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TWO POSSIBLE 3-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA-INSENSITIVE SITES IN PHOTOSYSTEM II OF SPINACH CHLOROPLASTS

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Summary

Two possible 3-(3,4-dichlorophenyl)-1,1-dimethylurea-insensitive sites were found in PS II of spinach chloroplasts, depending on the pH of the assay medium used. The low site (pH 6) can be inhibited by certain quinolines, such as 8-hydroxyquinoline at concentrations less than 50 μM. The high pH site (pH 8) can be inhibited by disodium cyanamide, folic acid, or 5,6-benzoquinoline at concentrations from 50 μM to 5 mM. With the exception of orthophenanthroline, which stimulates the high pH site but does not show much inhibition at low pH, all other inhibitors gave opposite effects at the pH values used, i.e., they stimulated at low pH or inhibited at high pH, or vice versa. Several mechanisms for the observed effects are discussed.

Introduction

An induction of 3-(3,4-dichlorophenyl)-1,1-dimethylurea-insensitive PS II activity by silicotungstic acid was first observed by Girault and Galmiche [1]. DCMU-insensitive silicomolybdic acid reduction by spinach chloroplasts was discovered by Giaquinta et al. [2] and by Barr et al. [3]. Detailed studies on the properties of this site were carried out by Giaquinta and Dilley [4,5] and by Zilinskas and Govindjee [6]. Berg and Izawa [7] described three silicomolybdate electron acceptor sites: (1) a DCMU-insensitive site; (2) a DCMU-sensitive site in PS II and (3) a DCMU-sensitive site in PS I in tobacco chloroplasts. In the presence of protective agents such as bovine serum albumin,

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS I, Photosystem I; PS II, Photosystem II; Mes, 4-morpholineethanesulfonic acid.

photophosphorylation with a P/e_2 ratio of 0.2 occurred on the silicomolybdate pathway. Rosa and Hall [8] could obtain a higher ATP/2e$^-$ ratio, namely 0.6—0.8, characteristic of site II photophosphorylation.

Up to this point, no specific inhibitor of the reaction was known, but chloroplasts could be uncoupled by NH_4Cl or various ionophores [4,5]. The present study was undertaken with the purpose of finding a specific inhibitor of the silicomolybdate pathway. Preliminary results by Crane and Barr [9,10] showed that bicarbonate inhibited silicomolybdate reduction. Some chelators, such as bathocuproine [11], also inhibit the DCMU-insensitive silicomolybdate reduction, whilst compounds resembling folic acid [12] stimulate it. The present study enlarges upon the idea of differential stimulation or inhibition of the DCMU-insensitive silicomolybdate pathway, with the result that two possible DCMU-insensitive sites, depending on the pH of the medium, were found in PS II of spinach chloroplasts.

Materials and Methods

Chloroplasts isolated from market spinach as previously described [9—11] were suspended in 0.4 M sucrose/NaCl (0.05 M). Chlorophyll was determined according to the method of Arnon [13], O_2 evolution or uptake was measured polarographically with a Clark-type electrode. Rates were recorded with a Sargent-Welch SRG recorder. Illumination was provided by a specially-built light source equipped with GE quartzline CBA lamp, filtered through a flask containing 1% Cu_2SO_4 solution. Light intensity on the outside of the water-jacketed reaction vessel was $2.5 \cdot 10^6$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}. Reaction mixtures for the various PS II reactions are given in the table and figure legends. All reaction mixtures were preincubated for 3 min in the dark to allow diffusion of inhibitors or stimulators into the thylakoid membrane. Water-insoluble compounds were added in ethanol, not to exceed 5% by vol., to avoid inhibition by ethanol itself. If, in certain cases, solubility in ethanol was poor, NaOH was added either to water or to ethanol, and heating to 50°C was also tried in extreme cases.

12-Molybdosilicate ($Na_4Mo_{12}O_{40}$) was purchased from Apache Chemicals, Inc., P.O. Box 126, Seward, IL 61077. Inhibitors or stimulators of silicomolybdate reduction were purchased from the Aldrich Chemical Co.

