Oligomycin Effects on ATPase and Photophosphorylation of Pea Chloroplast Thylakoid Membranes

PIERRE-YVES BOUTHYETTE and ANDRE T. JAGENDORF

Plant Biology Section, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Received for publication July 22, 1981 and in revised form November 10, 1981

ABSTRACT

Oligomycin inhibited the membrane-bound, Ca**+-dependent ATPase of pea (Pisum sativum var. Progress No. 9) chloroplasts up to 50%, but only after treating the membranes with trypsin, whether or not the trypsin step was needed for full activity. The energy-linked Mg**+-dependent (light- and dithiothreitol (DTT)-activated) ATPase of pea thylakoids could be inhibited up to 100% under specified conditions. The data indicate that oligomycin does not interfere with activation processes, and it failed to inhibit the ATPase of solubilized chloroplast coupling factor 1 under any circumstances. Photophosphorylation, previously thought insensitive to oligomycin, was inhibited 30% in the case of pea chloroplasts, and this increased to 50% inhibition after pretreating the chloroplasts with either trypsin or DTT. The nature of inhibition of phosphorylation was complex, with apparent small components of electron transport inhibition and uncoupling, as well as energy transfer inhibition.

Sensitivity of isolated pea thylakoid Mg**+-dependent ATPase to oligomycin was found during summer months but not during the winter. This was traced to a seasonal variation in relative humidity with values of 20% or less during the winter, leading to lack of inhibition in the isolated chloroplasts. The effect of low relative humidity during growth could be reversed by placing plants for 17 to 20 hours in a chamber at 50% relative humidity. The optimum humidity range for sensitivity to oligomycin was between 40 and 60%.

With chloroplasts potentially sensitive to oligomycin due to "permissive" growth conditions, the inhibition was modulated by a number of parameters during chloroplast activation or ATPase assay. Preincubation in high concentrations of KCl diminished oligomycin sensitivity, and this effect was reversed by valinomycin. The greatest sensitivity, as well as the highest ATPase rates, occurred only if thylakoids were activated by light and DTT at a relatively high temperature (35 to 40°C). Controlling the levels of uncouplers and running the reaction in darkness rather than in the light, both of which diminished the degree of membrane energization, increased the sensitivity to oligomycin. It is possible that accessibility of the oligomycin binding site is diminished by membrane energization. These results are contrasted with those from studies of variability in sensitivity of yeast mitochondria to oligomycin.

The proton-translocating membrane-bound ATPase systems of mitochondria (24) and of photosynthetic bacteria (6, 15) are known to be sensitive to the antibiotic oligomycin. However, the concentration needed for 50% inhibition depends very much on the enzyme. Rat liver and beef heart mitochondria are most sensitive with 50% inhibition occurring at 0.1 to 0.5 μg oligomycin per mg protein (8, 39); on the other end of the spectrum, as much as 10.0 μg oligomycin per mg protein is needed for 50% inhibition of some yeast mitochondrial ATPases (21, 27).

The analogous membrane-bound ATPase of chloroplasts was believed to be insensitive to this antibiotic. Baltshaefsky (5) observed that photophosphorylation in spinach chloroplasts was inhibited 20% in the presence of 3.5 μg oligomycin per mg protein, and this was considered negligible. Similar results were reported by others (3, 40). However, in the present work, we have defined conditions under which pea chloroplast ATPase shows a considerably greater degree of inhibition: 50% of photophosphorylation and up to 97% of the light- and DTT-induced, Mg**+-dependent ATPase activity are inhibited in the presence of 5.0 to 10.0 μg oligomycin per ml. The concentrations needed for these inhibitions are much higher than those used with the most sensitive mitochondrial enzymes but no more than those effective with yeast mitochondria. Our results confirm and extend observations made by Li et al. (25, 41), which came to our attention during preparation of this manuscript.

The degree of sensitivity to oligomycin is found to be variable in pea chloroplasts, as in other systems. In yeast mitochondria, for instance, the sensitivity of ATPase to oligomycin showed marked variations during the cell cycle (16, 27), and catabolite derepression of Saccharomyces pombe increased sensitivity 6-fold. Differences in sensitivity of yeast mitochondrial ATPase to oligomycin arose from different sources of enzyme, lipids associated with the complex, methods of purification, and genetic modification in work by Criddle and others (12, 23, 34, 36). The affinity for oligomycin was also changed by chemical modification of the enzyme (21).

In our present work, we found that pea thylakoid ATPase sensitivity to oligomycin was under a primary control resulting from growth of the plants under specified RH. The degree of inhibition was also affected by the temperature during activation of ATPase in the isolated chloroplasts, by pretreatment with K+ ions, and by the extent of membrane energization.

MATERIALS AND METHODS

Pea seeds (Pisum sativum var. Progress No. 9) were obtained from Agway Corporation. ATP, ADP, Tris, BSA, ascorbic acid, and PMS were purchased from Sigma Corporation. Oligomycin (Sigma) was prepared as a stock solution of 10 mg/ml in ethanol and stored at −20°C. Pyocyanin was obtained from Schwarz/Mann, sorbitol from Calbiochem, and 32P from New England

1 Supported in part by Grant GM-14479 from the National Institutes of Health.
2 Present address: Department of Plant Breeding, Cornell University, Ithaca, NY 14853.
3 To whom reprint requests should be addressed.

