A RAPID PROCEDURE FOR ISOLATING THE PHOTOSYSTEM I REACTION CENTER IN A HIGHLY ENRICHED FORM

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1. Introduction

Photochemical reaction center preparations have been isolated from several purple photosynthetic bacteria (reviewed [1–3]). Such preparations are probably devoid of all extraneous components and therefore represent the minimal size unit which can still perform on isolation the primary photochemical events exactly as they occur in situ. It is generally agreed that there are 6 chromophores (4 bacteriochlorophyll and 2 bacteriophaeophytins) in the unit (mol. wt 45 000–150 000). Much insight into the mechanism of the primary photochemical events in bacterial photosynthesis (e.g., the identity of the primary donor and acceptors, and the kinetics of charge separation and subsequent electron transport) has come from biochemical and biophysical examination of such preparations. On the other hand, an equivalent enrichment of the photosystem I and II reaction centers of plants has not been achieved. To date, photosystem I reaction center (P700) preparations have been isolated from Triton-, LDAO- or SDS-solubilized photosynthetic membranes by conventional protein separation techniques, and the products have contained about 40 antenna chlorophylls in addition to P700 [4–9]. A few short articles [10,11] have reported greater P700 enrichments in material obtained by solvent extraction of membranes or membrane fragments (photosystem I fractions), while a recent study has reported a greater enrichment using Triton and chromatographic procedures [12]. Although much useful information on the primary events occuring in photosystem I has been obtained from the existing P700 preparations, the presence of antenna pigments and extraneous proteins has prevented investigators from delineating as detailed an understanding of the biophysics and biochemistry of the plant primary reactions as is known for the purple bacterial reaction.

The aim of the present study was, therefore, to remove more antenna pigments from a P700 preparation than had been achieved heretofore, by using chromatographic fractionation of detergent extracts which, in comparison to solvent extraction, will yield a product containing fewer or no extraneous proteins. Since it is known (cf. [13]) that different detergents have different solubilization properties and since we previously had found [14–16] that some plant materials were far more suitable than others for fractionation by a specific detergent, we sought to combine the different properties of several detergents by using mixtures of them for solubilization and subsequent chromatography of those plant photosynthetic membranes which had proved to be the most amenable. As a result we were able to isolate rapidly, homogeneous material from some higher plants (see [16]), green

Abbreviations: chl, chlorophyll; LDAO, lauryl dimethyl amine oxide; SDS, sodium dodecyl sulfate

algae and cyanobacteria which was 2-fold more enriched in P700 (15–25 chl/P700) than the existing detergent preparations.

2. Materials and methods

2.1. *Plant material*

Maize (*Zea mays* L) and New Zealand spinach (*Tetragonia tetragonioides* Pall.) plants were grown from seed in greenhouses, and the fully expanded leaves of 4–6 week-old plants were harvested. *Scenedesmus obliquus* mutant 6E kindly provided by Dr N. I. Bishop was grown heterotrophically in the dark as in [17]. Vegetative and isolated heterocyst cells of *Anabaena cylindrica* [18] were provided by Dr E. Tel-Or, University of California, Berkeley. The culture of those plants or mutants (see section 3) that did not yield a chl/P700 ratio of 15–25:1 are too numerous to list.

2.2. *Preparation of photosynthetic lamellae*

Maize and New Zealand spinach leaves were homogenized in a Polytron Disintegrator (Brinkmann Instruments Inc.) in an isolation medium consisting of 0.5 M sucrose, 50 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA and 0.1 M NaCl, and a chloroplast pellet obtained by differential centrifugation [8]. The isolated chloroplast membranes were washed thoroughly with 50 mM Tris–0.1 mM EDTA (pH 8.0), to remove most of the water-soluble proteins (cf. [8]). Cells of *Scenedesmus* 6E, suspended in 50 mM Tris (pH 8.0), were broken by two passages through a French pressure cell at 16 000 p.s.i. The photosynthetic lamellae were isolated by centrifugation at 40 000 × *g* for 20 min followed by resuspension in 50 mM Tris–HCl buffer (pH 8.0) and recentrifugation. The *Anabaena* heterocyst cells were broken in a similar manner to those of *Scenedesmus* while the vegetative cells were broken during the separation of them from the heterocysts [18]. The photosynthetic membranes of both the vegetative and heterocyst cells were isolated as described above.

