Extraction and Reconstitution of Lyophilized Photosystem I Subchloroplast Fragments from Spinach Chloroplasts

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Lyophilized photosystem I subchloroplast fragments prepared from spinach chloroplasts were extracted and reconstituted. Hexane extraction eliminated 26% of the photosystem I activity without removing chlorophylls, and the reconstitution with β -carotene, an unknown lipid or chlorophyll *a* restored almost all the activity. Extraction with hexane-acetone (2:1, v/v) eliminated 81% of the activity with removal of 78% of the chlorophylls. In this case, reconstitution with chlorophyll *a* showed complete restoration of the activity, but β -carotene, the unknown lipid, and plastoquinone A caused a rather inhibitory effect. A possible explanation is that photosystem I reaction centers are closely surrounded by chlorophyll *a*; and β -carotene, the unknown lipid, and plastoquinone A function only through chlorophyll *a*.

Organic solvent extraction of lyophilized chloroplasts causes a decay of photosynthetic electron transport, and readdition of the lipid extract to the extracted chloroplasts restores the activity. By the extraction and reconstitution technique, several workers have shown that photosystem II activity can be recovered with plastoquinone^{1~3)} and more effectively with a mixture of plastoquinone and β -carotene.^{2,3)} Recently, a similar investigation on photosystem II subchloroplast fragment was carried out by Knaff *et al.*⁴⁾ They concluded that plastoquinone functions as the primary electron acceptor of photosystem II, and that β -carotene does not play a direct role in the primary photochemistry but is required for the C-550 absorbance change.

Photosystem I activity also was partially restored with the hexane-lipid extract or trilineolenin.⁵⁾ Bazynski⁶⁾ reported complete restoration of the activity with a large amount of α -tocopherol. In the previous paper,⁷⁾ we also revealed that β -carotene and a new lipid component, which was found in chloroplasts, stimulate preferentially the photosystem I reaction of

the extracted chloroplasts, while plastoquinone A and α -tocopherol reactivate both photosystem I and II reactions.

In this work, we prepared lyophilized photosystem I subchloroplast fragments and investigated the functional role of non-polar components around photosystem I by extracting and reconstituting the preparation.

MATERIALS AND METHODS

Preparation of chloroplasts and digitonin fractionation. Spinach class II chloroplasts were prepared in medium containing 0.35 M NaCl, 10 mM Tris-HCl (pH 8.0), and 5 mM MgCl₂ as described previously.⁷⁾ The chloroplasts were treated with 0.5% digitonin by modification of the method of Ohki and Takamiya.⁸⁾ The chloroplasts were resuspended in 150 mM KCl, 3 mM MgCl₂, 10 mM Tricine-KOH (pH 7.3), and 0.5% digitonin to give a final chloroplast suspension containing 0.5 mg chlorophyll per ml. After bubbling with nitrogen gas, the suspension was incubated in a stoppered Erlenmeyer flask at 0°C for 60 min with constant stirring by magnetic stirrer. The mixture was fractionated by differential centrifugation. The pellets, sedimented between 40,000 $\times g$ for 30 min and 200,000 $\times g$ for 60 min, were washed twice with distilled water by centrifugation to remove the residual digitonin. The resulting subchloroplast fragments were designated as PS-I fragments.

Lyophilization and lipid extraction of PS-I fragments. After adding saturated plastocyanin, the PS-I fragments

Abbreviations: MV, methyl viologen; DPCO, diphenylcarbazone; DCIP, 2,6-dichlorophenyl-indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

were suspended in distilled water to give a fragment suspension containing 80 μg chlorophyll per ml. The suspension was quickly frozen and lyophilized in vacuum desiccator filled with dried silica gel. In this case, if required, the suspension was divided into a certain amount of PS-I fragments (*e.g.*, corresponding to 40 μg chlorophyll) and then lyophilized. The extraction with organic solvents of the lyophilized PS-I fragments was carried out as follows. The solvents used were hexane, acetone, and mixtures of them (hexane-acetone: 2:1, 1:1, or 1:2, v/v). The lyophilized fragments (2 mg chlorophyll) were homogenized with one of these organic solvents (5 ml/mg chlorophyll) for 2 min at 10°C in a glass homogenizer. The extracted fragments were collected by centrifugation and reextracted three times with the same solvent. The resulting extracted PS-I fragments were then suspended in distilled water, divided into equal fractions, each of which contained the fragments corresponding to 40 μg chlorophyll, and finally lyophilized as described above. The lipid extracts were combined, dried in a rotary evaporator, and redissolved in a small volume of hexane or hexane-acetone (1:1, v/v) mixture.

