Inhibition of the Photosynthetic Activities of Isolated Spinach Chloroplasts by Phosphonate Compounds

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ABSTRACT

The effects of three closely related phosphate compounds on several photosynthetic activities of isolated chloroplasts were investigated. Phosphonoformic and phosphonoacetic acid were found to inhibit both CO2 fixation and the reduction of 3-phosphoglyceric acid, with CO2 fixation being more sensitive. In contrast, phosphonoacetic acid was only slightly inhibitory. The lack of inhibition appeared to be due to its inability to enter the stroma via the phosphate translocator. Measurements of changes in stromal metabolite levels following the inhibition of CO2 fixation by either phosphonoformic or phosphonoacetic acid indicated that the activity of ribulose bisphosphate carboxylase/oxygenase was reduced. Studies with the isolated enzyme confirmed that both of these compounds were effective competitive inhibitors of the carboxylase activity of the enzyme.

Considerable information is available on the inhibition of photosynthesis by inhibiting either electron transport (16) or photophosphorylation (12). Compounds that specifically inhibit enzymes involved in carbon metabolism are much less common. D,L-Glyceraldehyde inhibits the conversion of triose phosphate into RuBP by affecting the activity of phosphoribulokinase, but may also have other sites of action (24, 25). Halaocetal phosphates, which are structurally similar to dihydroxyacetone-P, can inactivate triose-P isomerase (9) but iodoacetol-P was found to irreversibly inactivate glyceraldehyde 3-P dehydrogenase at concentrations inhibitory for isolated chloroplasts (27). Xylolose 1,5-bisP and carboxyarabinol bisP are potent inhibitors of ribulose because of their similarity to RuBP (15, 19), but are not effective inhibitors with isolated chloroplasts because they cannot readily cross the outer envelope membranes. 4,4-Diisothiocyanato-2,2'-disulfonic acid stilbene was recently shown to inhibit the phosphate translocator and PGA reduction by isolated chloroplasts (22). Less specific inhibitors include iodoacetate and iodoacetamide that inhibit many enzymes by reacting with sulfhydryl groups (28) and sulfite which inhibits both ribulose and photophosphorylation (3). Finally, inhibition of the photorespiratory pathway with compounds like aminocetonictrile (5), or the absence of one of the pathway enzymes in mutant Arabidopsis plants (4), inhibits photosynthesis under photorespiratory conditions by reducing the activation of rubisco.

Phosphonates are analogs of naturally occurring phosphate esters (7) and therefore could be good candidates for being inhibitors of various enzyme activities (7, 8). For example, a phosphonate with similarities to dihydroxyacetone-P is actually a substrate for aldolase and glycerol-3-P dehydrogenase (26) and an analog of PGA is a substrate for phosphoglycerate kinase and glyceraldehyde 3-P dehydrogenase (17). Moreover, a well known phosphonate, N,N-bis-(phosphonomethyl)glycine, commonly known as glyphosate, is a potent herbicide that affects the synthesis of various aromatic amino acids (21).

The effects of phosphate compounds on isolated chloroplast photosynthesis have not been extensively studied. Perchorowicz et al. (18) fed high concentrations of PPA, an isosteric analog of 2-phosphoglycerate, to detached leaves through the stem and found that it inhibited photosynthesis but increased the activation state of rubisco. Hatch (10) also studied the effect of a few phosphonates as effectors of the activation of isolated rubisco and their ability to exchange with stromal phosphate. In this report, we investigated and compared the effects of a series of phosphonates on various photosynthetic activities of isolated chloroplasts and as a result of these studies also briefly examined their effects on the activity of the isolated rubisco enzyme.

MATERIALS AND METHODS

Plant Material. Spinach (Spinacia oleracea L., American Hybrid No. 424) was grown hydroponically under artificial light as described previously (20). Intact chloroplasts were obtained from fully expanded leaves following the procedure described by Salvucci et al. (23).

Chemicals. Radioactive sodium bicarbonate was obtained from New England Nuclear. RuBP was synthesized (13) from ribose 5-P (Sigma), PPA, PFA, and PAA were obtained from Alpha Products. Silicone oils were obtained from SWS Silicones Corp., Adrian, MI. 2-Hydroxy-4-phosphonobutyric acid was the kind gift of H. B. F. Dixon.

Rubisco Purification. The enzyme was isolated from stromal extracts of isolated chloroplasts as described by Salvucci et al. (23).

