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Photosynthetic Light Reactions in Chemically Fixed Spinach Thylakoids[1]

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Abstract. **After proper fixation with glutaraldehyde, spinach thylakoids retain both photosystem I and photosystem II activity. The photochemical activity of fixed thylakoids is highly resistant to 1 % triton. This indicates that fixed thylakoids may be useful starting material for isolation of functional components which can be related to the original structure of the membrane.**

Previous experiments showed that chloroplasts isolated from glutaraldehyde fixed spinach leaves retained photosystem II activity (6). However thylakoids fixed after isolation were totally inactive. In this paper we report a new method for fixing isolated spinach thylakoids which preserves activities of both photosystems I and II. After an initial loss of Hill reaction activity due to fixation, fixed thylakoids are quite resistant to damage by detergents such as triton. We anticipate that the isolated fixed thylakoids will be valuable starting material both for lipid extraction and reconstitution experiments, and for separating the thylakoid into subunits which can be referred to the intact structure.

Materials and Methods

Isolation of Thylakoids. Leaves of *Spinacia oleracea* L., obtained from the local market, were sliced and then homogenized in a cooled Waring Blendor for 60 sec in 0.05 M tricine buffer (pH 7.4) containing 0.5 M sucrose. The slurry was poured through 8 layers of cheesecloth and centrifuged for 5 min at 160*g* at 3°. The precipitate, containing mostly cell fragments was discarded. The supernatant, containing mostly class II chloroplasts was again centrifuged for 10 min at 900*g*. The pellet was washed 3 times with cold grinding buffer to remove most of the stroma material and centrifuged each time for 8 min at 1700*g*. After the final washing the pellet was resuspended in a small amount of cold grinding buffer.

Fixation. Glutaraldehyde was prepared by washing twice for about 20 min with animal bone charcoal and then titrating, if necessary, to pH 7.4 (1, 3). A 10 % (v/v) GA-solution made up in cold 0.5 M sucrose was added slowly during a period of 2 min to an equal volume of chloroplast suspension.

The suspension was stirred thoroughly in an icebath for 20 min and then was diluted with cold grinding buffer and centrifuged for 8 min at 2500*g*. The precipitate was resuspended in grinding buffer containing 0.2 M MA[3], again centrifuged and washed twice in grinding buffer with centrifugations at 1700*g*. The last precipitate was resuspended in a small amount of grinding buffer. Chlorophyll concentration was determined using the methods of Arnon (2) and MacKinney (5) and an average value of these 2 methods is reported. The chlorophyll *a*/*b* ratio was calculated after MacKinney (5).

Reagents used: DCMU (DuPont), DCPIP (Sigma), GA (Fisher Scientific), MA (Matheson), MV (California Chemical), tricine (Calbiochem), triton (Rohm and Haas).

Reaction Mixtures. The reaction mixture for photosystem II contained per ml: 0.03 μmole DCPIP, 100 μmoles MA, and 50 μmoles tricine. The chlorophyll content per ml was 4 μg for unfixed or 8 μg for fixed thylakoids. The total reaction mixture volume varied from 2.5 to 10 ml at pH 7.4.

The reaction mixture for photosystem I was modified from that of Kok (4) and contained per ml: 5 μmoles ascorbate, 5 μmoles MV, 0.1 μmole DCMU, 0.05 μmole DCPIP, and 50 μmoles of tricine. The chlorophyll content per ml was 10 to 25 μg for unfixed and 20 to 25 μg for fixed thylakoids. The total volume of the reaction mixture was 2 ml at pH 7.4.

The photosystem I reactions were performed in closed cuvettes which were flushed with N_2 for 60 sec before illumination. All reactions were per-

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[3] The following abbreviations are used: DCMU [3-(3,4-dichlorophenyl)-1, 1-dimethylurea]; DCPIP (2,6-dichlorophenolindophenol); GA (glutaraldehyde); MA (methylamine hydrochloride); MV (1,1'-dimethyl-4,4'-dipyridylium dichloride or methyl viologen); Tricine [N-tris (hydroxymethyl) methyl glycine]; Triton (Triton ×100).

formed at room temperature following a preillumination period. A Cary model 14 spectrophotometer, modified according to Sauer and Biggins (7) was used for measuring both light reactions and determining quantum requirements for the DCPIP Hill reaction. Photosystem II was assayed at 580 nm and photosystem I at 395 nm. Both reaction mixtures were illuminated by an actinic beam at 678 nm. Rates are presented as changes in optical density (OD). The maximum photosystem II rate in Fig. 1 is about 80 μmoles DCPIP reduced per mg chlorophyll per hr. The saturation rate is about twice this value (Fig. 3). The initial rate of photosystem 1 activity as reported in Fig. 2 is about 84 μequivalents per mg chlorophyll per hr.

