Short Communication

Site-specific Inhibition of Photophosphorylation in Isolated Spinach Chloroplasts by Mercuric Chloride

Received for publication June 4, 1973

DAVID A. BRADEN AND G. DOUGLAS WINGET
Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221
J. MICHAEL GOULD AND DONALD R. ORT
Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Photophosphorylation associated with noncyclic electron transport in isolated spinach (Spinacia oleracea) chloroplasts is inhibited to approximately 50% by low concentrations of HgCl₂ (less than 1 μmole Hg²⁺/mg chlorophyll) when the electron transport pathway includes both sites of energy coupling. Reactions involving only a part of the electron transport system can give a functional isolation of at least two sites coupled to phosphorylation. Only one of these sites, located between the oxidation of plastoquinone and the reduction of cytochrome f, is sensitive to mercuric chloride. The energy conservation site located before plastoquinone and close to photosystem II is unaffected by HgCl₂ concentrations up to 10-fold those required to inhibit phosphorylation by the coupling site after plastoquinone. This site-specific inhibition may reflect a mechanistic difference in the mode of energy coupling at the two coupling sites or a variable accessibility of HgCl₂ to these sites.

Concentrations of HgCl₂, which inhibit steady state phosphorylation, do not inhibit dark phosphorylation after illumination (Xₑ), suggesting that HgCl₂ affects a step in the coupling mechanism prior to the terminal step of ATP formation.

Recent data from several laboratories indicate that there are at least two sites of energy coupling associated with noncyclic electron transport in isolated chloroplasts (5–7, 11, 17–20). By utilizing various electron donor-acceptor systems in conjunction with several new inhibitors of electron transport, these energy-coupling sites can be functionally isolated and characterized. One site closely associated with photosystem II (5), differs in several respects from a second, well recognized site coupled to electron transport from plastoquinone to cytochrome f (3). The photosystem II-dependent energy coupling site exhibits no control over coupled electron transport and has a P/εₑ ratio of about 0.4 (5–7, 17, 18). Furthermore, this P/εₑ ratio is practically pH independent (5, 6). On the other hand, the coupling site between plastoquinone and cytochrome f, which is the rate-limiting step for the Hill reaction, exhibits control over electron transport and has a pH-dependent P/εₑ ratio of about 0.6 (pH 8.0–8.5) (6). Because of the apparent differences in the characteristics of the two coupling sites, it was of interest to study the effect of specific inhibitors of the phosphorylation reaction (energy transfer inhibitors) on each coupling site. In this communication, we report that the energy transfer inhibitor HgCl₂ (11) specifically inhibits ATP formation supported by the coupling site between plastoquinone and cytochrome f while not affecting ATP formation supported by the coupling site close to photosystem II.

MATERIALS AND METHODS

Spinach (Spinacia oleracea) chloroplasts were prepared as described previously (21), except TES-NaOH buffer replaced Tricine in the isolation media, since Tricine strongly binds Hg. Reactions were run in a thermostatted vessel at 19°C using strong light (>400 kerg/cm²·sec). In all cases, the chloroplasts were incubated with HgCl₂ for 30 sec before the addition of donors, acceptors, or inhibitors.

Electron transport using oxidized p-phenylenediamines, substituted p-benzoquinones, or ferricyanide as the electron acceptor was followed spectrophotometrically, as described elsewhere (19). MV reduction was measured as oxygen uptake (10) with a Clark-type membrane-covered electrode. ATP formation was assayed using a modified procedure of Avron (2). Radioactivity was measured using the Cerenkov technique of Gould et al. (4).

RESULTS AND DISCUSSION

Figure 1 shows the effect of low concentrations of HgCl₂ on electron transport and phosphorylation using three different electron donor-acceptor systems. As previously reported (11), the over-all Hill reaction with ferricyanide (Fig. 1A) or MV (Table I) as the electron acceptor is inhibited in direct proportion to the added HgCl₂ up to approximately 35 to 40 μmoles of HgCl₂/mg Chl. Although very high concentrations of HgCl₂ (greater than 1 μmole/mg Chl) result in nonspecific electron

difficulties with the addition of donors, acceptors, or inhibitors.

