INHIBITION OF PHOTOSYSTEM II-DEPENDENT PHOSPHORYLATION IN CHLOROPLASTS BY MERCURIALS

J. Michael Gould

Section of Biochemistry, Molecular and Cell Biology
Cornell University, Ithaca, New York 14853

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Summary

Photophosphorylation supported by the coupling site associated with Photosystem II electron transport (coupling site II) is 50 to 60 times less sensitive to the energy transfer inhibitor $HgCl_2$ than phosphorylation supported by the coupling site associated with Photosystem I electron transport (coupling site I). Coupling site II phosphorylation is only about 2 times less sensitive to the lipophilic mercurial p-hydroxymercuribenzoate (PHMB), however. Both coupling sites are equally sensitive to CF_1 antiserum. These results suggest that a portion of the energy conserving apparatus associated with coupling site II is in a more hydrophobic environment than the corresponding apparatus associated with coupling site I.

Introduction

The two energy conservation sites associated with the chloroplast electron transport chain exhibit different characteristics under certain circumstances. For example, the phosphorylation efficiency (P/e_2) attributable to the coupling site associated with Photosystem II electron transport (site II) is virtually independent of the medium pH over a wide range [1,2], whereas the P/e_2 attributable to the coupling site associated with Photosystem I electron transport (site I) is strongly dependent on the medium pH [2,3]. That is to say, the medium pH can act as an energy transfer inhibitor which is specific for coupling site I phosphorylation [15]. Similarly, low concentrations of the energy

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPPS, N-2-hydroxyethylpiperazine-N'-propanesulfonic acid; MV, methylviologen; DAD, diaminodurene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone.

transfer inhibitor $HgCl_2$ have been reported to specifically inhibit ATP formation associated with coupling site I [4]. These results can be taken to indicate that: (a) the energy transducing mechanisms at the two coupling sites are somehow different, or (b) the energy transducing reactions at site II are in an environment which is less accessible to the aqueous medium than the corresponding reactions at site I.

In an attempt to distinguish between these alternatives, the effects of $HgCl_2$ on phosphorylation associated with coupling site II have been reexamined. It was found that site II driven phosphorylation is in fact sensitive to $HgCl_2$ in a manner similar to site I driven phosphorylation, although much higher concentrations of $HgCl_2$ are required to inhibit site II driven phosphorylation. This and other evidence support the second alternative described above.

Materials and Methods

Chloroplasts were isolated from fresh market spinach as described elsewhere [3]. Electron transport and ATP formation were assayed by previously published techniques [2]. Coupling site II was assayed as the phosphorylation associated with the Photosystem II-dependent reduction of lipophilic acceptors such as oxidized diaminodurene (DAD_{ox}), oxidized p-phenylenediamine (PD_{ox}) [2] and DBMIB [1]. When DAD_{ox} or PD_{ox} was the acceptor a low concentration of DBMIB (0.5 μM) was added to block the Photosystem I dependent portion of the reduction [5]. Coupling site I was assayed as the phosphorylation associated with the Photosystem I-dependent oxidation of diaminodurene (plus ascorbate) in the presence of DCMU [3]. Since site I limits the rate of electron flux through the entire electron transport chain [2], the overall Hill reaction ($H_2O \rightarrow MV$) was also used as a convenient assay for site I.

ADP and p-hydroxymercuribenzoate were purchased from Sigma. $HgCl_2$ was from Allied Chemical. Diaminodurene and p-phenylenediamine (Aldrich) were purified as described elsewhere [3]. The synthesis of 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) was described earlier [5].

Results

Figure 1 shows the effect of $HgCl_2$ on the rate of ATP formation associated with the electron transport pathway $H_2O \rightarrow$ Photosystem II $\rightarrow$ DBMIB which includes only coupling site II [1]. It is clear that, contrary to a previous report [4], phosphorylation associated with coupling site II is sensitive to inhibition by $HgCl_2$. Indeed, phos-

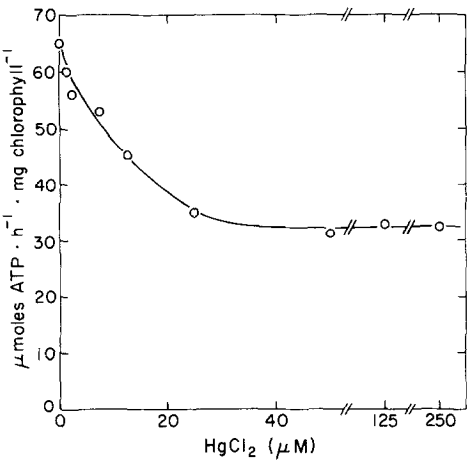

Figure 1. Effect of $HgCl_2$ on the rate of ATP formation associated with electron transport from H_2O to DBMIB. The reaction mixture (1.5 ml) consisted of 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM HEPPS-NaOH (pH 8) 6.7 mM $Na_2H^{32}PO_4$, 1.3 mM ADP, 20 μM DBMIB, 0.67 mM $K_3Fe(CN)_6$ and chloroplasts containing 20 μg chlorophyll. $HgCl_2$ was added in a small volume of water to the chloroplast suspension in the reaction vessel, followed immediately by the remainder of the reaction mixture. Notice that the phosphorylation associated with this electron transport pathway, which is supported by coupling site II only [1], is inhibited to a plateau of about 50%, as already noted for phosphorylation supported by coupling site I only [3,4].