2.3. *Solubilization and chromatography*

The well washed photosynthetic lamellae of maize, New Zealand spinach, *Scenedesmus* and *Anabaena*, were solubilized in a mixture of Triton X-100/LDAO/SDS (75/25/5, v/v/v of 1% solutions) in 50 mM Tris–HCl (pH 8.0) with a detergent/chlorophyll ratio of 40:1 (w/w). The solubilized lamellae were centrifuged at 40 000 × *g* for 20 min to remove starch and any other insoluble material; the pellet contained no green material. The resultant green solution was adsorbed to an equilibrated (0.01 M sodium phosphate, pH 7.0) hydroxylapatite column (3 × 2 cm). After loading, the column was washed with 0.01 M sodium phosphate (pH 7.0) until the eluate was colorless, then washed with the solubilization mixture, using at least 3 times the volume (generally 90–150 ml) of detergent mixture used to make the extract. More color was removed before the eluate again became colorless. A volume (20 ml) of 1% Triton (in 50 mM Tris) was run through the column followed by a similar volume of 0.01 M sodium phosphate (pH 7.0); no color was eluted by these washes. A P700-enriched fraction was then eluted with 0.2 M sodium phosphate (pH 7.0) and stored in this solution. Usually there was less than 1% total chlorophyll from the starting extract remaining on the column after the chromatography.

2.4. *Spectral analyses*

The room temperature (300 K) and chemical difference spectra, and light versus dark P700 difference spectra were recorded on an Aminco DW-2 Dual beam spectrophotometer. Light-induced oxidation of P700 and chemical difference spectra of P700 and cytochromes were measured as in [8].

2.5. *Pigment analyses*

Chlorophyll content was determined in the lamellae and Triton extracts as in [8]. The pigments in the preparation were extracted by acetone, partitioned into hexane, and separated by thin-layer chromatography [19].

2.6. *Electrophoretic analysis*

The isolated P700 material was dialysed, and then co-electrophoresed with the Triton X-100 [8] and SDS [5] preparations of the P700–chl *a*–protein (chl/P700 = 40:1) of higher plants and blue–green algae in a SDS–gel system [20] which allows the complexes to retain some of their chlorophyll during the run. Samples were delipidated, dissociated and electrophoresed under denaturing conditions as in [16].

2.7. *Chemicals*

LDAO, Ammonyx MCO, Ammonyx CO, and Ammonyx MO were gifts from the Onyx Chemical Co., NJ. Lubrol N5, Aerosol OT, Ferriopon T77 were gifts from the Plant Protection Service, Imperial Chemical Industries, Brackwell, Berks. SDS, SDBS, Lubrol WX and PX and Triton were obtained from Sigma Chemical Co., St Louis, MO.

3. Results

Prior to the more rigorous examination described here, we had found, as had some other investigators [7,8] that P700 enrichments greater than chl/P700 of 40:1 could be achieved under certain circumstances if thylakoid membranes were dissociated in Triton or LDAO and chromatographed as in [8]; we noted that maize gave greater enrichments more readily than other crop plants (e.g., tobacco, soybean, barley and cotton). Therefore we investigated the effectiveness of a variety of detergents (SDS; SDBS; Triton X-100, LDAO; BRIJ 35; BTC 50; Lubrol WX, PX N5, Aerosol OT; Ammonyx MCO, CO, and MO; Feriopon T77), used either alone or in combination, to reduce the chl/P700 ratio of the product chromatographically isolated from maize. We found no better combination than that of three of the most widely used detergents (Triton X-100, LDAO and SDS) nor any better chromatographic system (e.g., DEAE cellulose, controlpore glass beads) than hydroxylapatite. Since SDS had been found to destroy P700 activity [8], the SDS in the mixture had to be present in a low concentration, we therefore kept the SDS content at 5% of that of the sum of the other two, and varied the Triton and LDAO proportions from 90/10 to 10/90. The optimum detergent proportions for P700 enrichment in the product as well as for maximum recovery of P700 was 75/25/5 (w/w) with 60/40/5 (w/w) not being nearly so effective. The chl/P700 ratio in the product obtained was generally between 16:1–20:1; on a few occasions the ratio was around 25:1.

