The Distribution of Photosynthetic Reactions in the Chloroplast Lamellar System

II. Latent ATPase, Proton Pump, Cyclic Phosphorylation and Its Sensitivity towards Ammonia

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Chloroplast fragmentation, ATPase, Proton pump, Cyclic Phosphorylation, NH₄Cl-uncoupling

Cyclic phosphorylation and latent ATPase follow the distribution of photosystem I in the chloroplast membrane. Both activities are found higher in stroma lamellae than in grana. The extent of proton uptake is found to be higher in the grana. Since this uptake depends on internal volume and buffer capacity besides proton pump activity, the distribution of the proton pump proper remains to be elucidated.

Any fragmentation of the large inner compartment of the chloroplast lamellar system into smaller vesicles results in decreased sensitivity of cyclic phosphorylation to uncoupling by ammonium chloride. Consequently both, isolated grana and stroma lamellae, show decreased uncoupling by ammonium chloride. The effect can be explained by the action of a membrane potential in photophosphorylation which builds up during illumination and might be more stable in a system with the larger number of individual compartments just for statistical reasons. No assumption on changes in specific ion permeabilities during fragmentation of chloroplasts are needed.

The distribution of energy dependent reactions and of components, which catalyze them, in an energy transducing membrane system bears on the mechanism of energy conservation. The chemical mechanism requires a close neighbourhood of electron- and energy transfer, while an electrochemical mechanism does not. The electrochemical mechanism, on the other hand, postulates an obligate proton pump for phosphorylation. Proton pump and phosphorylation should not be separable by fractionation of the membrane system.

In this paper we investigate the distribution of coupling factor 1 **, measured as latent ATPase, of cyclic photophosphorylation and of the proton pump between preparations of grana and stroma lamellae, in comparison to the whole chloroplast lamellar system. We will show that there is some proton uptake in every membrane fraction as long as there is an appreciable rate of phosphorylation.

R. E. McCARTY first observed that ammonium chloride is a poor uncoupler of photophosphorylation in subchloroplast vesicles, in contrast to chloro-

** Abbreviations used: Tricine for (N-Tris-hydroxymethyl)-methyl)glycine, MES for 2-(A-morpholino)-ethane sulfonic acid, PMS for (N-methyl)-phenazonium methosulfate, CF₁ for coupling factor 1.
plasts. He tried to explain this finding by the assumption that subchloroplast vesicles should be less permeable to chloride than chloroplasts following a mechanism for amine uncoupling proposed by CROFTS. In this paper we extend these studies to isolated grana and stroma lamellar vesicles and will show that every fragmentation of the large compartment in chloroplasts into smaller units results in decreased sensitivity of photophosphorylation towards ammonia. We will suggest a mechanism which does not rest upon different anion permeabilities to account for the differential uncoupling effect in subchloroplast vesicles and chloroplasts. ARNTZEN et al. published similar experiments but arrived at a different conclusion.

Methods

Preparations

Class II chloroplasts were prepared as published. A more fragmented fraction was obtained by a second centrifugation of the first chlorophyll containing supernatant at 10,000 g (Table II). The fragmentation and fractionation of chloroplasts into grana and stroma lamellae by French-press or digitonin treatment has been described previously. A Sorvall Ribi cell fractionator, Model RF-1, at 4000 - 5000 psi, was used throughout this investigation. Sucrose, at 0.4 M, was found to protect photosynthetic reactions from damage during the fragmentation (see Table II). Sonication in the presence or absence of plastocyanin was performed as described in the accompanying paper. The sonication time was 30 sec.

Assays

Proton uptake and cyclic phosphorylation were measured according to McCARTY. The reaction mixtures are given in Table I. ATPase activity, activated by trypsin, was assayed by the method of VAMBUS and RACKER. The time of the trypsin incubation was varied from 5 to 30 min in 5 min intervals. The maximal ATPase activity obtained was taken for presentation.

Light intensity was measured with a YSI-Kettering radiometer. Chlorophyll was assayed according to ArnON.

