Effect of Pyrophosphate on Photosynthetic Electron Transport Reactions

Giorgio Forti and Emilia Maria Meyer
Laboratory of Plant Physiology, Istituto Botanico dell'Università Via Amendola 175, Bari, Italy

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Abstract. Inorganic pyrophosphate is found to inhibit the ferredoxin-dependent photoreduction of NADP by isolated chloroplasts. The reduction of ferricyanide is not inhibited, nor is the activity of photosystem 1 as measured with methyl viologen as the electron acceptor. All other ferredoxin-dependent reactions are inhibited, such as cytochrome c photoreduction and the reaction sequence: NADPH → Flavoprotein → ferredoxin → cytochrome c. The inhibition by pyrophosphate is, in all cases, competitive with ferredoxin and independent of NADP concentration. Pyrophosphate inhibition of the formation of the ferredoxin-flavoprotein complex is demonstrated spectrophotometrically.

Photosynthesis by isolated chloroplasts has been found to be stimulated by inorganic pyrophosphate (3, 9). The nature of this stimulation is, however, unknown, and some of the pertinent observations made in different laboratories seem to be in conflict with each other. For instance, Jensen and Bassham (9) reported that 32P-labeled pyrophosphate is not incorporated by isolated chloroplasts into any of the intermediates of carbon cycle nor into ATP or ADP during photosynthesis. In apparent contrast to this observation Cockburn et al. (4) reported that 1 pyrophosphate molecule can substitute for 2 molecules of orthophosphate to meet the requirement for phosphate by photosynthesizing isolated chloroplasts. Though it cannot be excluded that the apparent discrepancies might be due to the different preparative procedures employed, the former observation implies a catalytic (or regulatory) role of pyrophosphate, while the latter supports a stoichiometric role.

In an attempt to understand the role of pyrophosphate in photosynthesis, our attention was directed to its effects on electron transport reactions. The results indicate that pyrophosphate is an inhibitor of any ferredoxin-dependent reaction, and that the inhibition is competitive towards ferredoxin itself. Furthermore, it is demonstrated here by a spectrophotometric method that pyrophosphate inhibits the formation of the ferredoxin-flavoprotein complex.

Methods

Chloroplasts were prepared from spinach leaves as previously described (6), except that EDTA was now omitted from the grinding medium. In a number of preparations the buffer in the grinding medium was replaced by MES buffer of pH 6.1. This change did not affect the results obtained.

NADP photoreduction was measured as previously reported (5). Ferricyanide reduction was measured as the decrease of absorbancy at 420 nm, upon illumination of the chloroplasts suspensions, and cytochrome c reduction was followed at 550 nm. The light was provided by a slide projector, and filtered through 5 cm of water and a heat filter (Baltzers). Light intensity was $1.1 \times 10^6$ ergs $\cdot$ sec$^{-2}$ $\cdot$ cm$^{-2}$ at the level of the cuvettes. Cuvettes with a 10 mm light path were used.

Manometric experiments were performed in a conventional illuminated Warburg apparatus. Light intensity was 15,000 lux at the flasks level.

The difference spectra reported in Fig. 4 were similar to those recorded by Foust et al. (8), at 20°C, using the 0 to 0.1 absorbance units slide-wire of the Cary 14 spectrophotometer.

Ferredoxin was prepared from spinach leaves according to Boger et al. (1), and the chloroplast flavoprotein as previously described (7). The nucleotides and cytochrome c were from Sigma and all other reagents were analytical grade.

Results

Inhibition by Pyrophosphate of Chloroplast Reactions. The photoreduction of NADP is inhibited by pyrophosphate competitively with ferredoxin, (Fig. 1). The $K_i$ calculated from the double reciprocal plot is 9.7 mM. Changing the concentration of NADP does not affect the inhibition. A similar inhibition is observed when NADP is photoreduced in the presence of CMU, with the system ascorbate-DCPIP as the electron donor. On the other hand, pyrophosphate does not affect the activity of photosystem 1 per se, as shown by the fact that the reac-

1 Abbreviations used: MES, [2(N-morpholino)ethanesulphonic Acid]; HEPES, (N-2-hydroxyethylpiperezine-N'-2-ethanesulphonic Acid); CMU, p-chlorophenyl-dimethylurea; DCPIP, dichlorophenolindophenol.
viologen $\rightarrow$ O$_2$ is to according pH 8.3; was volume 1 ml. Temperature 22°; gas phase, air.

The inhibition of the photoreduction of cytochrome c, another well-known ferredoxin-dependent reaction, is shown in Table I. The inhibition in this case seems to be somewhat lower, probably because the reaction inhibited is a very fast one, and the over-all process is rate-limited by some other reaction in the sequence. The inhibition, however, increases during the progress of the reaction.

The competitive nature of pyrophosphate inhibition has also been demonstrated in a simpler system in which NADPH serves as the electron donor to cytochrome c through the flavoprotein and ferredoxin according to the sequence: NADPH $\rightarrow$ flavoprotein $\rightarrow$ ferredoxin $\rightarrow$ cytochrome c (7). Pyrophosphate inhibits this reaction competitively with ferredoxin; the $K_i$ calculated from this kind of experiment is 10 to 11 mM (Fig. 3).

