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PHOSPHORYLATION IN ISOLATED CHLOROPLASTS COUPLED TO DICHLOROPHENYLDIMETHYLUREA-INSENSITIVE SILICOMOLYBDATE REDUCTION

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SUMMARY

1. The electron transport in isolated chloroplasts with silicomolybdate as electron acceptor has been reinvestigated. The silicomolybdate reduction has been directly measured as ΔA_{750} or indirectly as O_2 evolution (in the presence or absence of ferricyanide).

2. Silicomolybdate-dependent O_2 evolution is inhibited to a similar extent by 3-(3,4-dichlorophenyl) 1, 1-dimethylurea (DCMU) or dibromothymoquinone (DBMIB), indicating the existence of two different sites of silicomolybdate reduction: one before the DCMU block (i.e. at Photosystem II) and one after the DBMIB block (i.e. at Photosystem I).

3. Silicomolybdate-dependent O_2 evolution is coupled to ATP synthesis with an ATP/2e$^-$ ratio of 1.0 to 1.1. The presence of ferricyanide inhibits this ATP synthesis (ATP/2e$^-$ ratio then is about 0.3).

4. Silicomolybdate-dependent O_2 evolution is also coupled to ATP-synthesis in the presence of DCMU with an ATP/2e$^-$ ratio of 0.6–0.8 characteristic of Site II; in this case the electron transport itself is not affected by uncouplers or energy-transfer inbihitors.

5. The data are interpreted as a further demonstration that the water-splitting reaction is responsible for the conservation of energy at Photosystem II.

INTRODUCTION

The introduction of the plastoquinone antagonist DBMIB [1] and of the

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Abbreviations: DBMIB, dibromothymoquinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DABS, diazonium benzene sulfonate; DAD, C-tetramethyl-*p*-phenylendiamin; MES, 2-(*N*-morpholino)ethane sulphonic acid; HEPES, *N*-2,hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid; Tris, tris (hydroxymethyl) amino methane.

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lipophilic Class III electron acceptors [2], has allowed the study, both in isolated chloroplasts and in whole cells [3, 4] of the electron transport which is confined to Photosystem II. There now exists substantial experimental support [5–9] for the existence of two sites of energy conservation associated with the non cyclic electron transport from H_2O to $NADP^+$. One of these sites (Site I) is associated with electron transport between plastoquinone and cytochrome *f* [10]; the other site (Site II), has recently been shown [5–9] to be associated with a Photosystem II-driven reduction of Class III electron acceptors. Phosphorylation at Site II has been shown to differ from phosphorylation at Site I, in that phosphorylation at Site II shows [8, 9, 11]: (i) a constant $ATP/2e^-$ ratio (about 0.5) over a range of pH values (pH 6–9); (ii) no, or little, [3, 5, 12] photosynthetic control and (iii) a different uncoupler sensitivity. It has been suggested [5, 9, 13] that the water-splitting reaction itself is responsible for the generation of a high-energy state at Photosystem II. This hypothesis is strongly supported by experiments [14–16] which show that protons derived from the oxidation of water are indeed released inside the thylakoid membrane, generating a trans-membrane H^+ gradient which could drive ATP synthesis [17]. On the other hand, Giaquinta et al. [18–22] have recently shown that the DCMU-insensitive, silicomolybdate-mediated reduction of ferricyanide by Photosystem II is associated with no detectable ATP synthesis, no proton accumulation, nor any DABS-detectable conformational change of Photosystem II; and these authors have concluded that, when the electron transport is limited to the Photosystem II sequence $H_2O \rightarrow$ silicomolybdate (+DCMU),the protons released from the oxidation of water are not confined to the inside of the thylakoid membrane. However,Giaquinta and Dilley [21] have observed a complete inhibition of O_2 evolution in the system $H_2O \rightarrow$ silicomolybdate (+DCMU) by an uncoupler-induced high internal pH. Such an inhibition has been accounted for by an internal localization of the water-splitting reaction [23, 24]. We have therefore reexamined the properties of the electron transport confined to the sequence $H_2O \rightarrow$ silicomolybdate and, in the absence of ferricyanide, it has been found that silicomolybdate acts as a direct electron acceptor, that the reduction of silicomolybdate is partially inhibited to a similar extent by DCMU and/or DBMIB, and that ATP synthesis is coupled to the abbreviated electron transport sequence $H_2O \rightarrow$ silicomolybdate (+DCMU).