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phorylation supported by both coupling sites exhibits the same 50% inhibition plateau characteristic of $HgCl_2$ inhibition [3,4,6]. However, as Table I shows, the amount of $HgCl_2$ required to obtain 50% inhibition is 50-60 fold higher for site II driven phosphorylation than for site I driven phosphorylation.

It should be pointed out that the very much higher levels of $HgCl_2$ required to inhibit site II driven phosphorylation are not a result of any direct interaction of the inhibitor with components of the reaction mixture, since identical inhibition curves were obtained at several different concentrations of the electron acceptor DBMIB (not shown). Furthermore similar results were obtained when DBMIB was replaced with oxidized p-phenylenediamine (PD_{ox}) as electron acceptor (Table I).

The other components of the reaction mixture were essentially the same for assays of both coupling sites.

The fact that both site I and site II phosphorylation are inhibited in a qualitatively similar manner by $HgCl_2$ suggests that there is probably no mechanistic difference between the two coupling sites. However, the substantial difference in the sensitivities of the two sites to $HgCl_2$ may be due to differences in the environments of the mercurial sensitive component at each site. Indeed when the more lipophilic mercurial p-hydroxymercuribenzoate (PHMB), also an energy transfer inhibitor in chloroplasts [6], is substituted for $HgCl_2$, the difference in sensitivity of the two coupling sites largely disappears (Table I). Both coupling sites are less sensitive to PHMB than to $HgCl_2$, but this probably is due to a lower relative affinity of PHMB for mercurial sensitive components. For instance, PHMB is also a less effective inhibitor of plastocyanin than $HgCl_2$ [6,20], although the relative sensitivities of phosphorylation and electron transport are approximately the same for both inhibitors (phosphorylation being about 10 times more sensitive) [6,11,20].

The apparent difference in the environments of the two coupling sites could result from a specific association of Photosystem II (and therefore coupling site II) with the grana lamellae and Photosystem I (and therefore coupling site I) with the stroma lamellae [7]. This idea was tested by incubating chloroplasts in an isoosmotic medium with rabbit antiserum prepared against chloroplast coupling factor (CF_1). It was found that the inhibition by a given level of antiserum was complete after about 10 minutes incubation at $4^{\circ}C$. As shown in Figure 2a, the anti-CF_1 serum behaved as an energy transfer inhibitor under these conditions. Furthermore, no tendency toward uncoupling or loss of "photosynthetic control" was observed in these unbroken chloroplasts,

TABLE I

EFFECT OF $HgCl_2$ AND p-HYDROXYMERCRIBENZOATE ON

ATP FORMATION ASSOCIATED WITH CHLOROPLAST COUPLING SITES

The reaction mixtures were the same as described in the legend to Figure 2b. When ferricyanide (FeCy) was the electron acceptor, the concentration was 0.5 mM. When oxidized p-phenylenediamine (PD_{ox}) was the electron acceptor, the concentration was 0.67 mM. Chloroplasts were incubated with p-hydroxymecuribenzoate (PHMB) for 4 minutes prior to illumination [6].

Mercurial	Electron Transport System	Coupling site involved	Rate of ATP formation (minus inhibitor)	Concentration giving 50% Inhibition
$HgCl_2$	$H_2O \rightarrow PD_{ox}$	Site II	225	30 µM
$HgCl_2$	DAD → MV	Site I	540	0.5 µM*
$HgCl_2$	H_2O → MV	Site II + Site I**	220	0.5 µM*
$HgCl_2$	H_2O → FeCy	Site II + Site I**	202	0.5 µM*
PHMB	$H_2O \rightarrow DAD_{ox}$	Site II	115	65-70 µM
PHMB	H_2O → MV	Site II + Site I**	131	40-45 µM

* For chloroplasts equivalent to 20 µg chlorophyll per 2 ml. This value is actually dependent upon the amount of chloroplasts added, and is more accurately stated as approximately 40 nmoles $HgCl_2$/mg chlorophyll [3,4,6,11].

** Actually an assay for Site I. see text and ref. 2.

indicating that the integrity of the grana had probably been preserved.* Figure 2b shows clearly that phosphorylation supported by both coupling sites is equally sensitive to the antiserum.

Discussion

Izawa and Good [6] showed that mercurials inhibit coupled electron

*Previous studies using CF_1 antiserum [17,18] have employed broken or swollen chloroplasts, where the grana configurations are largely destroyed [19].