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Abbreviations: PMS, phenazine methosulfate; STN, buffer made of 50 mM Tricine (pH 7.8), 0.3 M sorbitol, and 10 mM NaCl; CF1, chloroplast coupling factor 1; CCCP, carbonyl cyanide m-chlorophenylhydrazone; P/eo, ratio of ATP formed/pair of electrons transported.
Nuclear.

Isolation of Thylakoid Membranes. Ten- to 16-d-old plants grown in a greenhouse were homogenized in STN buffer: 0.3 mM sorbitol, 50 mM Tris-HCl (pH 7.4); and 10 mM NaCl. The brei was strained through four layers of cheesecloth and one layer of Miracloth and centrifuged for 15 min at 4,500 rpm in a Sorvall centrifuge with an SS-34 rotor. The pellet was resuspended and washed once in STN buffer, then two times in 10 mM NaCl with centrifugation at 10,000 rpm. Final resuspension was in 10 mM NaCl, and the concentration was adjusted to 0.5 mg Chl per ml.

In efforts to reduce any possible contamination by mitochondria, in some experiments, thylakoid membranes were collected from sucrose density gradients as described by Milfin and Beevers (30). A peak of succinic dehydrogenase was localized above the intact chloroplasts, and the chloroplasts could be collected without contamination by mitochondria.

Trypsin-Activated ATPase Measurement. Isolated, washed thylakoids containing either 25 μg Chl or 10 μl (4 to 5 μg) of solubilized CF₁ were incubated for 10 min at 25°C with 40 mM Tris-HCl (pH 8.0), 4 mM EDTA, 4 mM ATP, and specified concentrations of trypsin in a total volume of 110 μl (10). The trypsin digestion was stopped by addition of BSA (0.1 mg/ml), and ATP hydrolysis occurred in a medium containing 50 mM Tris-HCl (pH 9.0), 5 mM CaCl₂, 5 mM ATP, and 0.1 mg/ml BSA in a final volume of 1 ml (9). After a 5-min incubation at 30°C, the reaction was stopped by adding 1.0 ml of 5% TCA. Pi was measured either by the method of Taussky and Shorr (38) or by that of Ames (1), which is 7 times more sensitive.

Light-Activated ATPase. Thylakoid membranes (50 μg Chl) were incubated for 5 min at 25°C or at other temperatures, as indicated, in 30 mM NaOH-Tricine (pH 8.0), 40 mM KCl, 5 mM MgCl₂, 10 mM DTT, and 30 μM PMS under white light (50,000 lux); ATP to 5 mM was added immediately after the tubes were returned to the dark, and chloroplasts were incubated for an additional 5 min prior to adding acid and measuring Pi released (13). In some experiments, thylakoid membranes were pretreated for 1 h in the dark with DTT and the same buffer mixture; PMS was omitted in those activations. Without activation, the thylakoid Mg²⁺-dependent ATPase activity amounted to 7.8% (average of 14 experiments) of that found after activation.

Photophosphorylation and Electron Transport. For photophosphorylation, each reaction mixture (0.5 ml final volume) contained 50 μg Chl in 50 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 20 mM glucose, 4 mM ADP, 4 mM Pi (including 50,000 cpm [³²P]Pi), 92 units/ml of hexokinase, and 4 μM pyocyanine. The reaction was run at 25°C in 50,000 lux of white light from incandescent lamps for 2 min. The reaction was stopped, and Pi was precipitated by addition of HCl-molybdate-triethylamine (37) with 1% bromine water added. After centrifuging, aliquots of the supernatant solution containing [³²P]ATP were removed for counting in a Nuclear-Chicago planchet counter.

Electron transport was measured by the reduction of ferricyanide. Twenty μg Chl were incubated in the light in 1.0 ml of 50 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, 1 mM K₂Fe(CN)₆, and varying concentrations of oligomycin either in the presence or in the absence of 4 mM ADP and 4 mM Pi. After 2 min in the light (50,000 lux), an 0.8-ml aliquot was added to 0.2 ml of 5% (w/v) TCA and centrifuged, and the absorbancy was determined at 420 nm. The extinction coefficient used for fermin ferricyanide was 0.96 mm⁻¹.

Special Conditions. Preloading the thylakoids with K⁺ was attempted by incubating them for 45 min at 4°C in STN buffer containing 50 mM KCl. These were then washed in STN buffer without KCl and assayed for Mg²⁺-ATPase activity in either 50 mM KCl or 50 mM NaCl.

Effects of membrane energization were tested in some experiments, using thylakoids already activated by DTT in the light, by a further 20-min incubation at 25°C either under 10,000 lux of white light or in the dark with 5 mM ATP and a regenerating system (9) added. Ten-thousand cpn of [³²P]ATP were then added with 1 mM carrier ATP, and the incubation continued for 5 min before adding TCA, as above. In other assays, Chl concentrations were determined by the method of Arnon (2) and protein by the Lowry procedure (28).