These data are interpreted as supporting the concept that the internally localized water-splitting reaction is responsible for the conservation of energy at Photosystem II (see review of Trebst [25]).

METHODS AND MATERIALS

Chloroplasts from greenhouse grown spinach were essentially isolated as described by Heldt and Sauer [26] in 0.33 M sorbitol, 50 mM MES/NaOH (pH 6.2), 1 mM $MgCl_2$ and 10 mg/ml bovine serum albumin. After centrifugation, the chloroplasts were resuspended in a medium consisting of 0.33 M sorbitol, 50 mM HEPES/NaOH (pH 7.6), 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM sodium EDTA, 10 mg/ml bovine serum albumin and washed once in the same medium. The chloroplasts when isolated were Type A complete in the classification of Hall [27]. All procedures were carried out at 0–4 °C and the chloroplast suspension was maintained at 0 °C and used within 2–3 h. The chloroplasts were capable of fixing

CO_2 at rates of 50–100 μmol CO_2/h per mg. of chlorophyll. The chlorophyll concentration was measured according to Arnon [28]. Oxygen evolution was measured in a cell equipped with a Rank O_2 electrode, maintained at 17 °C and illuminated as described previously [29]. $^{32}P_i$ was obtained from the Radiochemical Centre, Amersham, Bucks., and phosphorylation measured according to Avron [30]. In all the experiments where DCMU and/or DBMIB were present, they were added immediately after the light was switched on. The rates of O_2 evolution were calculated from the initial slope (first 30–40 s) starting 10 s after the inhibitor addition. The rates of phosphorylation were measured in the first 40 s after the ADP addition. The ADP was also added just after the light was switched on and, where DCMU was also required in the experiment, immediately after DCMU. Dark controls were run in parallel and no ATP synthesis occurred in the absence of illumination. All the experiments reported in the present paper were carried out at least 4 times with different chloroplast preparations, over a period of 5 months. All chemicals used were of highest grade commercially available from B.D.H. Chemicals, Sigma Chemical Co. and Hopkin and Williams. Bovine serum albumin was Fraction V powder from Sigma Chemical Co. Silicomolybdate was kindly provided by Dr R. A. Dilley; silicomolybdate reduction was directly measured spectrophotometrically according to Barr et al. [31]. DAD and DBMIB were a gift of Professor A. Trebst.

RESULTS

As shown in Table I, the DCMU-sensitivity of O_2 evolution depends upon the conditions under which the DCMU is added. As previously reported [32] DCMU added in the light before silicomolybdate addition is a more effective inhibitor of O_2 evolution than DCMU added after silicomolybdate. It may also be seen (Table I) that the rate of O_2 evolution in the presence of ferricyanide was decreased by an addition of silicomolybdate (cf. refs 21, 32) and decreased to a greater extent by a preincubation in the dark with silicomolybdate, but in the latter case a greater proportion of the electron transport was resistant to DCMU. A possible explanation of this enhanced DCMU-resistance is that a preincubation with silicomolybdate enables ferricyanide to gain direct access to Q, the primary acceptor of Photosystem II [33]. However, as shown in Fig. 1, silicomolybdate can accept electrons even in the absence of ferricyanide, becoming reduced itself. In the absence of DCMU, O_2 evolution proceeds at a linear rate until 50–70 % of silicomolybdate is reduced; while when ferricyanide is also present, the linear rate of O_2 evolution is observed for a longer period of time, probably because ferricyanide reoxidises the silicomolybdate. However, in the presence of DCMU, O_2 evolution proceeds for only 30–60 s, either in the presence or in the absence of ferricyanide as previously reported [21, 32].

When silicomolybdate reduction was measured directly, it could be seen (Table II) that silicomolybdate itself acts as a Photosystem II electron acceptor. Furthermore, since the reduction of each mol of silicomolybdate is accompanied bythe evolution of one mol of O_2, it may be concluded that each mol of silicomolybdate accepts 4 electrons under the conditions of our experiments. It may also be observed (Table II) that both DCMU and DBIMB inhibit silicomolybdate reduction to a similar extent (about 50 %). This result leads to the conclusion that, when only

TABLE I

SILICOMOLYBDATE-MEDIATED OXYGEN EVOLUTION

The reaction mixture contained in 2 ml: 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM sodium EDTA, 10 mM NaCl, 5 mM KH_2PO_4 and 0.1 ml of chloroplast suspension equivalent to 70 μg of chlorophyll (chloroplasts in reaction mixture would be Type C [27]). Where indicated 0.5 mM ferricyanide, 50 μM silicomolybdate and 5 μM DCMU were added either before (dark) or immediately after the light was switched on.

