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RECONSTITUTION OF PHOTOSYSTEMS I AND II USING SPINACH SUBCHLOROPLAST FRAGMENTS FRACTIONATED BY TRITON TREATMENT*

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SUMMARY

Reconstitution of Photosystems I and II was demonstrated using small (approx. 10 nm diameter) and photochemically highly active subchloroplast fragments fractionated from spinach by Triton treatment and using diphenylcarbazide as a Photosystem-II electron donor. $NADP^+$-reduction activity of the reconstituted system was comparable to that of the unfractionated chloroplasts. Plastocyanin (or *Euglena* cytochrome 552) was an absolute requirement. Lecithin presumably served as a "binding agent".

INTRODUCTION

The concept of two photosystems in green-plant photosynthesis appears to have been well established from a large body of experimental evidence (see ref. 1 for a review). Physical separation of fragments representing Photosystems I and II from photosynthetic organelles by detergent fractionation[2], and, more recently, by mechanical means[3], lends further support to the concept. An earlier attempt to reconstitute the two fractions separated from spinach chloroplasts by Triton treatment was reported by Briantais[4]. The larger (Photosystem-II) particles obtained by Briantais were reported to give an oxygen burst upon illumination by the 654-nm light. A partial reconstitution was suggested by the observation that the reconstituted particle displayed an enhancement effect in oxygen evolution, which is usually observed with chloroplasts but not with separated particles[4]. Huzisige *et al.*[5] reported a partial reconstitution of two particles isolated from spinach chloroplasts by a combined treatment with digitonin, Triton and sonication, but the results were based on an $NADP^+$-reduction rate of 1.3 nmoles/mg chlorophyll per h. More recently, Arntzen *et al.*[6] reported an improved reconstitution of the Photosystem-I and -II particles fractionated from the grana lamellae of spinach chloroplasts by

Abbreviations: TSF-I, TSF-II and TSF-IIa represent Triton-fractionated subchloroplast fragments with Photosystem-I or -II activities; the TSF-IIa fragments are obtained from the TSF-II by further treatment. DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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digitonin treatment. They further showed that the Photosystem-I particles from the stroma lamellae were incapable of recombining with the grana Photosystem II particles.

This note reports the reconstitution of the Photosystem-I and -II fragments isolated from spinach chloroplasts by Triton fractionation. The reconstituted photochemical activity was assayed by $NADP^+$ reduction using diphenylcarbazide as an electron donor for Photosystem II. It is further shown that reconstitution with a highly purified, small Photosystem-II particle can yield an $NADP^+$-reduction rate comparable to that in unfractionated chloroplasts where water is the electron donor.

EXPERIMENTAL

The preparation procedures for TSF-I, TSF-II and TSF-IIa fragments were described previously[7–10]. Tris-washed chloroplasts were prepared according to Yamashita and Butler[11]. Reduction of the terminal electron acceptor, $NADP^+$, was used as the assay method for the reconstituted electron-transport activity. Red light isolated with a Corning 2403 filter, with an incident intensity of $2 \cdot 10^5$ ergs/cm^2 per s, was used for excitation. Other experimental details are described in the figure legends.

The enzymatic cofactors, ferredoxin, ferredoxin–$NADP^+$ reductase and plastocyanin were prepared by a modification of the method of Katoh *et al.*[12]. *Euglena* cytochrome 552 was prepared according to Perini *et al.*[13]. $NADP^+$ was purchased from Sigma; diphenylcarbazide from Eastman Kodak and recrystallized before use. The lecithin solution was prepared by suspending L-α-lecithin in distilled water (40 mg/ml) and sonicated for 45 min in a Raytheon sonic oscillator, followed by centrifugation at $27\,000 \times g$ for 20 min. The homogeneous suspension was used in the experiments and the precipitate was discarded.

RESULTS AND DISCUSSION

Photochemical activity of the Triton-fractionated subchloroplast fragments

Properties of fragments representing the two photosystems were reported earlier[14–16]. The TSF-I fragments are 7–8 nm thick, contain about one P700 per 100 chlorophyll molecules, and catalyze the reduction of $NADP^+$ by appropriate electron donors[14]. The TSF-II or -IIa fragments catalyze the photoreduction of 2,6-dichlorophenolindophenol (DCIP) by diphenylcarbazide[8–10]. For reference purpose, activities of fragments used in the present reconstitution studies are summarized in Tables I and II. To obtain optimum reconstitution of the two photosystems, Triton-fractionated subchloroplast fragments with high activities such as those shown in Tables I and II were found essential but not always sufficient; certain batches of TSF-I fragments, for unknown reasons, would occasionally not reconstitute. The $NADP^+$-reduction activity of TSF-I particles by ascorbate–DCIP had an absolute requirement for plastocyanin. Further confirming our previous reports[14], *Euglena* cytochrome 552 can substitute plastocyanin to yield a reasonable $NADP^+$-reduction activity.

