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A PARTIAL REACTION IN PHOTOSYSTEM II: REDUCTION OF SILICOMOLYBDATE PRIOR TO THE SITE OF DICHLOROPHENYLDIMETHYLUREA INHIBITION

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SUMMARY

Silicomolybdate functions as an electron acceptor in a Photosystem II water oxidation (measured as O_2 evolution) partial reaction that is 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) insensitive, that is, reduction of silicomolybdate occurs at or before the level of Q, the primary electron acceptor for Photosystem II. This report characterizes the partial reaction with the principal findings being as follows:

1. Electron transport to silicomolybdate significantly decreased room temperature Photosystem II fluorescence, and DCMU had no effect on the fluorescence level, consistent with silicomolybdate accepting electrons at or before Q.

2. In the absence of DCMU, silicomolybdate is also reduced at a site on the Photosystem I side of the DCMU block, prior to or at plastoquinone, since the plastoquinone antagonist dibromothymoquinone (DBMIB) did not affect the electron transport rate.

3. Electron transport from water to silicomolybdate (+DCMU) is not coupled to ATP formation, nor is there a measurable accumulation of protons within the membrane (measured by amine uptake). Silicomolybdate is not inhibitory to phosphorylation per se since neither cyclic nor post-illumination (X_E) phosphorylation were inhibited.

4. Uncouplers stimulated electron transport from water to silicomolybdate in the pH range of 6 to 7, but inhibited at pH values near 8.

These data are consistent with the view that when electron flow is through the abbreviated sequence of water to Photosystem II to silicomolybdate (+DCMU), conditions are not established for the water protons to be deposited within the membrane.

Experiments reported elsewhere (Giaquinta, R. T., Dilley, R. A. and Horton, P. (1974) J. Bioenerg. 6, 167–177) and these data, are consistent with the hypothesis that electron transport between Q and plastoquinone energizes a membrane confor-

Abbreviations: DBMIB, dibromothymoquinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; SiMo, silicomolybdate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, 4-trifluoromethoxycarbonyl cyanide; DABS, diazonium benzene sulfonate.

mational change that is required to interact with the water oxidation system so as to result in the deposition of water protons either within the membrane itself or within the inner osmotic space.

INTRODUCTION

Considerable information regarding the nature of photosynthetic electron transport and accompanying phosphorylation has been obtained through the study of various partial reactions or redox segments of the electron transfer sequence (c.f. Trebst [1]). Recently the introduction of various lipid-soluble (Class III) oxidants such as dimethylbenzoquinone, diaminodurene, phenylenediamine and dibromothymoquinone (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, DBMIB) [2–7] has allowed the separation and study of Photosystem II electron transfer apart from Photosystem I function. These studies indicate that photophosphorylation is energized by two regions of the electron transfer chain, one associated with each photosystem. While the mechanism of energy transduction is not completely understood, it is widely accepted that proton translocation and accumulation plays a major role [8–12] in the process. This has focused attention on water oxidation as generating the driving force for the Photosystem II energy conservation site through proton deposition on the inside of the grana membrane, while the energization step for the other site is thought to involve proton translocation via the plastoquinone-plastohydroquinone redox couple located between the two photosystems. Hauska et al. [13] suggested that cyclic cofactors such as phenazine methosulfate and pyocyanine generate an "artifical" energy conservation mode because of their ability to vectorially transport protons during their reduction-oxidation cycle, this conservation mode differing from the native plastoquinone-plastohydroquinone redox couple.

Our conceptual scheme for the mechanism whereby a proton motive force (the electrochemical potential gradient of H^+ ions) energizes the membrane centers around the protonation of membrane polyelectrolytes and subsequent conformational changes [10, 14, 15]. Thus, in the Photosystem II energy-conservation site the protons derived from water oxidation may interact with fixed charge groups in the membrane resulting in a conformational change necessary for energy transduction associated with Photosystem II.

More information on the role and nature of water oxidation (O_2 evolution) could be obtained if this process were studied apart from other Photosystem II reactions, that is, electron transfer to lipophilic oxidants encompasses the pathway from water to and including possibly plastoquinone. We have reported [16] that O_2 evolution can be studied using silicomolybdate as an electron acceptor. Electron transport to this acceptor, unlike conventional lipophilic Photosystem II electron acceptors, is largely insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This abbreviated electron transfer sequence seems to involve only the oxygen evolution apparatus, the photochemistry of Photosystem II and possibly the primary acceptor, Q, of Photosystem II. This report characterises the DCMU-insensitive O_2 evolution mediated by silicomolybdate in more detail and discusses the possible role of water oxidation in energy transduction associated with Photosystem II.

METHODS AND MATERIALS

Chloroplasts from *Spinacia oleracea* were isolated as described previously [16] in 0.4 M sucrose, 20 mM Tricine/KOH (pH 7.8), 3 mM $MgCl_2$, 10 mM KCl, 3 mM ascorbate and bovine serum albumin (2 mg/ml final volume). After differential centrifugation the chloroplasts were resuspended in the above medium lacking ascorbate and bovine serum albumin. Electron transport, either O_2 evolution or consumption, was measured polarographically using a YSI oxygen electrode. Photophosphorylation was measured by the incorporation of ^{32}P into ATP according to Neumann et al. [17]. Ferricyanide-supported pH changes [18] and amine uptake [19] were measured according to described techniques. Details of these procedures are given in the figure and table legends.

Chloroplast fluorescence was determined on an Aminco model SPF-125 fluorometer using a 685-nm setting on the detecting side and exciting with 547-nm radiation isolated with a Balzers interference filter.

RESULTS

Effect of silicomolybdate on electron transport

The DCMU insensitivity of O_2 evolution [16] as a function of silicomolybdate (SiMo) concentration is shown in Fig. 1. In the presence of DCMU, SiMo can restore the O_2 evolution rate to approximately 75 % of the control rate, measured from water to ferricyanide. Maximum restoration occurred in the range of 50–150 μM SiMo. We routinely used 25–50 μM SiMo for electron transport studies. The O_2 evolved was derived from Photosystem II water oxidation since no SiMo-mediated O_2 evolution was observed when Tris-treated chloroplasts (lacking water oxidation) were employed.

