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A FRACTION FROM *ANABAENA VARIABILIS* ENRICHED IN THE REACTION CENTER CHLOROPHYLL P700*

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SUMMARY

Extraction of carotenoids from lyophilized membrane fragments of *Anabaena variabilis* followed by treatment with Triton X-100 and sucrose density gradient centrifugation yields a bluish-green membrane fragment which is enriched in P700. This high-P700 fragment, denoted HP700, contains three P700 molecules per 100 chlorophyll *a*; the ratio is three times higher than that of the Photosystem I fragment (obtained by treatment with Triton X-100 alone). Examination of the HP700 fragment by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate reveals the presence of only one chlorophyll–protein complex band. The protein content in the HP700 fragment is about half that of the Photosystem I fragment on a chlorophyll *a* basis.

The HP700 fragment shows the characteristic absorbance change of P700 and the related electron paramagnetic resonance signal upon illumination. Compared with the Photosystem I fragment, the HP700 fragment shows a slower rate of bleaching of P700 upon illumination. These two fragments also differ in their fluorescence properties. The intensity of fluorescence at 685 mμ is higher for the HP700 fragment at 25°. At −196° the long-wavelength fluorescence is found at a shorter wavelength (722 mμ) in the HP700 fragment and is lower in intensity than that observed with the Photosystem I fragment. These data indicate that there is less efficient energy transfer between the light-harvesting chlorophyll *a* and P700 in the HP700 fragment. The electron micrograph of the HP700 fragment shows the presence of small membrane fragments and some strands.

Exposure of the HP700 fragment to ferricyanide or to light causes an oxidation of the P700 chlorophyll. Accompanying this change is a decrease in absorbance at 680 mμ. This change may represent another chlorophyll species which is part of the reaction center complex.

INTRODUCTION

KOK[1] discovered the chlorophyll complex absorbing at 700 mμ, P700, which acts as the reaction center of Photosystem I of chloroplasts[2]. Partial purification of the

Abbreviations: EPR, electron paramagnetic resonance; PMS, phenazine methosulfate.

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P700 has also been reported by KOK[3], who obtained a pellet containing P700 in the ratio of 1.4 P700 per 100 chlorophylls, by extracting chloroplasts with 72% aqueous acetone to remove up to 85% of the chlorophyll[4]. The P700 content of the pellet so obtained was approximately the same as that of the Photosystem I fragment prepared from spinach chloroplasts by the action of Triton X-100[5]. Further attempts to isolate a reaction center fraction containing only P700 have not succeeded because of the difficulties encountered in attempting to remove bulk chlorophylls and other constituents from Photosystem I.

In this paper we will describe a method successfully utilized to isolate a fragment which has a high concentration of the reaction center, P700. For this investigation we have chosen *Anabaena variabilis*, a blue-green alga whose photosynthetic system is amenable to fractionation with Triton X-100. Of the total chlorophyll in cells of this alga, approx. 80% is found in the Photosystem I fragment which has P700 in the ratio of one P700 to 100 chlorophylls[6]. Extraction of carotenoids from the lyophilized membrane fragments of *A. variabilis*, followed by a treatment with Triton X-100 and a sucrose density gradient centrifugation, yields a bluish-green fragment. A ratio of three P700 to 100 chlorophylls is obtained for this fragment. The low concentration of protein found in this fragment shows that protein as well as chlorophyll is removed during the isolation of this bluish-green fragment. The present paper describes the physical properties and structure of this fragment.

EXPERIMENTAL

Isolation of high-P700 (HP700) fragment

The procedure for the preparation of the chlorophyll-containing membrane fragment from intact *A. variabilis* cells was the same as reported previously[6]. The isolation procedure for the HP700 fragment is shown diagramatically in Fig. 1. All steps were carried out at 0°, and 0.01 M Tris–HCl buffer (pH 7.5) was used where indicated. Lyophilized membrane fragments (200 mg) were extracted 5 times with hexane, and the pellet collected by low-speed centrifugation was extracted by 40% aqueous ethanol (v/v). Higher concentrations of aqueous ethanol were also used for comparison. A bluish-green pellet, obtained by centrifuging the aqueous ethanol suspension at 10000 × *g* for 10 min, was suspended in buffer. A solution of 1% Triton X-100 in buffer was added to an equal volume of this suspension (0.3 mM in terms of chlorophyll concentration) and the mixture was kept at 0° for 30 min. Centrifugation of the suspension at 10000 × *g* for 15 min gave a pellet, which was resuspended in a solution of 5% Triton X-100 in buffer, and the mixture was kept at 0° for 30 min. The final concentration of chlorophyll in the suspension was 0.15 mM. Portions of the suspension (3 ml) were layered onto tubes containing discontinuous sucrose density gradients of ten layers formed from 3-ml portions of 30–3% sucrose solution. Centrifugation was at 131000 × *g* for 18 h in the SW27 rotor of Spinco Model L2-65 ultracentrifuge.