Results and Discussion

Fig. 1 shows a typical recording of DCPIP reduction by 2.5 % GA-fixed thylakoids. The variations in slope are a result of varying incident light intensity and illustrate the intensity dependence of the photosystem II reaction. Fig. 2 shows a typical recording for MV reduction by spinach thylakoids fixed in 2.5 % GA. In GA-fixed material this photochemical activity decreases only slowly with time and is, under favorable storage conditions, retained for weeks.

This retention of both photosystems I and II in isolated spinach thylakoids after proper aldehyde fixation suggests that such membranes may be useful

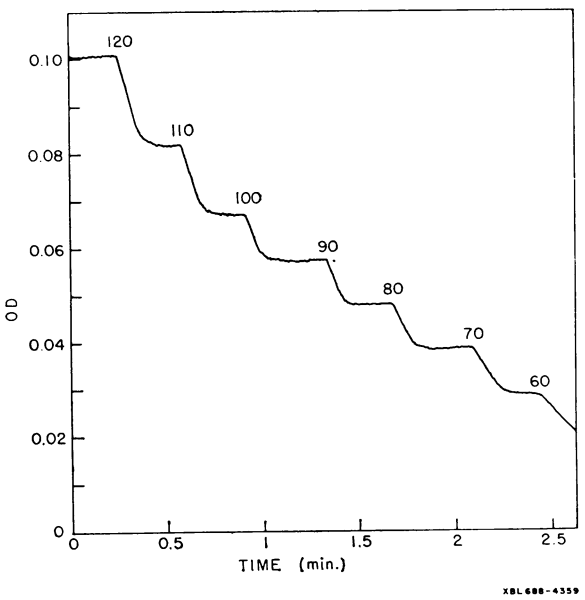

FIG. 1. Photosystem II activity of isolated spinach chloroplasts fixed in 2.5 % (v/v) GA. Reaction mixture is given under methods. Chlorophyll (as fixed chloroplasts) 15 μg/ml. Total volume 2.5 ml, pH 7.4. Ten min fixation with 2.5 % GA (pH 7.4). The reaction mixture was illuminated with 675 nm actinic light at voltages given in the figure.

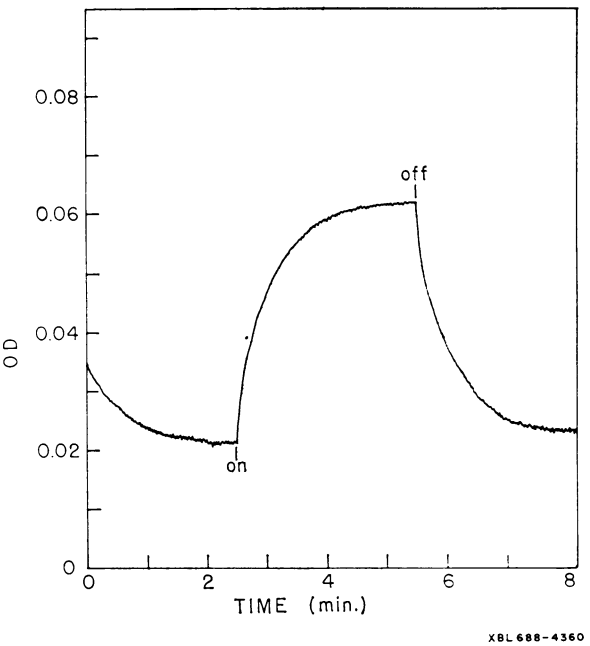

FIG. 2. Photosystem I activity of isolated spinach chloroplasts fixed in 2.5 % GA. Reaction mixture is given under methods. Chlorophyll (as fixed chloroplasts) 23 μg chlorophyll/ml. Total volume 2.5 ml, pH 7.4. The reaction mixture was illuminated with 678 nm light.

starting material for separation of the thylakoid into its functional components. Crosslinking of the proteins by glutaraldehyde would yield fragments which retain the original protein associations and can be related to structures found in the starting material. We started such experiments by investigating the effect of the detergent triton on GA-fixed spinach thylakoids (Fig. 3). We treated unfixed and GA-fixed thylakoids with a 1 % (v/v) triton solution and studied its effect on the photosystem II activity. After investigating the photosystem II rates as a function of actinic light intensity for 10 μg chlorophyll per ml (exception see below), we made reciprocal plots shown in Fig. 3. Maximum photosystem II rates are calculated from the intercept on the vertical axis. The increasing slope of the curves in detergent treated material is most easily explained by uncoupling of chlorophyll from the photosystem II reaction sites. Though this chlorophyll is still present in the reaction mixture, light absorbed by it is ineffective in driving the Hill reaction.