Reconstitution of the extracted PS-I fragments. Appropriate amounts of lipid extract or lipid-soluble components dissolved in hexane or hexane-acetone (1:1, v/v) were added to the extracted PS-I fragments (corresponding to 40 μg chlorophyll as the original contents, 0.56 mg protein). After being mixed well, the solvent was removed under vacuum. Then, the reconstituted fragments were dispersed in 1.5 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 0.2 M sucrose and saturated plastocyanin by a Vortex mixer. To the mixture was added the reagent solution (1.5 ml) for DPCO disproportionation, and then it was subjected to photoactivity measurements as described below. When another method for the activity measurement was adopted, the corresponding buffer was employed for the dispersion of the reconstituted fragments.

Measurements of photochemical activities. Photosystem I activity was measured by two methods, MV-mediated oxygen uptake ($\text{DCIPH}_2 \rightarrow \text{MV}$) and DPCO disproportionation. MV-mediated oxygen uptake was determined polarographically with a YSI 4004 Clark oxygen electrode. The assay mixture (3 ml) contained, in final concentrations, 50 mM Tris-KOH (pH 8.0), 10 mM NaCl, 5 mM MgCl_2 , 0.4 M sucrose, 0.1% Triton X-100, 0.2 mM NaN_3 , 0.1 mM DCIP, 5 mM ascorbic acid, 10 μM DCMU, 0.1 mM MV, saturated plastocyanin, and the fragments (40 μg chlorophyll). DPCO disproportionation was measured according to the method described by Vernon.⁹⁾ The mixture (3 ml) contained, in final concentrations, 50 mM phosphate buffer (pH 7.8), 0.1% Triton X-100, 0.2 M sucrose, 2.5 mM DPCO, 10 μM DCMU, saturated plastocyanin, and

the fragments (40 μg chlorophyll).

Separation and preparation of lipid and pigment components from chloroplasts. The lipid extract from chloroplasts was separated on a silica gel column by using hexane, benzene, chloroform, and mixtures of them as the eluting solvents. Further purification and identification were carried out by silica gel thin-layer chromatography as described in the previous paper.⁷⁾ By this technique, β -carotene, plastoquinone A, and an unknown lipid (a new lipid⁷⁾ were separated in chromatographically pure forms. Chlorophyll *a* and chlorophyll *b* were also prepared by preparative thin-layer chromatography.¹⁰⁾ Quantitative separation and estimation of the crude lipid extract were performed by thinchromatography¹¹⁾ (a quantitative thin-layer chromatography using a new apparatus equipped with a flame ionization detector, Iatroscan TH-10 TLC Analyser). The solvent systems used were hexane-diethyl ether-acetone (60:30:20, v/v) and hexane-benzene (1:1, v/v).

RESULTS

The first problem encountered in this work was the preparation of lyophilized subchloroplast fragments which have a high photochemical activity and extractable with non-polar organic solvents. Among several possible techniques tested, a procedure of digitonin treatment and subsequent differential centrifugation was most convenient and suitable, because it is possible to deal with a large amount of chloroplasts, and also pre-washing of the fragments with distilled water before lyophilization, which is required for maintaining the photoactivity during lyophilization, can be effectively carried out. Investigation of the lyophilized PS-I fragments obtained in this way showed that they have MV-mediated oxygen uptake of 500~550 μmol per mg chlorophyll per hr and no significant photosystem II activity.

The second problem was quantitative handling of the dried PS-I fragments as experimental samples, because the fragments must be used in the dried state and the amount of them required for each experiment is too small to weigh. This was overcome by resuspending the fragments in distilled water, dividing into the amounts required and then lyophilizing as

described in METHODS.

Figure 1 shows the effects of solvent extractions on the photoactivity of the lyophilized PS-I fragments. Hexane extraction caused about 30% loss of the original photosystem I activity. With increasing acetone in the solvent mixture, the photoactivity decreased rapidly, and most of the activity was eliminated

Fig. 1. Effects of Organic Solvent Extraction on Photosynthetic Activity of Lyophilized PS-I Fragments.

Lyophilized PS-I fragments (LF) were extracted with hexane (H), hexane-acetone (H/A, 2: 1, 1: 1, and 1: 2, v/v) or acetone (A). The original and extracted PS-I fragments were subjected to measurements of photosystem I activity ($\text{DCIPH}_2 \rightarrow \text{MV}$). The values are shown as percentages of the original value in the lyophilized fragments. The original value was 539 μmol oxygen consumed/mg chlorophyll $\cdot$ hr.

by extraction with acetone.