Carboxylase Assay. Carboxylase activity was assayed at 25°C by following 14CO2 incorporation into acid-stable products. The assay medium contained 50 mm tricine-NAOH (pH 8), 10 mm MgCl2, 10 mm NaHCO3 (1 μCi 14C/μmol), 4 mm 2-mercaptoethanol, and 0.4 mm RuBP in a final volume of 0.5 ml. The reaction was initiated with 10 μg ribulose previously fully activated and terminated after 30 s by the addition of 4 N formic acid in 1 N

1 On leave from A.R.O., The Volcani Center, Bet Dagan, Israel.
2 Abbreviations: RuBP, ribulose 1,5-bisphosphate; ribulose, ribulose-1,5-bisphosphate carboxylase/oxygenase; PFA, phosphonooformic acid; PAA, phosphonoacetic acid; PPA, phosphonopropionic acid; PGA, 3-phosphoglyceric acid.

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HCl. Samples were dried overnight at 60°C and radioactivity determined by scintillation spectroscopy. The inhibition by phosphonates was determined over the range of 0.5 to 8 mM. The RuBP concentrations for determining the Km were over the range of 10 to 400 μM.

**CO₂ Fixation.** Isolated chloroplasts (30 μg Chl) were added in the dark to the assay medium which contained 0.33 mM sorbitol, 50 mM Hepes-NaOH (pH 7.8), 5 mM NaHCO₃ (0.2 μCi ¹⁴C/μmol), 0.3 or 1 mM PGA, 1 or 3 mM Pi and 172 IU catalase in a final volume of 1 ml. When present, the phosphonate concentration was between 2 to 10 mM. After 2 min of dark preincubation the samples were illuminated (500 μE m⁻² s⁻¹, time 0) and aliquots were removed at appropriate times and quenched with acid.

**Backexchange of ³²P.** Chloroplasts equivalent to 1.5 mg Chl were incubated with 2 mM Pi and 20 μCi ³²P in 1 ml (0.33 mM sorbitol, 50 mM Hepes [pH 7.6]) for 5 min at 0°C. Five ml of the same buffer were added and the mixture centrifuged for 2 min at 900g. The supernatant was removed and the pellet was resuspended in a small amount of the same buffer. Chloroplasts were diluted to 0.1 mg chloroplast/ml and layered on 100 μl silicone oil (AR20:AR200, 1:4) which overlayed 20 μl 10% HClO₄. Pi or the various phosphonates were rapidly mixed into the suspension, and after 10 s the chloroplasts were centrifuged through the oil into the acid with a Beckman microfuge. Aliquots of the incubation mixture and the pellet were assayed for ³²P.

**O₂ Evolution.** CO₂ and PGA-dependent O₂ evolution of chloroplasts was measured with a Clark-type O₂ electrode (Hansatech, Norfolk, UK) at 25°C with illumination by 1400 μE m⁻² s⁻¹ white light. The assay medium contained 0.33 mM sorbitol, 50 mM Hepes-NaOH (pH 7.8), 172 IU catalase, Pi and PGA concentrations as indicated, and 75 μg Chl in a final volume of 1 ml. When CO₂ fixation was followed, 10 mM NaHCO₃ was added to the assay mixture.

**Measurement of Stromal Metabolite Levels.** Stromal metabolites were labeled with ³²Pi, separated from external metabolites and quantitated by HPLC as previously described (2).

**RESULTS AND DISCUSSION**

Three commercially available phosphonates, which differ only in the number of carbons between the phosphorous and carboxylate groups, were selected for initial study. Phosphonofluoridic acid, the simplest, was the most effective inhibitor of the CO₂ fixation activity of isolated chloroplasts (Fig. 1A). In the presence of 0.3 mM PGA and 1 mM Pi, only 50% of the control activity remained with a concentration of 2 mM PFA and almost complete inhibition occurred with 10 mM PFA. A slightly larger phosphonate, PAA, was almost without effect as it inhibited CO₂ fixation by only 16% at 10 mM under similar conditions. However, an even larger phosphonate, PPA, was moderately inhibitory as it reduced CO₂ fixation by 18 and 84% at concentrations of 2 and 10 mM, respectively. We also tested the effect of three other phosphonates: methyl phosphonic acid (no carboxylate moiety), phosphonobutyric acid, and 2-hydroxy-4-phosphono-butyric acid, an analog to PGA (6). These compounds were about as ineffective as PAA and were not investigated further. These results indicated that small changes in structure markedly changed the effectiveness of these compounds in inhibiting CO₂ fixation.