Results

Table I summarizes our data on the distribution of latent ATPase, proton uptake and cyclic phosphorylation among grana and stroma lamellae prepared by either French-press or digitonin treatment. As shown previously, the trypsin-activat-

<table>
<thead>
<tr>
<th>Treatment of chloroplasts</th>
<th>Membrane Fraction</th>
<th>ATPase Activity (uMoles Pi esterified per mg chlorophyll per hr)</th>
<th>Cyclic Phosphorylation (μMoles Pi/mg chlorophyll per hr)</th>
<th>Proton Uptake (μMoles H+ per mg chlorophyll per hr)</th>
<th>0.4 M sucrose in the isolation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>French press grana</td>
<td>280</td>
<td>15</td>
<td>0.060</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>French press stroma l.</td>
<td>490</td>
<td>5</td>
<td>0.020</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Digitonin grana</td>
<td>270</td>
<td>180</td>
<td>0.210</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Digitonin stroma l.</td>
<td>540</td>
<td>240</td>
<td>0.140</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table I. Latent ATPase, Cyclic Phosphorylation and Proton Uptake in Preparations of Grana and Stroma Lamellae.

The chloroplast activities were measured according to the Methods. The reaction mixture for cyclic phosphorylation contained in 1 ml, 50 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, 3 mM ADP, 2 mM P₁ with 2 x 10⁶ to 10⁸ ipm ³²P₁, 1 mg defatted bovine serum albumin, 0.05 mM PMS and chloroplasts or subchloroplast vesicles equivalent to 10 μg chlorophyll. The samples were illuminated in a water bath at RT with white light of 10⁷ ergs/cm² and sec intensity. The reaction medium for proton uptake contained in 2.8 ml, 2 mM MES-NaOH (pH 6.5), 50 mM NaCl, 0.01 mM PMS and chloroplasts or subchloroplast vesicles corresponding to 150 to 200 μg chlorophyll. The suspension was illuminated with white light (2 x 10⁷ ergs/cm² and sec) at 13⁰ and pH 6.5. The electrodes were protected from illumination by aluminium foil. ATPase and cyclic phosphorylation are expressed in μmoles P₁/mg chlorophyll per hr, the proton uptake in μ equivalents protons per mg chlorophyll.

<table>
<thead>
<tr>
<th>Treatment of chloroplasts</th>
<th>μmoles P₁ esterified per mg chlorophyll and hr</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation (2000 g)</td>
<td>306</td>
<td>33</td>
</tr>
<tr>
<td>Isolation (10000 g)</td>
<td>208</td>
<td>75</td>
</tr>
<tr>
<td>Ultra sound</td>
<td>52</td>
<td>40</td>
</tr>
<tr>
<td>Ultra sound + plastocyanin</td>
<td>148</td>
<td>130</td>
</tr>
<tr>
<td>French-press</td>
<td>125</td>
<td>62</td>
</tr>
<tr>
<td>Digitonin</td>
<td>188</td>
<td>155</td>
</tr>
</tbody>
</table>

Table II. Uncoupling of Cyclic Phosphorylation by Ammonia in Fragmented Chloroplasts.

Chloroplasts and chloroplast fragments were prepared as cited under Methods. The total chlorophyll containing fraction was tested for photophosphorylation as described in Table I. NH₄Cl was added as indicated.

ed Ca²⁺-ATPase, which reflects coupling factor 1, is found in both fractions. An isotonic concentration of sucrose in the medium during fragmentation of chloroplasts has not much effect. The ATPase remains bound also in the absence of sucrose, except
in stroma lamellae isolated after incubation in 0.5% digitonin, which lose about one third of the activity. Even at 1.5% digitonin not more than half of the ATPase is removed from the chloroplast membrane. This is in apparent disagreement with Wessels, electron microscopic results.

The ATPase is somewhat enriched in the stroma lamellae and diminished in the grana compared to chloroplasts. We do not think that the enrichment of stroma lamellae in ATPase reflects cosedimentation of CF₁ at a high centrifugal force, because in the presence of sucrose during fragmentation and sedimentation the density of the medium is increased and particulate chlorophyll remained in the supernatant, but the enrichment of ATPase in the stroma lamellae is even more pronounced.