Control of Humidity during Plant Growth. In experiments which required prior incubation of pea plants under conditions of constant humidity, an apparatus was used in which air was allowed to pass through a cylindrical lucite air-tight chamber (32 cm high and 28.5 cm in diameter) after being bubbled through H₂O in a 2-L sidearm flask that was kept at a given temperature by immersion in a constant temperature water bath. In these conditions, the RH in the chamber was given by:

\[
\text{RH} = \frac{D_A}{D_w}
\]

where D_A is the water-vapor density of air at the ambient temperature in the room and D_w is the water-vapor density of the air at the set temperature of the water bath. RH in the chamber was monitored with a Luft HTAB-176 hygrometer.

RESULTS

Unlike solubilized CF₁ from spinach, where ATPase is latent and will appear only after treatment with trypsin, DTT, or heat (26), the soluble CF₁ from pea chloroplasts showed considerable Ca²⁺-dependent ATP hydrolysis. With CF₁ from pea plants harvested in the summer, full activity was seen in the absence of added trypsin (Fig. 1); with CF₁ from winter-harvested plants, trypsin treatment increased the ATPase 2- to 6-fold. Higher levels of trypsin inhibited ATPase of winter enzyme 99% but inhibited that from summer-harvested plants only 40%. At no time did we observe inhibition of the solubilized CF₁ from pea chloroplasts by oligomycin, whether the enzyme was activated by trypsin (26), by DTT (29), or by heating (18).

Fig. 1. Effect of trypsin on soluble Ca²⁺-dependent ATPase activity from thylakoid membranes of winter-grown and summer-grown peas. An EDTA extract of chloroplasts was prepared and assayed, according to published procedure (19), during summer (○) or winter (●) months. Ca²⁺-dependent ATPase activity was measured after treatment with increasing concentrations of trypsin. The ATPase activity is shown as percentage of the maximum rates, which were 184 (winter) or 176 (summer) μmol Pi formed per h per μg original Chl.
Membrane-Bound Ca\textsuperscript{2+}-ATPase. The membrane-bound Ca\textsuperscript{2+}-dependent ATPase exhibited some activity without trypsin (15 to 50% of the fully activated enzyme), but we did not detect seasonal variations in its activation by trypsin. In several attempts, both summer and winter, we could find almost no Mg\textsuperscript{2+}-dependent ATPase of the membranes without prior activation of some sort.

The remarkable sensitivity of pea thylakoid-bound CF\textsubscript{1} to both activation and inhibition by trypsin, compared to the lack of sensitivity of CF\textsubscript{1} on spinach thylakoids, is shown in Figure 2. This experiment also illustrates the fact that the membrane-bound pea CF\textsubscript{1} was not inhibited by oligomycin until after treatment with trypsin. Once trypsin has been used, the inhibition by 6 \mu g/ml oligomycin ranged from 25 to 50% in numerous experiments. The degree of inhibition reached a maximum at 6 \mu g/ml oligomycin per ml (i.e. 18 \mu g/mg protein) (Fig. 3) after the chloroplast were pretreated with trypsin at 5 \mu g/ml. The potentiating effect of trypsin reached a maximum at 5 \mu g trypsin per ml in the activation stage (data not shown); at no time did oligomycin inhibit untreated thylakoid Ca\textsuperscript{2+}-ATPase. At higher concentrations of trypsin, the ATPase was inhibited considerably (Fig. 1) but the sensitivity to oligomycin did not increase further. To guard against the possibility that we were measuring sensitivity to oligomycin of contaminating mitochondrial ATPase, thylakoids were purified on sucrose density gradients (30) to remove mitochondria more rigorously, and similar results were obtained.

After solubilization at low ionic strength, the ATPase of CF\textsubscript{1} was not inhibited by oligomycin, whether activated by trypsin (26), DTT (29), or heat (18).

Photophosphorylation. Photophosphorylation was also sensitive to oligomycin. Before trypsin treatment, inhibition reached 32% (Table I) at an oligomycin concentration of 6 \mu g/ml (18 \mu g/mg protein). Under the same conditions, photophosphorylation by spinach thylakoids was inhibited 20%, in good agreement with results reported originally by Baltschefsky (5). However, if pea thylakoids were pretreated for 10 min at 25\textdegree C with 2 \mu g trypsin per ml, although the phosphorylation rates decreased about 20%, the maximum inhibition by oligomycin reached 53% (Table I).

![Fig. 2. Effect of trypsin on thylakoid Ca\textsuperscript{2+}-dependent ATPase in the presence and in the absence of oligomycin (3.3 \mu g/ml). Pea or spinach chloroplast thylakoids were assayed for Ca\textsuperscript{2+}-dependent ATPase activity, according to "Material and Methods," with variable concentrations of trypsin as shown. For control (○, □) and oligomycin-treated (3.3 \mu g/ml) (●, ■) samples, ATPase activity is expressed as \mu mol Pi formed per h per mg Chl.](image)