When TSF-II fragments were purified further, first by centrifugation followed by passing the supernate through a "Bioglas" column[9,10] to remove excess inactive chlorophyll solubilized by Triton, a Photosystem-II fraction with a high chlorophyll

TABLE I

NADP+-REDUCTION ACTIVITY OF TSF-I FRAGMENTS BY ASCORBATE–DCIP

(a) TSF-I particles diluted with 0.05 M phosphate buffer (pH 6.6) to a total volume of 2 ml; chlorophyll concn, 5 μg/ml. Other components were: ferredoxin and ferredoxin–NADP+ reductase, in saturating amounts; NADP+, 0.3 mM; plastocyanin, 3.0 μM, ascorbate, 2.5 mM; DCIP, 0.05 mM. NADP+ reduction was measured by the absorption increase at 340 nm. (b) Same as in (a) except plastocyanin was replaced by *Euglena* cytochrome 552 at 3.3 μM.

	Activity (μmoles/mg chlorophyll per h)
(a) Complete system	1150
minus plastocyanin	0
(b) Complete system	620

TABLE II

DCIP-REDUCTION ACTIVITY OF TSF-IIa, TSF-II FRAGMENTS AND TRIS-WASHED CHLOROPLASTS

(a) TSF-IIa particles diluted with 0.05 M phosphate buffer (pH 6.4) to a final volume of 2 ml; chlorophyll concn, 2.4 μg/ml. Other components were: DCIP, 0.05 mM; diphenylcarbazide, 0.5 mM DCIP-reduction activity was measured by the absorption decrease at 590 nm. (b) and (c) Same as in (a) except TSF-IIa was replaced by TSF-II (chlorophyll concn, 3.5 μg/ml) and Tris-washed chloroplasts (chlorophyll concn, 6 μg/ml), respectively. Concns of diphenylcarbazide and DCIP same as in (a).

	Activity (μmoles/mg chlorophyll per h)
(a) Complete system with TSF-IIa	1190
plus 10^{-5} M DCMU	52
(b) Complete system with TSF-II	230
(c) Complete system with Tris-washed chloroplasts	230
minus diphenylcarbazide	0

a/b ratio (TSF-IIa) was obtained. Thus, on a total chlorophyll basis, the TSF-IIa fragments have a much higher DCIP-reduction activity than TSF-II fragments when diphenylcarbazide[8–10] was used as the electron donor. Tris-washed chloroplasts have a DCIP-reduction activity comparable to that of the TSF-II fragments. Unlike unfractionated chloroplasts, TSF-IIa is somewhat less sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibition. However, more than 95% of the electron transport was inhibited at 10^{-5} M DCMU (see Table II).

Photochemical activity of reconstituted Photosystem-I and -II subchloroplast fragments

Since the electron for the reduction of NADP+ in complete photosynthesis originates from water, a recombination of fragments representing the two photosystems can be demonstrated only by using an electron donor whose entry site lies on the oxidizing side of Photosystem II. In almost all Photosystem-II fragments prepared by detergent treatment, however, the water oxidation site is inactivated, and a substitute electron donor must be used. The usual difficulty is that few electron donors are specific enough for this purpose. We have therefore used the more specific

Photosystem-II electron donor, diphenylcarbazide, in the reconstitution experiments.

In making up the reaction mixture, the following order of adding the reagents was found to yield optimum rates. Without dilution, the first portion consisted of 25 μl TSF-IIa, 50 μl lecithin, 25 μl plastocyanin, 25 μl ferredoxin, 25 μl ferredoxin–$NADP^+$ reductase and 10 μl TSF-I; then 10 μl diphenylcarbazide (0.1 M in methanol), 40 μl $NADP^+$ (0.015 M) and 0.2 ml concentrated phosphate buffer (0.5 M) *plus* 1.6 ml water, to form another portion; and finally the two portions were combined into the reaction mixture of 2 ml final volume at 0.05 M phosphate concentration and pH 6.6. Experimental results on the reconstitution activity are summarized in Table III.