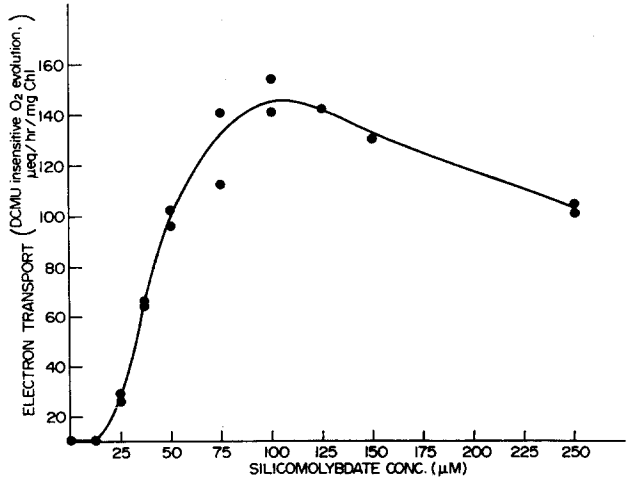

Fig. 1. Effect of SiMo on O_2 evolution in the presence of DCMU. The reaction mixture contained in 2 ml, 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine/KOH (pH 8.2), 0.5 mM ferricyanide, 5 μM DCMU and chloroplasts equivalent to 50 μg chlorophyll. Control rate ($H_2O \rightarrow$ ferricyanide) was 201 μequiv/h per mg chlorophyll. Illumination was with heat filtered white light of $2 \cdot 10^5$ ergs/cm^2 per s intensity. The temperature of the stirred reaction mixture was maintained at 18 °C.

TABLE I

EFFECT OF SILICOMOLYBDATE ON ELECTRON TRANSPORT FROM WATER TO FERRICYANIDE IN THE PRESENCE OF DBMIB

The reaction mixture contained in 2 ml, 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine/KOH (pH 8.0), 0.5 mM ferricyanide (FeCy), and chloroplasts equivalent to 40 μg chlorophyll. The concentration of DCMU was 5 μM, DBMIB, 0.5 μM and SiMo, 33 μM. Illumination was with heat-filtered white light of approximately $2 \cdot 10^5$ ergs/cm^2 per s intensity.

Assay	O_2 evolution (μequiv/h per mg chlorophyll)
1 $H_2O \rightarrow$ FeCy	234
2 $H_2O \rightarrow$ FeCy+DBMIB	130
3 $H_2O \rightarrow$ FeCy+SiMo	388
4 $H_2O \rightarrow$ FeCy+SiMo+DBMIB	367
5 $H_2O \rightarrow$ FeCy+SiMo+DCMU	112

In the absence of DCMU, SiMo stimulated electron transfer from $H_2O \rightarrow$ ferricyanide (Table I). The increased rate of electron transport with SiMo (minus DCMU) was not sensitive to the plastoquinone antagonist, DBMIB (Table I) which prevents the oxidation of reduced plastoquinone [20, 21]. The addition of DCMU does inhibit the increased rate of O_2 evolution caused by SiMo alone, but this DCMU insensitive rate is 50–75 % of the control rate (Table I and Fig. 1). The DCMU and DBMIB results indicate there are two sites of SiMo reduction, one prior to the DCMU inhibition site, at or before the primary acceptor *Q*, and the other site between Q and the DBMIB-inhibition site (probably plastoquinone [20]).

The pH dependence of SiMo-catalyzed O_2 evolution is shown in Fig. 2. Without DCMU, O_2 evolution (plus SiMo) shows a pH dependence similar to that

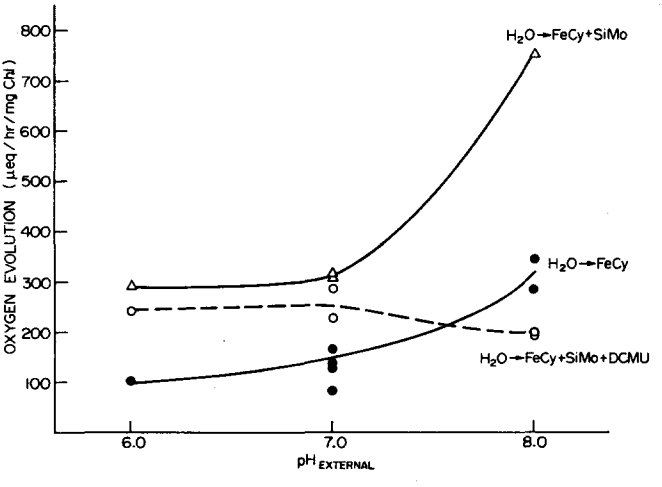

Fig. 2. pH dependence of O_2 evolution in the presence of SiMo and DCMU. Conditions as described in Fig. 1. SiMo and DCMU concentrations were 50 and 5 μM, respectively. MES, HEPES, and Tricine buffers at 20 mM were used to maintain the pH at 6, 7 and 8, respectively.

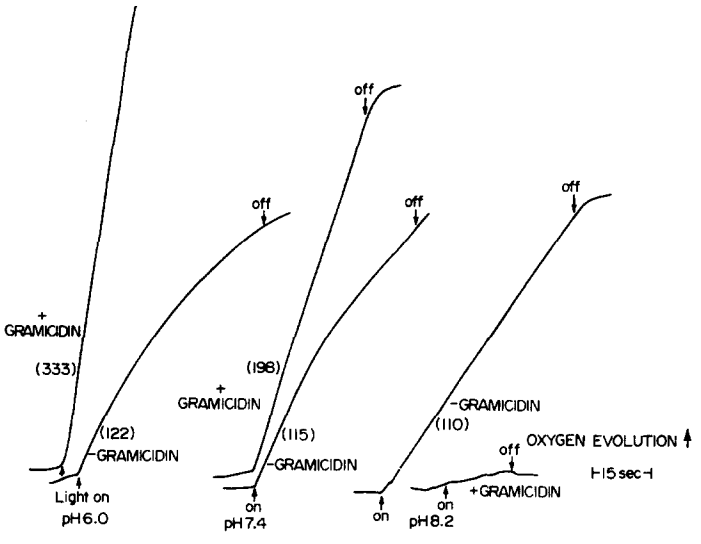

Fig. 3. O_2 evolution traces as a function of pH in the presence of gramicidin D. Conditions as in Figs 1 and 2. Gramicidin concentration was 2 μM. Rates of electron transport (μequiv/h per mg chlorophyll) in parentheses.

of $H_2O \rightarrow$ ferricyanide electron flow, in that electron flow increases with increasing external pH. The major difference between the curves for electron flow from water $\rightarrow$ ferricyanide$\pm$SiMo is that the plus SiMo condition results in faster electron transfer rates over the pH 6–8 range consistent with data in Table I. In the presence of DCMU and SiMo, O_2 evolution is nearly independent of external pH. At pH 6 and 7 O_2-evolution is essentially similar in the SiMo$\pm$DCMU case, however at pH 8.0 in the presence of DCMU, there is no increase in the O_2-evolution rate as found in the minus DCMU condition. In fact, at pH 8.0 the rate is slightly but consistently decreased compared to pH 7.0. This point deserves some clarification. The rates for DCMU-insensitive SiMo-mediated O_2 evolution were calculated using the initial slope of O_2 production (first 15–30 s). After this period, the slopes deviate from linearity. This non-linear rate of O_2 evolution is pronounced at the lower pH values of 6 and 7 (Fig. 3). At pH 8 however, the rate of O_2 evolution appears linear for at least 1 min. The reason for the non-linearity of slopes at the lower pH values is not readily apparent. Further addition of SiMo or ferricyanide did not increase this slower rate and thus this time dependent inhibition may possibly reflect irreversible damage to Photosystem II.

