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PATHWAYS OF SILICOMOLYBDATE PHOTOREDUCTION AND THE ASSOCIATED PHOTOPHOSPHORYLATION IN TOBACCO CHLOROPLASTS

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SUMMARY

Three sites of silicomolybdate reduction in the electron transport chain of isolated tobacco chloroplasts are described. The relative participation of these sites is greatly influenced by the particular reaction conditions. One site (the only site when the reaction medium contains high concentrations of bovine serum albumin (> 5 mg/ml)) is associated with Photosystem I, since it supports phosphorylation with a P/e_2 value close to 1 and the reaction is totally sensitive to both plastocyanin inhibitors and 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Two other sites of silicomolybdate reduction are associated with Photosystem II. One site is 3-(3,4-dichlorophenyl)-1,1-dimethylurea insensitive and supports phosphorylation when the reaction mixture contains dimethyl sulfoxide and glycerol (protective agents). The P/e_2 value routinely observed is about 0.2. Bovine serum albumin (1–2 mg/ml) can also act as a protective agent, but the efficiency of Photosystem II phosphorylation observed is lower. Silicomolybdate reduction supports virtually no phosphorylation, regardless of the reduction pathway, when the reaction mixture contains no protective agents. This is due to irreversible uncoupling by silicomolybdate itself. The silicomolybdate uncoupling is potentiated by high salt concentrations even in the presence of protective agents. Exposure of chloroplasts to silicomolybdate in the absence of protective agents rapidly inactivates both photosystems.

INTRODUCTION

Silicomolybdate and other heteropolyanions have been of recent interest as Hill oxidants due to their ability to accept electrons before the site of DCMU inhibition [1–8]. There is evidence which suggests that the DCMU-insensitive site of silicomolybdate reduction is at the primary electron acceptor (Q) of Photosystem II [4]. Thus, silicomolybdate provides an important reaction system for the study of energy coupling at Photosystem II.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; P/e_2, the ratio of the number of ATP molecules formed to the number of pairs of electrons transported.

Several lines of evidence indicate the involvement of a chemiosmotic-type mechanism in Photosystem II energy coupling. For instance, proton translocation has been shown to occur during Photosystem II electron transport [13], and experiments with artificial electron donors to Photosystem II produced results which tend to substantiate the hypothesis that the protons produced when water is oxidized create the proton gradient which drives phosphorylation [9, 10]. Since silicomolybdate supports water oxidation (and presumably the production of protons) in the presence of DCMU, one would reasonably expect that silicomolybdate reduction in DCMU-poisoned chloroplasts would support Photosystem II energy coupling much as do standard Photosystem II reactions in the absence of DCMU. Early attempts to demonstrate this DCMU-insensitive Photosystem II phosphorylation yielded negative results [2, 5, 6]. However, we have recently shown with tobacco chloroplasts [8] that the DCMU-insensitive silicomolybdate reduction does support phosphorylation if the reaction mixture contains dimethyl sulfoxide and glycerol. The observed phosphorylation efficiency (P/e_2) was 0.20–0.25, values which are reasonably close to the well documented phosphorylation efficiency of Photosystem II phosphorylation in Class II chloroplasts (P/e_2, 0.3–0.4; e.g. refs. 11–13).

In this paper we describe more completely the DCMU-insensitive silicomolybdate reduction and the associated phosphorylation, as well as the other two pathways of silicomolybdate reduction and phosphorylation.

MATERIALS AND METHODS

Chloroplasts were prepared by grinding fresh leaves of greenhouse-grown tobacco (*Nicotiana tabacum*) in a solution containing 0.3 M NaCl; 0.03 M Tricine/NaOH, pH 7.7; and 3 mM $MgCl_2$; in a Waring Blendor at 4 °C. The homogenate was filtered through cheesecloth and centrifuged at $2500 \times g$ for 30 s. The chloroplasts were quickly and gently resuspended with a small paint brush into a 0.2 M sucrose solution containing 20 mM Tricine/NaOH or Tricine/lysine (see below), pH 7.5; and 3 mM $MgCl_2$. The resulting suspension was centrifuged at $1000 \times g$ for 1 min to remove cell debris, followed by a centrifugation at $2000 \times g$ for 5 min to pellet the chloroplasts. This pellet was resuspended as before in the same sucrose medium except that bovine serum albumin (2 mg/ml) was added to the final suspension. The chlorophyll concentration of this suspension was about 1 mg/ml.

Silicomolybdic acid (α-12-molybdosilicic acid; $H_4SiMo_{12}0_{40}$) was a generous gift of Dr. G. A. Tsigdinos (Climax Molybdenum Co., Ann Arbor, Mich.). The acid was dissolved in either water or in a 50 % dimethyl sulfoxide solution to 50 mg/ml. Small amounts of insoluble material were removed by centrifugation or filtration. Exact concentrations of silicomolybdate were determined spectrophotometrically by the methods of Strickland [14]. Both solutions seemed stable for several days at room temperature.

Electron transport was measured as O_2 evolution using Clark-type O_2 electrode at 20 °C. The basic reaction components are listed in Fig. 1. In routine experiments reactions were initiated by simultaneously turning on the actinic light (580–700 nm; approx. 500 kergs $\cdot s^{-1} \cdot cm^{-2}$) and injecting 20 μl of the silicomolybdate solution into the reaction mixture. Unless otherwise noted, the reaction time was 1–1.5 min.