The Photosystem I fragment was isolated from the membrane fragment by the method previously reported[6].

Spectrophotometry and fluorometry

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. The electron paramagnetic resonance (EPR) spectra were determined with a Varian Model 4500 ESR spectrometer operated at 2.5 mW microwave power (100 kcycles field modulation) with the necessary accessory equipment[7].

Fluorescence measurements were made according to the procedure described previously[6]. 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.

Assay of chlorophyll a and proteins

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 mμ[8]. Protein was determined by the method of LOWRY *et al.*[9].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out using polyacrylamide gel (5%), and 0.02 M borate buffer (pH 10.3)[10,11]. Protein in the gel was stained with 0.1% amido black 10B in methanol–water–acetic acid (4:5:1, by vol.).

Electron microscopy

Electron micrographs were obtained with a Philips EM-200 electron microscope. Negative staining with phosphotungstic acid at pH 5.9 in the presence of 5 mM $MgCl_2$ was used to prepare the samples.

RESULTS

Isolation of HP700 fragment

The procedure used for isolating the HP700 fragment from the membrane fragment of *A. variabilis* is shown diagramatically in Fig. 1. Most of the β-carotene in the membrane fragment was extracted by hexane, which also partly extracted echinenone and zeaxanthin. A subsequent extraction with 40% aqueous ethanol and exposure to 0.5% Triton X-100 removed most of the residual carotenoids (echinenone, zeaxanthin, myoxthanthophyll and the trace of β-carotene) and 60–70% of the bulk chlorophyll. Higher concentrations of aqueous ethanol (50–70%) extracted more chlorophyll, but the total amount of chlorophyll extracted by aqueous ethanol (40–70%) and 0.5% Triton X-100 was nearly constant. The pellet obtained after 0.5% Triton X-100 treatment was dissolved in 5% Triton X-100 and centrifuged through sucrose density gradient at 131000 $\times$ *g* for 18 h. The bluish-green fragment obtained after sucrose density gradient centrifugation contained 16% of the total chlorophyll and is designated a high-P700 fragment because of the relatively high P700: chlorophyll ratio in this fragment. The HP700 fragment sedimented to the layer containing 9% sucrose, showing that the density of the HP700 fragment is much lower than that of the Photosystem I fragment of *A. variabilis* reported previously[6].

A green band was located near the top of the tube. The high fluorescence intensity of this band indicated the presence of free chlorophyll and carotenoids. Protein was not found in this band.

The absorption spectra of the HP700 and Photosystem I fragments are shown in Fig. 2. The absorption spectrum of the HP700 fragment (Curve A) did not show any peak or shoulder around 495 mμ due to carotenoid absorption, while a shoulder at 495 mμ was present in the spectrum of the Photosystem I fragment (Curve B). The HP700 fragment is, therefore, free from carotenoids. The absorption maxima of the red and Soret bands of chlorophyll *a* in the HP700 fragment were located at 676 and 438 mμ, respectively, while those in the Photosystem I fragment were located at 680 and 440 mμ, respectively.

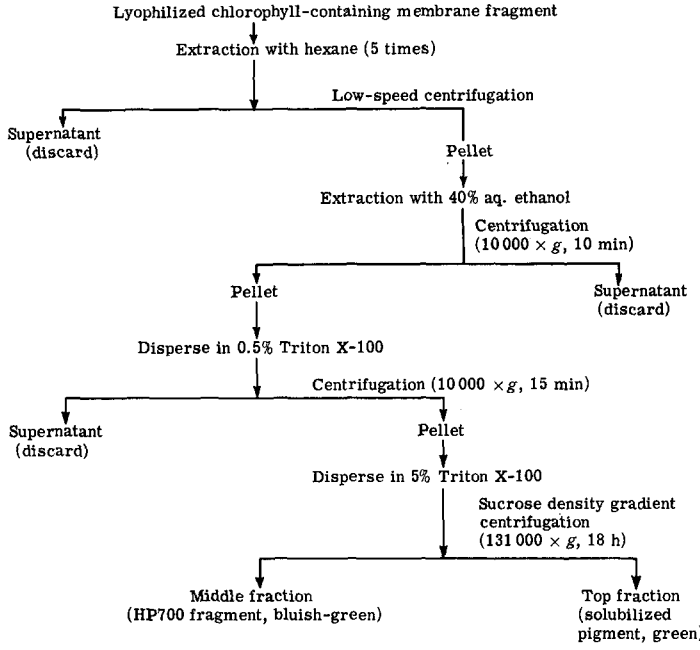


Fig. 1. Isolation procedure for the HP700 fragment.