The complete system yielded an $NADP^+$-reduction activity of 67 μmoles/mg chlorophyll per h. As indicated in Table III, this rate was obtained by correcting the observed apparent rate by subtracting the contribution of an absorption increase at 340 nm due to diphenylcarbazide oxidation to diphenylcarbodiazone. When the millimolar extinction of the diphenylcarbazide oxidation product was taken as 5.4 at 300 (and 4.2 at 340 nm) and that of NADPH as 6.2, and assuming a stoichiometric relationship, a factor of 0.6 was found necessary for correcting the $NADP^+$-reduction rate calculated from the absorption increase measured at 340 nm. It should be noted that Arntzen *et al.* have applied a correction factor of 0.66 in a similar reaction[6].

In order to ascertain that the 340-nm absorption increase was indeed contributed by $NADP^+$ reduction as well as diphenylcarbazide oxidation, but not due entirely to the latter reaction, we have made further qualitative checks and found the above correction to be reasonable. One experiment was to use excess ferredoxin as the electron acceptor (*i.e.* complete system (a) in Table III *minus* the reductase

TABLE III

RECONSTITUTION OF PHOTOSYSTEM-I AND -II SUBCHLOROPLAST FRAGMENTS MEASURED BY $NADP^+$ REDUCTION BY DIPHENYLCARBAZIDE

(a) The complete reaction mixture contained: TSF-I (chlorophyll concn, 2.5 μg/ml); TSF-IIa (chlorophyll concn, 6 μg/ml), in 0.05 M phosphate buffer (pH 6.6) in a final volume of 2 ml; ferredoxin, ferredoxin–$NADP^+$ reductase, $NADP^+$, and plastocyanin same as in Table I; diphenylcarbazide same as in Table II; lecithin, 0.5 mg/ml (also see text for other details). (b) Same as in (a) except plastocyanin was replaced by *Euglena* cytochrome 552 at 3.3 μM. (c) Same as in (a) except TSF-IIa was replaced by TSF-II (chlorophyll concn, 7 μg/ml). (d) The reaction mixture contained: Tris-washed chloroplasts (chlorophyll concn, 7 μg/ml); ferredoxin, $NADP^+$, and plastocyanin (same as in Table I); diphenylcarbazide, 0.5 mM.

	Activity * (*μmoles/mg chlorophyll per h*)
(a) Complete system with TSF-IIa	67
plus 10^{-5} M DCMU	8
minus lecithin	29
minus plastocyanin	0
minus TSF-I	7
(b) Complete system	41
(c) Complete system with TSF-II	22
minus TSF-I	7
(d) Complete system with Tris-washed chloroplasts	82
minus diphenylcarbazide	8

* All rates corrected for contribution of an absorption increase at 340 nm due to diphenylcarbazide oxidation; see text for a more detailed discussion.

and $NADP^+$), only a slight absorption increase at 340 nm was observed upon illumination. Under this condition the expected light-induced absorption increase at 340 nm due to diphenylcarbazide oxidation was presumably in part cancelled by an absorption decrease expected for ferredoxin reduction. Upon addition of the reductase and $NADP^+$, however, normal absorption increase was observed at 340 nm upon illumination.

Another experiment was to place the complete reaction mixture in both the sample and reference cuvettes, but only the sample cuvette was illuminated, and the light-induced absorption increase at 340 nm with time was recorded. At the end of illumination, the difference spectrum between the illuminated sample and the unilluminated reference was also recorded from 400 to 280 nm. While the absorption increase at 340 nm represented a combined rate of $NADP^+$ reduction and diphenylcarbazide oxidation, the difference spectrum would represent the actual amount of NADPH and diphenylcarbodiazone formed in the light reaction. By carefully and gradually adding successive small amounts of a dilute hypochlorite solution to the reference cuvette to oxidize diphenylcarbazide and then taking the difference spectrum after appropriate additions, it was possible to chemically form an equivalent amount of diphenylcarbodiazone in the reference cuvette to compensate that generated by light in the sample cuvette until the difference spectrum representing the net amount of light-reduced NADPH appeared. It should be pointed out that although such an experiment convincingly demonstrated that $NADP^+$ was indeed reduced in the reconstituted system, the procedure was not practicable for a quantitative estimation of the reduction rate.