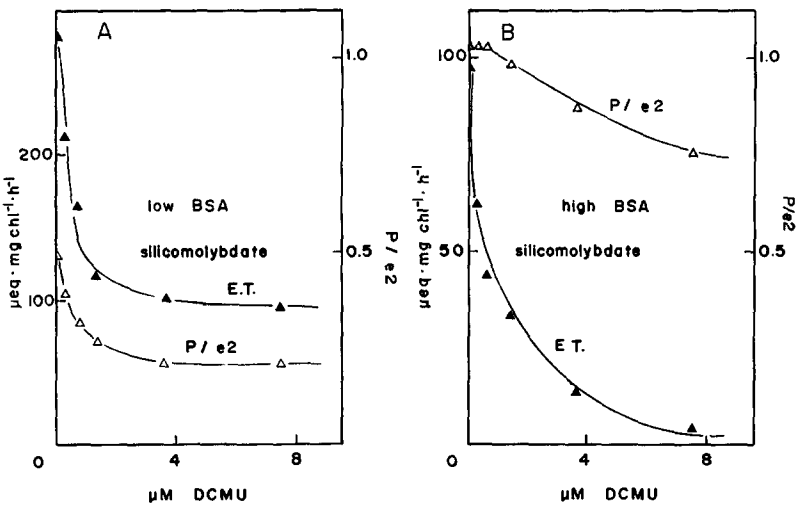

Fig. 1. The effects of increasing concentrations of DCMU on the silicomolybdate-mediated Hill reaction at low (0.2 mg/ml) and high (7 mg/ml) bovine serum albumin (BSA) concentrations in chloroplasts under phosphorylating conditions. 20 μl of silicomolybdate (50 mg/ml) dissolved in 50% dimethyl sulfoxide in water (v/v) were injected into the reaction mixture at the time of illumination to initiate the reaction. The final reaction mixture (2 ml) contained: 100 mM sucrose, 30 mM *N*-2-hydroxyethylpiperazine propane sulfonic acid (HEPPS/NaOH) pH 8.0, 3 mM $MgCl_2$, 5% (v/v) glycerol, 5 mM $NaH^{32}PO_4$, 0.75 mM ADP; 0.5% (v/v) dimethyl sulfoxide (carried over with silicomolybdate), 270 μM silicomolybdate, and chloroplasts equivalent to 100 μg chlorophyll (chl). For other conditions and methods, see Materials and Methods. E.T., electron transport.

After O_2 determination, a 1 ml aliquot of the reaction mixture was analyzed for [^{32}P] ATP by a modification of the method of Avron [15].

Photosystem I-inhibited chloroplasts were prepared by a modification of the methods of Yocum [16]. Chloroplasts as prepared above were resuspended into a solution containing: 50 mM KCN; 90 mM *N*-2-hydroxyethylpiperazine propane sulfonic acid (HEPPS), pH 8.0; 50 μM $K_3Fe(CN)_6$; 100 mM sucrose; 3 mM $MgCl_2$; and $HgCl_2$ added to give a final concentration of 1 mol Hg/3 mol chlorophyll (chlorophyll concentration, 0.3 mg/ml). This suspension was incubated in the dark at 4 °C for 1 h, followed by centrifugation to pellet the chloroplasts and resuspension into the sucrose bovine serum albumin buffer from above. The methyl viologen Hill reaction of these chloroplasts was completely inhibited. KCN [17, 18] and Hg^{2+} [19] are known to inactivate plastocyanin in situ.

Since there was considerable cation-stimulated uncoupling of the silicomolybdate-mediated electron transport reactions (see Results) attempts were made to minimize and standardize the amounts of Na^+ in the reaction mixtures. Therefore, buffers prepared for the pH studies were neutralized with lysine (free base) instead of NaOH. Control experiments showed that the variable lysine concentration did not affect the electron transport rates or coupling efficiency as did the Na^+.

RESULTS

The effects of bovine serum albumin and KCN/Hg^{2+} treatment

During our attempts to determine factors which govern the phosphorylation

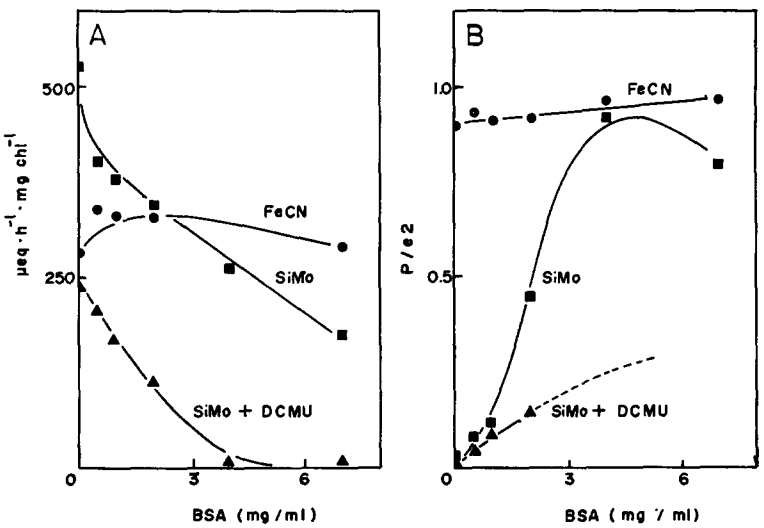

Fig. 2. The effects of bovine serum albumin (BSA) on the silicomolybdate (SiMO)- and ferricyanide (FeCN)-mediated Hill reaction. Reaction conditions were like those in Fig. 1. The ferricyanide and DCMU concentrations are 0.4 mM and 5 μM, respectively, Chl. chlorophyll.