Electron acceptor	Conditions under which electron acceptor(s) added	Conditions under which DCMU added	Rate of O_2 evolution μequiv/mg chlorophyll/h
Ferricyanide	Dark	–	188
Ferricyanide	Light	–	188
Ferricyanide	Dark	Light	0
Ferricyanide+silicomolybdate	Dark	–	122
Ferricyanide+silicomolybdate	Light	–	122
Ferricyanide+silicomolybdate	Dark	Light	45
Ferricyanide+silicomolybdate	Dark	Dark	45
Ferricyanide+silicomolybdate	Light	Light (before silicomolybdate)	10
Ferricyanide+silicomolybdate	2 min preincubation in the dark	–	100
Ferricyanide+silicomolybdate	2 min preincubation in the dark	Light	78

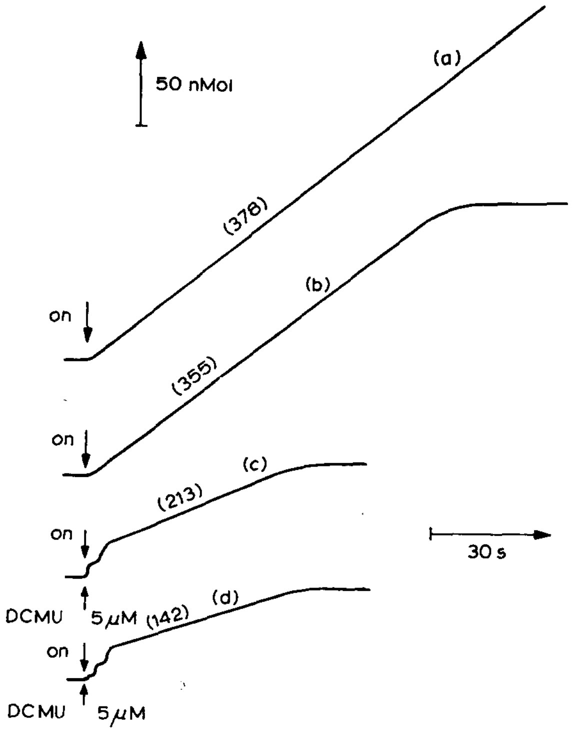

Fig. 1. O_2 evolution traces. Conditions as in Table I with 100 μM silicomolybdate present in all cases. Chlorophyll concentration was 35 μg/ml. In (a) and (c), 0.5 mM ferrycianide was also present. The numbers in brackets are the rate of O_2 evolution expressed in μequiv/h per mg chlorophyll.

TABLE II

SILICOMOLYBDATE-DEPENDENT ELECTRON TRANSPORT

Conditions as described in Table I with 100 μM silicomolybdate present. Chlorophyll concentration was 27.5 μg/ml. The rates of silicomolybdate reduction were calculated over the first 30 s of illumination by measuring the ΔA_{750} on the basis of an extinction coefficient of 8 $mM^{-1} \cdot cm^{-1}$ according to Barr et al. [31].

Additions	Rate of O_2 evolution (μmol/h per mg chlorophyll)	Rate of silicomolybdate reduction (μmol silicomolybdate/h per mg chlorophyll)
None	58	60
DCMU (5 μM)	33	30
DBMIB (1 μM)	35	35