Recent data from several laboratories indicate that there are at least two sites of energy coupling associated with noncyclic electron transport in isolated chloroplasts (5–7, 11, 17–20). By utilizing various electron donor-acceptor systems in conjunction with several new inhibitors of electron transport, these energy-coupling sites can be functionally isolated and characterized. One site closely associated with photosystem II (5), differs in several respects from a second, well recognized site coupled to electron transport from plastoquinone to cytochrome f (3). The photosystem II-dependent energy coupling site exhibits no control over coupled electron transport and has a P/εₑ ratio of about 0.4 (5–7, 17, 18). Furthermore, this P/εₑ ratio is practically pH independent (5, 6). On the other hand, the coupling site between plastoquinone and cytochrome f, which is the rate-limiting step for the Hill reaction, exhibits control over electron transport and has a pH-dependent P/εₑ ratio of about 0.6 (pH 8.0–8.5) (6). Because of the apparent differences in the characteristics of the two coupling sites, it was of interest to study the effect of specific inhibitors of the phosphorylation reaction (energy transfer inhibitors) on each coupling site. In this communication, we report that the energy transfer inhibitor HgCl₂ (11) specifically inhibits ATP formation supported by the coupling site between plastoquinone and cytochrome f while not affecting ATP formation supported by the coupling site close to photosystem II.

MATERIALS AND METHODS

Spinach (Spinacia oleracea) chloroplasts were prepared as described previously (21), except TES-NaOH buffer replaced Tricine in the isolation media, since Tricine strongly binds Hg. Reactions were run in a thermostatted vessel at 19°C using strong light (>400 kerg/cm²·sec). In all cases, the chloroplasts were incubated with HgCl₂ for 30 sec before the addition of donors, acceptors, or inhibitors.

Electron transport using oxidized p-phenylenediamines, substituted p-benzoquinones, or ferricyanide as the electron acceptor was followed spectrophotometrically, as described elsewhere (19). MV reduction was measured as oxygen uptake (10) with a Clark-type membrane-covered electrode. ATP formation was assayed using a modified procedure of Avron (2). Radioactivity was measured using the Cerenkov technique of Gould et al. (4).

RESULTS AND DISCUSSION

Figure 1 shows the effect of low concentrations of HgCl₂ on electron transport and phosphorylation using three different electron donor-acceptor systems. As previously reported (11), the over-all Hill reaction with ferricyanide (Fig. 1A) or MV (Table I) as the electron acceptor is inhibited in direct proportion to the added HgCl₂ up to approximately 35 to 40 μmoles of HgCl₂/mg Chl. Although very high concentrations of HgCl₂ (greater than 1 μmole/mg Chl) result in nonspecific electron
transport inhibition (11, 13), the low levels of HgCl₂ used here (less than 1 μmole/mg Chl) inhibit ATP formation (and that portion of the electron transport dependent on phosphorylation) to a plateau of approximately 50%. Contrary to the results of Miles et al. (16), however, we find the same degree of inhibition by HgCl₂ when electron transport is measured spectrophotometrically or as oxygen evolution. Neither basal (−P) nor uncoupled (+methylamine) electron transport is significantly affected by HgCl₂, indicating that HgCl₂ does act as an energy-transfer inhibitor rather than an electron-transport inhibitor at these concentrations (11). Chloroplasts which have been uncoupled by EDTA treatment, which removes C₇ (1), are also insensitive to HgCl₂.

DCIPH₂ (in the presence of DCMU and ascorbate) donates electrons at a point before the rate-limiting coupling site on the electron transport chain (i.e. before cytochrome f) (6, 9, 15). It has recently been shown that the photosystem I-dependent partial reaction DCIPH₂ → MV includes only the rate-limiting coupling site after plastoquinone and not the coupling site before plastoquinone (6). Figure 1B shows that HgCl₂ affects the partial reaction DCIPH₂ → MV and the over-all reaction H₂O → FeCy similarly, indicating that both pathways include the HgCl₂-sensitive site. Moreover, since this coupling site constitutes the primary rate-limiting step for both electron transport reactions, a similar 50% sensitivity to HgCl₂ should be observed for both systems (compare Fig. 1, A and B).