Equipped with a formula that yielded material containing half the antenna chlorophyll present in other detergent-prepared P700 fractions, several other plant species were examined. Only a few gave this same enrichment. One was a mutant of *Scenedesmus* 6E, which lacks colored carotenoids, and another was *Anabaena* (vegetative or heterocyst cells). The following species did not consistently yield a product with a chl/P700 ratio less than 25:1 though enrichments better than 40:1 were obtained: 20 virescent mutants of barley, soybean, bush bean, jack bean, cotton, tobacco, swiss chard, pine, maple, wild type *Scenedesmus, Tribonema, Glenodinium, Gonyaulax* and *Nostoc*. It was observed that when freshly isolated, rather than stored, chloroplasts from maize and New Zealand spinach leaves or from *Scenedesmus* 6E or *Anaebena* cells were used the higher P700 enrichments were more readily obtained.

The chromatographic procedure described in section 2 consistently yielded material from maize and New Zealand spinach with a room temperature absorption spectrum (fig.1) indistinguishable from that reported for the Triton–P700–chl *a*–protein of higher plants [8], except that the shoulder around A_{490} in the Triton preparation is absent from that made with a mixture of detergents. This shoulder is believed [8] to be contributed by β-carotene. It was observed by thin-layer chromatography that β-carotene was consistently absent from the mixed-detergent preparations of *Anabaena* and *Scenedesmus*, and absent the majority of the time from preparations of maize and New Zealand spinach. The same absorption spectra were exhibited by *Scenedesmus* 6E and *Anabaena* vegetative and heterocyst preparations. All the isolated complexes have a red A_{677} max with Soret bands at 437 nm and 420 nm at 300 K. The isolated complex from maize and New Zealand spinach routinely represented a 20-fold enrichment of P700 (chl/P700, 15–25:1) over the starting material. The same chl/P700 ratio was present in preparations from *Anabaena* vegetative cells, whereas *Scenedesmus* 6E and *Anabaena* heterocyst preparations consistently gave 20:1 or lower.

The photo-oxidation kinetics (fig.1) of the mixed-detergent preparation were rapid in the absence of electron acceptors, while the dark-reduction kinetics were slow in the absence of electron donors; both were increased by such additions (cf. [8]). The photobleaching of P700 was fully reversible, and no loss in the extent of the P700 absorbance changes occurred upon repeated oxidation and reduction. Maximum bleaching was at 697 nm for both higher plant species and *Scenedesmus*, while it was at 702 nm for *Anabaena* heterocysts and vegetative cells. The P700 prepara-