Results and Discussion

Silicomolybdate reduction by chloroplasts is normally assayed as O_2 evoluton at near neutral pH [2—5], although it is known that PS II reactions show maximum rates in the pH 6—7 range [14]. When a pH-dependence curve was constructed in this study on SN chloroplasts (Fig. 1), it was found that silicomolybdate in the presence of DCMU gave rise to two maxima (between pH 6.0—6.5 and 7.5—8.0) with 25 mM Tris-Mes buffer. These results suggested the presence of two different DCMU-insensitive silicomolybdate reduction sites. Silicomolybdate concentration curves at pH 6 and 8 (Fig. 2) confirmed the above assumption. According to Fig. 2, smaller amounts of 12-molybdosilicate (85 μM at pH 6 vs. 250 μM at pH 8) are required for the maximum rate of O_2

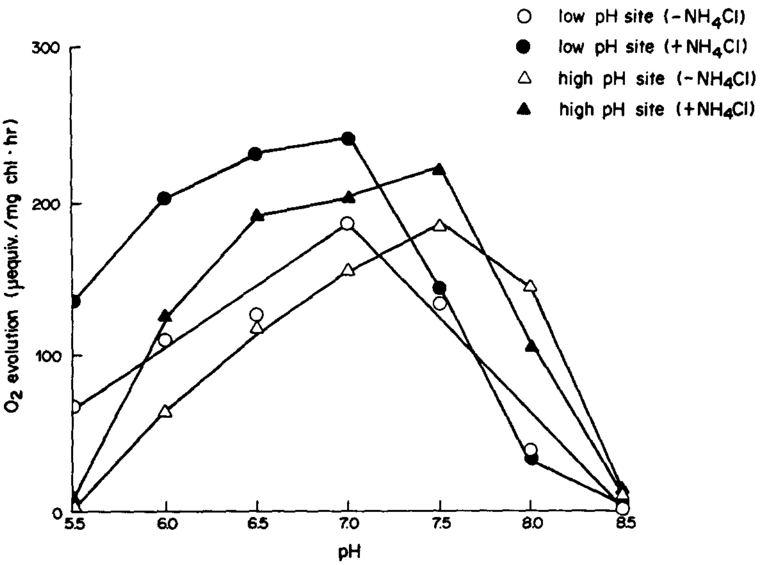

Fig. 1. pH requirements of DCMU-insensitive silicomolybdate reduction in photosystem II of spinach chloroplasts. For the low pH site, the reaction mixture contained chloroplasts (50 μg, Chl), 25 mM Tris-Mes at various pH values as indicated, 2 μM DCMU, 2 mM NH_4Cl where indicated and 85 μM silicomolybdate. For the high pH site all reaction components as above, except silicomolybdate concentration increased to 250 μM.

evolution in the presence of 2 μM DCMU at low pH.

To provide further evidence for the presence of two separate DCMU-insensitive sites in PS II of spinach chloroplasts, it was decided to concentrate on the following two approaches: (1) to study the effect of ions on these sites, to