Ferricyanide (0.5 mM) was generally present in addition to SiMo when measuring O_2 evolution to maintain the SiMo in the oxidized form. SiMo does, however, accept electrons irrespective of the presence of ferricyanide [22] then becoming fully reduced. This can be seen in Fig. 4a which shows the effect of added SiMo on O_2 consumption during the Mehler-type reaction accompanying non-cyclic electron flow from water $\rightarrow$ anthraquinone sulfonate. Upon addition of SiMo to this oxygen consuming reaction a transient O_2 evolution occurs lasting 30–60 s followed by resumption of the anthraquinone sulfonate-catalyzed oxygen consumption. The length of time the transient O_2 evolution lasts is proportional to the concen-

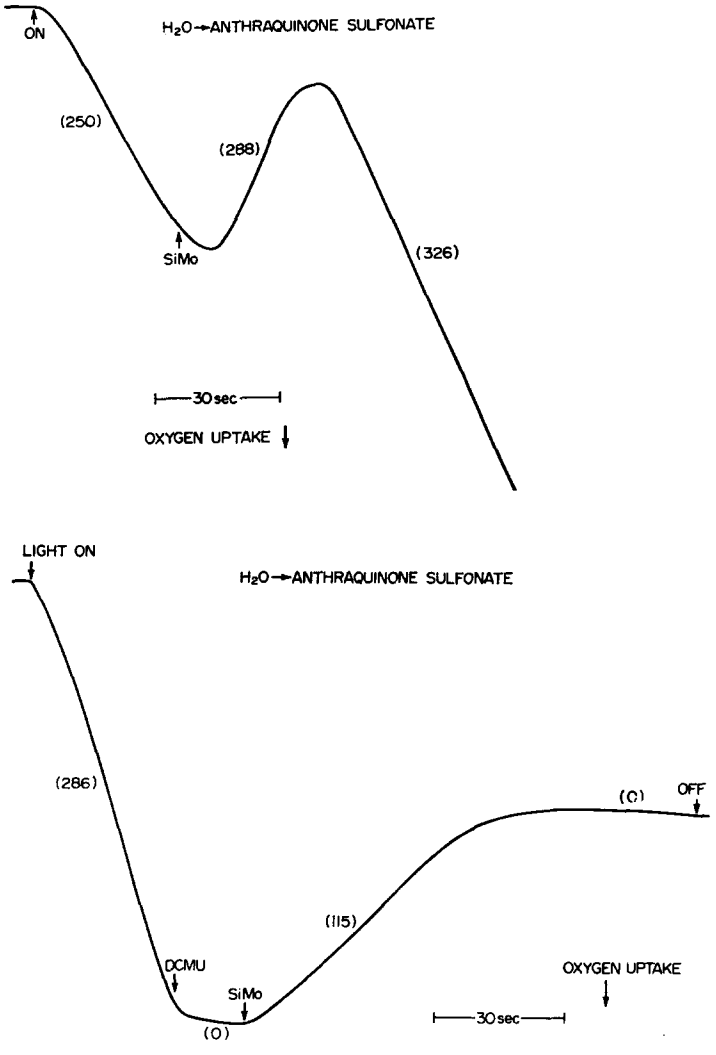

Fig. 4. Effect of SiMo addition on electron transport from water to anthraquinone sulfonate. Fig. 4a reaction mixtures contained in 2 ml, 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine/KOH (pH 8.0), 0.2 mM anthraquinone sulfonate, 0.5 mM NaN_3 and chloroplasts equivalent to 50 μg chlorophyll. SiMo concentration was 35 μM. Electron transport rates (μequiv/h per mg chlorophyll) in parenthesis. Fig. 4b, same as above except 5 μM DCMU was added where indicated.

tration of SiMo added. This transient O_2 evolution is consistent with SiMo intercepting electrons prior to the site of anthraquinone sulfonate reduction resulting in a net O_2 evolution from water oxidation. After SiMo becomes fully reduced, electrons flow to the anthroquinone sulfonate acceptor site with resumption of O_2 uptake. This interpretation is supported by the observation that when the SiMo is first reduced with ascorbate prior to addition, no O_2 evolution occurs upon its addition to the anthraquinone sulfonate assay.

The oxygen electrode trace in Fig. 4b demonstrates that the observed DCMU insensitivity of Photosystem II O_2 evolution in the presence of SiMo is not simply due to removal of DCMU from its inhibitory site by the SiMo. Fig. 4b shows the rate of oxygen consumption during non-cyclic electron transfer from water to anthraquinone sulfonate and its inhibition by DCMU. Addition of SiMo to the reaction results in O_2 evolution until the SiMo is reduced. Unlike Fig. 4a where DCMU is absent, no resumption of oxygen consumption occurs in the presence of DCMU, indicating that DCMU is still at its inhibitory site, preventing the continuance of electron flow to anthraquinone sulfonate.