In contrast to the work of the Kettering group, we want to emphasize that in every fraction, which is able to catalyse photophosphorylation, we can observe proton uptake during illumination. As seen from Table I this uptake is lower in grana preparations than in chloroplasts and still less in the preparations of stroma lamellae. More protons move into the membrane vesicles obtained by French-press treatment than into those obtained by incubation with digitonin, if comparable rates of phosphorylation are retained.

Cyclic phosphorylation seems to be concentrated in the stroma lamellae like other photosystem I activities. The digitonin treated fractions usually yielded higher rates. Sucrose was necessary to protect phosphorylation and proton uptake from damage during fragmentation of chloroplasts.

In Fig. 1 we show decreased sensitivity of cyclic phosphorylation towards ammonia for grana as well as for stroma lamellae, compared to chloroplasts. Least sensitive are stroma lamellae prepared by incubation in digitonin followed by the corresponding grana fraction. The membrane vesicles obtained by French-press treatment exhibit a higher degree of uncoupling in the same order. Our data do not suggest that there are different areas of ion permeabilities within the chloroplast lamellar system, as concluded by Arntzen et al. We think that the phenomenon of decreased sensitivity towards ammonia in chloroplast fragments is related more directly to the fragmentation itself. This is once more supported by the data from Table II, which show that every treatment which breaks down the large, interconnected (cf. to Ref.) lamellar system of the chloroplast, results in decreased uncoupling by ammonium chloride. This already holds for chloroplast fragments sedimented between 2000 and 10 000 g after routine isolation of chloroplasts. Very effective is brief sonication in hypotonic media (cf. to Ref. 4). As shown previously, the rate of phosphorylation can be partially protected from damage if a high amount of plastocyanin is present during sonication, but this higher rate again was nearly insensitive towards ammonia. Table II also shows that the decreased efficiency of uncoupling by ammonia in French-press — and digitonin-treated chloroplasts is not dependent on subsequent isolation of grana and stroma lamellae. Again chloroplasts treated with digitonin are less sensitive than those passed through the French-press. Total uncoupling in every chloroplast membrane fraction was obtained if 2 μM valinomycin was present in addition to ammonium ions.

Discussion

The photosynthetic activities tested are not evenly distributed between isolated grana and stroma lamellae. In agreement with Arntzen et al., the latent ATPase is higher in stroma lamellae than in grana preparations. In accordance we previously found that phosphorylating stroma lamellae exhibit a lot of membrane spheres upon negative stain in the electron microscope. These had been identified as CF₁, the latent ATPase. The corresponding grana preparations looked almost smooth. Since the
ATPase is activated by trypsin, the observed higher activity in the stroma lamellae might just reflect a more exposed surface structure, so that more could be activated. However, the total inhibition of photophosphorylation by an antibody against coupling factor 1, under hypotonic as well as isotonic conditions (unpublished experiments by R. Berzborn) makes a structurally buried CF$_1$ in the grana regions unlikely. Therefore, the possibility exists that there are areas in the grana regions which lack CF$_1$. It remains to be demonstrated that photosynthetic electron flow occurs in these areas before we can discuss how the energy would be transferred from such electron flow to a spatially separated CF$_1$ (R. Berzborn, in preparation).

Cyclic photophosphorylation, like other photosystem I reactions, is higher in the stroma lamellae than in isolated grana. The light-induced proton uptake is distributed the other way round, but is already lower in grana preparations than in chloroplasts. A decreased proton uptake needs not reflect a less active proton pump, because the number of protons pumped from the outside to the inside to build a certain pH-difference across the membrane is proportional to the internal volume and the internal buffer capacity. The former naturally decreases by fragmentation of Chloroplasts and the latter might also be lowered. We want to emphasize that whenever a good rate of cyclic phosphorylation was retained in a subchloroplast preparation we also could observe some proton uptake during illumination.