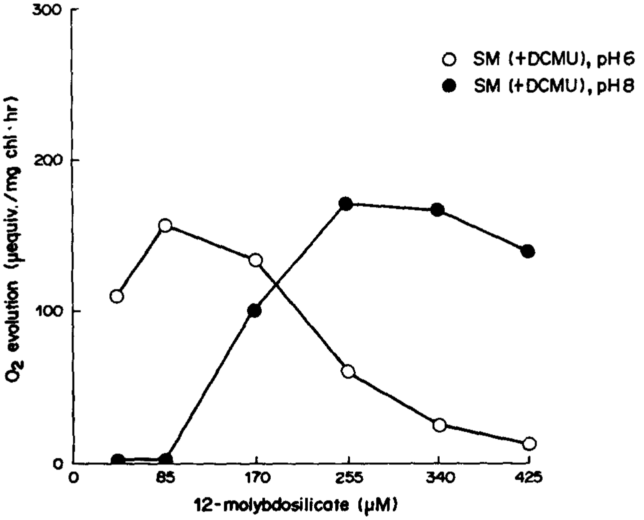

Fig. 2. Optimum concentrations of silicomolybdate required for DCMU-insensitive silicomolybdate reduction in photosystem II of spinach chloroplasts. Different silicomolybdate concentrations gave maximum rates of silicomolybdate reduction at pH 6 and 8. The reaction mixture contained chloroplasts (50 μg Chl), 25 mM Tris-Mes (pH 6 or 8), 2 μM DCMU, 2 mM NH_4Cl and silicomolybdate (85 μM at pH 6, 250 μM at pH 8). SM, silicomolybdate.

find out how topographical changes in membrane conformation affected each of the two silicomolybdate reduction sites and (2) to find specific inhibitors for one or the other of these two sites. Table I summarizes ion effects on silicomolybdate reduction at pH 6 and 8. It can be seen that the pH 6 silicomolybdate site is stimulated by the addition of low concentrations of bicarbonate (2 mM), but is inhibited by the same concentration of $MgCl_2$ or $CaCl_2$. The effect is reversed at pH 8. The high pH site is inhibited by low concentrations of bicarbonate, but is stimulated by low concentrations of $MgCl_2$ or $CaCl_2$. Such results seem to indicate that the pH 8 silicomolybdate reduction site is associated with the forward electron transport pathway, since $MgCl_2$ and other ions facilitate energy transfer ('spillover') from PS II to PS I, as shown by the studies of Gross and associates [15,16]. It is contrary to the data presented by Bose et al. [17] who show stimulation of silicomolybdate reduction in PS II at pH 6.8 by 5 mM $MgCl_2$. We see similar stimulation of silicomolybdate reduction in PS II in winter or with low-light-grown spinach, but in the present study summer or high-light-grown spinach was used; hence, different sites may be involved and the two sets of data may be reconcilable with the above conditions in mind.

The search for specific inhibitors for the pH 6 or 8 DCMU-insensitive silicomolybdate reduction site in PS II has yielded several compounds which inhibit one site, but not the other (Figs. 3—5). With the exception of orthophenanthroline in Fig. 3, which gives almost no inhibition of the reaction at pH 6 but stimulates at pH 8, all other compounds inhibit the reaction at one pH and stimulate at the other. Thus, certain quinolines inhibit the pH 6 reaction and stimulate the pH 8 reaction (Fig. 3). Other quinolines, such as 5,6-benzoquinoline (Fig. 4) have the opposite effect; 5,6-benzoquinoline inhibits the pH 8 reaction and stimulates at pH 6. Silicomolybdate reduction at pH 6 is also stimulated by folic acid, as shown by Leonard et al. [12] and by disodium cyanamide at high concentrations (100% stimulation at pH 6 by 5 mM disodium cyanamide, Fig. 4). At pH 8, however, the same concentrations of these compounds show 50—100% inhibition of the high pH DCMU-insensitive silicomolybdate site. The best stimulator at pH 6 is bathocuproine, a copper

TABLE I

THE EFFECT OF VARIOUS IONS ON DCMU-INSENSITIVE SILICOMOLYBDATE REDUCTION IN PHOTOSYSTEM II OF SPINACH CHLOROPLASTS

O_2 evolution rates are expressed as μequiv./mg Chl per h; reaction mixtures as in Fig. 1; + indicates percent stimulation; — indicates percent inhibition of control rate.