efficiency of silicomolybdate reduction, we found that bovine serum albumin (albumin) has profound effects on silicomolybdate reduction and the associated phosphorylation. Fig. 1 shows the effect of DCMU on the silicomolybdate Hill reaction with chloroplasts exposed to either high albumin concentrations (7 mg/ml) or low albumin concentrations (0.2 mg/ml). Fig. 1A shows that in the absence of large amounts of albumin, DCMU is incapable of completely inhibiting the electron transport from water to silicomolybdate. It is also clear from this figure that the DCMU-insensitive electron transport is coupled to ATP synthesis with a P/e_2 of about 0.2. Fig. 1B shows, however, that silicomolybdate behaves very much like ferricyanide in the presence of high concentrations of albumin; the silicomolybdate reduction is now totally sensitive to DCMU and it supports phosphorylation with a P/e_2 near 1. Clearly the presence of high concentrations of albumin provides conditions which favor the reduction of silicomolybdate at Photosystem I and hence through two sites of energy coupling.

The above experiments were conducted using reaction media which contained 0.5 % dimethyl sulfoxide (carried over with the silicomolybdate) and 5 % glycerol. These agents are highly effective in improving the poor efficiencies of the phosphorylation associated with silicomolybdate reduction especially in the presence of DCMU [8]. The experiments of Figs. 2A and 2B demonstrate that albumin alone can act as a protective agent. As the increasing concentration of albumin diminishes the rate of silicomolybdate reduction, the phosphorylation efficiency (P/e_2) is dramatically increased: from near zero to 0.9 in the absence of DCMU and from zero to 0.15 in the presence of DCMU. These inverse relationships between the rate of electron transport and phosphorylation efficiency have also been noted for dimethyl sulfoxide and glycerol [8]. The ferricyanide-like behavior of silicomolybdate at high albumin concentrations (total sensitivity to DCMU and P/e_2 approaching 1) is again clearly seen in Fig. 2.

TABLE I

THE EFFECT OF BOVINE SERUM ALBUMIN AND DCMU ON THE SILICOMOLYBDATE (SiMo) HILL REACTION IN PHOTOSYSTEM I-INHIBITED CHLOROPLASTS

Chloroplasts were inhibited with KCN/Hg^{2+} as described in Materials and Methods. The inhibited chloroplasts were unable to reduce methyl viologen. Assay conditions were like those described in Fig. 1A except the DCMU concentration was 5 μM and the temperature was 20 °C. FeCN, ferricyanide.

Bovine serum albumin (mg/ml)	μequivalents/mg chlorophyll per h		
	FeCN	FeCN+SiMo	FeCN+SiMo+DCMU
0.2	4	160	35
7.0	5	5	3

The experiment of Table I provides evidence that at high albumin concentrations silicomolybdate reduction indeed takes place only at Photosystem I. In this experiment, chloroplasts were pre-treated with KCN/Hg^{2+} to inhibit Photosystem I activity [16]. It can be seen from the table that with low albumin concentrations, the silicomolybdate reaction still occurs (through Photosystem II), but when albumin is present, the silicomolybdate cannot act as an electron acceptor for Photosystem II and virtually no O_2 production occurs. The presence of ferricyanide in the reaction medium and the absence of any significant water to ferricyanide reaction, offers assurance that Photosystem I is indeed inhibited. It is also important to note that the KCN/Hg^{2+}-resistant Photosystem II reduction of silicomolybdate under low albumin conditions is partially sensitive to DCMU. We are thus led to conclude that there are at least three sites of silicomolybdate reduction in the photosynthetic electron transport chain: a Photosystem I site and two Photosystem II sites, one before and one after the DCMU-sensitive site. The accessibility of the Photosystem I reduction site to silicomolybdate is only mildly hindered by high concentrations of albumin (Fig. 2A) but the two Photosystem II sites are completely "shielded" in the presence of high concentrations of albumin (Table I).