However, when electrons from water reduce lipophilic acceptors (class III acceptors [19]), such as oxidized p-phenylenediamine (PD₃), a different effect of HgCl₂ is observed. These lipophilic oxidants (in the presence of the plastocyanin inhibitors KCN or poly-L-lysine (17) or the plastocyanin antagonist dibromothymoquinone (12, 20)) accept electrons at a point in the electron transport chain before plastoquinone (5). Thus the partial reaction H₂O → PD₃ includes the photosystem II-dependent phosphorylation site, but not the rate-determining site after plastoquinone. As Figure 1C shows, electron transport and phosphorylation associated with the partial reaction H₂O → PD₃ is completely insensitive to HgCl₂ inhibition. Several other class III acceptors were also tested for HgCl₂ inhibition with similar results (Table I).

The absence of inhibition by HgCl₂ with class III electron acceptors is probably not a result of fortuitous reaction conditions that mask the inhibition. In all cases, chloroplasts were incubated with HgCl₂ for 30 sec before the addition of the acceptor system. Since SH-compounds can reverse HgCl₂ inhibition (11), it seems likely that Hg²⁺ is reacting with a membrane sulfhydryl group. Thus it is unlikely that binding between Hg⁴⁺

![Image](https://via.placeholder.com/150)

**Fig. 1. Effect of HgCl₂ on electron transport (E.T.) and phosphorylation associated with various electron transport pathways.** The reaction mixture (2 ml) contained 50 mM HEPPS-NaOH buffer (pH 8.2), 2 mM MgCl₂, 0.1 mM sucrose, 1 mM ADP, 5 mM NaH₂PO₄, chloroplasts containing 40 μg of chlorophyll, and the indicated donor-acceptor system. These systems were: A, 0.4 mM ferricyanide; B, 0.4 mM DCIPH₂, 2.5 mM l-ascorbate and 50 μM MV; C, 0.5 mM p-phenylenediamine (PD₃) plus 1.4 mM ferricyanide. When added, methylamine was 10 mM. In the DCIPH₂ → MV system (B), 1 μM DCMU was added to block electron transport from photosystem II. When PD₃ was the electron acceptor (C), 0.5 mM DBMIB was added to block the photosystem I component of PD₃ reduction (1). Note that only the H₂O → PD₃ system, which does not utilize the rate-determining coupling site after plastoquinone, is insensitive to inhibition by HgCl₂. Rates of electron transport and ATP formation are given in μmoles/hr·mg chlorophyll.

**Table I. Effect of HgCl₂ on Photophosphorylation in Spinach Chloroplasts with Various Electron Acceptors**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Electron Acceptor</th>
<th>Phosphorylation Rate</th>
<th>Micromoles HgCl₂ added/mg Chl</th>
<th>μmoles ATP/hr·mg Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td>0.05</td>
</tr>
<tr>
<td>1</td>
<td>MV</td>
<td></td>
<td>200</td>
<td>102</td>
</tr>
<tr>
<td>2</td>
<td>DBMIB</td>
<td></td>
<td>47</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>DMQ</td>
<td></td>
<td>71</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>DADₑₓ</td>
<td></td>
<td>217</td>
<td>200</td>
</tr>
</tbody>
</table>

transport inhibition (11, 13), the low levels of HgCl₂ used here (less than 1 μmole/mg Chl) inhibit ATP formation (and that portion of the electron transport-dependent on phosphorylation) to a plateau of approximately 50%. Contrary to the results of Miles et al. (16), however, we find the same degree of inhibition by HgCl₂ when electron transport is measured spectrophotometrically or as oxygen evolution. Neither basal (−P) nor uncoupled (+methylamine) electron transport is significantly affected by HgCl₂, indicating that HgCl₂ does act as an energy-transfer inhibitor rather than an electron-transport inhibitor at these concentrations (11). Chloroplasts which have been uncoupled by EDTA treatment, which removes C₇ (1), are also insensitive to HgCl₂.

DCIPH₂ (in the presence of DCMU and ascorbate) donates electrons at a point before the rate-limiting coupling site on the electron transport chain (i.e. before cytochrome f) (6, 9, 15). It has recently been shown that the photosystem I-dependent partial reaction DCIPH₂ → MV includes only the rate-limiting coupling site after plastoquinone and not the coupling site before plastoquinone (6). Figure 1B shows that HgCl₂ affects the partial reaction DCIPH₂ → MV and the over-all reaction H₂O → FeCy similarly, indicating that both pathways include the HgCl₂-sensitive site. Moreover, since this coupling site constitutes the primary rate-limiting step for both electron transport reactions, a similar 50% sensitivity to HgCl₂ should be observed for both systems (compare Fig. 1, A and B).