The residual lipid contents after the extractions were estimated by thinchromography as described in METHODS. The results are summarized in Table I. Extraction with hexane removed 73% of the β -carotene and plastoquinone fraction, but almost all of the chlorophylls and polar lipids remained in the fragments. In contrast, the extraction with hexane-acetone (2: 1, v/v) removed a large portion of the chlorophylls in addition to β -carotene and plastoquinone. On acetone extraction, furthermore, almost all of the non-polar lipids and pigments and also an appreciable of polar lipids were eliminated.

It is well known that the photoactivity of chloroplasts or subchloroplast fragments is enhanced in the presence of detergents, such as Triton X-100, probably due to dispersion and exposure of the active sites. Figure 2 shows the effect of Triton X-100 on the photoactivity of the lyophilized PS-I fragments and the extracted fragments. Both samples indicated the maximum activity in the presence of 0.1% Triton X-100. Thus, all measurements of photosystem I activity were carried out in the presence of 0.1% Triton X-100.

Figure 3 shows the reconstitution of the extracted PS-I fragments with various amounts of the lipid extract. The maximum restor-

TABLE I. THE CONTENTS OF LIPID-SOLUBLE COMPONENTS IN PS-I FRAGMENTS AND THEIR REMOVAL WITH SOLVENT EXTRACTIONS

Lyophilized PS-I fragments were extracted with hexane, hexane-acetone (2: 1, v/v) or acetone. The lipid-soluble components in PS-I fragments and the extracted fragments were extracted quantitatively with water-saturated *n*-butanol, and estimated by thinchromography as in METHODS. The original contents of lipid-soluble components in PS-I fragments are shown as percentages of total lipid extracts, and the amounts of lipids extracted with solvents are presented as percentages of the original contents of the total or each component, respectively.

Lipid-soluble components	Original contents %	Extracted with		
		Hexane	Hexane-acetone (2: 1, v/v)	Acetone
Total	100	13	48	73
Non-polar lipids, pigments	59	23	75	99
β -carotene, plastoquinone A ^a	30	73	97	99
chlorophyll <i>a</i> , chlorophyll <i>b</i>	13	trace	78	99
others	15	trace	45	99
Polar lipids	41	trace	10	35

^a This fraction contains also sterol ester and hydrocarbones.

FIG. 2. Effects of Triton X-100 on the Photoactivity of Lyophilized PS-I Fragments and the Extracted Fragments.

Lyophilized PS-I fragments (the amount corresponding to 40 μ g chlorophyll) were suspended in 3 ml of reaction mixture containing Triton X-100 at the concentrations indicated, and photosystem I activity (MV-mediated oxygen uptake) was measured. The hexane-extracted fragments (corresponding to 40 μ g chlorophyll as the original content) were also measured in the same way. $\circ$ — $\circ$, lyophilized fragments; $\triangle$ — $\triangle$, hexane-extracted fragments.

FIG. 3. Reconstitution of the Extracted PS-I Fragments by Adding the Lipid Extract.

Lyophilized PS-I fragments were extracted with hexane-acetone (2:1, v/v). The lipid extract was readded to the extracted fragments (corresponding to 40 μ g chlorophyll as the original content) in various amounts. The amounts of added lipids are shown as percentages of total lipid extract from the fragments corresponding to 40 μ g chlorophyll. Photosystem I activity was measured by DPCO disproportionation, and expressed as μ mol DPCO reacting/14 mg protein·hr. Fourteen mg protein corresponds to the amount of the protein of the original fragments containing 1 mg chlorophyll.

ration of photosystem I activity was obtained with an amount of lipid extract similar to that present in the original fragments. The addition of excess lipid-extract repressed the rate.

Table II shows the reconstitution of the hexane-extracted PS-I fragments with crude lipid extract and pure isolated lipid components. Extraction with hexane decreased the activity to 74% of the original. As shown in the Table, the reconstitutions with crude lipid extract, β -carotene, an unknown lipid or chlorophyll a completely restored the original photosystem I activity. These results suggest that the role of β -carotene and the unknown lipid can be replaced by chlorophyll a under the conditions tested. Although plastoquinone A also restored the activity, the rate was less than those of other components.

TABLE II. RECONSTITUTIONS OF HEXANE-EXTRACTED PS-I FRAGMENTS

Lyophilized PS-I fragments were extracted with *n*-hexane. The crude lipid extract and pure isolated components were added to the extracted fragments in the same amount as present in the original fragments. Photosystem I activity was measured by DPCO disproportionation as described in METHODS.