The effects of DBMIB

It has been reported that 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) has no effect on silicomolybdate reduction [2, 7]. As with other silicomolybdate reactions, however, the inhibition of silicomolybdate reduction by DBMIB has been found to be strongly dependant on the reaction conditions. Figs. 3A and 3C show that DBMIB does inhibit both silicomolybdate reduction and ferricyanide reduction in the presence of high concentrations of albumin. However, the concentrations of DBMIB required for the inhibition (10 μM) are about 10 times higher than those normally required, suggesting that there is a considerable binding of DBMIB by albumin. In the absence of high concentrations of albumin, 1–2 μM DBMIB inhibits ferricyanide reduction strongly though not completely (Fig. 3D; see also ref. 11). In contrast, the low concentrations of DBMIB do not inhibit silicomolybdate reduction under the same low albumin conditions, confirming the previous report [2]. It should be noted that the low albumin conditions employed here (0.2 mg/ml albumin with dimethyl sulfoxide and glycerol) are the same as the phosphorylating conditions

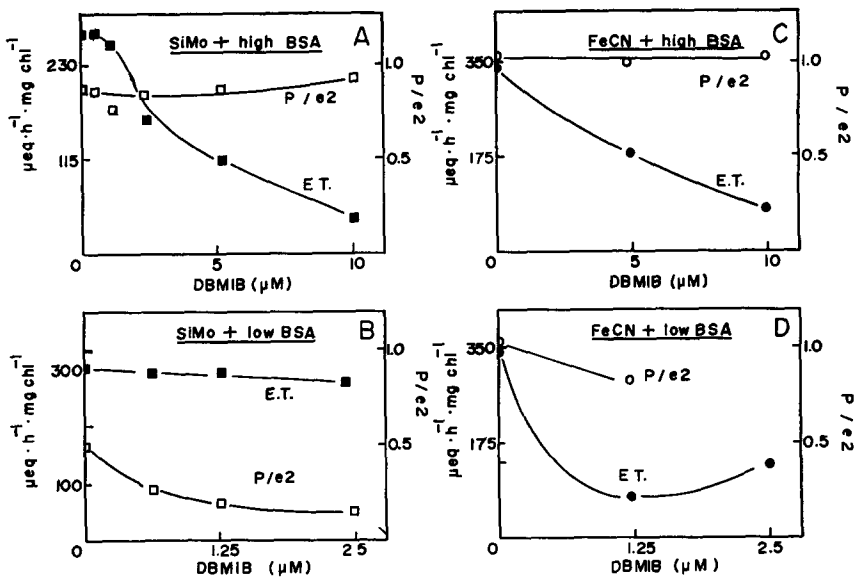

Fig. 3. The effect of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) on silicomolybdate (SiMo)- and ferricyanide (FeCN)-mediated Hill reaction under low and high bovine serum albumin (BSA) conditions. Ferricyanide and DCMU were 0.4 mM and 5 μM, respectively. Reactions media for "high BSA" contained 7 mg bovine serum albumin/ml; "low BSA", 0.2 mg bovine serum albumin/ml. For other conditions see Fig. 1, and Materials and Methods. E.T., electron transport; Chl, chlorophyll.

employed for the DCMU experiment of Fig. 1A. As Fig. 3B shows, although the electron flow rate is little affected by DBMIB, the P/e_2 level drops from 0.5 to 0.2 just as in the case of DCMU inhibition (Fig. 1A). Thus, if one ignores the electron transport data, the shift in P/e_2 can be readily explained by assuming that, even under low albumin conditions, part of silicomolybdate reduction still takes place through Photosystem I and therefore through both sites of energy coupling. (Indeed, in our chloroplast preparations the P/e_2 of 0.5 observed at zero DBMIB concentration seems too high to be ascribed to Photosystem II energy coupling alone.) The plastoquinone analog DBMIB [20] would certainly block the Photosystem I electron transport component together with Photosystem I energy coupling (hence the drop in P/e_2). This blocking, however, might not be expressed as a partial inhibition of overall electron flow if, for instance, there had been a kinetic competition between Photosystem II silicomolybdate reduction and the electron transport towards Photosystem I, for a common electron source in the electron transport chain between the photoacts.

The effects of pH, salts, and temperature

As we have amply documented already, the DCMU-insensitive silicomolybdate reduction does support phosphorylation under appropriate conditions. The effects of pH on the DCMU-insensitive Hill reaction and the associated phosphorylation are shown in Fig. 4. The data show that both the electron transport and the phosphorylation are relatively pH insensitive between 6.5 and 8.5 except for the steep decline of the electron transport rate above pH 8. This pH profile of electron transport agrees

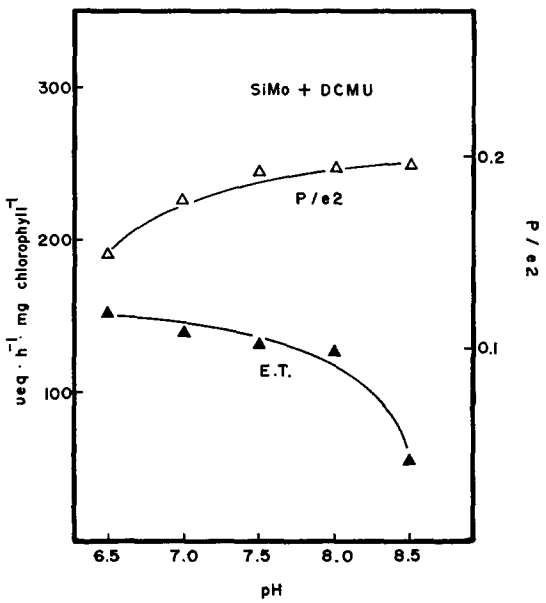

Fig. 4. The effect of pH on the DCMU-insensitive silicomolybdate (SiMo)-mediated electron transport (E.T.) and phosphorylation efficiency (P/e_2). Reaction conditions were like those of Fig. 1A except that the buffer was 50 mM HEPPS adjusted to the indicated pH with lysine (free base) as described in Materials and Methods to minimize salt uncoupling. Chl, chlorophyll.