We reported previously [16] that SiMo had no effect on methylviologen-catalyzed oxygen consumption, a Mehler reaction similar to the anthraquinone sulfonate reduction reported above. The reason for the discrepancy between the two sys-

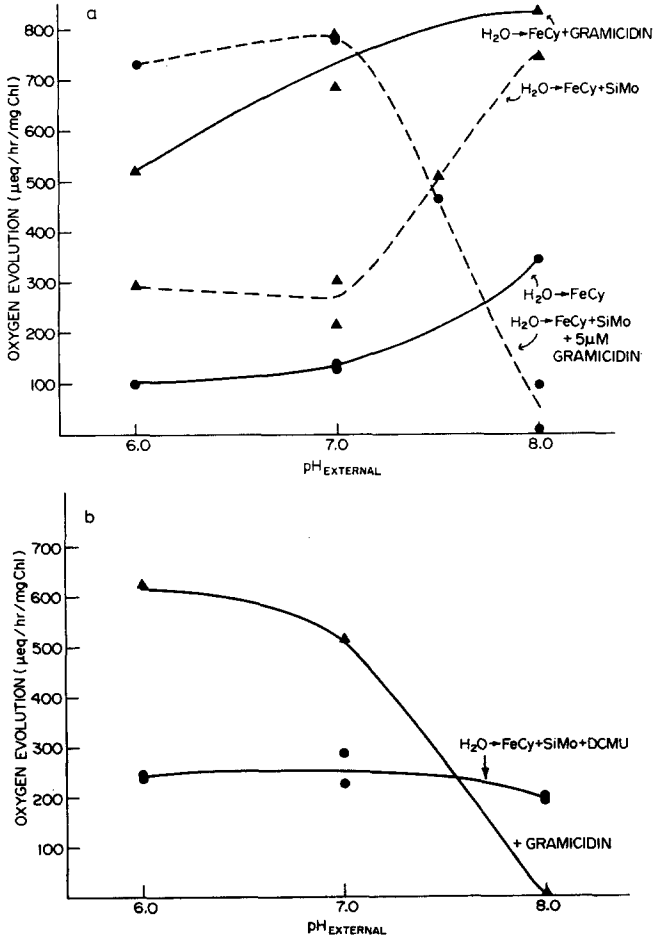

Fig. 5. Effect of Gramicidin D on SiMo-catalyzed electron transport (±DCMU) as a function of pH. Fig. 5a, reaction conditions as in Fig. 2. Concentration of ferricyanide was 0.5 mM; SiMo 50 μM; gramicidin D, 5 μM. Fig. 5b same as 5a except 5 μM DCMU was included in assay.

tems (anthraquinone sulfonate and methylviologen) became apparent after we discovered that the methylviologen complexes and precipitates the added SiMo (visualized by a faint cloudiness in the reaction mixture not readily observed in the presence of chloroplasts). Thus the methylviologen at 0.5 mM was complexing the SiMo at 50 μM, thereby effectively eliminating the SiMo from the reaction resulting in no observable effect on oxygen consumption. Therefore anthraquinone sulfonate was substituted as the acceptor for all non-cyclic electron transport assays and all added cofactors were monitored for precipitation with SiMo.

Effect of uncouplers on SiMo reduction

The pH dependence of uncoupler stimulation of electron transport from water to ferricyanide is shown in Fig. 5a (solid line). That the uncoupler gramicidin stimulates electron flow between pH 6 and 8 is well documented and is shown here for comparison with its effects on SiMo-mediated O_2 evolution.

The pH dependence for the effect of gramicidin on electron flow from water to SiMo in the absence and presence of DCMU is shown in Figs 5a and 5b, respectively. In contrast to the control ($H_2O \rightarrow$ ferricyanide), gramicidin in the presence of SiMo$\pm$DCMU markedly inhibits O_2 evolution above pH 7.5. Fig. 3 shows the O_2 evolution traces for the SiMo+DCMU reaction in the presence of gramicidin, again illustrating that at pH 8.2 O_2 evolution is almost completely inhibited by gramicidin. Interestingly, the non-linear slopes characteristic of SiMo reduction (+DCMU) at pH 6 and 7 approach linearity with gramicidin present. The gramicidin sensitivity of O_2 evolution at pH 8.0 in the presence of SiMo seems to apply to several uncouplers tested (Table II). Both NH_4Cl and 4-trifluoromethoxy carbonyl cyanide (FCCP) mimic gramicidin; at pH 6 and 7 they stimulate O_2 evolution and near pH 8.0 inhibition occurs. Similar results were also observed for nigericin (K^+) and carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP). Valinomycin plus K^+ had no effect on electron transport at pH 6 and 6.8, but did slightly stimulate O_2 evolution near pH 8.0.

TABLE II

pH DEPENDENCE OF UNCOUPLERS OR IONOPHORES ON ELECTRON TRANSPORT FROM WATER TO SILICOMOLYBDATE IN THE PRESENCE OF DCMU

Reaction mixture contained in 2 ml: 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM ferricyanide, 5 μM DCMU, 50 μM SiMo, chloroplasts equivalent to 20 μg chlorophyll per ml and 20 mM MES, HEPES or Tricine buffers at pH 6.0, 6.8 or 7.8, respectively. Rates of O_2 evolution expressed as μequiv/h per mg chlorophyll. Control = $H_2O \rightarrow$ ferricyanide+SiMo+DCMU.

pH	Control	NH_4Cl (5 mM)	FCCP (2 μM)	Valinomycin (3 μM)
6.0	205	287	349	205
6.8	212	349	370	205
7.8	102	30	34	165

Effect of SiMo on photophosphorylation

The DCMU-insensitive electron transfer from water to SiMo does not result in ATP formation [16]. The efficiency of phosphorylation measured by the *P*/2e ratio, was less than 0.1 in the $H_2O \rightarrow$ SiMo+DCMU reaction compared to near 1.0

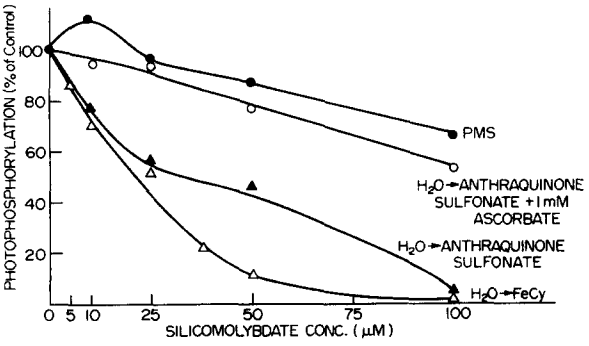

Fig. 6. Effect of SiMo on non-cyclic and cyclic photophosphorylation. The basic reaction mixture contained in 2 ml, 100 mM KCl, 5 mM $MgCl_2$, 20 mM Tricine/KOH (pH 8.2), 0.8 mM ADP, 3 mM phosphate with approximately 10^6 cpm of carrier-free ^{32}P and chloroplasts equivalent to 40 μg chlorophyll. Concentration of ferricyanide was 0.5 mM; anthraquinone sulfonate, 0.2 mM; ascorbate, 1 mM: and phenazine methosulfate (PMS), 30 μM. Illumination was for 1 min with strong white light (heat filtered) of 10^6 ergs/cm^2 per s intensity. Control rates of ATP formation (μmol/h per mg chlorophyll) for ferricyanide reduction were 421; anthraquinone sulfonate, 666; and phenazine methosulfate, 920.

for the control ($H_2O \rightarrow$ ferricyanide) even though comparable electron transfer rates occurred in both reactions.