According to the chemiosmotic theory of energy conservation, an electrochemical proton potential across the membrane drives ATP synthesis. This potential consists of an osmotic and an electrical part. The relative sizes of these parts may vary, the sum remaining constant at a given electron transport rate. The first uncouplers of photophosphorylation investigated were ammonium salts. Based on several experimental facts Crofts proposed a mechanism for the uncoupling action of ammonium chloride in isolated chloroplasts, which is generally accepted. According to this free ammonia is the active species, which permeates through the membrane and decreases the pH-difference, i.e. the osmotic part of the proton potential. The membrane potential, i.e. the electrical part, is neutralized by permeation of ions, e.g. chloride.

If the pH-difference during illumination were the same in chloroplasts and subchloroplast vesicles, despite a decreased proton uptake in the latter, as discussed above, ammonium chloride should be a potent uncoupler in both systems. This is not the case, a first shown by McCarty. Every subchloroplast vesicle preparation shows decreased sensitivity towards ammonium chloride compared to chloroplasts. Complete uncoupling of photophosphorylation in these preparations is obtained by ammonium chloride in the presence of valinomycin. Valinomycin is able to complex ammonium ions and carry them through the lipid barrier of the membrane (cf. to Ref. 5), collapsing any membrane potential. The decreased sensitivity of photophosphorylation towards ammonium chloride and the synergistic uncoupling effect of valinomycin in subchloroplast vesicles suggest that the membrane potential part of the electrochemical proton potential is larger than in chloroplasts.

The relative sizes of electrical and osmotic part of the proton potential could be controlled by specific ion permeabilities of the chloroplast membrane. Chloride is thought to be permeable in chloroplasts, because they swell in the light in the presence of ammonium chloride. Subchloroplast vesicles generally show much less light-induced swelling (G. A. Hauska, preliminary experiments). Thus decreased chloride permeability could account for decreased uncoupling of photophosphorylation by ammonium chloride. It is not necessary to separate grana from stroma lamellae to observe this effect. Moreover our experiments show that phosphorylation in isolated grana is less sensitive to ammonia than in chloroplasts. In addition Shuldiner and Avron wave demonstrated that uncoupling of photophosphorylation in chloroplasts by ammonium salts is not dependent on the anion in the salt, while light-induced swelling and ammonium uptake is dependent on the presence of a permeable anion, e.g. chloride. Chloride permeability therefore, cannot be exclusively responsible for the observed differential uncoupling effect of ammonium chloride in chloroplasts and subchloroplast.
vesicles. We think that the fragmentation of the large compartment of a chloroplast into smaller vesicular structures itself is responsible (Fig. 2).

Fig. 2. Schematic Fragmentation of the Chloroplast Lamellar-System. $\Delta p$ denotes membrane potential, $\Delta pH$ the pH-difference across the membrane.

Every membrane phase is in a dynamic state and will have transient permeability changes ("holes") statistically distributed with time and membrane area. It is reasonable to assume that a membrane potential would collapse much faster through such "holes" than a concentration gradient would equilibrate. Consequently the relative size of the membrane potential in the steady state of the proton potential during illumination will depend on the number of compartments, besides the influence of specific ion permeability and the number of "holes" per unit time and membrane area (membrane stability). It is obvious from Fig. 2 that one permanent hole in a chloroplast would collapse all of the membrane potential. In the corresponding ten subchloroplast vesicles only one tenth of the membrane potential would be dissipated by one hole. If transient holes are formed the steady state value of the membrane potential will depend on the rate of membrane potential formation relative to the relaxation time of the "holes", but still would be higher in a membrane system with more compartments. Since ammonia cannot dissipate the membrane potential it will be a poorer uncoupler in membrane systems with a higher membrane potential during illumination, i.e. in a system with more compartments.

In that context we want to point out that the procedure of chloroplast isolation from the leaf already causes some fragmentation. The harsher the procedure the higher the fragmentation of the chloroplasts and the lower the expected uncoupling of photophosphorylation by ammonium chloride might be, as indeed demonstrated (in Table II). Therefore, we would be cautious in the interpretation of a comparatively low uncoupling efficiency of ammonium chloride in isolated agranal chloroplasts\(^7\), which could be an isolation artefact.

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