silicomolybdate is present, it can accept electrons at two different sites: one before the DCMU block and one after the DBMIB block. While Giaquinta and Dilley [20,21] have previously observed a partial inhibition by DCMU of a silicomolybdate mediated reduction of ferricyanide, these authors did not observe any significant inhibition of this reaction by DBMIB. Thus we measured the effect of DCMU and/or DBIMB on the electron transport from H_2O to ferricyanide in the presence of catalytic amounts of DAD (a Class III electron acceptor) and/or silicomolybdate. It was found (Table III) that silicomolybdate mediated electron transport was always inhibited to the same extent by DCMU and/or DBMIB, while, as expected, DAD-mediated electron transport was only slightly inhibited by DBMIB, but completely inhibited by DCMU. The data of Table III provide further evidence that silicomolybdate can accept electrons at only one site on the Photosystem II side of the DBMIB block, since it is shown that an addition of silicomolybdate on top of DAD decreases the rate of electron transport to the level of silicomolybdate reduction and makes it insensitive to DCMU. It seems that the O_2 evolution observed in the presence of both DAD and silicomolybdate is due to silicomolybdate-mediated electron transport without any significant participation of DAD because, in the presence of DBMIB, the rate of O_2 evolution resembles that dependent upon silicomolybdate mediated electron transport and differs from that dependent upon DAD-mediated electron transport in its insensitivity to NH_4^+ (Table III) [6].

As previously reported by Giaquinta and Dilley [21] we found (data not shown) that NH_4Cl at high pH completely inhibits silicomolybdate-dependent O_2 evolution (±DCMU). As mentioned above and discussed at length by Reimer and Trebst [24], an inhibition of O_2 evolution at high pH by uncouplers, such as NH_4Cl, necessitates an internal localization of the water-splitting reaction. For these authors, the internal localization is attributable to a light-activated conformational change [24]. However, Giaquinta and Dilley [21] have suggested that the water-splitting reaction is externally located in the system $H_2O \rightarrow$ silicomolybdate (+DCMU). Therefore it was of interest to determine whether, under our experimental conditions, phosphorylation was coupled to electron transport in the systems $H_2O \rightarrow$ silicomolybdate (+DCMU) since it has been suggested that the internal production of protons by the water-splitting reaction is responsible for the generation of a high energy state at Photosystem II [25].

The data of Table IV show that with Type C chloroplasts [27] in the reaction mixture, phosphorylation with an ATP/$2e^-$ ratio of 1.0 was obtained coupled to either ferricyanide reduction or to silicomolybdate reduction, showing that silicomolybdate per se does not affect the phosphorylation. However, when ferricyanide and silicomolybdate were both present, we obtained an ATP/$2e^-$ ratio of 0.3 which is in agreement with the value reported by Giaquinta et al. [22]. Significantly phosphorylation was also coupled to the DCMU-insensitive reduction of silicomolybdate. Again, the ATP/$2e^-$ ratio was much higher in the absence of ferricyanide (ATP/$2e^-$ ratio of 0.8) than in the presence of ferricyanide (ATP/$2e^-$ ratio of 0.3). It may be seen (Table IV) that controls in the presence of DCMU obviated the possibility that the phosphorylation measured was not dependent upon silicomolybdate reduction. If we subtract from the ATP obtained in the presence of silicomolybdate+DCMU, the ATP obtained in the presence of DCMU alone, we still obtain an ATP/$2e^-$ ratio of 0.6 which is characteristic of Site II phosphorylation measured

TABLE III

EFFECT OF DCMU AND DBMIB ON ELECTRON TRANSPORT FROM H_2O TO FERRICYANIDE CATALYSED BY SILICOMOLYBDATE AND DAD

Conditions as described in Table I with 0.5 mM ferricyanide present. Chlorophyll concentration was 35 μg/ml. In parentheses: relative rates from expt. in Table VII.

Additions	Rate of O_2 evolution (μequiv/h per mg chlorophyll)			
	No inhibitor	DCMU 5 μM	DBMIB 1 μM	DCMU 5 μ M + DBMIB 1 μM
None	156	0	46	–
Silicomolybdate 50 μM	160	72	72	–
Silicomolybdate 100 μM	200	160	168	160
Silicomolybdate 100 μM + NH_4Cl 4 mM	(305)	(145)	150	–
DAD 0.1 mM	320	0	250	0
DAD 0.1 mM + NH_4Cl 4 mM	350	–	60	–
DAD 0.1 mM + silicomolybdate 100 μM	220	160	168	152
DAD 0.1 mM + silicomolybdate 100 μM + NH_4Cl 4 mM	310	–	148	140

TABLE IV

PHOSPHORYLATION COUPLED TO SILICOMOLYBDATE-DEPENDENT ELECTRON TRANSPORT

Conditions as in Table I with 1 mM ADP present. 5 mM KH_2PO_4 contained 3.5 10^6 cpm $^{32}P_i$. Chlorophyll concentration was 37.5 µg/ml.