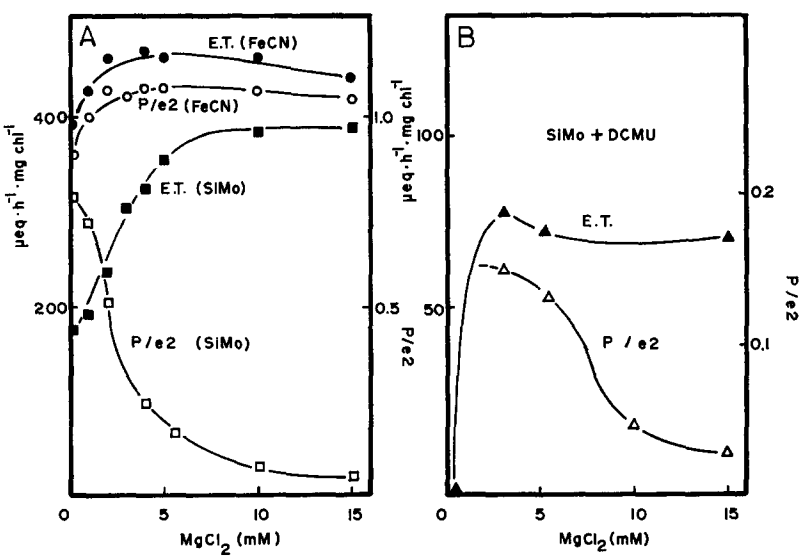

Fig. 5. The effect of $MgCl_2$ on the silicomolybdate (SiMo)- and ferricyanide (FeCN)-mediated electron transport (E.T.) and phosphorylation efficiency (P/e_2). Reaction conditions were like Fig. 1A except that DCMU was 5 μM and ferricyanide was 0.4 mM where indicated. Chl, chlorophyll.

rather well with that reported by Barr et al. [3]. The relatively pH-independent phosphorylation efficiency has been considered as characteristic of Photosystem II energy coupling [13].

Mg^{2+} (as $MgCl_2$) has striking effects on silicomolybdate reduction and the associated phosphorylation (Fig. 5). In the absence of DCMU, there is a complete uncoupling of silicomolybdate reduction at a Mg^{2+} concentration of 10 mM. The electron transport rates are strongly stimulated by the same concentrations of Mg^{2+} which uncouple phosphorylation (Fig. 5A). In contrast, ferricyanide reduction and the associated phosphorylation are practically independent of Mg^{2+} over the range tested. It should be pointed out, however, that no effort to exhaustively remove Mg^{2+} from the chloroplasts or from the ADP was made, and so even at the "zero" Mg^{2+} concentration indicated, there was certainly some endogenous Mg^{2+} present. DCMU-insensitive silicomolybdate reduction is even more sensitive to Mg^{2+} than is the sensitive reaction (Fig. 5B). At Mg^{2+} concentrations less than 0.5 mM, no or very little electron transport is seen. The reduction rate rises very sharply with the increase in Mg^{2+} concentration and reaches a plateau at about 2–3 mM Mg^{2+}. Further increase in Mg^{2+} concentration induces uncoupling.

NaCl also stimulates silicomolybdate reduction and induces uncoupling although the required concentrations are approx. 10 times higher than those for $MgCl_2$ (Fig. 6). Chloride salts of other alkali metals (KCl, LiCl) were as effective as NaCl, and so were sodium salts of other anions (Na_2SO_4, $NaHCO_3$) when compared on the basis of the Na^+ concentration. We conclude, therefore, that the cations are primarily responsible for the salt effects.

As is clear from the experiments described so far, silicomolybdate reduction (with or without DCMU) is not coupled or is only feebly coupled unless precautions are taken to minimize uncoupling by silicomolybdate (addition of protective agents

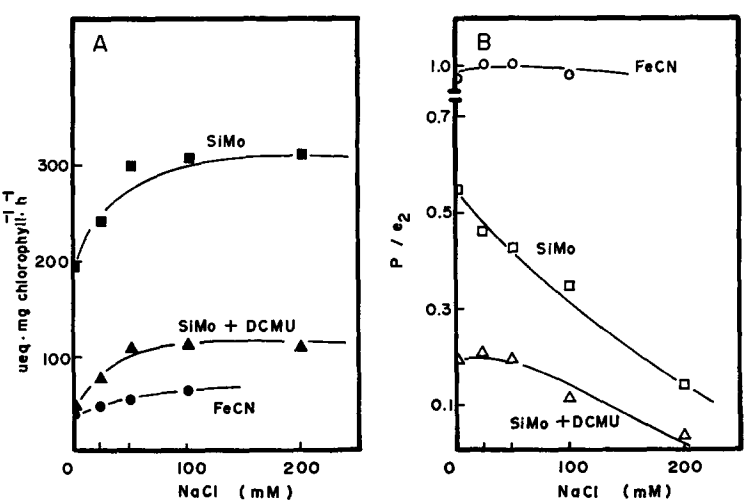

Fig. 6. The effect of NaCl on ferricyanide (FeCN)- and the DCMU-insensitive silicomolybdate (SiMo)-mediated electron transport (E.T.) and phosphorylation efficiency (P/e_2). Reaction conditions are like those of Fig. 1 except that in A the medium contained 0.4 mM ferricyanide and in B, 5 μM DCMU. Chl, chlorophyll.