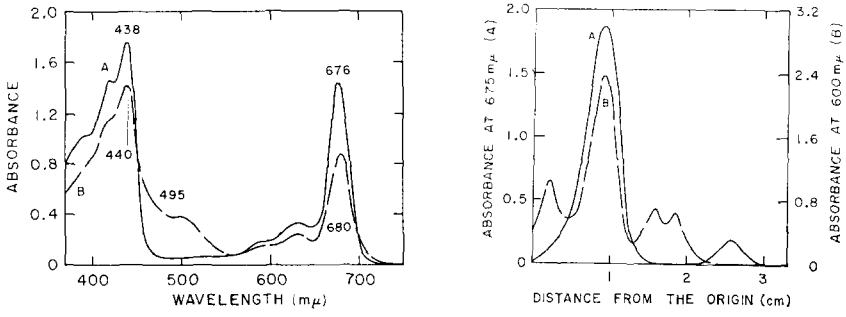


Fig. 2. Absorption spectra of the HP700 (Curve A) and Photosystem I (Curve B) fragments.

Fig. 3. The electrophoretic patterns of sodium dodecyl sulfate-solubilized HP700 fragment. Curve A was recorded at 675 mμ before staining, and Curve B at 600 mμ after staining the protein with amido black 10B. The electrophoresis on polyacrylamide gel was conducted at pH 10.3 for 1.5 h.

Electrophoresis

The HP700 fragment was solubilized with sodium dodecyl sulfate and examined by electrophoresis on polyacrylamide gel. Fig. 3 shows electrophoretic patterns of sodium dodecyl sulfate-solubilized HP700 fragment before and after staining.

The pattern recorded at 675 mμ before staining (Curve A) shows the presence of two bands. Only the band with lower mobility ($4.4 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$), which contained 93% of the chlorophyll, was colored when stained in the gel, as seen from the pattern recorded at 600 mμ after staining (Curve B). The small amount of chlorophyll in the lesser band is free chlorophyll released from the HP700 fragment by the action of sodium dodecyl sulfate. This action of detergents has been observed previously[10–12]. The HP700 fragment was free from pigment protein of Photosystem II, as seen from Curve A. Curve B also shows the presence of three colorless protein bands. 40% of the protein in the HP700 fragment was such colorless protein.

P700 and protein concentrations

The concentration of P700 in each fragment was calculated from the difference spectrum between a ferricyanide-oxidized and dithionite-reduced sample. Solid curves in Fig. 4 show the difference spectra obtained by chemical oxidation and reduction of each sample, showing a good coincidence with the light-*minus*-dark difference spectra measured in the presence of 1 mM ascorbate (dotted curves). The maximal absorbance change of P700 in the HP700 fragment occurred at 701 mμ, whereas the Photosystem I fragments showed a maximal absorbance change at 705 mμ. Another bleaching was observed, with maximal bleaching at 684 and 680 mμ for the Photosystem I and HP700 fragments, respectively.

The P700 contents of each fragment are presented in Table I, which shows an almost 4-fold concentration of the reaction center chlorophyll in the HP700 fragment.

The protein content of each fragment is given in the last column of Table I. The protein content of the HP700 fragment was less than that of the Photosystem I and the original membrane fragments. This shows that considerable protein was removed from the membrane fragment, and from the Photosystem I fragment, during the isolation of the HP700 fragment.

The data obtained by gel electrophoresis show that 40% of the protein in the HP700 fragment was colorless protein. Therefore, the protein to chlorophyll ratio of the pigment–protein complex in the HP700 fragment, although not determined, must be smaller than that of the HP700 fragment shown in Table I.