The lack of inhibition by pyrophosphate of photosystem 2 activity is shown by the fact that it does not inhibit ferri cyanide reduction (Table II). A slight but reproducible stimulation of the ferricyanide reduction is observed (Table II). In the presence of

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Table I. Effect of Pyrophosphate on Cytochrome c Photoreduction

<table>
<thead>
<tr>
<th>Time in light</th>
<th>Control</th>
<th>50 mM</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>33.4</td>
<td>19.0</td>
<td>43</td>
</tr>
<tr>
<td>2</td>
<td>32.0</td>
<td>16.0</td>
<td>50</td>
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<tr>
<td>3</td>
<td>33.4</td>
<td>13.2</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>9.2</td>
<td>70</td>
</tr>
</tbody>
</table>

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Table II. Effect of Pyrophosphate on Ferricyanide Reduction

<table>
<thead>
<tr>
<th>Additions</th>
<th>1 Min</th>
<th>2 Min</th>
<th>3 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.060</td>
<td>0.110</td>
<td>0.155</td>
</tr>
<tr>
<td>P-P 33 mM</td>
<td>0.085</td>
<td>0.155</td>
<td>0.215</td>
</tr>
<tr>
<td>ADP, P</td>
<td>MgCl$_2$</td>
<td>0.095</td>
<td>0.165</td>
</tr>
<tr>
<td>ADP, P</td>
<td>MgCl$_2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ P-P 33 mM</td>
<td>0.070</td>
<td>0.130</td>
<td>0.185</td>
</tr>
</tbody>
</table>
of ADP, Mg$^{2+}$ and P$_1$ a small inhibition appears. Though a full explanation of these minor effects must await further investigation, it is evident that the major site of pyrophosphate inhibition is at the ferredoxin level.

*Inhibition by Pyrophosphate of the Ferredoxin-Flavoprotein Interaction.* Ferredoxin reacts with its immediate electron acceptor, the flavoprotein, forming a complex which can be demonstrated directly by spectrophotometric measurement (8,10). The effect of pyrophosphate on this reaction is shown in Fig. 4. The difference spectra were recorded as by Foust et al. (8) using dual compartment silica cells. The light path in each compartment was 4.5 mm and the total volume in them was 0.9 ml. An equal volume of ferredoxin solution was pipetted into the front compartment of each cuvette together with an equal volume of buffer and pyrophosphate where indicated. An equal volume of buffer was added to the rear compartment of each cell. The cells were then balanced by means of the multipotentietometers on the spectrophotometer, and a baseline was recorded. Equal volumes of the flavoprotein solution were then pipetted into the front compartment of the sample cell and the rear compartment of the reference. Buffer was added to the front compartment of the reference cell in the same amount as the flavoprotein addition. After mixing accurately the content of each compartment, the difference spectrum was recorded. It can be seen that pyrophosphate suppresses almost completely (Fig. 4, curve 2) the difference spectrum clearly shown (Fig. 4, curve 1) in the presence of phosphate. This demonstrates that pyrophosphate inhibits the formation of the ferredoxin-flavoprotein complex.

**Discussion**

The results reported above demonstrate that pyrophosphate inhibits any ferredoxin-dependent reaction of chloroplasts competitively with ferredoxin. It also inhibits the formation of the ferredoxin-flavoprotein complex, which is probably required for NADP reduction by the chloroplast. On the other hand, it is well-known that pyrophosphate stimulates photosynthesis by isolated chloroplasts, a process which requires the production of NADPH and ATP (3,9). Jensen and Bassham found (9) that 0.7 mM pyrophosphate produces continued stimulation for over 30 min, while 5 mM pyrophosphate which gives maximal initial stimulation of CO$_2$ fixation, subsequently inhibits. These authors also reported that pyrophosphate does not merely protect the chloroplast preparation, but its actual presence in the reaction mixture is required to give its effect. This observation is paralleled by ours that pyrophosphate inhibition of ferredoxin is completely reversible: when the inhibitor is removed, complete reactivation of ferredoxin is observed.

The apparent contradiction between the opposite effects of pyrophosphate on NADP reduction and CO$_2$ fixation might be resolved, in one of 2 ways.
a) The partial inhibition of electron transfer from ferredoxin at the concentrations of pyrophosphate which stimulate CO₂ fixation could increase the concentration of reduced ferredoxin, which was reported to activate fructose 1,6-diphosphatase (2). If this last enzyme is a rate-limiting step in the photosynthetic carbon reduction cycle, while the rate of electron transport is not limiting (as is the case in strong light), one would expect the observed effects of pyrophosphate. b) A second, highly speculative explanation could be that the pyrophosphate effects would be due to the fact that reduced ferredoxin can be used directly as the reductant for CO₂ assimilation. In this case, inhibition by pyrophosphate of NADP reduction would prevent the loss of reduced ferredoxin (and the drop of electrochemical potential from -0.43 to -0.32 volts) and stimulate CO₂ assimilation, on the assumption that the direct utilization of ferredoxin as a reductant for CO₂ is not inhibited by pyrophosphate.

Acknowledgment

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Literature Cited