The effect of SiMo concentration (in the absence of DCMU) on non-cyclic and cyclic phosphorylation is shown in Fig. 6. Phosphorylation accompanying ferricyanide reduction shows a 50–60 % inhibition at 25 μM and 90 % inhibition at 50 μM SiMo. Cyclic phosphorylation catalyzed by phenazine methosulfate is relatively insensitive to SiMo at all but the highest concentrations employed ,100 μM. Non-cyclic anthraquinone sulfonate mediated ATP formation also was inhibited approximately 50 % at 25 μM and for this reason 25 μM was used in all subsequent assays since this concentration gave a marked distinction between non-cyclic (50–60 % inhibition) and cyclic (no inhibition) ATP formation. It should be noted, however, that the rates of ATP formation in Fig. 6 were derived from reactions illuminated for 1 min in the presence of SiMo. The time course of SiMo inhibition of ATP synthesis is discussed below.

Since we have shown above that (1) electron transport from $H_2O \rightarrow$ SiMo (minus DCMU) was not inhibited by DBMIB (Table I) and (2) addition of SiMo to the oxygen consuming electron transfer reaction of $H_2O \rightarrow$ anthraquinone sulfonate caused a transient O_2 evolution, it may be that the observed inhibition of non-cyclic ATP synthesis (Fig. 6) is caused by SiMo-intercepting electrons at a point prior to the coupling site of ATP synthesis. This is supported by the observation that when SiMo is first reduced with ascorbate (or dithioerythritol), there was a significant protection of non-cyclic phosphorylation ($H_2O \rightarrow$ anthraquinone sulfonate) over a wide range of SiMo concentrations (Fig. 6) and complete protection of ATP synthesis at 25 μM. The same condition (i.e. reduced SiMo) did not result in the transient O_2 evolution shown in Fig. 4a, suggesting that SiMo is not accepting electrons in this state. Therefore, it seems that non-cyclic ATP formation only shows inhibition when SiMo is present in the oxidized form, a form capable of accepting electrons in either

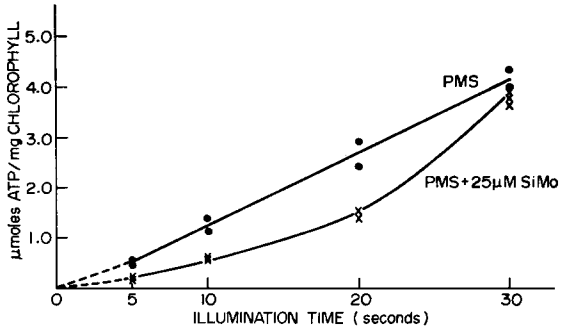

Fig. 7. Time course of phenazine methosulfate (PMS)-catalyzed ATP formation in the presence of SiMo. Reaction conditions as described in Fig. 6. The reaction mixture was illuminated with white light (10^6 ergs/cm^2 per s) for the times indicated. The amount of ATP formed after 60 s of illumination for phenazine methosulfate alone was 6.25 μmol/mg chlorophyll and for phenazine methosulfate +25 μM SiMo was 7.5 μmol/mg chlorophyll.

a DCMU or DBMIB insensitive manner.

If SiMo accepts electrons at Photosystem II, it is expected that phenazine methosulfate-catalyzed phosphorylation (minus DCMU) would show inhibition at short illumination times since Photosystem II electrons would not be available to reduce the cyclic cofactor. Fig. 7 shows the amount of ATP formed as a function of illumination time during phenazine methosulfate phosphorylation±SiMo. In the presence of SiMo, the amount of ATP produced in the first 20 s was significantly lower than the control. After 30 s of illumination the amount of ATP formed in the presence of SiMo was nearly equal (in some experiments it was actually greater than the control) and by 60 s was routinely more than the amount formed with phenazine methosulfate alone (see Fig. 7 legend and Table III). Neither DCMU nor exogenous reductant was present in these experiments. These results are consistent with oxidized SiMo accepting electrons from Photosystem II and thus momentarily preventing Photosystem II electron transfer from "poising" the cyclic cofactor. The observation that after approximately 30 s (the time needed for SiMo reduction (Fig. 4)) the amount of ATP formed exceeds the control perhaps reflects a more favorable redox poising

TABLE III

EFFECT OF SILICOMOLYBDATE ON PHOTOPHOSPHORYLATION MEDIATED BY PHENAZINE METHOSULFATE AND DIAMINODURENE

Reaction mixture contained in 2 ml, 100 mM KCl, 5 mM $MgCl_2$, 3 mM K_2HPO_4, 25 mM Tricine/KOH (pH 8.1), 0.8 mM ADP, and chloroplasts equivalent to 20 μg chlorophyll per ml. Concentration of diaminodurene was 0.5 mM; DCMU, 5 μM; and phenazine methosulfate, 30 μM.

Assay	Phosphorylation (μmol/h per mg chlorophyll)
Diaminodurene+DCMU	681
Diaminodurene+DCMU+25 μM SiMo	673
Phenazine methosulfate	728
Phenazine methosulfate+25 μM SiMo	881

in this system when SiMo is present. SiMo had no effect on cyclic phosphorylation catalyzed by diaminodurene (+DCMU) (Table III). Diaminodurene, however, is able to reduce SiMo chemically and therefore the SiMo present in this system has no accepting properties.

Acid-base phosphorylation and post-illumination ATP formation (X_E)

Since the inhibition of ATP formation is correlated with the accepting properties of SiMo it is important to determine whether the oxidized but not reduced form of SiMo is inhibitory per se to phosphorylation, that is, either acting as a transient (while it is in the oxidized state) uncoupler or energy transfer inhibitor. To determine the effect of oxidized SiMo on phosphorylation independent of its electron-accepting properties, we investigated its effect on ATP synthesis induced by (1) a dark acid to base transition and (2) post-illumination ATP synthesis (X_E). SiMo (plus ferricyanide) present in the acid stage of the acid-bath phosphorylation resulted in a 33–35 %

TABLE IV

EFFECT OF SILICOMOLYBDATE ON ACID-BASE PHOSPHORYLATION

After isolation in sucrose buffer [16], chloroplasts were washed and resuspended in 10 mM NaCl. Approximately 0.2 mg chlorophyll was incubated in 1 ml of 15 mM succinate (pH 4.0) for 20 s and then rapidly injected into a 1-ml reaction mixture containing 0.1 M Tricine/KOH, pH 9.0, 5 mM $MgCl_2$, 0.2 mM ADP, 2 mM K_2HPO_4, 5 μM DCMU and 10^6 cpm of ^{32}P. Final pH of the mixture was 8.3–8.5. After 20 s the reaction was terminated with trichloroacetic acid and the amount of ^{32}P incorporated into ATP was determined [17]. SiMo and ferricyanide (FeCy) concentrations were 25 and 50 μM respectively.