TABLE II

IRREVERSIBLE INHIBITION OF ELECTRON TRANSPORT AND UNCOUPLING BY SILICOMOLYBDATE IN THE ABSENCE OF PROTECTIVE AGENTS

Chloroplasts were washed with 270 μM silicomolybdate or buffer containing no silicomolybdate as described in the text. Reaction conditions were as described in Fig. 1 except that 0.4 mM ferricyanide, 1 mM diaminodurene, 50 μM methyl viologen, were present where indicated. The reaction medium for the diaminodurene reaction contained 5 μM DCMU.

Chloroplast treatment	e^- transport reaction	μequivalents/ mg chlorophyll per h	P/e_2
Silicomolybdate-washed chloroplasts	$H_2O \rightarrow$ ferricyanide	40	0
	diaminodurene $\rightarrow$ methyl viologen	75	0
	$H_2O \rightarrow$ silicomolybdate	0	–
Control-washed chloroplasts	$H_2O \rightarrow$ ferricyanide	190	0.8
	diaminodurene $\rightarrow$ methyl viologen	500	0.26*
	$H_2O \rightarrow$ silicomolybdate	213	0.15

* P/e_2 uncorrected for superoxide oxidation.

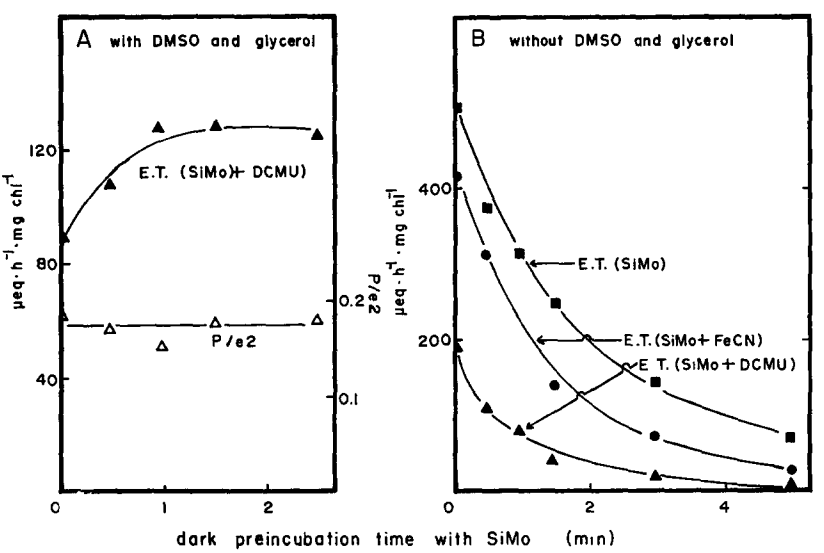

Fig. 7. The effect of dark preincubation of chloroplasts with silicomolybdate (SiMo) on the subsequent silicomolybdate reduction and associated phosphorylation. In A, complete reaction mixtures as described in Fig. 1A were injected with 20 μl of silicomolybdate (in 50 % dimethyl sulfoxide) at zero time in the dark. The indicated times elapsed before the onset of illumination. In B, glycerol was omitted from the reaction medium and aqueous solution of silicomolybdate was injected. When present, ferricyanide and DCMU were 0.4 mM and 5 μM, respectively. FeCN, ferricyanide; DMSO, dimethyl sulfoxide; E.T., electron transport; Chl, chlorophyll.

and adjustment of salt concentrations). Experiments were conducted to learn whether or not the silicomolybdate uncoupling was reversible. Chloroplasts were exposed to the concentration of silicomolybdate regularly used for electron transport study (270 μM), centrifuged quickly (2 min), and then washed free of silicomolybdate with the suspending medium. The activities of these silicomolybdate-washed chloroplasts can be seen in Table II. Clearly, the uncoupling action of silicomolybdate is irreversible. Moreover, all the reactions tested showed strong inhibition of electron transport as well as uncoupling. Even Photosystem I, which is usually less susceptible to inhibition than is Photosystem II is largely inhibited (see data for diaminodurene reactions). Silicomolybdate reduction itself is completely inhibited by this silicomolybdate wash. No such inhibition or uncoupling was observed when chloroplasts were exposed to silicomolybdate in the presence of protective agents (Fig. 7A; in these experiments, assays were performed in situ, rather than after a silicomolybdate wash). Interestingly, there is some increase in the rate of DCMU-insensitive silicomolybdate reduction with the incubation time (1 min). Although not shown, the increase in the DCMU-insensitive electron transport is accompanied by a shortening of the lag phase that precedes the linear phase of electron transport [8]. The parallel preincubation experiments of Fig. 7B depict the time courses of the rapid inactivation of chloroplasts that occurs during exposure to silicomolybdate in the absence of protective agents (dimethyl sulfoxide and glycerol).