![Fig. 3. Effect of oligomycin on the thylakoid Ca\textsuperscript{2+}-dependent and Mg\textsuperscript{2+}-dependent ATPase activities. Thylakoid membranes were pretreated with 5 \mu g trypsin per ml and assayed for Ca\textsuperscript{2+}-dependent ATPase activity in the presence of increasing concentrations of oligomycin. ATPase activity in the control assay (minus oligomycin) was 250 \mu mol Pi formed per h per mg Chl. Thylakoid membranes were pretreated with light and 10 mM DTT, then assayed for Mg\textsuperscript{2+}-dependent ATPase activity with increasing concentrations of oligomycin. The Mg\textsuperscript{2+}-ATPase activity in the control (minus oligomycin) was 180 \mu mol Pi per h per mg Chl.](image)

Table I. Inhibition of Photophosphorylation by Oligomycin

<table>
<thead>
<tr>
<th>Oligomycin</th>
<th>Control</th>
<th>Trypsin-Treated</th>
<th>DTT-Treated</th>
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<tbody>
<tr>
<td></td>
<td>Rate</td>
<td>Inhibition</td>
<td>Rate</td>
</tr>
<tr>
<td>\mu g/ml</td>
<td>\mu mol Pi esterified/h·mg Chl</td>
<td>%</td>
<td>\mu mol Pi esterified/h·mg Chl</td>
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<tr>
<td>0</td>
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<td>450</td>
<td>711</td>
</tr>
<tr>
<td>1.0</td>
<td>525</td>
<td>355</td>
<td>21</td>
</tr>
<tr>
<td>2.0</td>
<td>470</td>
<td>319</td>
<td>29</td>
</tr>
<tr>
<td>12.0</td>
<td>374</td>
<td>211</td>
<td>53</td>
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</table>

Pretreatment of the thylakoid membranes with DTT (10 mM) did not affect phosphorylation rates but increased oligomycin inhibition of photophosphorylation to a maximum of 50%.

To investigate further the nature of inhibition by oligomycin, measurements were made of its effect on photoreduction of ferri-cyanide and the accompanying esterification of 32P (Fig. 4). Inhibition of ATP synthesis was somewhat more sensitive than the simultaneous electron transport rate in the presence of ADP and Pi, leading to a drop in the P/\epsilon\textsubscript{2} ratio from 0.96 in the controls to 0.70 at the high concentration of 10 \mu g oligomycin per ml. In several other experiments, phosphorylation rates were inhibited between 18 and 28% more than were the rates of coupled electron transport (data not shown).
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Fig. 4. Effect of oligomycin on the Hill reaction under phosphorylating and nonphosphorylating conditions. Thylakoids were illuminated with ferricyanide with varying concentrations of oligomycin as shown, either with (○, ○) or without (●) addition of ADP and Pi as detailed in "Materials and Methods." The loss of absorbancy at 420 nm was measured, and results are shown in equivalents ferricyanide reduced per h per mg Chl. Ferricyanide reduction was also measured in the presence of 3 μm gramicidin (uncoupled rates). Photophosphorylation was measured simultaneously in assay mixtures supplemented with 32Pi. The control rate of photophosphorylation (no oligomycin) was 300 μmol ATP per mg Chl per h.

Oligomycin also inhibited electron transport per se, in the uncoupled Hill reaction with 3 μm gramicidin present, to a maximum of 28% (Fig. 4). If the same degree of electron flow inhibition occurred under coupled conditions, it might have made the calculated P/O ratio too high. However, at least for the Mg2+-dependent ATPase, it was shown that oligomycin was a more effective inhibitor and may have had its binding sites more accessible, under slightly uncoupled conditions, than when the membranes were highly energized (see below). Thus, it is not at all certain that the 28% inhibition of electron transport seen with uncoupler present applied quantitatively to the electron flow supporting photophosphorylation.

A role as energy transfer inhibitor can be inferred from the fact that oligomycin inhibited phosphorylation and its accompanying electron flow more severely (up to 45%) than it did the uncoupled electron flow (28%) (Fig. 4). To the extent that the inhibition of uncoupled electron flow reflected tighter binding of oligomycin than under coupled conditions, the energy transfer inhibition may have been even stronger than that indicated.

There was no apparent effect of oligomycin on basal electron transport (as would be expected from a simple energy transfer inhibitor). In this case, the uncoupling effect and the inhibition of electron transport might have been in fortuitous balance.

Membrane-Bound ATPase. The final, and most oligomycin-sensitive, activity was the proton-pumping, membrane-bound, light- and DTT-activated Mg2+-dependent ATPase. This ATPase was inhibited 78% by oligomycin (6 μg/ml or 18 μg/mg protein) in the experiment shown in Figure 3, and inhibition was virtually complete by 12 μg oligomycin per ml. In six repetitions of this experiment during the summer months, the degree of inhibition varied between 53 and 78% when using the standard concentration of 6 μg oligomycin per ml; in only one other experiment was the inhibition 13%. Chloroplast preparations purified on sucrose gra-

Dants (31) showed very similar levels of ATPase activity and of inhibition by oligomycin.