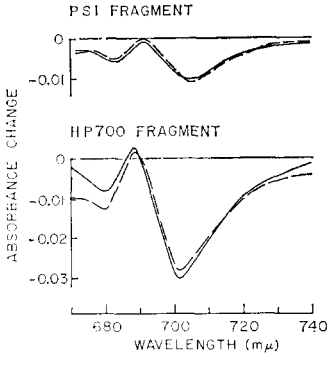
TABLE I

P700 AND PROTEIN CONTENTS OF ANABAENA FRAGMENTS

Fragment	*P700**	*Protein***
Membrane fragment	0.8	0.81
Photosystem I fragment	1.0	0.52
HP700 fragment	3.1	0.23

* P700 in moles/100 moles chlorophyll *a*.
** Protein in mg/100 mμmoles chlorophyll *a*.

Table II shows the relationship of P700 recovery to the ethanol concentration in the aqueous ethanol used for the initial extractions. The recovery of P700 obtained by using 40% aqueous ethanol extraction was 63% of that found in the original



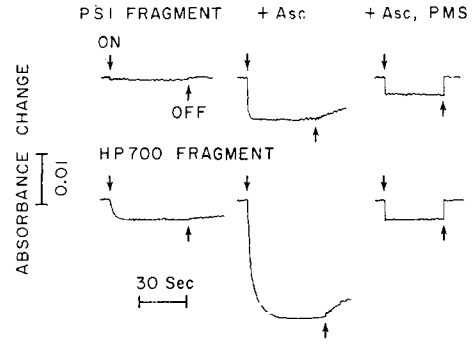


Fig. 4. Difference spectra of ferricyanide oxidized-*minus*-dithionite reduced samples (———) and light-*minus*-dark difference spectra measured in the presence of 1 mM ascorbate (– – – –). Each sample had a chlorophyll content of 16.2 μg chlorophyll *a*/ml and an absorbance of 1.0 at the absorption maximum of the red band. PSI, Photosystem I.

Fig. 5. Light-induced absorbance changes related to P700 in the HP700 and Photosystem I fragments. The absorbance changes were followed at 701 and 705 mμ for the HP700 and Photosystem I fragments, respectively. The fragments were suspended in 0.01 M Tris–HCl buffer (pH 7.5). The chlorophyll content of each sample was 16.2 μg/ml. The samples were examined first without any addition (left tracings), with 1 mM ascorbate (middle tracings) and then with ascorbate *plus* 10 μM PMS present (right tracings). The actinic light was from a tungsten lamp and reached the sample after passing through a filter (Corning CS-5-60). The intensity of the actinic light at the sample was $2.0 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}.

TABLE II

EFFECT OF ETHANOL CONCENTRATION ON RECOVERY OF P700 IN THE HP700 FRAGMENT

Ethanol (%)	*Recovery of P700* (%)	*P700**	*Protein***
40	63	3.1	0.23
50	57	2.8	0.24
60	23	3.0	0.20

* P700 in moles/100 moles chlorophyll *a*.
** Protein in mg/100 mμmoles chlorophyll *a*.

membrane. The rest of P700 was found in the pellet obtained by sucrose density gradient centrifugation. Extraction with higher concentration of aqueous ethanol decreased the recovery. With 60% aqueous ethanol the recovery was 23%, and 70% aqueous ethanol extraction gave much less recovery. The decrease of the recovery is due to a decrease in the solubility of the pellet, obtained after aqueous ethanol extraction, in 5% Triton X-100. When 60 or 70% ethanol was used, most of the bluish-green material was located at the bottom of the tube after sucrose density gradient centrifugation. The results summarized in Table II also show that the ratios of P700 and protein to chlorophyll *a* were nearly constant at different ethanol concentrations, despite the rather great variation in the percent recovery of P700.

Physical properties

Fig. 5 shows the light-induced absorbance changes related to the photooxidation of P700, obtained with the HP700 and Photosystem I fragments in the absence and presence of electron donors (ascorbate, ascorbate and phenazine methosulfate (PMS)). The P700 absorbance changes were followed at 701 and 705 mμ for the HP700 and Photosystem I fragments, respectively. Both fragments showed only small absorbance changes related to P700 oxidation upon illumination, showing that most of the P700 in these fragments was present initially in an oxidized state. Addition of ascorbate to these fragments caused a larger light-induced absorbance change. The magnitude of the light-induced absorbance change of the HP700 fragment was 3 times higher than that of the Photosystem I fragment.