Assay	ATP formation (nmol ATP/mg chlorophyll)	% inhibition
Control ± FeCy	177	0
SiMo + FeCy in acid stage	117	34
SiMo + FeCy in base stage	159	10

TABLE V

EFFECT OF SILICOMOLYBDATE ON POST-ILLUMINATION ATP FORMATION (X_E)

Ferricyanide (FeCy), SiMo or NH_4Cl were present in the dark phosphorylation stage only. Chloroplasts equivalent to 140 mg chlorophyll were illuminated for 30 s with heat-filtered white light (approximately 10^6 ergs/cm per s) in a continuously stirred reaction mixture containing in 2 ml, 0.1 M sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 10 mM MES buffer (pH 6.0) and 37 μM pyocyanine. Immediately after shutting off the light, 1 ml of a mixture containing 0.15 M Tricine buffer (pH 8.3), 3 mM ADP and 10 mM $NaH_2{}^{32}PO_4$ was injected into the reaction mixture. After 20 s 0.1 ml of 50 % trichloroacetic acid was added to terminate the reaction. Final concentrations of FeCy, SiMo or NH_4Cl were 35 μM, 34 μM and 7 mM respectively.

Additions (dark stage)	ATP formation (nmol ATP/mg chlorophyll)	% inhibition
FeCy	99	0
FeCy + SiMo	87	12
FeCy + NH_4Cl	3	97

inhibition of phosphorylation whereas it was not inhibitory when present in the base stage (Table IV). SiMo added in the dark stage had essentially no effect on the post-illumination ATP formation while the uncoupler NH_4Cl, added in the dark stage, markedly inhibited the X_E-induced phosphorylation (Table V).

Effect of SiMo on proton and amine uptake

Ferricyanide supported pH changes and amine uptake were employed to determine if the electron transfer sequence of $H_2O \rightarrow$ SiMo+DCMU resulted in net proton accumulation within the grana membranes. In the presence of ferricyanide a characteristic reversible pH change occurs which is superimposed on a net acidification of the medium (Fig. 8). Both the initial proton uptake and post illumination proton efflux are indicative of net proton accumulation. The irreversible net acidification of the medium is a measure of the steady-state electron transfer rate resulting from protons released during water oxidation [18]. As expected, DCMU completely inhibits both these pH changes. In the presence of SiMo only the irreversible acidification of the medium is observed with no indication of either the initial pH rise or post illumination efflux characteristic of proton accumulation (Fig. 8). The net acidification of the media occurs irrespective of the presence of ferricyanide illustrating that SiMo itself is accepting electrons.

Similar results were obtained when using energy-dependent amine uptake [19] as a monitor for net internal acidification of the grana membranes (Table VI). These results (Fig. 8 and Table VI) indicate that no proton accumulation occurs during electron transfer from water to SiMo in the presence of DCMU.

Reduced SiMo was without effect on phenazine methosulfate-catalyzed cyclic proton uptake (Table VII), affecting neither the extent, nor any parameters of the post illumination H^+ efflux, suggesting that the reduced form does not induce significant membrane leakiness, consistent with the phosphorylation data. It is more difficult to

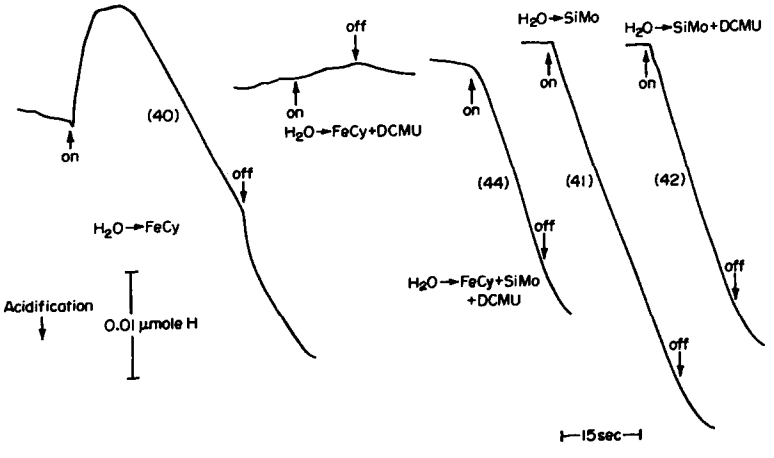

Fig. 8. Light-induced pH changes in the presence of SiMo and DCMU. The reaction mixture contained in 3 ml, 100 mM KCl, 5 mM $MgCl_2$. 0.5 mM MES buffer (pH 6.2), 33 μM SiMo, 5 μM DCMU and 0.5 mM ferricyanide (where indicated). Rates of electron transport (μequiv/h per mg chlorophyll) in parentheses.

TABLE VI

EFFECT OF SILICOMOLYBDATE ON AMINE UPTAKE

The reaction mixture contained in 3 ml, 30 mM lysine/HCl, 0.01 M Tris/Tricine buffer (pH 7.0), 0.33 mM NH_4Cl, and chloroplasts equivalent to 72 μg chlorophyll. Concentration of ferricyanide (FeCy) was 0.1 mM; DCMU, 5 μM; SiMo, 33 μM. Sensitivity of assay was 0.03 μmol NH_4^+ per mg chlorophyll.

Assay	Amine uptake (μmol/mg chlorophyll)
1 $H_2O \rightarrow$ FeCy (control)	0.16
2 $H_2O \rightarrow$ FeCy+DCMU	0–0.03
3 $H_2O \rightarrow$ FeCy+DCMU+SiMo	0–0.03

TABLE VII

EFFECT OF REDUCED SILICOMOLYBDATE ON PHENAZINE METHOSULFATE-CATALYZED PROTON ACCUMULATION

Reaction mixture contained in 2 ml, 100 mM KCl, 5 mM $MgCl_2$. 1 mM MES buffer (pH 6.0), 1 mM dithioerythritol and chloroplasts equivalent to 42 μg chlorophyll. The k_d is the rate constant for the proton efflux reaction. Phenazine methosulfate was 30 μM.