Ion added	Concentration (mM)	pH 6 rate		pH 8 rate	
		O_2	% of control	O_2	% of control
None	—	160	—	170	—
$MgCl_2$	2	134	—16	226	+33
$MgCl_2$	20	0	—100	0	—100
$CaCl_2$	2	124	—22	203	+19
$CaCl_2$	20	0	—100	0	—100
Bicarbonate	2	169	+6	68	—60
Bicarbonate	20	90	—44	0	—100

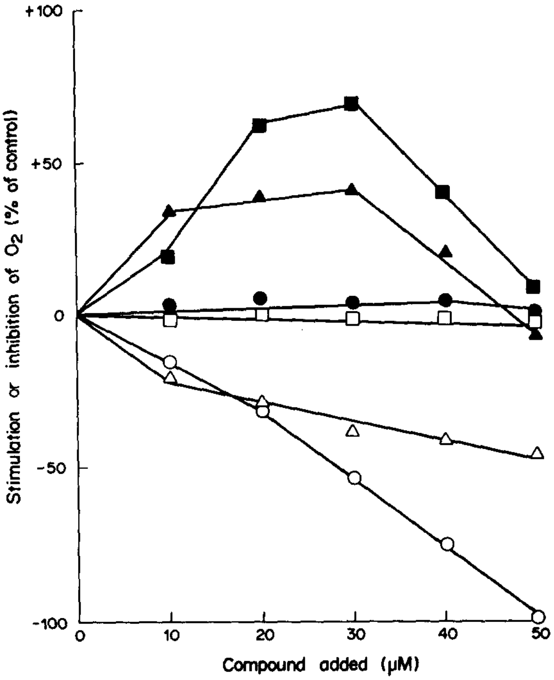

Fig. 3. Inhibition of DCMU-insensitive silicomolybdate (SM) reduction at pH 6. (○——○) $H_2O \rightarrow$ SM (+DCMU), pH 6, plus 8-mercaptoquinoline; (●——●) same at pH 8; (△——△) $H_2O \rightarrow$ SM (+DCMU), pH 6, plus 8-hydroxyquinoline; (▲——▲) same at pH 8; (□——□) $H_2O \rightarrow$ SM (+DCMU), pH 6, plus orthophenanthroline; (■——■) same at pH 8. The control rate was 50 μequiv./mg Chl per h at pH 6 and 150 μequiv./mg Chl per h at pH 8. +indicates stimulation; −indicates inhibition of rate compared to control.

chelator (Fig. 5). It can stimulate the pH 6 site by 200% and inhibit the pH 8 site by more than 50%.

Several explanations for the observed effects are possible. The well-known spillover phenomenon of energy distribution between the two photosystems [15,16] could account for Mg^{2+} stimulation of silicomolybdate reduction at pH 8, if the high pH site of DCMU-insensitive silicomolybdate reduction was somehow associated with this phenomenon. However, since high light intensities ($2.5 \cdot 10^6$ ergs $\cdot$ $cm^{-2} \cdot s^{-1}$) were used, it is unlikely that spillover is involved in the stimulation by Mg^{2+} observed in Table I. It is also known that high Mg^{2+} concentrations [18] cause grana stacking, which could result in apparent inhibition, or loss of activity, as with 20 mM Mg^{2+} observed in this study (Table I). Another explanation to account for stimulation of the DCMU-insensitive silicomolybdate reduction at high pH and inhibition at low pH, or vice versa, might be differential solubilities of the compounds used. This may be so for folic acid, which is more soluble at pH 8 than at pH 6. If silicomolybdate itself were responsible, by dissociating at a pH higher than 8, no stimulation of the high pH site would be possible which disagrees with the data presented here. Another explanation is that differences in the DCMU-insensitive silicomolybdate reduction according to low or high pH arise from a conformational change in chloroplast membranes. Such a change was proposed by