The Mg2+-dependent ATPase of chloroplast membranes needs continuing activation by the high energy state and reducing agents during ATP hydrolysis to keep the enzyme active. To test the possibility that oligomycin works on activation rather than on activity, membrane-bound CF1 was activated (by light and DTT) with or without oligomycin present, then detached by 0.75 mM EDTA (23). The solubilized CF1 was assayed for Ca2+-dependent ATPase without further activation; the activated condition on the membrane apparently carries over to the solubilized enzyme and is not readily reversible. It is apparent (Table II) that the activation process, reflected in 70% higher activities of the solubilized CF1, was not inhibited by oligomycin even though expression of the ATPase by the membrane-bound enzyme was inhibited. Consistent with a lack of effect on activation was the fact that we found considerable inhibition when oligomycin was added to the Mg2+-dependent ATPase assay after activation was complete (31%, as compared to 50% if added before activation).

Modulating Oligomycin Sensitivity of the Mg2+-ATPase: Effects of Humidity during Growth. Since the Mg2+-dependent ATPase activity of the thylakoids was the most sensitive to oligomycin, further studies on conditions affecting the oligomycin inhibition were made exclusively with this reaction. Originally the Mg2+-ATPase was found to be seasonal; strong inhibitions were found in the summer, but almost none were found in the winter using greenhouse-grown plants. Temperature during growth, photoperiod, and RH were altered individually to see which of these factors might be responsible for the seasonal variation; only RH had any effect. Plants grown or incubated under the narrow range of 40 to 60% RH in a constant relative humidity chamber yielded a thylakoid Mg2+-ATPase sensitive to oligomycin. Drier (below 40% RH) or more humid growth conditions (above 60% RH) resulted in decreased sensitivity (Fig. 5). The results shown in Figure 5 were compiled over a 6-month period, and, although the RH did not affect the activity of the Mg2+-ATPase, its effect on oligomycin sensitivity proved to be very reproducible. This finding explains the earlier seasonal variation, since the RH in heated buildings in the wintertime in Ithaca is on the order of 20%, and thylakoids from plants grown under this condition were completely insensitive to oligomycin.

The length of time of incubation at designated RH needed to change oligomycin sensitivity has not yet been investigated sys-

Table II. Oligomycin Does Not Prevent Activation of ATPase

<table>
<thead>
<tr>
<th>Chloroplasts (0.2 mg Chl) were treated with 10 mM DTT for 5 min in the light at 35°C. Aliquots were assayed directly for Mg2+-dependent ATPase in the dark or they were diluted in 0.75 mM EDTA to remove CF1, and centrifuged, and the supernatant was assayed for Ca2+-dependent ATPase without further activation. The ATPase assays contained 30 mM Tricine (pH 8.0), 5 mM ATP, and either 5 mM MgCl2 for the membrane-bound enzyme or 5 mM CaCl2 for the soluble enzyme. Incubation was for 5 min at 37°C (Ca2+) or at 25°C (Mg2+). Oligomycin (5 μg/ml) was added either before the first activation step or afterwards in the ATPase assay as shown. Controls were kept in the dark without DTT.</th>
<th>Activation</th>
<th>Oligomycin Added</th>
<th>ATPase Rates</th>
</tr>
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<tr>
<td>Membrane-bound</td>
<td>Soluble</td>
<td>μmol Pi liberated/mg Chl-h</td>
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<tr>
<td>Before</td>
<td>After</td>
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</tr>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>77</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>30</td>
</tr>
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</table>
Figure 5 illustrates the effect of RH prior to harvest on the sensitivity of Mg$^{2+}$-ATPase to oligomycin. Greenhouse-grown pea plants were incubated for 17 h at constant RH, and their chloroplasts were subsequently isolated and assayed for Mg$^{2+}$-dependent ATPase activity (for procedure, see "Materials and Methods"). The ATPase sensitivity to 5 μg oligomycin per ml was plotted as a function of previous RH. Each point represents the result from a different experiment over the course of several months.

Effect of pH on Sensitivity to Oligomycin. The Mg$^{2+}$-ATPase had a moderately sharp optimum at 7.5. Rates with oligomycin were depressed throughout the range from pH 7 to pH 8.3, with somewhat less inhibition at pH 7.6 and pH 9.0 (Fig. 6). These results contrast with the high sensitivity to pH of oligomycin inhibition in the case of yeast mitochondria (21).

Effect of the Preincubation Temperature. Activation of the Mg$^{2+}$-dependent ATPase by light and DTT was conducted at different temperatures, and the resulting enzyme activity showed different sensitivities to oligomycin (Fig. 7). In all cases, ATP hydrolysis was measured at 25°C. Without oligomycin the peak of activity was reached after activation at 35°C, and these rates were 3.5 times faster than those obtained after activation at 25°C. In the presence of oligomycin, the optimum for activation occurred at 30°C. Thus, the inhibition by oligomycin was less than 30% at temperatures below 25°C but rose rapidly to 80% at 40°C (Fig. 7B). Since the peak for activation of the ATPase was 35°C, this temperature was used routinely.

Effect of Uncouplers. Low concentrations of uncouplers stimulate the Mg$^{2+}$-ATPase of pea thylakoid membranes (Fig. 8) just as they do those of spinach or lettuce (20). While the stimulation was usually 2- to 3-fold, it was larger in the experiment shown in Figure 8. Gramicidin similarly increased the rate of ATPase (Fig. 9). These uncoupler-stimulated ATPase rates were inhibited more by oligomycin than were the reactions lacking uncoupler. In the case of NH$_4$Cl, the addition of 10 μg/ml oligomycin abolished completely the stimulation of ATPase by uncoupler (Fig. 8).