Treatment of PS-I fragments	Photosystem I activity	
	μ mol/hr/14 mg protein ^a	Relative values
Fresh	325	—
Lyophilized	285	100
Extracted	210	74
Addition to the extracted		
crude lipid extract	290	102
β -carotene	290	102
unknown lipid ^b	290	102
plastoquinone A	260	91
chlorophyll a	295	104

^a 14 mg protein; the amount of PS-I fragments corresponding to 1 mg chlorophyll.

^b A new lipid component stimulating photosystem I activity.⁷⁾

Table III shows the reconstitution of the hexane-acetone (2:1, v/v)-extracted PS-I fragments. Extraction with the hexane-acetone mixture caused a decrease of activity to 19% of the original. Reconstitution with the crude extract recovered the activity up to 68%.

TABLE III. RECONSTITUTIONS OF HEXANE-
ACETONE (2:1, v/v)-EXTRACTED
PS-I FRAGMENTS

Lyophilized PS-I fragments were extracted with hexane-acetone (2:1, v/v). Other techniques of reconstitution and measurements of the photoactivity were the same as described in Table II.

Treatment of PS-I fragments	Photosystem I activity	
	$\mu\text{mol/hr/14}$ mg protein^a	Relative values
Lyophilized	285	100
Extracted	55	19
Addition to the extracted		
crude lipid extract	195	68
chlorophyll <i>a</i>	315	111
chlorophyll <i>b</i>	265	93
chlorophyll <i>a+b</i> (3:1)	295	104
β -carotene	35	12
unknown lipid ^b	40	14
plastoquinone A	50	18
chlorophyll <i>a</i> +crude lipid extract	235	82
chlorophyll <i>a</i> + β -carotene	265	93
chlorophyll <i>a</i> +plastoquinone A	275	96

^a 14 mg protein; the amount of PS-I fragments corresponding to 1 mg chlorophyll.

^b A new lipid component stimulating photosystem I activity.⁷⁾

Complete restoration occurred on reconstitution with chlorophyll *a* or a mixture of it and chlorophyll *b* (3:1). Reconstitution with chlorophyll *b* alone also caused high restoration, but it was less than that of chlorophyll *a* alone. On the contrary, the reconstitution with β -carotene, the unknown lipid or plastoquinone A indicated a rather inhibitory effect. This result was also in contrast with that observed of the hexane-extracted fragments. It was further noted that the combination of chlorophyll *a* with crude lipid extract, β -carotene or plastoquinone A caused less restoration than chlorophyll *a* alone.

DISCUSSION

In the present work, lyophilized PS-I fragments, retaining most of the original photoactivity, were prepared, and extraction and

reconstitution experiments on this preparation could be carried out. As MV-supported oxygen uptake ($\text{DCIPH}_2 \rightarrow \text{MV}$) is sensitive and convenient for measurement of photosystem I activity, it was preferentially used in the early period of this study. However, during the reconstitution experiments, it was observed that a large amount of oxygen uptake occurred in the $\text{DCIPH}_2 \rightarrow \text{MV}$ assay mixture containing free chlorophylls. Thus, DPCO disproportionation was exclusively used in the reconstitution experiments.

In the previous paper, we reported that plastoquinone A and α -tocopherol reactivated both photosystem I and II reactions, while β -carotene and the unknown lipid stimulated preferentially the photosystem I reaction of the lyophilized chloroplasts. A similar result was obtained in the present work using the PS-I fragments. Namely, β -carotene and the unknown lipid reactivated the photoactivity of the fragments and completely restored the activity lost by hexane extraction. However, the results obtained with the fragments extracted with hexane-acetone (2:1, v/v) were very different. They showed that chlorophyll *a* and a mixture of it and chlorophyll *b* completely restored the activity of the fragments, but β -carotene, the unknown lipid, and plastoquinone A caused rather a slight inhibition.

A possible explanation for these results is that photosystem I reaction centers are closely surrounded by chlorophyll *a*, and the components such as β -carotene, the unknown lipid, and plastoquinone A may function only through chlorophyll *a* in photosystem I. The hexane-extracted fragments lose most of the β -carotene and plastoquinone but not chlorophylls. In contrast, the hexane-acetone-extracted fragments lose a large part of the light-harvesting chlorophylls with other non-polar and polar components, suggesting that the reaction centers are considerably exposed. Thus, the observed inhibitory effect of β -carotene *etc.* is probably due to their random occupation of the normal positions of chlorophyll *a* near the reaction centers. In the same way, the facts that mixtures of chlorophyll *a*

with other components caused less restoration than chlorophyll *a* alone, may be attributed to that the mixed components hinder chlorophyll *a* from returning to the normal positions.

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