Assay	Extent of accumulation (μmol H/mg chlorophyll)	Proton efflux rate (μmol H/mg chlorophyll per h)	k_d (s^{-1})
1 Control+dithioerythritol	0.52	65	0.04
2 25 μM SiMo+dithioerythritol	0.55	79	0.05

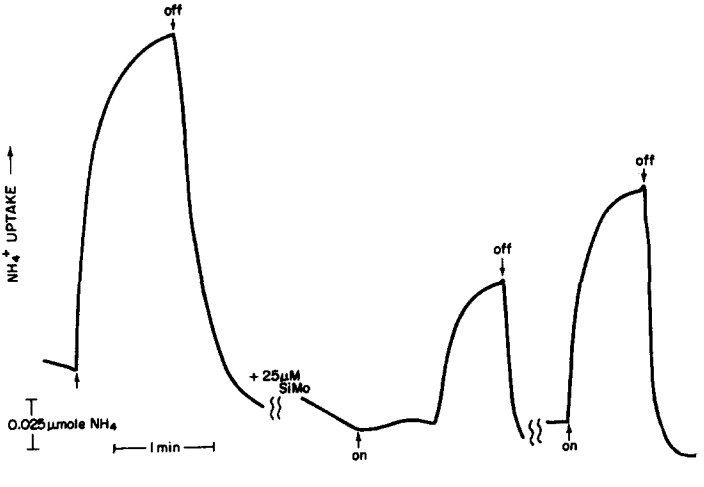

Fig. 9. Effect of SiMo on phenazine methosulfate-catalyzed amine uptake. Reaction mixture contained in 3 ml, 10 mM Tris/Tricine buffer (pH 7.0), 0.33 mM NH_4Cl, 30 mM lysine/HCl, and chloroplasts equivalent to 40 μg chlorophyll. Illumination was with heat filtered red light (Corning filter No. 2403) of $2 \cdot 10^5$ ergs/cm^2 per s. Amine uptake was measured with a Beckman cationic electrode. Concentration of phenazine methosulfate was 30 μM.

ascertain the effect of oxidized SiMo on the phenazine methosulfate-catalyzed proton uptake because of the SiMo acting as an electron acceptor at Photosystem II causing a net acidification of the medium upon illumination. Fig. 9 however demonstrates the oxidized to reduced conversion of SiMo on phenazine methosulfate catalyzed amine uptake, a measure of internal acidification. In the presence of SiMo little amine uptake occurred initially. After a lag period of approximately 30 s (needed for SiMo reduction), amine uptake commences and subsequent cycles of illumination result in substantial amine uptake. When SiMo is first reduced with dithioerythritol, both proton accumulation (Table VII) and amine uptake occur as in the control. These results are consistent with SiMo (+DCMU) accepting electrons (not coupled to internal acidification) until it becomes reduced, followed by resumption of phenazine methosulfate catalyzed proton and amine uptake.

Fluorescence

Fig. 10 shows that SiMo significantly reduces the room temperature chloroplast fluorescence even in the presence of DCMU, consistent with the compound accepting electrons prior to or at the primary acceptor, Q, of Photosystem II.

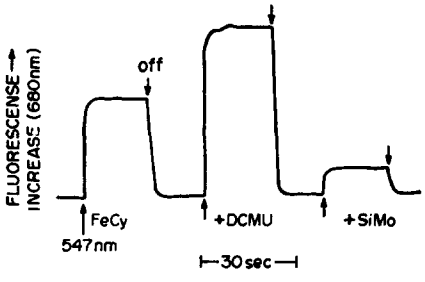

Fig. 10. Effect of SiMo phenazine methosulfate on Photosystem II fluorescence. Reaction mixture contained in 2 ml, 100 mM KCl, 5 mM $MgCl_2$, 20 mM tricine, pH 8.0, 0.5 mM ferricyanide, and 35 μg chlorophyll. Concentrations of SiMo and DCMU were 20 and 5 μM, respectively. Details given in Methods.

DISCUSSION

This study demonstrates that in the presence of DCMU, SiMo can accept electrons from water oxidation resulting in unimpaired Photosystem II O_2 evolution. The DCMU insensitivity of this reaction is not caused by displacement of DCMU from its inhibitory site (Fig. 4b) and the O_2 evolved is derived from Photosystem II water oxidation, since Tris treatment abolishes the effect. In the absence of DCMU, SiMo can also accept electrons prior to the site of DBMIB inhibition and thus presumably prior to or from plastoquinone. SiMo itself is functioning as an electron acceptor since SiMo reduction (heteropoly blue formation) can be measured spectrophotometrically [22]. The observation that SiMo (10–25 μM) significantly decreases Photosystem II fluorescence in the presence of DCMU is consistent with SiMo accepting electrons prior to the DCMU inhibition site, thus maintaining Q in the oxidized form and a low fluorescence level.

There have been two other reports indicating a DCMU-insensitive Photo-

system II electron transport, Miles et al. [23] using $HgCl_2$ and Girault and Galmiche [24] using silicotungstic acid.

Effect of uncouplers

Experiments designed to measure the effect of internal pH on electron transport have led to the concept that the internal pH optimum for electron transport is near 5.5, while the external optimum is near 8.5 [25–28]. The proton gradient (ΔpH) of approximately 3 established during electron transport generates the optimum acidic pH within the membrane [28]. The observation that uncouplers at high external pH inhibit electron transport is consistent with this concept, since the uncouplers would prohibit the internal acidification thus keeping the internal pH closer to the inhibitory external alkaline pH of the media [28]. Harth et al. [29] have recently proposed that the O_2-evolution apparatus is the site of inhibition by uncouplers at high external pH. The DCMU-insensitive electron transport from $H_2O \rightarrow$ SiMo+DCMU provides a means to test this hypothesis since in this partial reaction the O_2-evolution apparatus can be functionally isolated from electron transfer beyond Q, the Photosystem II primary acceptor. Fig. 5 and Table II demonstrate that several uncouplers markedly inhibit O_2 evolution only at the higher pH values. This observation can be explained in terms of the internal pH-regulating electron flow. That is, since water to SiMo+DCMU electron flow does not contribute to internal proton deposition from water oxidation (Fig. 8 and Table VI), uncouplers equilibrate the unfavorable external alkaline pH with the internal regions. At lower external pH values uncouplers stimulated SiMo-catalyzed O_2 evolution since equilibration with the external medium does not shift the pH beyond the favorable range. The results with SiMo support the contention of Harth et al. [29] that the internal pH regulates the water-oxidation reaction.