Sensitivity of the Mg$^{2+}$-ATPase to low concentrations of oligomycin was enhanced strikingly by the presence of an uncoupler. In one experiment with 3 μM gramicidin present, inhibition by 0.01, 0.10, and 1.0 μg/ml oligomycin amounted to 19, 30, and 38% respectively. None of these concentrations inhibited control chloroplasts lacking the uncoupler. Only at 5 μg oligomycin per ml, the control activity was inhibited 44% and that with gramicidin present was inhibited 72%. Thus (for comparative purposes), the I$_{50}$ (μg oligomycin per mg protein causing 50% inhibition) was lowered from 22 in the control chloroplasts to 8 when the reaction occurred with 3 μM gramicidin present. Higher concentrations of oligomycin tended to inhibit control and uncoupler-supplemented reactions equally, perhaps due to the weak uncoupling action of oligomycin itself (see above).

Effect of Illumination on the Oligomycin Sensitivity of the Mg$^{2+}$-ATPase. Thylakoids were assayed for Mg$^{2+}$-ATPase by a modification of the method of Carmeli and Avron (13) (Table III). It is apparent that the ATPase is faster in the dark than it is in the light, unless gramicidin is added. This rate differential was not due to rephosphorylation of released $^{32}$Pi, since it was also seen when 10 mM Pi was added to dilute out the pool of $^{32}$P available for phosphorylation. The sensitivity to oligomycin was much greater in the dark than it was in the light, and no inhibition can be seen unless gramicidin is present for the reaction in the light. This is not true for the ATPase occurring in darkness.

The gramicidin dependency of ATPase oligomycin sensitivity in illuminated chloroplasts is shown in Figure 9. There was no sensitivity to 5 μg/ml oligomycin without gramicidin (data point not on the graph), but 20% inhibition occurred at 0.003 μM gramicidin. The response to gramicidin was approximately linear with log of its concentration, both with and without oligomycin. The inhibition by oligomycin was maximal (40% in this case) at 1 μM gramicidin. Sensitivity to oligomycin in the light was also permitted by addition of 90 μM valinomycin or 5 μM CCCP in place of gramicidin.

Effects of K$^+$ and Valinomycin. Preloading the thylakoids with K$^+$ by prior incubation in 50 mM KCl decreased the oligomycin sensitivity of the Mg$^{2+}$-ATPase (Table IV). This effect was not shared by adding KCl at the last minute just before the reaction.
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Fig. 7. Effect of temperature (during DTT activation) on Mg\(^{2+}\)-dependent ATPase activity and its sensitivity to oligomycin. The thylakoids were isolated and assayed for Mg\(^{2+}\)-dependent ATPase activity by the procedure described in "Materials and Methods." B. During DTT activation, six different temperature were tested in the absence (○) and in the presence (□) of 5 μg oligomycin per ml. The ATPase activity was expressed in μmol of Pi formed per h per mg Chl. A. The inhibition (%) resulting from addition of oligomycin was plotted as a function of temperature (B).

Fig. 8. Effect of NH\(_4\)Cl on Mg\(^{2+}\)-dependent ATPase activity, in the presence and in the absence of oligomycin. Chloroplast thylakoid membranes were DTT- and light-activated at 25°C in the presence (□) or in the absence (○) of 10 μg oligomycin per ml and assayed, in the dark, for Mg\(^{2+}\)-dependent ATPase activity. The ATPase activity, expressed as μmol Pi formed per h per mg Chl, was plotted as a function of NH\(_4\)Cl concentration.

Begun, and it was not duplicated by prior incubation in 50 mM NaCl. These ATPase reactions were performed in the dark, so that the control thylakoids would show maximal inhibition by oligomycin.

Consistent with a role for internal K\(^+\) in decreasing sensitivity, addition of 3 μM valinomycin to preloaded thylakoids restored or enhanced the response to oligomycin (Table IV). However, the valinomycin effect was not prevented by having external KCl at 50 mM, so a transmembrane K\(^+\) gradient cannot be assigned a primary role at this point. Also, 3 μM valinomycin, by itself, with nonpreincubated thylakoids, increased the extent of oligomycin inhibition (from 71 to 92% in a typical experiment).

**DISCUSSION**

The ATPase of pea chloroplast CF\(_1\) is activated by about the same concentration of trypsin when it is membrane-bound (Fig.

**Table III. Effect of Dark Incubation and Gramicidin on the Oligomycin Sensitivity of the Mg\(^{2+}\)-Dependent ATPase**

Thylakoid membranes were isolated from greenhouse-grown pea plants preincubated at 45 to 55% RH for 17 h prior to harvest. The thylakoids were assayed for Mg\(^{2+}\)-dependent ATPase activity by the procedure described in "Materials and Methods." DTT- and light-activation was carried out in the presence or in the absence of 6 μg oligomycin per ml. The ATPase assay was performed in the light (15,000 lux) or in the dark, in the presence or in the absence of 3 μM gramicidin, immediately after activation.