Electron acceptor	No inhibitor		
	Rate of O_2 evolution (µequiv/h per mg chlorophyll))	Rate of ATP formation (µmol/h per mg chlorophyll	ATP/2e$^-$ ratio
None	–	45	–
Ferricyanide 0.5 mM	600	260	0.9
Silicomolybdate 100 µM	324	171	1.1
Silicomolybdate 100 µM + ferricyanide 0.5 mM	392	67	0.3

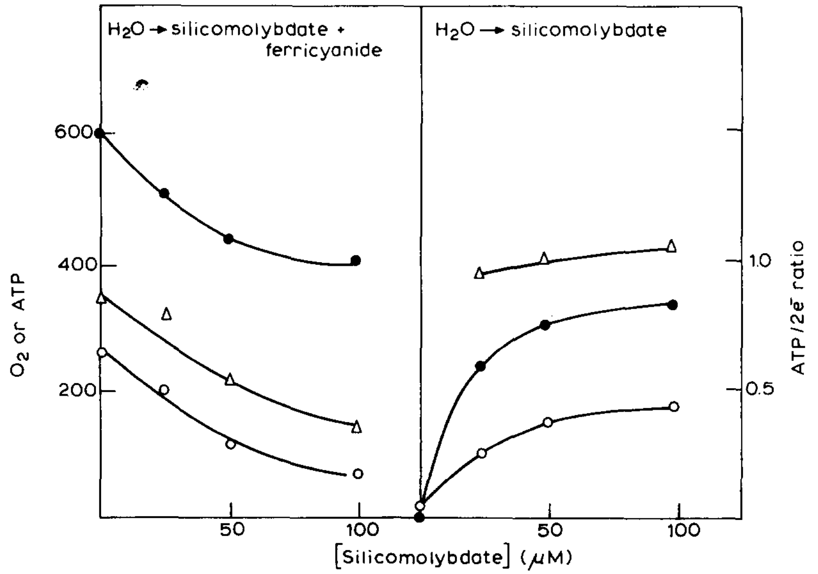

Fig. 2. Electron transport, phosphorylation and ATP/2e$^-$ ratio as function of silicomolybdate concentration. Conditions as in Table IV. Where indicated 0.5 mM ferricyanide was added. Chlorophyll concentration was 37.5 µg/ml. ● — ●, O_2 evolution expressed in µequiv/h per mg chlorophyll. ○ — ○, ATP formation expressed in µmol/h per mg chlorophyll. △ — △, ATP/2e$^-$ ratio.

DCMU 5 μM			DBMIB 1 μM		
Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	ATP/2e$^-$ ratio	Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	ATP/2e$^-$ ratio
0	18	–	0	17	–
0	10	–	120	17	0.3
160	67	0.8	184	67	0.7
208	27	0.3	240	28	0.2

with Class III electron acceptors [5–9]; instead if we apply the same correction to the system where ferricyanide is also present the ATP/2e$^-$ ratio drops to a very low level (about 0.1). Essentially similar results were observed when DBMIB was used instead of DCMU showing that there is only one site before the DBMIB block where silicomolybdate accept electrons.

Fig. 2 shows the relationship between silicomolybdate-dependent phosphorylation and the concentration of silicomolybdate. It may be noted that in the presence of ferricyanide, increasing the concentration of silicomolybdate results in a decrease in the ATP/2e$^-$ ratio from 0.9 to 0.3; while in the absence of ferricyanide, varying the concentration of silicomolybdate over a similar range has no significant effect on the ATP/2e$^-$ ratio of 1.0.