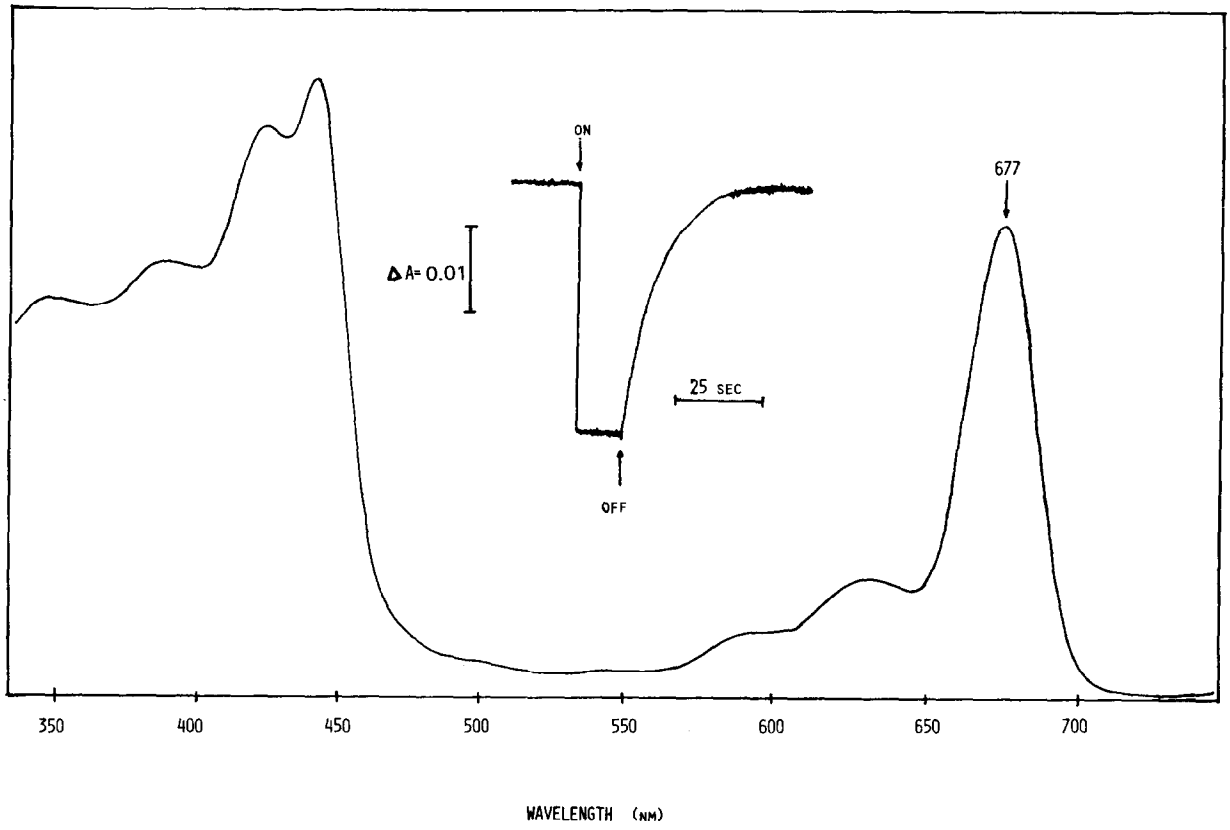

Fig.1. Room temperature absorption spectrum of the mixed detergent–*P*700–chl *a*–protein (chl/P700 = 20:1) from maize (chlorophyll conc. 8.6 μM). Also shown are the kinetics of the photo-oxidation and dark-reduction of P700 in the presence of sodium ascorbate and methyl viologen in the isolated complex (chlorophyll conc. 0.86 μM).

tions from *Scenedesmus* 6E and *Anabaena* did not show the presence of any cytochromes; on occasions cytochrome *f* but not b_6 (cf. [8]), co-eluted with the maize and New Zealand spinach preparations.

Electrophoresis under non-dissociating conditions (see section 2) of the P700-enriched fractions showed they had the same electrophoretic mobility as the SDS– and Triton–P700 chl *a*–protein isolated from higher plants or from blue–green algae. Preliminary results indicate that upon delipidation and heating at 100°C in the presence of SDS, urea and mercaptoethanol, the complex displays a subunit structure similar to that reported for the Triton–P700–chl *a*–protein of blue–green algae, i.e., two polypeptides (one minor) around mol. wt 50 000 and two very small molecular weight polypeptides [15].

4. Discussion

Using a mixture of detergents for solubilization of certain plant photosynthetic membranes, highly enriched P700 preparations (chl/P700, 15–25:1) can be obtained, starting from whole leaves or algal cells, in less than 3 h. Four plant species of the many examined consistently yielded this highly enriched product; some other species may prove equally or more useful for such fractionation. Cells of two of the four most useful species (*Scenedesmus* 6E and heterocysts of *Anabaena*) have a low carotenoid content and lack chlorophyll *b*. Interestingly, carotenoid-less mutants of a purple bacterium yielded the first true reaction center preparation [1–3]. Furthermore, it is our experience that LDAO is very effective in extracting

carotenoids from membrane-bound pigment–protein complexes. There is no rational explanation for why low or no carotenoid content facilitates fractionation of the reaction center from the antenna unless we surmise that carotenoid acts as a 'glue' between antenna– and reaction center–protein complexes. Since all of the most enriched preparations lacked colored carotenoids, and since P700 activity in such preparations is not destroyed by repeated light oxidation and dark reduction, then the β-carotene present in the SDS– and Triton–P700–chl *a*–protein preparations apparently does not play a critical role in P700 activity (cf. [21]). The rapid kinetics of P700 oxidation (fig.1) indicate that the native primary electron acceptor(s) are still present (cf. 8,22]) in the product, and thus the amount of SDS used here has not removed or altered the relative orientation of the primary reactants as occurs with higher SDS concentrations [16,22]. It is interesting to note one model for the P700–chl *a*–protein [16] proposes that the preparation is composed of equal proportions of two slightly different types of trimers (one containing ~20 chl + 1 P700 entity; the other containing ~20 chl only). It could be that the product obtained here is solely the P700-containing trimer. We did not succeed in obtaining P700 in an equivalent state of enrichment with respect to antenna pigments as P870, and thus the question still remains whether such an equivalent enrichment is possible. Almost certainly there must be some P700-containing species or mutant that will show P700 can be even further enriched.

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