The kinetics of Fig. 5, with or without addition of ascorbate, show that the rate of P700 bleaching in the HP700 fragment was slower than that in the Photosystem I fragment. The addition of ascorbate and PMS caused both a faster photoreaction and a faster decay rate for both of these fragments. A larger light-induced absorbance change was observed for both fragments with ascorbate alone than with ascorbate and PMS. This might be due to a faster back reaction in the presence of ascorbate and PMS (reaction of reduced PMS with oxidized P700) which would change the equilibrium point between oxidation and reduction of P700 to a more reduced state.

Another physical property of the HP700 fragment which might be related to its P700 content is the light-induced EPR signal[13–15]. Fig. 6 shows the characteristics of the EPR signals observed for the HP700 and Photosystem I fragments, which show a characteristic signal I with a g value of 2.00251. Without the addition of any electron donors, the HP700 and Photosystem I fragments exhibited an EPR signal (left tracings, solid lines) without illumination. No attempt has been made, however, to rigorously exclude room light from these samples prior to the run. No change in the signal was observed for these fragments upon illumination. Addition of electron donors, ascorbate or ascorbate and PMS, decreased the signals (left tracings, dotted lines). In the presence of ascorbate, both the HP700 and Photosystem I fragments demonstrated a light-induced EPR signal and the magnitude of the EPR signal of these fragments in the light was the same as that measured in the dark without addition of any electron donors. The magnitude of the light-induced EPR signal of the HP700 fragment was 3 times higher than that of Photosystem I fragment and, as shown in Table I, this is consistent with the P700 contents of these fractions. Therefore, there seems to be a close relationship between the content of P700 and the magnitude of the light-induced EPR signal. In the presence of ascorbate and PMS, no light-induced EPR signal was observed for either of these fragments. The HP700 fragment showed a different response in terms of light-induced absorbance change and production of an EPR signal when ascorbate and PMS were present. Although the absorbance change in the presence of ascorbate and PMS was less than that observed with ascorbate alone, there was no light-induced EPR signal observed. The presence of PMS allows a much faster decay of the P700 signal to take place, thus decreasing the steady-state level of the signal in both cases. Although the light intensity employed in the EPR measurement was only slightly less than that employed in the measurements of absorbance change, the much higher concentration of chlorophyll (20 times) in the EPR measurements could result in a much lower

effective light intensity in the cavity, resulting in the inability of the actinic light to drive the reaction faster than the back reaction in the presence of PMS.

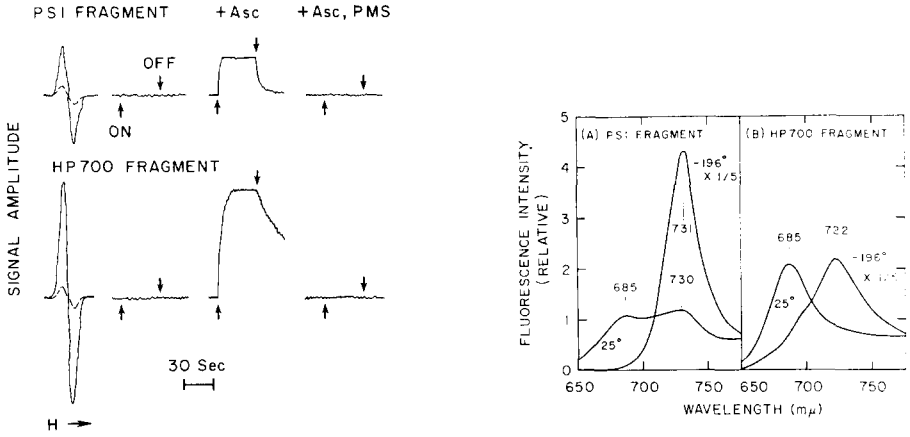


Fig. 6. Light-induced EPR signals for the HP700 and Photosystem I (PSI) fragment at 12 Gauss modulation and 1000 amplitude setting. The fragments were suspended in 0.01 M Tris–HCl buffer (pH 7.5). The chlorophyll contents of each sample were 0.3 mg/ml. The complete EPR spectra are shown (left tracings) in the absence (solid lines) and presence of 1 mM ascorbate (dashed lines). The kinetic traces were obtained without any addition (left tracings), with 1 mM ascorbate (middle tracings) and then with ascorbate *plus* 10 μM PMS present (right tracings). The actinic light was from a tungsten lamp and reached the sample after passing a filter (Baird Atomic) which passed light above 640 mμ. The light intensity of the actinic light at the sample was $1.4 \cdot 10^4$ ergs·cm^{-2}·sec^{-1}.