To exclude the possibility that the phosphorylation measured in the system $H_2O \rightarrow$ silicomolybdate (+DCMU) was due to a silicomolybdate-stimulated endogenous cyclic phosphorylation and not coupled to a simultaneous O_2 evolution, chloroplasts were washed with a hypotonic medium to produce Type E chloroplasts and so decrease the rate of the endogenous cyclic phosphorylation [34]. As shown in Table V, this washing procedure decreased the rate of the endogenous cyclic phosphorylation without affecting the ATP/2e$^-$ ratio of 1.0 obtained with either silicomolybdate or ferricyanide in the absence of DCMU. Nor did the washing procedure affect the ATP/2e$^-$ ratio of 0.6 with silicomolybdate in the presence of DCMU, if in this latter case the contribution of cyclic phosphorylation was substracted from the total phosphorylation (not calculated in Table V). A further demonstration that the phosphorylation observed with silicomolybdate in the presence of DCMU, is dependent upon electron transport from H_2O, is provided by the data shown in Table VI. These data show that by washing the chloroplasts with Tris [35] the O_2 evolution in the presence of silicomolybdate and DCMU is completely inhibited and correspondingly the phosphorylation is decreased to the low level observed in the presence of DCMU alone, which is probably due to the endogenous cyclic phosphorylation. But it may be seen that in our conditions (Table VI), the Tris-washed chloroplasts still retained some Photosystem II activity (about 30 %) in the

TABLE V

EFFECT OF HYPOTONIC WASHING OF CHLOROPLASTS ON DCMU-INSENSITIVE SILICOMOLYBDATE-DEPENDENT PHOSPHORYLATION

Conditions as in Table IV. Chlorophyll concentration in the reaction mixture was 37.5 μg/ml. Broken chloroplasts (Type E) were prepared by diluting 1 ml of isolated chloroplasts (750 μg of chlorophyll) with 10 ml of 50 mM HEPES /NaOH(pH 7.6) containing 10 mM NaCl. After 10 min at 0 °C the diluted chloroplast were sedimented at 20 000 × *g* for 10 min and resuspended in 0.33 M Sorbitol, 50 mM HEPES/NaOH (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM sodium EDTA, 10 mM NaCl and 10 mg/ml bovine serum albumin.

Additions	Control chloroplasts (Type C)			Broken chloroplasts (Type E)		
	Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	ATP/$2e^-$	Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	ATP/$2e^-$ ratio
Ferricyanide 0.5 mM	444	220	1.0	460	221	1.0
DCMU 5 μM	0	11	–	0	3	–
Silicomolybdate 100 μM	316	157	1.0	308	168	1.1
Silicomolybdate 100 μM + DCMU 5 μM	60	28	0.9	80	25	0.6

TABLE VI

EFFECT OF TRIS-WASHING ON THE SILICOMOLYBDATE-DEPENDENT OXYGEN EVOLUTION AND PHOSPHORYLATION

Conditions as in Table IV. In the reaction mixture, chlorophyll concentration was 35 μg/ml. Tris-washed chloroplasts were prepared essentially according to Yamashita and Butler [35]. Type A chloroplasts were centrifuged and resuspended in 0.8 M Tris/HCl (pH 8) at a chlorophyll concentration of 0.1 mg of chlorophyll/ml. After 10 min at 0 °C the chloroplasts were sedimented at 1000 × *g* for 3 min and resuspended in 0.33 M Sorbitol, 50 mM HEPES/NaOH (pH 7.6), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM sodium EDTA, 10 mM NaCl and 10 mg/ml bovine serum albumin.

Additions	Control chloroplasts			Tris-washed chloroplasts		
	Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	ATP/2e$^-$ ratio	Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	ATP/2e$^-$
Ferricyanide 0.5 mM	312	136	0.9	108	40	0.8
DCMU 5 μM	0	10	–	0	10	–
Silicomolybdate 100 μM	316	132	0.8	72	28	0.8
Silicomolybdate 100 μM + DCMU 5 μM	152	46	0.6	0	10	–

TABLE VII

EFFECT OF NH_4Cl AND PHLORRHIZIN ON SILICOMOLYBDATE-DEPENDENT OXYGEN EVOLUTION AND PHOSPHORYLATION

Conditions as in Table IV. Chlorophyll concentration was 40 μg/ml.

