HP700 fragment (representing the absorbance maximum of P700) have the maximal change at a longer wavelength (702 nm) than that of the fluorescence (700 nm). Preliminary experiment showed, however, that illumination of the HP700 fragment of Scenedesmus mutant 6E prior to cooling with liquid N_2 decreased the fluorescence intensity at 700 nm, suggesting that the 700 nm fluorescence in the HP700 fragment of Scenedesmus mutant 6E could originate from P700.

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