<table>
<thead>
<tr>
<th>Gramicidin</th>
<th>Light</th>
<th></th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligomycin</td>
<td>Inhibition</td>
<td>Oligomycin</td>
<td>Inhibition</td>
</tr>
<tr>
<td>μmol Pi/mg Chl-h</td>
<td>%</td>
<td>μmol Pi/mg Chl-h</td>
<td>%</td>
</tr>
<tr>
<td>18</td>
<td>26</td>
<td>78</td>
<td>51</td>
</tr>
<tr>
<td>3 μM</td>
<td>120</td>
<td>97</td>
<td>98</td>
</tr>
</tbody>
</table>

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Table IV. Effects of K* and Valinomycin on Sensitivity of Chloroplast ATPase to Oligomycin

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Oligomycin</th>
<th>Control</th>
<th>Preloaded with K*</th>
<th>Preloaded with K* and Assayed with 3 μM Valinomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>52</td>
<td>17</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>76</td>
<td>56</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>27</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>38</td>
<td>27</td>
<td>60</td>
</tr>
</tbody>
</table>

2) as after it is solubilized (Fig. 1). This is an interesting contrast to spinach chloroplast CF₁, which needs about 10 times higher trypsin concentration for adequate activation when it is on the membrane (Fig. 2) than when it is solubilized. The fact that solubilized pea CF₁ has fully active Ca²⁺-dependent ATPase in the summer months (Fig. 1) might have been due to its unusual sensitivity to an endogenous protease. However, the membrane-bound pea ATPases (Ca²⁺-dependent and trypsin-activated, and Mg²⁺-dependent and light- and DTT-activated; see Table III) are not fully expressed until after an activation process, even during the summer months. The Ca²⁺-ATPase of the membranes (a relatively weak activity) was 15 to 50% active, and the Mg²⁺-ATPase of the membranes was only 8% active, in untreated thylakoids. Thus, alternative explanations must be found for the 100% activity of solubilized pea CF₁ during summer months. Further exploration of these winter-summer differences and comparison to spinach chloroplast CF₁ may contribute to a better understanding of the activation process.

The data presented in this paper show that photophosphorylation and thylakoid ATPase can be inhibited significantly by oligomycin. Pea chloroplasts are intrinsically a little more sensitive than are spinach thylakoid membranes (5), and the extent of inhibition is increased by pretreating the membranes with trypsin or with DTT (Table I). With these pretreatments, photophosphorylation and the membrane-bound Ca²⁺-dependent ATPase can be inhibited up to 50% by oligomycin. Artifacts due to contaminating mitochondria can be ruled out, since sucrose gradient-purified thylakoid membranes (30) had the same responses to oligomycin; and both the light-dependent ATP synthesis and light-triggered Mg²⁺-ATPase (clearly chloroplast reactions) are inhibited significantly by oligomycin.

Although the membrane-bound Ca²⁺-ATPase was active without trypsin, inhibition by oligomycin was completely dependent on prior treatment with trypsin. Similarly, photophosphorylation inhibition was increased from 30% before to 50% after trypsin treatment. This suggests that the oligomycin binding site in chloroplasts may be partly obscured by a shielding protein, at least somewhat trypsin-digestible. Preincubation of chloroplasts with DTT (Table I) also increased oligomycin sensitivity, perhaps due to a conformation change in CF₁ caused by the highly reducing conditions which can also activate ATPase (29).

It was tempting initially to ascribe the inhibition of photophosphorylation by oligomycin to an inhibition of the proton channel as in mitochondria (8). Although some of the electron transport data (Fig. 4) supports a role as energy transfer inhibitor, the situation is obviously more complex, because oligomycin can also inhibit electron transport to some extent and acts in part as a weak uncoupler at high concentrations. The quantitative aspects of its action are made more uncertain, because the effectiveness of oligomycin as an ATPase inhibitor (Figs. 8 and 9; Table III) varies with the degree of membrane energization, and this factor might affect its other functions as well. These complications may be the cause of our failure to see oligomycin inhibition of H⁺ leakage from illuminated chloroplasts put into the dark (data not shown).

A further reason for putting at least one action of oligomycin at a site other than blockage of proton pumping is its inhibition of the membrane-bound, trypsin-activated, Ca²⁺-dependent ATPase. Table V summarizes characteristics of the ATPase activities examined in thylakoids; the membrane-bound Ca²⁺-ATPase is not associated with proton pumping, and its rate is not stimulated by uncouplers. Thus, oligomycin inhibits, in this case, a scalar reaction. Nevertheless, some association of CF₁ with the membranes is required for oligomycin inhibition, since it had no effect on the solubilized enzyme (Table V).

The Mg²⁺-dependent ATPase was the most sensitive function examined, with inhibitions reaching nearly 100% at 20 µg oligomycin per ml and 6 µg/ml causing inhibitions from 50 to 80%. This ATPase can be thought of as a poised enzyme system, requiring the high energy state of the membrane both for original activation and for maintenance of activity (the protons pumped inward during ATP hydrolysis are necessary for continuing ATP hydrolysis). However, oligomycin does not inhibit the activation process (Table II), and its inhibition cannot be ascribed to uncoupling, because control rates are stimulated a great deal by controlled addition of uncouplers (Figs. 8 and 9). Also, under proper conditions, inhibition by oligomycin appears at concentrations too low (i.e. 0.03 to 1.0 µg/ml) for it to have an uncoupling effect (Fig. 4). The reason for much greater sensitivity of the Mg²⁺-ATPase than of other chloroplast functions, thus, is not known.