However, when electrons from water reduce lipophilic acceptors (class III acceptors [19]), such as oxidized p-phenylenediamine (PD₃), a different effect of HgCl₂ is observed. These lipophilic oxidants (in the presence of the plastocyanin inhibitors KCN or poly-L-lysine (17) or the plastocyanin antagonist dibromothymoquinone (12, 20)) accept electrons at a point in the electron transport chain before plastoquinone (5). Thus the partial reaction H₂O → PD₃ includes the photosystem II-dependent phosphorylation site, but not the rate-determining site after plastoquinone. As Figure 1C shows, electron transport and phosphorylation associated with the partial reaction H₂O → PD₃ is completely insensitive to HgCl₂ inhibition. Several other class III acceptors were also tested for HgCl₂ inhibition with similar results (Table I).

The absence of inhibition by HgCl₂ with class III electron acceptors is probably not a result of fortuitous reaction conditions that mask the inhibition. In all cases, chloroplasts were incubated with HgCl₂ for 30 sec before the addition of the acceptor system. Since SH-compounds can reverse HgCl₂ inhibition (11), it seems likely that Hg²⁺ is reacting with a membrane sulfhydryl group. Thus it is unlikely that binding between Hg⁴⁺

![Image](https://via.placeholder.com/150)

**Table II. Effect of HgCl₂ on Postillumination ATP Formation (Xₑ) **

<table>
<thead>
<tr>
<th>Addition (dark stage)</th>
<th>ATP Formed</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>HgCl₂ (20 nmole/mg Chl)</td>
<td>58</td>
<td>9.5</td>
</tr>
<tr>
<td>HgCl₂ (200 nmole/mg Chl)</td>
<td>52</td>
<td>18</td>
</tr>
<tr>
<td>Triphenyltin chloride (10 μM)</td>
<td>8.8</td>
<td>87</td>
</tr>
<tr>
<td>Methylamine hydrochloride (5 mM)</td>
<td>12</td>
<td>81</td>
</tr>
</tbody>
</table>
and the quinonediimides or substituted benzoquinones used here as class III acceptors would be strong enough to reverse the inhibition. Furthermore, UV spectra of DMQ, DAD, PD, and DBMIB remained virtually unchanged in the presence of high concentrations (33 μM) of HgCl₂, again suggesting that little or no binding is occurring. This evidence suggests that HgCl₂ is not interacting chemically with the class III acceptors used here and supports our contention that the energy-coupling site close to photosystem II is insensitive to HgCl₂.

Table II presents evidence that HgCl₂ acts on an early step of energy conservation rather than a terminal reaction of ATP formation. Concentrations of HgCl₂, which strongly inhibit steady state phosphorylation (e.g. H₂O → MV), were demonstrated to have virtually no effect on the chloroplast's ability to synthesize ATP in the dark after brief illumination (Xₑ) (8). However, methylamine, an uncoupler, and triphenyltin chloride, an energy transfer inhibitor which acts on a terminal reaction of ATP formation, decrease the yield of Xₑ significantly (8; unpublished observations of J.M.G.). The idea that HgCl₂ affects an early stage of energy transfer is also supported by the fact that the trypsin activated ATPase activity of CF₁, and whole chloroplasts is not affected by the low levels of HgCl₂ used here (data not shown).

The results presented herein lend strong support to the argument that the two known sites of energy coupling associated with noncyclic electron flow in chloroplasts may exhibit mechanistic differences in their mode of energy transfer (5, 6). It has previously been shown that these coupling sites differ in their response to pH (5, 6), uncouplers, ADP + P, (7), and in phosphorylation efficiency (P/e₅ ratio) (6). In addition, we have now demonstrated that the energy-transfer inhibitor HgCl₂ is specific for the rate-determining coupling site after plastoquinone and before cytochrome f. This selectivity may be due to a variable accessibility of HgCl₂ to the coupling sites, or, alternatively, to a basic difference in the mechanism of the early steps of energy conservation at the two sites.

A discussion of why the HgCl₂ sensitive coupling site is only partially sensitive (50%) to the inhibitor is beyond the scope of this report. In a subsequent publication, a more detailed study of HgCl₂ inhibition of chloroplast reactions will be presented, with emphasis on the nature of HgCl₂ inhibition and the significance of the 50% inhibition plateau.

Acknowledgments—The authors wish to thank S. Izawa for many valuable suggestions.

LITERATURE CITED