Short Communication

Detergent Fractionation of Glutaraldehyde-fixed Spinach Thylakoids

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In 1969, Hallier and Park (5) showed that glutaraldehyde-fixed spinach thylakoids retained appreciable Hill reaction activity after extraction with 1% Triton X-100. The resistance of the fixed membranes to detergent destruction led them to suggest that such membranes might be favorable starting material for subsequent detergent fractionation studies. They postulated that cross-linking of membrane proteins would allow isolation of the larger membrane subunits identified by electron microscopy (8, 9). This paper reports an exploration of this suggestion. We investigated whether fixed membranes fragment differently from unfixed membranes when treated with various detergents and whether the fragments so liberated could be related morphologically to structures observed in the intact thylakoid.

MATERIALS AND METHODS

Preparation of Thylakoids. All experiments were performed on EDTA-washed thylakoids prepared from market spinach according to the method of Howell and Moudrianakis (6). The thylakoids collected from the final step of the Howell-Moudrianakis procedure were washed once with 0.01 M Na-borate buffer, pH 8.0, and once with 0.05 M borate and were finally suspended in 0.05 M borate. The thylakoids were examined by electron microscopy after negative staining with ammonium molybdate to ensure that they were devoid of ATPase particles (6). Freeze fracturing and deep etching of these thylakoids showed characteristic faces and surfaces (9).

Fixation with Glutaraldehyde. The thylakoids washed with EDTA were fixed by passage through a sucrose density gradient containing 5% glutaraldehyde. Solutions of sucrose in borate buffer were made to obtain densities of 1.28 g/cc and 1.1 g/cc. Another solution of sucrose in borate buffer containing 5% glutaraldehyde, purified as described by Hallier and Park (5), was made to a density of 1.05. The solutions were layered on the top of each other in a 32-ml centrifuge tube so that the tube contained 15 ml of solution with density 1.28, 10 ml of solution with density 1.10, and 5 ml of 5% glutaraldehyde solution with density 1.05. A suspension of thylakoids in 0.05 M borate was layered on top. This preparation was then centrifuged at 24,000 rpm for 20 min in an SW 25-1 rotor. The fixed thylakoids sediment to the boundary between the two sucrose layers. The green material was removed, washed once with 0.01 M borate and then with 0.05 M borate, pH 8.0, and was finally taken up in 0.05 M borate. The unfixed thylakoids were subjected to a similar density gradient centrifugation except that 5% glutaraldehyde was excluded from the 1.05 density solution.

Treatment with Detergents. Fixed or unfixed thylakoids were taken up in 0.05 M borate. An equal volume of detergent solution was added to obtain a desired final detergent concentration. The solutions were incubated at 1 to 2°C for 30 min and then centrifuged at 10,000 g or 20,000 g for 30 min. The precipitate and supernatant fractions from such a separation were further analyzed.

Sonication. Sonication was carried out in a Raytheon 200 watt oscillator (cup type) at maximal power. The suspension of thylakoids (5 ml) was sonicated for a total period of 5 min, 1 min at a time at an interval of 1 min. The chamber of the oscillator was cooled by circulating water at 0°C around it.

Chemical Determinations. Chlorophyll concentrations were measured according to the method of Arnon (1). Nitrogen determinations were made by a standard microKjeldahl method with urea as a standard.

Electrophoresis. Electrophoresis was carried out according to the method described by Clarke (3) with the modification that the electrolyte contained 0.2% sodium dodecyl sulfate and larger gel columns were used for preparative purposes. The protein-chlorophyll complexes separated on the gel were extracted by cutting and macerating the desired portion with a mortar and pestle in the presence of buffer.

Ultracentrifugation. The ultracentrifugation studies were performed in a Spinco Model E ultracentrifuge at 20°C and at 59,780 rpm. The material prior to ultracentrifugation was dialyzed against detergent (0.2% SDS) for SDS extractions, 1% Triton for Triton extractions, 1% Tween for Tween extractions, and 0.5% digitonin for digitonin extractions) in borate buffer for about 70 hr. The solutions used for ultracentrifugation contained about 0.5 mg of protein per ml and were analyzed by schlieren optics. The S values have not been corrected for the densities and viscosities and are expressed as S 20, w.

RESULTS

Distribution of Nitrogen. The distribution of nitrogen after treating the fixed and unfixed thylakoids with a strong ionic detergent, 0.02 M SDS, is given in Figure 1. SDS dissolves more than 98% of the unfixed membrane nitrogen. The fixed membranes show a much greater resistance towards the action of SDS. Only 73% of the total nitrogen is solubilized by a single extraction. Subsequent sonication in the presence of detergent further solubilizes the nitrogen from the fixed precipitate leaving 3.7% nitrogen in the residue. If the thylakoids are not washed with

Abbreviations: chl: chlorophyll; SDS: sodium dodecylbenzenesulfonate; SDS: sodium dodecyl sulfate.

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EDTA and are then solubilized in 0.02 M SDS and sonicated for 5 min, then about 95% of the nitrogen is solubilized from the unfixed membranes whereas only 86% of the nitrogen is solubilized from the fixed membranes. It is apparent that EDTA washing increases the sensitivity of the thylakoid to SDS solubilization.