Curve C, the photosystem II activity of unfixed thylakoids has a very low intercept (less than 0.3 or a change in OD per min of about 3), which corresponds to a rate of greater than 100 μmoles DCPIP reduced per hr per mg chlorophyll when corrected for the assay wavelength of 580 nm. This rate is approached at relatively low light intensity.

Unfixed thylakoids treated with triton for 3 hr at 0° show the opposite extreme. The slope of this curve, C+T, indicates that compared with the un-

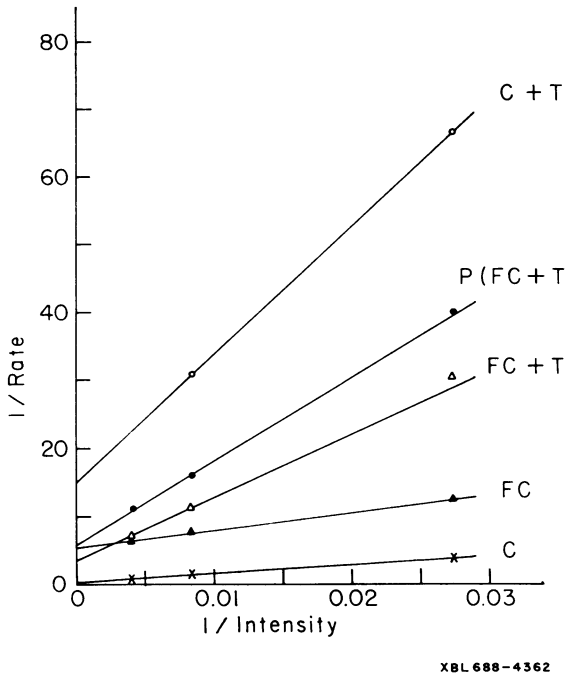

FIG. 3. Effect of GA-fixation and treatment with triton X-100 on the photosystem II activity of isolated spinach chloroplasts. Reaction mixtures are given under methods. Chlorophyll (as chloroplasts) 8 μg/ml except for sample P(FC + T) which contained 5 μg/ml. Total volumes 2.5 ml, pH 7.4. Treatments: C = unfixed chloroplasts. C + T = unfixed chloroplasts in 1 % (v/v) triton. FC = fixed chloroplasts (20 min fixed in 5 % GA, pH 7.4). FC + T = fixed chloroplasts in 1 % triton. P(FC+T) = washed precipitate of FC + T. The reaction mixtures were illuminated with 678 nm light at the relative intensities shown. For comparison, the rates presented in this plot are based on original thylakoid material containing 10 μg chlorophyll. Intensity is given in millivolts output from the silicon photo diode. Fifty millivolts (0.02 on the reciprocal scale) correspond to an incident intensity of 3.2 nanoeinsteins cm^{-2} sec^{-1}. Rates are given as ΔOD/min. Thus an intercept of 10 corresponds to a ΔOD/min of 0.1.

treated thylakoids a very high amount of light energy is necessary to approach the maximum rate in triton treated material. The intercept with the vertical axis yields a maximum rate only about 2 % that of untreated thylakoids. High intensity rates were very erratic with this material since it was rapidly destroyed under high intensity conditions.

Very different from these 2 extremes are the data from GA-fixed thylakoids. The intercept value for the fixed thylakoids, FC, is about 5. This maximum rate, though much lower than that of unfixed material, is not changed significantly by 1 % triton treatment, FC + T. Centrifugation of fixed, triton treated material at 36,000*g* for 20 min left 24 % of the chlorophyll in the supernatant. Since the calculations for the other samples were based on the amount of chlorophyll as an indication of the number of reaction sites, the calculations for the GA-fixed, triton extracted sample had to be corrected for chlorophyll loss. The results for this extracted sample are based on 7.6 μg chlorophyll/ml rather than 10 μg chlorophyll per ml. In this extracted sample, P(FC+T), part of the antenna chlorophyll was dissolved, but the number of reaction sites in comparison to the other samples retained the same. This loss of antenna chlorophyll leads to higher amounts of energy necessary for reaching the maximum rate, but the rate itself, even after the detergent treatment, is the same as for the 2 other GA-fixed samples, FC and FC + T. The rate of the washed and resuspended sample is less than that of the sample in triton at low light intensities. This suggests that still more chlorophyll has become uncoupled from the photosystem II sites by washing.

These results indicate that the cross linking of the proteins by glutaraldehyde fixation makes the protein structure of the thylakoids largely resistant to the destructive effects of triton while still allowing appreciable solubilization of the thylakoid lipids. The fixed material after triton extraction is much less rapidly destroyed by high intensity light than its unfixed counterpart.

We anticipate that further fractionation of the glutaraldehyde fixed thylakoids may provide active particle preparations which can be readily related to the particle structure of intact thylakoids.

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