The presence of higher external concentrations of PGA and Pi, which would more closely simulate conditions in vivo, reduced the ability of these compounds to inhibit CO₂ fixation (Fig. 1B). At 1 mM PGA and 3 mM Pi, CO₂ fixation was inhibited by only 27% at 2 mM and 82% at 10 mM PFA. Similarly, only a 40% inhibition was observed by 10 mM PPA under these conditions as compared to 84% obtained with the lower concentrations of PGA and Pi. These results indicate that the inhibition of CO₂ fixation appears to be competitive in nature and that large amounts of these phosphonates would have to be present in the cytoplasm of leaf cells to be effective at the whole plant level.

Since phosphonates are analogs of phosphate and phosphate esters, it is obvious that the large differences in the effectiveness of the phosphonates in inhibiting CO₂ fixation might be due to one or more of several factors. They may differ in their ability to inhibit the translocation of phosphorylated intermediates between the medium and the stroma. They may differ in their ability to enter the stroma. Finally, if they can enter the stroma, they may differ in their ability to interfere with carbon metabolism.

The transport and access of Pi and other phosphorylated intermediates to the stroma is controlled at the chloroplast envelope by a specific protein known as the phosphate translocator (11). Of all the phosphorylated intermediates in the stroma, only Pi, PGA, and dihydroxyacetone-P are transported rapidly and with high affinity. The effect of the phosphonates on Pi transport was examined by measuring their effects on ³²Pi uptake, and their ability to enter the stroma was examined by backexchange in chloroplasts preloaded with ³²Pi. All three appeared to competitively inhibit Pi uptake at 4°C when present at a concentration of 2 mM (data not shown). A detailed kinetic analysis was not undertaken, since almost no transport into the stroma (measured as backexchange of Pi) could be measured at this temperature. When measured at 25°C, the same temperature as used in the CO₂ fixation experiments, a reasonable rate of transport in exchange for Pi could be measured (Fig. 2). When added at a concentration of 2 mM, PFA and PPA were transported at initial
Inhibition by Phosphonates

INHIBITION BY PHOSPHONATES

Fig. 2. Time course of stromal Pi release into the medium upon addition of 2 mM Pi (●), PFA (△), PPA (○), and PAA (□) to intact chloroplasts at 25°C.

![Graph showing time course of stromal Pi release](image)

Fig. 3. The concentration dependence of backexchange of 32Pi with PFA (△), PPA (○), and PAA (□) at 25°C. Extent of release 10 s after addition is shown. Lines represent calculated values using estimated kinetic constants of $V_{\text{max}} = 69 \pm 5 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{Chl} \cdot \text{h}^{-1}$ and $K_m = 1.05 \pm 0.15 \text{mm}$ for PPA; $V_{\text{max}} = 77 \pm 11 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{Chl} \cdot \text{h}^{-1}$ and $K_m = 2.44 \pm 0.36 \text{mm}$ for PFA.

rates of about 11 and 20 $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{Chl} \cdot \text{h}^{-1}$ and equilibrium reached after 1 to 2 min. The entry of PAA was less than 1 $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{Chl} \cdot \text{h}^{-1}$. The concentration dependence of the exchange of these phosphonates was examined in more detail and is shown in Figure 3. PPA exchanged more rapidly at all concentrations with a calculated $V_{\text{max}}$ of 69 $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{Chl} \cdot \text{h}^{-1}$ and a $K_m$ of 1.0 mm. PFA had slightly higher $V_{\text{max}}$ of 77 $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{Chl} \cdot \text{h}^{-1}$ but a much higher $K_m$ of 2.4 mm. The exchange of PAA was very low at all concentrations examined. From these data it is clear that the inability of PFA to inhibit CO$_2$ fixation is due to its inability to enter the stroma via the phosphate translocator. Since both PPA and PFA entered the chloroplasts to nearly the same extent after 2 min (Fig. 2), the greater effectiveness of PFA in inhibiting CO$_2$ fixation must be related to a greater effect on one or more of the stromal enzymes.

The inhibition of CO$_2$ fixation by these phosphonates was further characterized by examining their effects on PGA reduction, which involves only three stromal enzymes. Because of the slow exchange rate, the chloroplasts were incubated for 2 min with the phosphonate in the dark before the reaction mixture was illuminated. Both PGA and Pi were present in these experiments to ensure stromal ATP/ADP was not reduced by Pi limitation (14).