To compare the sensitivity of pea chloroplasts with that of other membrane-bound ATPases, values of I₅₀ (the amount of oligomycin in µg/mg protein needed for 50% inhibition) were compiled from published data with various mitochondrial and chromatophore ATPases (Table VI). In the case of chromatophores and chloroplasts, only Chl levels were measured. For Table VI, these were converted to protein values using published conversion factors, and the I₅₀ values are, therefore, only estimates for purposes of comparison. Rat liver and beef heart mitochondrial ATPases are the most sensitive to oligomycin, with I₅₀ between 0.1 and 0.5 µg/ml protein. Chloroplast thylakoid ATPase on the other hand is the least sensitive, but its I₅₀ is in the range of sensitivities exhibited by some yeast mitochondrial and bacterial chromatophore ATPases. Under carefully controlled conditions, the thylakoid ATPase can show some inhibition by concentrations of oligomycin as low as 0.03 µg/mg protein.

Table V. Manifestations of ATPase Activity of CF₁

<table>
<thead>
<tr>
<th>Location</th>
<th>Activation</th>
<th>Cat-</th>
<th>Proton-</th>
<th>Overt in</th>
<th>Maximum Inhibition by Oligomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>Light, DTT</td>
<td>Mg²⁺</td>
<td>+</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Membrane</td>
<td>Trypsin</td>
<td>Ca²⁺</td>
<td>0</td>
<td>+*</td>
<td>50</td>
</tr>
<tr>
<td>Soluble</td>
<td>Trypsin</td>
<td>Ca²⁺</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

* Prior to activation, rates ranged from 15 to 50% of those after activation; no seasonal correlation was observed.
OLIGOMYCIN EFFECTS ON CHLOROPLAST ATPASE

The strong degree of oligomycin inhibition of the Mg\textsuperscript{2+}-ATPase permitted exploration of parameters that modify its effectiveness. The failure to see any inhibition in the winter months was traced to the low RH during the period of plant growth no more than 18 h prior to harvest (Fig. 5). The molecular basis for this effect remains to be explored. However, it will be of interest to see whether some previously reported cases of seasonal variation in the response of isolated chloroplasts to inhibitors (32) or activators (33) have a similar basis.

Once chloroplasts are obtained from plants at a “permissive” RH, other parameters during the ATPase reaction affect the amount of inhibition by oligomycin quite strongly. A relatively high temperature during activation by light and DTT favors oligomycin sensitivity (Fig. 7). Internal K\textsuperscript{+} (but not Na\textsuperscript{+}) ions decreased sensitivity to oligomycin, and this effect was reversed by valinomycin (Table IV). Our results with pea thylakoids seem to be the opposite of those found by Johnson et al. (22), using yeast mitochondria, where external KCl increased ATPase sensitivity to oligomycin. Also, valinomycin increases the sensitivity of pea thylakoids, while, with yeast mitochondria, valinomycin decreased sensitivity to oligomycin.

A final controlling factor for oligomycin sensitivity may be the degree of membrane energization. This can be inferred from the loss of oligomycin sensitivity when Mg\textsuperscript{2+}-ATPase operates in the light (Table III) and increases due either to controlled addition of uncouplers (Figs. 8 and 9) or to running the reaction in darkness (Table III). In the latter case, ATP hydrolysis causes some membrane energization but not enough to prevent oligomycin inhibition. Even in darkness, the addition of gramicidin was stimulatory. Presumably, the ATPase complex undergoes conformational shifts at different levels of energization, leading to greater or lesser exposure of the oligomycin binding site (or, perhaps, to variations in affinity of the site for oligomycin).

If we ascribe differential sensitivity to oligomycin to different conformational states of the system, it is clear that two different resistant configurations can be obtained. The parameters functioning in activation or reaction mixtures can modulate the degree of inhibition, presumably causing formation of greater or lesser proportions of a resistant configuration. However, they are never able to restore sensitivity to chloroplasts taken from the 20% RH-grown plants, which must, therefore, be in a different, completely intractable configuration.

The effects of membrane energization differ radically from those seen with the yeast mitochondrial ATPase, in which oligomycin was shown to interact preferentially with energized membranes (8, 17, 21, 35). Bertina et al. (8) proposed that two conformations of the oligomycin-sensitive site existed and that oligomycin specifically bound to the conformation that was involved in the induction of respiratory control. In chloroplasts, even though the high energy state of the thylakoid membranes affects oligomycin sensitivity and the thylakoid ATPase has been shown to go through one or more conformational changes during membrane energization (Ref. 20, review) it is not certain if these conformational changes are responsible for the modulation of oligomycin sensitivity. Furthermore, in view of the opposing results reported in mitochondria and chloroplasts, it remains to be determined whether the site of action of the antibiotic is altogether different in the two systems. In any event, it is clear that chloroplasts should no longer be considered to be completely insensitive to oligomycin.

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