In order to further confirm the reconstitution reaction and $NADP^+$ reduction by a separate independent method, we have utilized the fluorescence property of NADPH for a qualitative assay. The fluorescence assay would have the advantage of being specific with respect to reduced NADP and free from interference such as that encountered in absorption-change measurements. The technique used is similar to that described by Amesz[17]. The kinetic spectrofluorometer consists of a fluorescence excitation source, an excitation source for photosynthesis, and a detection circuit. The ultraviolet lines (predominantly at 313, 334 and 366 nm) from a high-pressure mercury arc lamp (Quarz-lampen, Hanau, Germany) was isolated by a 3-mm Schott UG-11 filter and used for exciting the NADPH fluorescence; the same red light described earlier was used to induce photosynthesis. The ultraviolet light beam was modulated at 400 Hz by a Princeton Applied Research (PAR) chopper. The fluorescence in the blue region (isolated by a 420–440-nm interference filter (Baird Atomic) and a Corning 3-73 cut-on filter) was detected by an EMI 9558 photomultiplier and was processed by a PAR Model-200 modular lock-in amplifier. The light-induced appearance of NADPH fluorescence in a typical reconstitution reaction is presented in Fig. 1. Separate assay of $NADP^+$ reduction by measuring the 340-nm absorption increase showed a corrected rate of about 40 μmoles/mg chlorophyll per h. The somewhat lower reconstitution rate here was due to a slightly lower activity of the TSF-IIa particles which were prepared during an off season and had been stored for an extended period of time. In any event, the results in Fig. 1 unequivocally demonstrated that $NADP^+$ was reduced in the reconstituted two photosystems utilizing electrons from the Photosystem II-specific donor, diphenylcarbazide.

Other evidences of support for the reconstitution reaction are also listed in

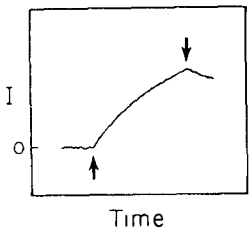

Fig. 1. Light-induced appearance of NADPH fluorescence in a reconstitution reaction mixture (composition similar to that of mixture (a) of Table III). Relative fluorescence intensity I is plotted against time. Arrows indicate light on and off; illumination period, 1 min. See text for other details.

Table III. The reconstitution reaction was sensitive to DCMU, and the degree of sensitivity to DCMU was characteristic of the TSF-IIa fragments[8–10]. The reconstituted $NADP^+$-reduction activity, like that for the partial Photosystem-I reaction (see Table I), had an absolute requirement for plastocyanin. It was further shown that *Euglena* cytochrome 552 can substitute for plastocyanin in the reconstitution reaction, with an efficiency comparable to that in the partial Photosystem-I reaction of $NADP^+$ reduction (*cf.* Table I). The presence of a "binding agent", lecithin, also appears necessary; in its absence, the activity decreased to about 25%.

By substituting the small TSF-IIa fragments (approx. 10 nm diameter) with the relatively larger TSF-II fragments (approx. 500 nm diameter), the reconstitution activity decreased to about 30%. This decrease in activity may be attributable to the lower basal activity of the TSF-II fragments (*cf.* Table II) as well as a physically less intimate contact between the TSF-I and the relatively large TSF-II fragments.

It is also worth noting that the $NADP^+$-reduction rate obtained for the reconstituted Photosystems I and II, even after appropriate correction, is quite comparable to that of unfractionated chloroplasts, which is usually about 100 μmoles/mg chlorophyll per h [5,6,18,19]. The electron-transport rate in the reconstituted fragments is presumably subject to the rate limitation by the same electron-transport intermediates as in unfractionated chloroplasts. This rate must be considered as reasonable, since no attempt was made to optimize the quantity of the two fragments used in the recombination, and thus the chlorophyll contents.

Although these reconstitution experiments are consistent with the currently generally accepted two-photosystem scheme for green-plant photosynthesis, they offered no further insights into the nature of some of the intermediate electron carriers lying between the two photosystems. For instance, Huzisige *et al.*[5] reported in their reconstitution experiments that both plastocyanin and plastoquinone were absolutely required, while we found only plastocyanin was required. The function of plastoquinone in TSF-IIa has not yet been investigated. Cytochrome b_{559} is known to undergo a photoreduction in the TSF-IIa fragments[10]. It may be assumed that cytochrome b_{559} serves as one link in the reconstituted electron transport, but this remains to be demonstrated. The question of cytochrome f being an intermediate electron carrier also cannot be demonstrated, since cytochrome f contained in the TSF-I fraction is known to have co-sedimented during the centrifugation step and is not an integral part of the fragments[15]. However, it has been established that there

is an absolute requirement for plastocyanin in either the partial Photosystem-I reaction or the reconstituted reaction for $NADP^+$ reduction, and that TSF-I is a fragment highly enriched in P700.

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