The silicomolybdate mediated electron transport rates, with or without DCMU, and the ferricyanide reduction rates, all increase with the reaction temperature in familiar ways (Fig. 8A). However, Arrhenius plots reveal that the thermodynamic properties of silicomolybdate reduction in the presence of DCMU are much different from those of silicomolybdate reduction in the absence of DCMU (Fig. 8B). In the absence of DCMU, silicomolybdate reduction has an activation energy (E_a) of

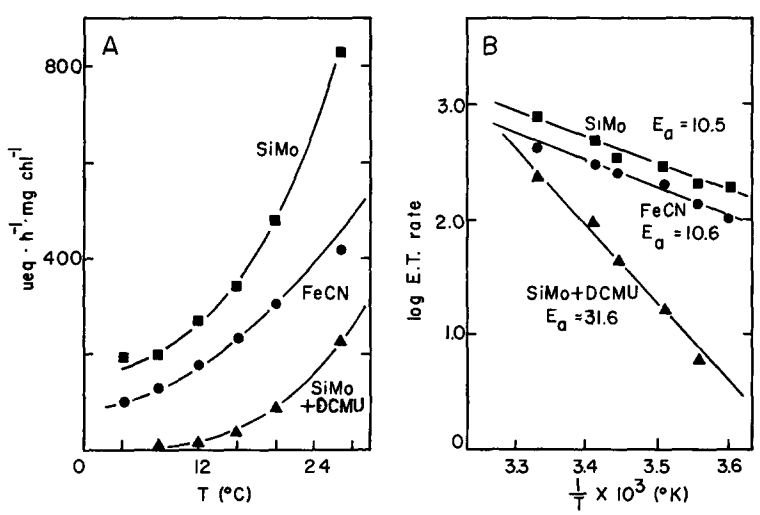

Fig. 8. The effect of temperature on the silicomolybdate (SiMo)- and ferricyanide (FeCN)-mediated Hill reaction. Reaction conditions were as in Fig. 1A. Ferricyanide and DCMU were 0.4 mM and 5 μM, respectively, when used. All reaction buffers were adjusted to pH 8 at their respective reaction temperature. Chl, chlorophyll.

about 10 kcal which is very similar to the E_a calculated for the ferricyanide reduction. The E_a of the DCMU-insensitive silicomolybdate reduction was calculated to be about 30 kcal. Apparently the DCMU-insensitive reaction is not the favored reaction thermodynamically. The implication of these data will be discussed below.

DISCUSSION

From the data of Fig. 1B and of our previous report [8] it is clear that the water to silicomolybdate reaction in the presence of DCMU can support phosphorylation. This result is in apparent contradiction to the results of Giaquinta et al. [2, 5, 6] who found that the same reaction was completely non-phosphorylating. However, the experimental conditions employed in this study are much different from those of Dilley 's group and it seems clear that it is our use of protective agents which makes the observation of ATP formation possible. The protective agents used, (or the stabilizing agents we referred to in our previous paper), were dimethyl sulfoxide (as a solvent for silicomolybdate) and glycerol. Omission of either one of these markedly lowers the phosphorylation efficiency [8]. In the absence of both of these agents, silicomolybdate reduction in our tobacco chloroplasts is almost totally nonphosphorylating regardless of the presence or absence of DCMU.

Giaquinta and Dilley [2, 6] have previously concluded that silicomolybdate had no uncoupling activity. The concentrations of silicomolybdate they tested (33 μM or below) are too low to support measurable DCMU-insensitive silicomolybdate reduction in our "protected" system with tobacco chloroplasts. Although the direct comparison of data are impossible, it is clear that the concentrations of silicomolybdate we used (200–270 μM) were strongly uncoupling (see Table II) and that this uncoupling is prevented by the addition of the protective agents. The prevention, however, does not seem perfect since the maximum phosphorylation efficiency observed for DCMU-insensitive silicomolybdate reduction (P/e_2 of 0.2 in this study, but could reach 0.27; see ref. 8) is not quite as high as the efficiency of standard Photosystem II phosphorylation (P/e_2 of 0.3–0.4 in Class II chloroplasts; see refs. 11–13). High concentration of bovine serum albumin completely protect chloroplasts from silicomolybdate uncoupling (P/e_2 near 1 without DCMU). The high albumin, however, completely blocks Photosystem II reduction of silicomolybdate (Fig. 2B). A similar phenomenon can be seen when the dimethyl sulfoxide concentration in the reaction mixture is increased to 2 % (data not shown). It thus seems that the conditions which quench silicomolybdate uncoupling are also conditions which severely limit the access of silicomolybdate to Photosystem II. In other words, conditions which permit silicomolybdate to accept electrons efficiently from Photosystem II, might inevitably be conditions which permit partial (or complete) silicomolybdate uncoupling. Consequently, the true phosphorylation efficiency (P/e_2 of 0.3–0.4) might only be estimated as an extrapolated value, as exemplified by the albumin experiment of Fig. 2B.