Effect on phosphorylation and proton gradients

The abbreviated electron transport sequence from water to SiMo +DCMU does not result in ATP synthesis (Fig. 6 and ref. 16) nor does it result in internal proton accumulation (Fig. 8 and Table VI) as measured by amine uptake. The absence of internal acidification of the grana membranes cannot be explained by the protons generated from water oxidation being neutralized during reduction of SiMo (SiMo serving as a proton sink), since in the electron flow sequence $H_2O \rightarrow$ SiMo+DCMU there is rapid net acidification of the medium (Fig. 8) indicating water protons are liberated into the external media and not bound to SiMo during its reduction.

SiMo does not appear to be inhibitory to ATP formation per se since phenazine methosulfate cyclic phosphorylation was not affected (Fig. 6 and Table III). Non-cyclic phosphorylation shows inhibition only when SiMo is present in the oxidized form (Fig. 6), a form capable of accepting electrons. That inhibition can be explained by assuming that oxidized SiMo accepts electrons prior to the DCMU-inhibition site, this abbreviated electron transfer from $H_2O \rightarrow$ SiMo not being coupled to ATP formation. In the absence of DCMU there is some ATP synthesis occurring in the $H_2O \rightarrow$ SiMo electron transport pathway which is only partially inhibited by DBMIB. This ATP synthesis apparently requires electron transport after the DCMU inhibition site and may reflect the ATP formation accompanying reduction of conventional Photosystem II acceptors [1–7] which are inhibited by

DCMU but not by the plastoquinone analogue DBMIB. Using short illumination times (10 s) and SiMo concentrations from 10 to 15 μM, electron transport from $H_2O \rightarrow$ SiMo in the presence of DBMIB supported phosphorylation with an efficiency ($P/2e$) of 0.35 compared to the control ($H_2O \rightarrow$ ferricyanide) which gave a $P/2e$ of approximately 1.1. A similar phosphorylation efficiency for $H_2O \rightarrow$ SiMo was obtained when KCN-treated chloroplasts (lacking Photosystem I) were used. The magnitude of rates of phosphorylation and electron transport in the $H_2O \rightarrow$ SiMo+DBMIB reaction or in KCN-treated chloroplasts were on the order of 30 and 170 μmol or μequiv/h per mg chlorophyll, respectively. Thus based on the P/2e ratio, silicomolybdate in the absence of DCMU acts similar to the conventional lipophilic Photosystem II acceptors [1–7]. That the $H_2O \rightarrow$ SiMo+DBMIB or $H_2O \rightarrow$ SiMo (using KCN-treated chloroplasts) phosphorylates with this efficiency also argues against SiMo acting as an inhibitor of ATP synthesis per se. Experiments further characterizing this $H_2O \rightarrow$ SiMo minus DCMU electron transport will be presented elsewhere. When ATP synthesis occurs in the $H_2O \rightarrow$ SiMo+DBMIB case, some internal acidification can be measured by amine uptake.

The possibility arose that there may be two populations of membranes present, one of intact vesicles responsible for $H_2O \rightarrow$ SiMo (−DCMU) phosphorylation and another population of damaged particles which displayed a DCMU insensitive electron transport with no phosphorylation. To determine whether this hypothesis was feasible, the relative quantum requirements of $H_2O \rightarrow$ SiMo±DCMU electron transport were determined. One would predict different quantum requirements for these reactions if two populations of particles existed. Similar quantum requirements (calculated by intensity/velocity versus intensity plots) were found for both electron transfer reactions ($H_2O \rightarrow$ SiMo±DCMU) indicating we are dealing with a single population of membranes (data to be published elsewhere).

ATP synthesis induced by an acid-base transition was sensitive to SiMo when present in the acid stage only (Table IV). We do not believe, however, that this reflects uncoupling, because Polya and Jagendorf [30] have demonstrated that various polyanions when present in the acid stage during an acid → base transition significantly inhibited subsequent ATP formation by denaturing the coupling factor enzyme. It is likely therefore that the inhibition of ATP formation during the acid-base transition by SiMo, a heteropolyanion, reflects this low pH polyanion inhibition of the coupling factor. The oxidized form of SiMo (added to the dark stage) did not inhibit post illumination ATP formation (X_E) (Table V) compared to an uncoupler which did inhibit as predicted from past work [37, 38] indicating lack of uncoupling or energy transfer inhibition by oxidized SiMo. The lack of inhibition of phenazine methosulfate and diaminodurene cyclic phosphorylation by SiMo also argues against SiMo acting as an uncoupler.

Correlation between Photosystem II membrane conformational change and energy transduction

Recently, using the non-permeant chemical modifier, diazonium benzene sulfonate (DABS) as a means to study membrane conformational change, we showed that activation of electron transfer from water to SiMo+DCMU did not result in an increased incorporation of the diazonium reagent to the membranes [31]. An increased amount of covalently bound DABS being used as an indicator of increased accessi-

bility of membrane components to the water soluble reagent, i.e. a conformational change. Activation of electron transfer from water to Class III lipophilic oxidants [31] such as DBMIB (accepting electrons at the level of plastoquinone [32]) did, however, result in the incremental DABS binding to the membranes suggesting that electron transfer through the region between the primary acceptor of Photosystem II and plastoquinone potentiated a membrane conformational change. This electron transfer-dependent DABS incorporation resulted in the conversion of cytochrome *b*-559 from its high potential to low potential form. As described previously [33, 34] the electron transfer dependent DABS incorporation is not dependent on net proton accumulation since uncouplers did not prevent the incremental DABS binding. Thus the proposed conformational change is not synonymous with conformational changes attendent on massive H^+ accumulation. Since electron transfer from $H_2O \rightarrow$ SiMo+ DCMU was coupled to neither (1) ATP synthesis, nor (2) proton accumulation, nor (3) membrane conformational changes [31], it may be inferred that an obligatory relationship exists between these events during energy transduction. It is well documented [1–7] that Photosystem II electron flow from H_2O to lipophilic Class III acceptors does result in ATP formation and proton accumulation [4] and a conformational change revealed by DABS binding [31]. It may be that during normal Photosystem II electron flow, the vectorial release of water protons into either the internal grana space or into localized regions within the membrane [15. 16, 35, 36] is mediated through a conformational change activated by electron transport between Q and plastoquinone, possibly involving cytochrome *b*-559. When the conformational change is not energized, as seems to be the case for the water $\rightarrow$ SiMo+DCMU reaction, water oxidation occurs, but it releases water protons to the external media or to a region in the membrane where they are not effective in energizing the membrane.

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