Fig. 7. Fluorescence spectra of the Photosystem I (PSI) fragment (A) and the HP700 fragment (B) at room (25°) and liquid N_2 temperature (−196°).

Photochemical activity

Neither the HP700 nor the Photosystem I fragment showed $NADP^+$ reduction upon illumination in the presence of the appropriate enzymes (ferredoxin, ferredoxin–$NADP^+$ reductase and plastocyanin) prepared from spinach. Further experiments performed with the enzymes from Anabaena are necessary.

Fluorescence measurements

The fluorescence spectra at room (25°) and liquid N_2 temperature (−196°) for the HP700 and Photosystem I fragments are shown in Fig. 7. These measurements were made with samples having the same total concentration of chlorophyll *a*. The fluorescence spectrum of the Photosystem I fragment at 25° had two maxima at 685 and 730 mμ, with relatively high intensity at 730 mμ. Cooling of the Photosystem I fragment with liquid N_2 resulted in a marked increase of the fluorescence intensity at 731 mμ. At 25° the fluorescence spectrum of the HP700 fragment showed a peak only at 685 mμ; the 730 mμ fluorescence intensity was lower in the Photosystem I fragment. The HP700 fragment did not contain free chlorophyll, since the fluorescence spectrum did not show a peak or shoulder at 678 mμ, the emission peak of solubilized chlorophyll *a*. The changes in the fluorescence properties of the HP700 fragment most likely reflect a decrease in the efficiency of energy transfer between the light-absorbing chlorophyll *a* and the species responsible for the long-wavelength fluorescence.