Additions	Control		
	Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	ATP/$2e^-$ ratio
Ferricyanide 0.5 mM	396	222	1.2
DCMU 5 μM	0	14	–
Silicomolybdate 100 μM	288	141	1.0
Silicomolybdate 100 μM+DCMU 5 μM	172	54	0.6
Ferricyanide 0.5 μM+silicomolybdate 100 μM	308	40	0.3
Ferricyanide 0.5 mM+silicomolybdate 100 μM +DCMU 5 μM	160	20	0.3

presence of ferricyanide or silicomolybdate alone (cf. [35]). Finally, we determined the effect of the energy-transfer inhibitor 4,4′-6′ trihydroxy-2′-glucosidodihydrochalcone (phlorrhizin) and the uncoupler, NH_4Cl, on the DCMU-insensitive electron transport (Table VII). As previously observed [8, 9] with the DBMIB-insensitive electron transport from H_2O to Class III electron acceptors, neither an energy-transfer inhibitor nor an uncoupler had any effect on the rates of the DCMU-insensitive electron transport, while these agents completely abolished phosphorylation (Table VII).

DISCUSSION

Extensive studies [5–9, 11, 12] have already been made of the phosphorylation coupled to the DBMIB-insensitive electron transport from H_2O to Class III electron acceptors such as DAD. Furthermore, a DCMU-insensitive electron transport in the presence of silicotungstate [36], Hg^{2+} ions [37] or silicomolybdate [18,–22, 32] has been observed. In the present study we have been able to observe a phosphorylation coupled to this DCMU-insensitive electron transport from water to silicomolybdate.

The DCMU-insensitive reduction of silicomolybdate described in the present paper is accompanied by phosphorylation with an ATP/$2e^-$ ratio of 0.9–0.6 (depending upon the subtraction of the endogenous cyclic phosphorylation), but is not affected by uncouplers or energy-transfer inhibitors (Table VII) indicating a lack of photosynthetic control. Similarly an ATP/$2e^-$ ratio of 0.5 and a lack of photosynthetic control have been found in previous studies [8, 9] of Photosystem II-associated phosphorylation, where, however, Class III electron acceptors were employed, and where electron transport was sensitive to DCMU, i.e. when electron transport between Q and plastoquinone was presumable involved.

It is well established that phosphorylation requires an internal acidification

+NH_4Cl 4 mM			+Phlorrhizin 1 mM		
Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	$ATP/2e^-$ ratio	Rate of O_2 evolution (μequiv/h per mg chlorophyll)	Rate of ATP formation (μmol/h per mg chlorophyll)	$ATP/2e^-$ ratio
780	52	0.1	88	20	0.5
–	–	–	–	–	–
440	27	0.1	164	20	0.2
156	0	–	160	0	–
420	10	0.1	172	7	0.1
176	0	–	148	0	–

of the thylakoid [38]. With the limited electron transport chain involved in the system $H_2O \rightarrow$ silicomolybdate (+DCMU), the most likely source of the protons accumulated inside the thylakoid is the oxidation of water per se. Thus, we conclude that in the system $H_2O \rightarrow$ silicomolybdate (+DCMU), the oxidation of water occurs at the inner surface of the thylakoid membrane. Further, since electron transport from Q to plastoquinone does not occur in the presence of DCMU, it may be concluded that electron transport from Q to plastoquinone is not necessary for an internal localization of the water-splitting reaction. This conclusion is at variance with that of Giaquinta and Dilley [21] who did not report any phosphorylation coupled to the electron transport in the system $H_2O \rightarrow$ silicomolybdate (+DCMU). It would be of interest to determine to what extent this discrepancy between our results and those of Giaquinta and Dilley [21] can be accounted for by the different methods employed in isolating the chloroplasts which result in chloroplasts with different phosphorylation coupling abilities [26, 39]. Our conclusion is, however, compatible with the findings of Riemer and Trebst [24], who concluded that electron transport through Photosystem II is necessary for an internally localized water oxidation.

Similar rates of electron transport, phosphorylation, and similar $ATP/2e^-$ ratios were observed when the electron transport to silicomolybdate was partially inhibited either by DCMU or by DBMIB. Thus it may be concluded that silicomolybdate accepts electrons at two sites: one site before the DCMU block, the other site after the DBMIB block. Furthermore, we conclude that energy conservation is associated with electron transport before the DCMU block. This site of energy conservation may be identified with the water oxidizing reaction.

There are two main features of silicomolybdate reduction which are at present unexplained and which clearly require further study. Firstly, silicomolybdate is reduced only for a limited period of time in the presence of DCMU. Secondly, when silicomolybdate or ferricyanide are added singly an $ATP/2e^-$ ratio of 1.0 is observed, but when silicomolybdate and ferricyanide are added together, an ATP/2e$^-$ ratio of about 0.3 is observed.

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