Concentrations of 6 mM PFA and 10 mM PPA were selected since they severely inhibited CO$_2$ fixation at low Pi and PGA concentrations (Fig. 1A). With only 1 mM Pi and 0.3 mM PGA, PGA-dependent O$_2$ evolution was reduced to 15 to 16% in both cases (Table I). Increasing the PGA concentration progressively reduced the inhibition such that with 1 mM Pi and 3 mM PGA the rates were 60% of the control with PFA and 86% with PPA. Conversely, increasing the Pi concentration from 1 to 3 mM with PGA maintained at 1 mM, did not significantly alleviate the inhibition by either PFA or PPA.

The effect of these two phosphonates on O$_2$ evolution in the presence of HCO$_3$ was also determined in the same experiment (Table I). In all cases, significantly more inhibition occurred as compared to when HCO$_3$ was absent. This result suggested that CO$_2$ fixation was more sensitive to the presence of the phosphonates than was PGA reduction alone. Other experiments with 6 mM PFA (data not shown) in which both O$_2$ evolution and $^14$CO$_2$ fixation were followed, indicated that CO$_2$ fixation could be inhibited by over 90% with only about 65% inhibition of PGA reduction.

The ability to alleviate the inhibition of PGA reduction with increasing the PGA concentration is consistent with an effect of both PFA and PPA on the PGA kinase reaction. However, the greater inhibition of CO$_2$ fixation indicated that these compounds must also inhibit one or more of the enzymes involved in the conversion of dihydroxyacetone phosphate to RuBP and/or the carboxylation reaction itself. To identify possible sites of inhibition leading to reductions in CO$_2$ fixation that could not be attributed to PGA reduction alone, we examined the changes in stromal intermediates following the addition of either PFA or PPA to intact chloroplasts actively fixing CO$_2$ (Table II). In this experiment both PPA and PFA reduced the instantaneous rate of CO$_2$ fixation by about 30% at the time the samples were taken for analysis (i.e. 3 min 45 s). As compared to either the control or the levels measured before the addition of the phosphonate, total stromal Pi and the level of all intermediates except RuBP and ATP declined, reflecting the entry of the phosphonates in exchange for PGA, dihydroxyacetone phosphate, and Pi. The increased level of RuBP indicated a greater reduction in the activity of rubisco relative to the rate of RuBP regeneration. Presumably, PGA reduction was also inhibited following the addition of the phosphonate (Table I). However, the PGA level declined, as would be expected if the phosphonates had a greater effect on

Table I. Inhibition of O$_2$ Evolution by PFA and PPA in the Presence and Absence of Bicarbonate

Experimental conditions as described in "Materials and Methods." Control rates varied between 71 to 91 $\mu$mol O$_2$ mg$^{-1}$ Chl h$^{-1}$ without HCO$_3$ and 114 to 128 with HCO$_3$.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Percent of Control Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>No HCO$_3^-$</td>
<td>+ 6 mM PFA</td>
</tr>
<tr>
<td>1 mM Pi + 0.3 mM PGA</td>
<td>16</td>
</tr>
<tr>
<td>1 mM Pi + 1 mM PGA</td>
<td>45</td>
</tr>
<tr>
<td>1 mM Pi + 3 mM PGA</td>
<td>60</td>
</tr>
<tr>
<td>3 mM Pi + 1 mM PGA</td>
<td>49</td>
</tr>
<tr>
<td>+ 10 mM HCO$_3^-$</td>
<td>1 mM Pi + 0.3 mM PGA</td>
</tr>
<tr>
<td>1 mM Pi + 1 mM PGA</td>
<td>28</td>
</tr>
<tr>
<td>1 mM Pi + 3 mM PGA</td>
<td>53</td>
</tr>
<tr>
<td>3 mM Pi + 1 mM PGA</td>
<td>31</td>
</tr>
</tbody>
</table>
Table II. Effect of Addition of PFA or PPA on Stromal Metabolite Levels