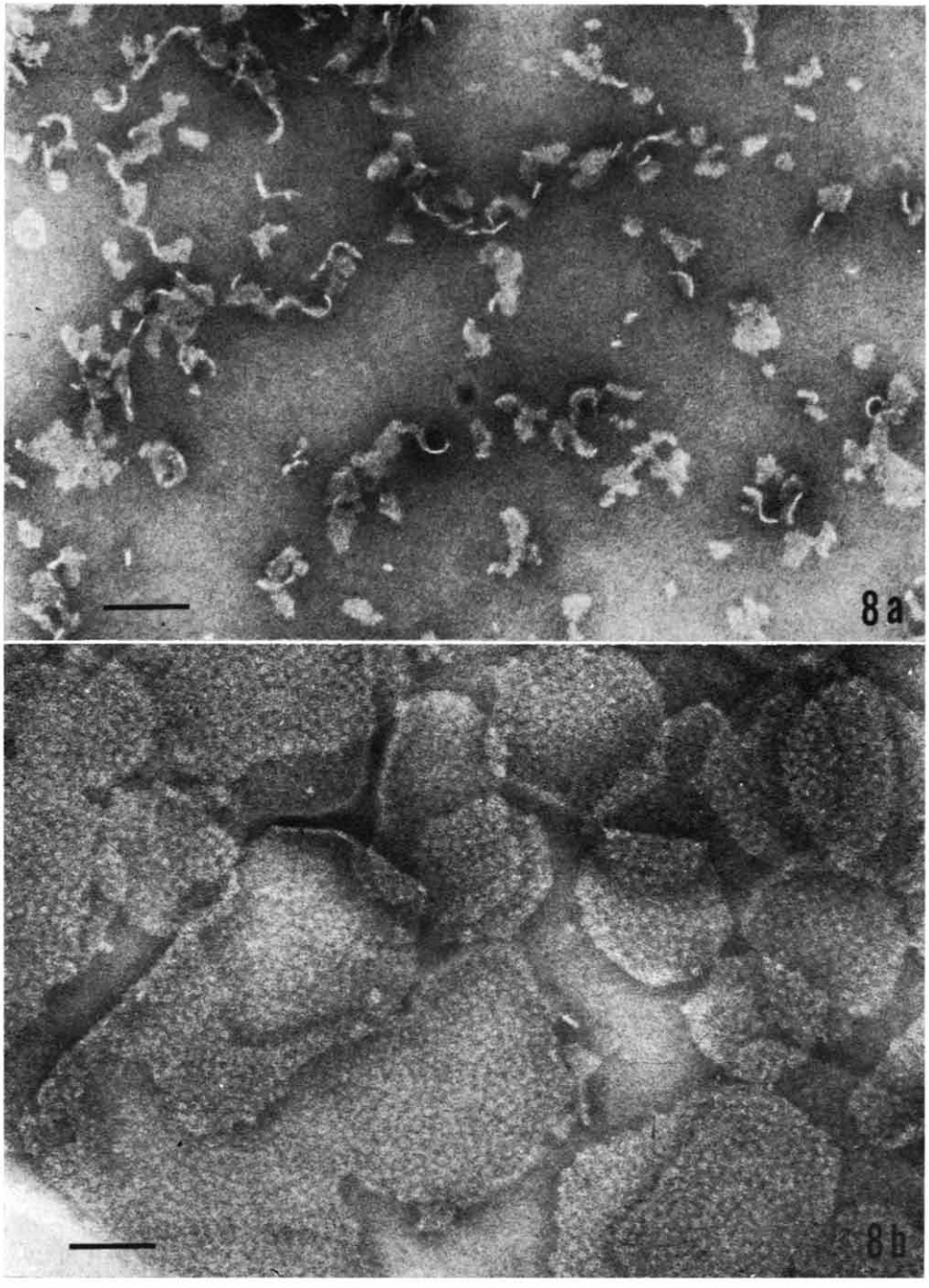


Fig. 8. Electron micrographs of (a) the HP700 fragment and (b) Photosystem I fragment. Negative staining with phosphotungstic acid at pH 5.9 in the presence of 5 mM $MgCl_2$ was used to prepare the samples. The bars represent 1000 Å.

The fluorescence intensity of the HP700 fragment was enhanced at −196°. The enhancement of the fluorescence intensity at longer wavelength was much lower for the HP700 fragment than for the Photosystem I fragment. At −196° the fluorescence spectrum of the HP700 fragment showed a peak located at 722 mμ and a shoulder at about 700 mμ. The appearance of the shoulder at 700 mμ and the difference in the liquid N_2 temperature fluorescence maximum of the HP700 fragment from that of the Photosystem I fragment show that the state of the residual chlorophyll in the HP700 fragment is not the same as that of the majority of the chlorophyll in the Photosystem I fragment. Again these data are consistent with a decreased efficiency of energy transfer between the chlorophyll species in the HP700 fragment.

Structure

The HP700 fragment has been examined in the electron microscope, using the technique of negative staining. Fig. 8a shows a micrograph of the HP700 fragment, showing the presence of small, irregular membrane fragments as well as some small strands 50–60 Å in width. Fig. 8b shows the structure of a Photosystem I fragment prepared in the usual way with Triton X-100 (no extraction with hexane). This fragment, which has been described previously[6] shows the presence of 100-Å particles arrayed across the surface of the membrane fragment. Such 100-Å subunits are not evident in the HP700 fragments, which appear as smaller, smooth, irregular membrane fragments, with an occasional stranded structure. Preliminary electron microscopic investigation was made of the HP700 fraction prepared by gel electrophoresis. The micrographs so obtained showed the presence of rather spherical particles, which were smaller than the fragments shown in Fig. 8a, but there is a possibility that small aggregates of the gel used in the electrophoresis procedure were carried over into the extracted material used for electron microscopy.

DISCUSSION

The detergent Triton X-100 has been used extensively in the preparation of membrane fragments from the photosynthetic tissues of higher green plants, algae and bacteria. In the case of higher plants and algae, two fragments are produced which correspond to the two photosystems which are operative in plant photosynthesis. The fragment which derives from Photosystem I contains the reaction center P700 along with the appropriate light-harvesting chlorophyll. In the case of *A. variabilis*, this Photosystem I fragment contains P700 in a ratio of one P700 per 100 chlorophyll *a* molecules, and increasing the concentration of detergent used in the fragmentation procedure does not have a significant effect upon this ratio. Therefore, it was not possible to remove additional light-harvesting chlorophyll *a* molecules, and thus concentrate the P700, by manipulation of the Triton X-100 concentration.

In the case of some photosynthetic bacteria, Triton X-100 readily fragments the photosynthetic membrane to produce two fragments[16,17,20,21], one of which contains the reaction center bacteriochlorophyll along with some light-harvesting bacteriochlorophyll. The work of REED AND CLAYTON[18] showed that it is possible to remove all of the light-harvesting bacteriochlorophyll from a carotenoidless mutant of *Rhodopseudomonas spheroides*, producing a complex whose pigment is exclusively the bacteriochlorophyll (P800 and P870) which constitute the reaction center.