The $S_{20}$ values of solubilized material from fixed membranes (see below) indicated we had not obtained the large membrane subunits we had hoped to find. Therefore, we continued these experiments with weaker detergents. Both fixed and unfixed thylakoids were treated with 1% Tween, 0.5% digitonin, and 1% Triton for 30 min. The suspensions were centrifuged for 30 min at 10,000g. The distribution of nitrogen is given in Table I.

As observed for SDS treatment, all the detergents solubilized considerably higher amounts of nitrogen from unfixed membranes. Tween is the mildest detergent we used, solubilizing only 7.4% of the nitrogen from the fixed and 36.4% of the nitrogen from the unfixed membranes. Triton is much more effective and solubilizes 34% of the nitrogen from the fixed and 95% from the unfixed membranes. The action of digitonin is intermediate and solubilizes 12 and 84% of the nitrogen from the fixed and unfixed membranes respectively.

**Electrophoretic Properties.** The supernatants from SDS extraction of fixed and unfixed membranes as well as the supernatants and precipitates from digitonin and Triton treatments were analyzed by electrophoresis. The supernatants were applied directly to the gel. The precipitates were solubilized with 0.2% SDS prior to electrophoresis.

Only the unfixed precipitates and supernatants previously treated with SDS or digitonin entered the gel column. The supernatant from SDS treatment of unfixed material separated into two major bands, each containing a protein-chlorophyll complex. The slow moving band gave a high chl a/chl b ratio (5-6), and the fast moving band gave a chl a/chl b ratio of 1 to 1.5. The unfixed material previously treated with digitonin separated into three bands. The slow moving band had a high chl a/chl b ratio (5-6) and probably corresponds to photosystem I as suggested by Thornber et al. (12) and shown by Sane et al. (10). Bands two and three ran close to each other and gave a low chl a/chl b ratio ranging from 1.2 to 1.5. These bands probably correspond to photosystem 2. The electrophoretic behavior is similar to that observed by Thornber et al. (12). In both cases a small amount of free pigment appears as an additional fast moving band.

The unfixed material previously treated with Triton and the fixed material previously treated with any detergent did not enter the gel column. This could be due to either increased size of the fragments or to decreased charge. We investigated the first possibility by studying the sedimentation behavior of the supernatant fractions.

**Sedimentation Behavior of Supernatants.** The supernatants from fixed and unfixed material obtained by SDS extraction (Fig. 1) after extensive dialysis were examined in the ultracentrifuge. Both the supernatants sedimented with one symmetrical peak. Contrary to our expectations, the $S_{20}$ for unfixed material was 3.2, supernatant I from fixed material was 1.6, and for supernatant II it was 1.4. These $S_{20}$ values show that the fixed membranes after SDS treatment yield smaller fragments than the unfixed membranes.

The other detergents behaved differently from SDS, yielding fragments of similar size from both fixed and unfixed thylakoids. We conclude that the $S_{20}$ values of the solubilized fixed membranes for a given detergent are similar or smaller than those of unfixed membranes (Table I). It appears that glutaraldehyde fixation results in both inter- and intramolecular linkage and does not allow subsequent selective fragmentation to yield the larger membrane subunits we had initially hoped to isolate. The fact that supernatant fractions from fixed membranes did not enter polyacrylamide gels on electrophoresis is apparently not due to large fragment size. It may be due to a decreased number of ionizable groups following glutaraldehyde fixation (4).

The $S_{20}$ value observed by us for digitonin-treated unfixed membranes is similar to that reported by Chiba (2) but differs from those reported by Smith and Pickles (11). We observed only one boundary in the ultracentrifuge whereas Smith and Pickles (11) and Chiba (2) observed more boundaries in the ultracentrifuge. It appears that additional boundaries observed by these workers may be due to contaminating proteins which we have removed by EDTA washing. A similar difference exists between our results and those of Thornber et al. (12). Whereas Thornber et al. (12) observed two boundaries in SDSBS solubilized material, we ob-

**Table I. Percentage of Nitrogen Solubilized and $S_{20}$ Values of 10,000g Supernatant on Treatment with Different Detergents**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Unfixed $N$</th>
<th>Unfixed $S_{20}$</th>
<th>Fixed $N$</th>
<th>Fixed $S_{20}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween, 1%</td>
<td>36.4%</td>
<td>1.5</td>
<td>7.4%</td>
<td>1.8</td>
</tr>
<tr>
<td>Digitonin, 0.5%</td>
<td>83.8%</td>
<td>5.9</td>
<td>11.9%</td>
<td>6.4</td>
</tr>
<tr>
<td>Triton, 1%</td>
<td>95.4%</td>
<td>1.8</td>
<td>34.4%</td>
<td>1.8</td>
</tr>
<tr>
<td>Digitonin super. +0.2% SDS</td>
<td>3.0%</td>
<td>3.0</td>
<td>2.5%</td>
<td></td>
</tr>
</tbody>
</table>
served only one boundary. Our S_{20} values observed for SDS treated unfixed membranes agree well with those observed by Itoh et al. (7) for dodecyl benzene sulfonate treated material.

These experiments extend the observations of Hallier and Park (5) that glutaraldehyde fixation stabilized thylakoids against detergent action. They also indicate that detergent treatment of these membranes yield fragments similar to or smaller than those from untreated membranes. Though the detergent fractionation experiments reported here failed to yield large membrane subunits from fixed membranes, other fractionation procedures may be successful in achieving this goal.

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