Chloroplasts (70 μg Chl) were added to medium which contained 0.33 mM sorbitol, 50 mM Hapes-NaOH (pH 7.8), 10 mM NaHCO₃, 0.4 mM Pi (30 μCi ³²P/μmol), 0.1 mM EDTA and 170 units catalase in a final volume of 1 ml and incubated for 5 min in the dark. Samples were removed for assay at 2:30 and 3:45 min after illumination. PFA and PPA were added immediately after the 2:30 sample was removed. Stromal metabolites were determined as described in “Materials and Methods.” Measurements of O₂ evolution in separate samples indicated a reduction of about 30% in the instantaneous rate at 3:45 in both cases, as compared to the control (95 μmol mg⁻¹ Chl h⁻¹).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>+2 mM PFA</th>
<th>+2 mM PPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar monophosphates</td>
<td>2:30</td>
<td>3:45</td>
<td>2:30</td>
</tr>
<tr>
<td>Triose-P</td>
<td>2:30</td>
<td>3:45</td>
<td>2:30</td>
</tr>
<tr>
<td>Pi</td>
<td>2:30</td>
<td>3:45</td>
<td>2:30</td>
</tr>
<tr>
<td>PGA</td>
<td>2:30</td>
<td>3:45</td>
<td>2:30</td>
</tr>
<tr>
<td>Sugar bisphosphates</td>
<td>2:30</td>
<td>3:45</td>
<td>2:30</td>
</tr>
<tr>
<td>RuBP</td>
<td>2:30</td>
<td>3:45</td>
<td>2:30</td>
</tr>
<tr>
<td>ATP</td>
<td>2:30</td>
<td>3:45</td>
<td>2:30</td>
</tr>
<tr>
<td>Total stromal Pi</td>
<td>282</td>
<td>303</td>
<td>319</td>
</tr>
</tbody>
</table>

Table III. Effect of Different Phosphate Concentrations on the Kₘ (RuBP)

The phosphonates were added to the assay media and the reaction was initiated with RuBP.

<table>
<thead>
<tr>
<th>Phosphonate Concentration</th>
<th>Kₘ (RuBP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>PFA</td>
</tr>
<tr>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>0.5</td>
<td>73</td>
</tr>
<tr>
<td>1</td>
<td>119</td>
</tr>
<tr>
<td>2</td>
<td>218</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>8</td>
<td>634</td>
</tr>
<tr>
<td>Kₘ (mm)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The rate of PGA production by rubisco. The maintenance of a high ATP level in the phosphate samples indicated that PGA reduction was not limited by an effect of the phosphonates on photophosphorylation, but more likely by effects on PGA kinase as suggested earlier. From this kinetic analysis of the inhibition, carboxylation appears to be the most sensitive site. Perchorowicz et al. (18) in their studies which showed that CO₂ fixation was inhibited by feeding PPA to leaves but that the activation state of the enzyme was increased, inferred that PPA must be reducing the overall activity of the enzyme in vivo, but did not measure RuBP levels.

In order to demonstrate further that rubisco is a major site for the inhibitory effects of these phosphonates on CO₂ fixation, we investigated the effects of these compounds on the activity of the isolated enzyme (Table III). All three compounds increased the apparent Michaelis constant for RuBP at mM concentration but also began to affect the Vₘₐₓ at very high concentrations (not shown). From Dixon plots the apparent Kₛ were calculated to be 0.4 mM for PPA, 1.7 mM for PGA, and 2.2 mM for PAA. The inhibition by these phosphonates is similar to that reported for PGA (0.84 mM) and Pi (0.9 mM) (1). Furthermore, the positive effect of these compounds on activation reported both in vitro (10) and in vivo (18) is unlikely to result in an increase in activity in vivo since the assay of activation involves dilution.

When all of the data that we obtained is considered, it appears that the greater effectiveness of PFA in inhibiting CO₂ fixation as compared to PPA may be due to the fact that it is a good inhibitor of both PGA reduction and rubisco activity. While PPA inhibits rubisco activity better than PFA, it inhibits PGA reduction much less than PFA at equivalent concentrations. Part of the difference in effectiveness may also be due to inhibition at other sites which were not revealed by the types of experiments that we performed. There may also be significant differences in the stromal concentrations of PFA and PPA under the conditions utilized for our CO₂ fixation experiments in that competition between Pi, PGA, and the phosphonate for entry into the stroma exists. We could not determine uptake directly under similar conditions, but could only utilize measurements of backexchange of Pi. A lack of information on the actual stromal concentration of these compounds and the free concentration of RuBP precluded using some of our data more quantitatively to compare with the measured effects of these compounds. PFA does not inhibit CO₂ fixation or PGA reduction very well and this is largely explained by its limited ability to enter the stroma. Furthermore, it was also found to be the least effective in inhibiting rubisco activity.

LITERATURE CITED

3. CERONIC ZG, R KAIZEC; M PLEIMICK 1982 The role of phosphophylylation in SO₂ and SO₃⁻ inhibition of photosynthesis in isolated chloroplasts. Planta 156: 249–254
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