Experiments performed in our laboratory indicated that the successful preparation of such reaction center complexes from the photosynthetic bacteria depends upon the absence of carotenoids in the membrane. In translating this information to systems which contain carotenoids in the photosynthetic membrane, it should be possible to remove the carotenoids by solvent extraction, and a subsequent treatment with Triton X-100 could remove more of the light-harvesting chlorophyll *a* than would be removed by use of the detergent alone. The experiments reported above proceeded from this assumption.

The prior removal of carotenoids from the membrane system of *A. variabilis* does facilitate the removal of more chlorophyll *a* when such preparations are subsequently exposed to Triton X-100. In terms of P700 concentration, purifications approaching 4-fold are routinely obtained, which give a ratio of chlorophyll *a*:P700 of approx. 30. We have not been successful in proceeding beyond this point, in terms of removal of light-harvesting chlorophyll *a*. The small fragment so obtained has a P700 which appears normal in most respects, showing the usual light-induced absorbance changes and EPR spectra, although such preparations will not photoreduce $NADP^+$. Examination of the kinetics of formation of oxidized P700 shows that this light-induced reaction is slower in the HP700 fragment. This is probably not due to the fact that a majority of the light-harvesting chlorophyll *a* has been removed from the HP700 fragment. Since the total chlorophyll concentrations are the same for the two preparations used in the experiments reported in Fig. 5, one would expect that the rate of P700 oxidation might be lower in the HP700 fragment. However, the intensity of the actinic light used in these experiments is well above saturation[15], which should compensate for the lowest concentration of light-harvesting chlorophyll. A more likely explanation is less efficient transfer of excitation energy from the light-harvesting chlorophyll *a* to the P700 in HP700 fragments. This is in agreement with the fluorescence properties of these fragments.

One significant difference between the HP700 fragment and the Photosystem I fragment is the pattern of fluorescence obtained, both at 25 and −196°. The HP700 fragment has less of the long-wavelength fluorescence, and more of the short-wavelength fluorescence. The decreased long-wavelength fluorescence indicates that either the long-wavelength fluorescence observed in chloroplasts and preparations of Photosystem I does not originate from the reaction center chlorophyll itself, or that the efficiency of energy transfer from the light-harvesting chlorophyll *a* to the P700 is lowered in the HP700 preparations. The latter explanation is more logical, since it explains the decreased long-wavelength fluorescence and the increased short-wavelength fluorescence, as well as the decreased rate of formation of oxidized P700 in HP700 fragments upon illumination.

It seems important to us that all the variations of solvent treatment yield preparations which are fairly constant in terms of chlorophyll *a*:P700 ratio. This can be explained if the light-harvesting chlorophyll *a* is in at least two environments in the original membrane structure, and that the residual 30 chlorophyll *a* molecules which remain to serve each P700 are in an environment which makes them inaccessible to the detergent. Perhaps those 90 light-harvesting chlorophyll *a* molecules which are removed by the Triton X-100 are complexed in the original membrane system with the carotenoids, and removal of the carotenoids by hexane extraction leaves these chlorophylls vulnerable to the action of Triton X-100. The residual 30 chloro-

phyll *a* molecules would be complexed to some other lipid, which is not removed by the hexane extraction, and therefore these chlorophyll *a* molecules are protected from the action of the detergent. The exact nature of binding of the residual 30 light-harvesting chlorophylls (as well as the P700) to the protein in the HP700 fragment remains to be determined.

The data of Fig. 4 shows that at least two distinct absorbance changes are involved in both the chemical and photochemical reactions of the HP700 fragment. In all preparations made to date the component showing the change at 680 mμ accompanies the one showing the change at 701 mμ. The absorbance change at 680 mμ does not appear to be caused by the bleaching of a secondary band of the P700 molecule, since the band at 701 mμ is skewed and the steep part is on the short-wavelength side. It appears, rather, that the 680-mμ change is due to a red shift of the absorption band of some other chlorophyll component in the fragment. Since light causes an absorbance change for both components and since P700 is involved in the photochemistry of Photosystem I of chloroplasts[15], it appears that the 680-mμ components are also intimately involved in the reaction center of Photosystem I, perhaps in a manner analogous to P800 in the reaction center of bacterial systems[18]. A similar light-induced change at 682 mμ in a Photosystem I fragment prepared from spinach chloroplasts with digitonin has been reported by DÖRING *et al.*[19], who used 256 repetitive flashes to allow for the detection of the small absorbance change in the relatively impure preparation. In the present case, removal of the majority of the chlorophyll molecules allows for a direct demonstration of this 680-mμ component.

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