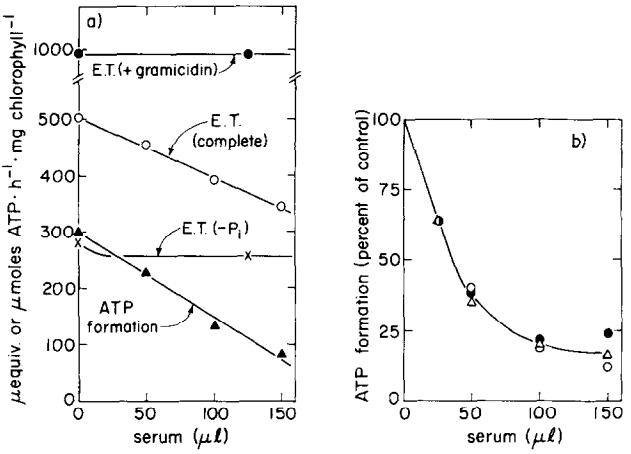

Figure 2. (a) Inhibition of ATP formation and noncyclic electron transport in unswollen chloroplast by rabbit antiserum against chloroplast coupling factor (CF_1). The reaction mixture (2.0 ml) consisted of 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM tricine-NaOH (pH 8.2), 100 μM methylviologen, 1 mM ADP, 5 mM $Na_2H^{32}PO_4$ and chloroplasts containing 40 μg chlorophyll. When added, gramicidin was 4 μg/ml. (b) Effect of rabbit anti-CF_1 serum on ATP formation associated with various electron transport pathways. The reaction mixtures (1.5 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM tricine-NaOH (pH 8.2), 6.7 mM $Na_2H^{32}PO_4$, 1.3 mM ADP, chloroplasts containing 10 μg chlorophyll, and the appropriate electron transport donors and acceptors. These cofactors were: $H_2O \rightarrow$ MV (○), 133 mM methylviologen; $H_2O \rightarrow DAD_{ox}$ (●), 0.67 mM diaminodurene plus 2 mM $K_3Fe(CN)_6$; DAD → MV (△), 2.67 mM diaminodurene, 3.3 mM ascorbate and 133 μM methylviologen. In the $H_2O \rightarrow DAD_{ox}$ system, 0.5 mM DBMIB was added to block the Photosystem I component of DAD_{ox} reduction. In the DAD → MV, system, 1 μM DCMU was added to block the flow of electrons from Photosystem II. Note that phosphorylation supported by all three pathways is equally sensitive to the antiserum. (Control serum taken from the same rabbit prior to immunization had no effect on electron transport or ATP formation in any of the systems studied.) Control (100%) rates of ATP formation (in μmoles ATP·h^{-1}·mg chlorophyll^{-1}) were: $H_2O \rightarrow DAD_{ox}$, 165; and DAD → MV, 992.

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transport and ATP formation in a manner characteristic of energy transfer inhibitors. Energy transfer inhibitors are believed to block phosphorylation by interfering with the terminal enzymatic steps of ATP synthesis, perhaps by binding to the coupling factor or associated membrane proteins (as in mitochondria [8]). Indeed, the amount of $HgCl_2$ required to attain the 50% inhibition plateau at coupling site I in chloroplasts (1 atom Hg^{++}/40-50 chlorophyll molecules or about 10 atoms Hg^{++}/electron

transport chain [9]) is in the same order of magnitude as the number of CF_1 molecules associated with the thylakoid [10].

It has been suggested that $HgCl_2$ and other mercurials inhibit by binding to essential sulfhydryl residues [6,11] since $HgCl_2$ inhibition is relieved by cysteine but not by other chelators of Hg^{++} [6]. Further evidence that a sulfhydryl may be involved comes from the unusual effect of $HgCl_2$ on electron transport in the absence of both ADP and P_i. Under these conditions the rate of electron transport is stimulated by $HgCl_2$ to the level of coupled electron flow in the absence of $HgCl_2$ [6]. The only other energy transfer inhibitor reported which exhibits this property is N-ethylmaleimide (NEM) [12], which reacts with residues on the γ-subunit of CF_1 [13].

Bradeen et al [4] concluded that $HgCl_2$ probably did not inhibit CF_1 directly since the formation of ATP in the dark after an illumination (X_E) [14] was much less sensitive to the inhibitor than steady-state phosphorylation. However, this phenomenon has been observed for other energy transfer inhibitors as well, and probably reflects the introduction of other rate-limiting steps in X_E-type phosphorylation (J. M. Gould, unpublished observations). In any event, it seems likely that mercurials, like other energy transfer inhibitors, interfere with phosphorylation at a point close to the terminal enzymatic processes [6,12,16,21].

The simplest way to explain the coupling site-specific effects of mercurials [11] and pH [2,15] is to assume that there is a complete set of ATP synthesizing enzymes specifically associated with the energy conserving reactions at each coupling site. The apparent coupling site-specific effects can then be understood by postulating that the mercurial and pH sensitive sites are located in some region of the energy conserving complex (i.e. the CF_1 molecule(s) and hydrophobic proteins) which is in a more hydrophobic environment at coupling site II than the corresponding region of the energy conserving complex associated with coupling site I.

This conclusion is supported by the observation that the lipophilic mercurial PHMB is far less site-specific than $HgCl_2$. At least a part of each of the CF_1 molecule(s) associated with each coupling site must be exposed to the aqueous medium, however, since antibody against CF_1 does not exhibit coupling site-specificity.

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