We have as yet no clear idea of how dimethyl sulfoxide and glycerol prevent uncoupling by silicomolybdate. Very probably these agents can form complexes with silicomolybdate and retard its hydrolytic degradation. But they also might serve as a "shield" for the chloroplast and could thus protect sensitive regions of the membrane. Also unsolved is the question of why the prevention of silicomolybdate un-

coupling by the protective agents is abolished when the salt concentration ($MgCl_2$, NaCl, etc.) is increased (Fig. 6). Almost certainly the uncoupling by silicomolybdate is not a common type as exemplified by amines or by phenolic substances. Experiments are in progress to clarify the mechanism of silicomolybdate uncoupling.

Giaquinta et al. [2] and Ben-Hayyim and Neumann [7] found that DBMIB had no effect on silicomolybdate reduction and concluded that there was no electron flow past the region of plastoquinone. As is already clear, this conclusion does not apply when chloroplasts are completely "protected" by high concentrations of albumin. Furthermore, our phosphorylation data (Figs. 1A and 3B) suggest that even when Photosystem II is relatively accessible to silicomolybdate, part of the reduction still takes place at Photosystem I, in much the same way as with standard Class III acceptors [11, 17]. However, the conclusion of the above two groups of workers may well be valid under their own conditions. With the complexity and the variability of the interactions of silicomolybdate with chloroplasts, it is almost impossible for one to assess critically the observations of others based on his own. Not surprisingly, the effects of silicomolybdate wash reported by Ben-Hayyim and Neumann [7] are also very much different from ours (Table II).

The temperature experiments are interesting because they clearly show that the DCMU-insensitive reaction (E_a about 30 kcal) is a thermodynamically less favored reaction than is the DCMU-sensitive reaction (E_a about 10 kcal). This suggests that the DCMU-insensitive reaction might not occur unless forced by a blocking of electron flow after the primary electron acceptor of Photosystem II (Q). It is also possible that some kind of thermal perturbance of the membrane is required if silicomolybdate is to react with Q. (Note in Fig. 8 that virtually no DCMU-sensitive reaction occurred below 10 °C.) The appreciable lag observed for the DCMU-insensitive reaction [8] and the increasing rate of the DCMU-insensitive reaction with the increasing preincubation time (Fig. 8), support such a possibility. Other kinetic differences between

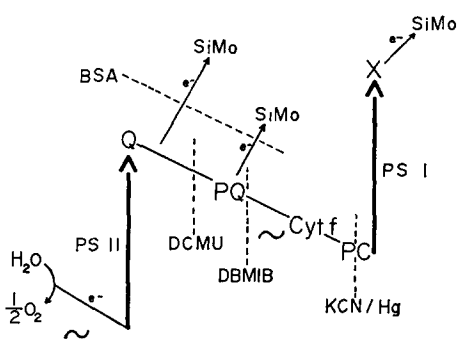

Fig. 9. Three sites of silicomolybdate (SiMo) reduction in the photosynthetic electron transport chain in tobacco chloroplasts. Reduction through two Photosystem II sites (separated by DCMU) is associated with Photosystem II energy coupling only while reduction through Photosystem I is associated with two sites of phosphorylation. "Squiggles" (~) represent energy coupling sites. The broken lines indicate inhibition. Abbreviations: PS II, Photosystem II; Q, primary acceptor of Photosystem II: SiMo, silicomolybdate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PQ, plastoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; cyt *f*, cytochrome *f*; PC, plastocyanin; PS I, Photosystem I; X, primary acceptor of Photosystem I; BSA, bovine serum albumin.

the DCMU-sensitive and the DCMU-insensitive silicomolybdate reduction have been emphasized by Giaquinta et al. [5].

Some of our conclusions from the study of silicomolybdate reactions in tobacco chloroplasts are summarized in Fig. 9 in the form of a diagram. There is certainly a site of silicomolybdate reduction before the DCMU block [4, 6] and the pathway of silicomolybdate reduction through this site is coupled to phosphorylation (ref. 8 and this paper). There is another site of reduction between the DCMU block and the DBMIB block, and the silicomolybdate reduction through this site is similarly coupled to ATP synthesis at Photosystem II (see also ref. 8). Finally, there is a third site of reduction at Photosystem I. This is the only accessible site under high albumin conditions and silicomolybdate reduction at the site can support phosphorylation with a P/e_2 of 1.

As the results of this paper clearly show, silicomolybdate is a Hill oxidant which should be used only with extreme caution, especially under phosphorylating conditions. Besides its uncoupling activity, its demonstrable ability to complex or otherwise interact with common reagents such as methyl viologen and phenazine methosulfate, also needs strict attention in designing and interpreting experiments.

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ADDENDUM

After the submission of the manuscript of this paper, a report by Rosa and Hall (Biochim. Biophys. Acta 449, 23–36, 1976) has appeared which deals with the phosphorylation associated with silicomolybdate reduction in spinach chloroplasts. A P/e_2 value as high as 0.6 (or 0.3 in the presence of ferricyanide) has been reported by these authors for the DCMU-insensitive silicomolybdate reduction in their chloroplast preparation which most closely corresponds to our preparation. We have also been informed recently that Ben-Hayyim and Neumann (Eur. J. Biochem. in the press) have come to the conclusion that DCMU-insensitive silicomolybdate reduction is potentially coupled to phosphorylation.

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