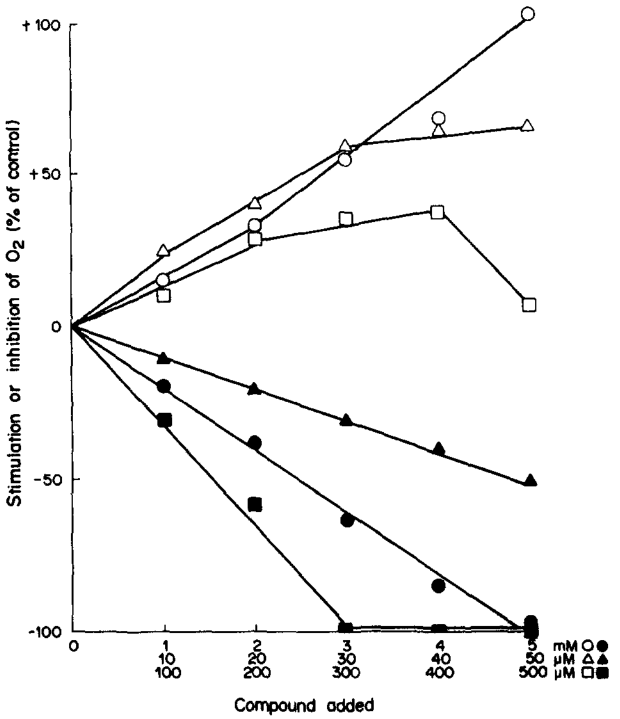

Fig. 4. Inhibition of DCMU-insensitive silicomolybdate (SM) reduction at pH 8. (○——○) $H_2O \rightarrow$ SM (+DCMU), pH 6, plus disodium cyanamide; (●——●); same at pH 8; (△——△) $H_2O \rightarrow$ SM (+DCMU), pH 6, plus folic acid; (▲——▲) same at pH 8; (□——□) $H_2O \rightarrow$ SM (+DCMU), pH 6, plus 5,6-benzoquinoline; (■——■) same at pH 8. The control rate was 61 μequiv./mg Chl per at pH 6 and 153 μequiv./mg Chl per h at pH 8. +indicates stimulation; —indicates inhibition of rate compared to control.

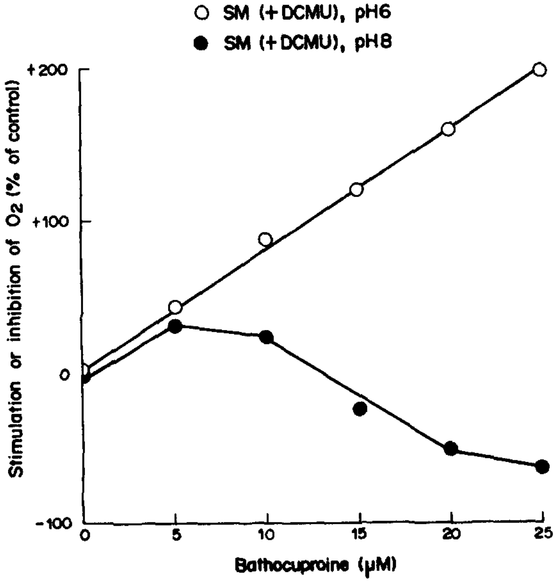

Fig. 5. Stimulation of DCMU-insensitive silicomolybdate reduction at pH 6 by bathocuproine. The control rate was 51 μequiv./mg Chl per h at pH 6 and 145 μequiv./mg Chl per h at pH 8. +indicates stimulation; —indicates inhibition of rate compared to control.

Dilley and associates to explain differences in radioactive *p*-diazoniumbenzenesulfonic acid [19] or iodoacetic acid [20] labeling patterns in the Q region in light or dark samples. Since the first DCMU-insensitive site was associated with the Q region of chloroplasts, as shown by Giaquinta et al. [2] and confirmed by subsequent investigations in other laboratories [5,6], it is possible that this conformational change can also be induced by changes in external pH in the light samples. If that is the case, two DCMU-insensitive sites may be present in PS II of spinach chloroplasts. However, Q reduction alone in forward electron transport is not sufficient to cause silicomolybdate reduction, which indicates that the DCMU-insensitive silicomolybdate reduction in PS II may require additional components besides Q.

TABLE II

THE EFFECT OF VARIOUS UNCOUPLERS ON DCMU-INSENSITIVE SILICOMOLYBDATE REDUCTION IN PHOTOSYSTEM II OF SPINACH CHLOROPLASTS

O_2 evolution rates are expressed as μequiv. O_2/mg Chl per h; reaction mixtures as in Fig. 2 except various uncouplers in place of NH_4Cl as indicated below; + shows percentage stimulation; — shows percentage inhibition of control rate. SM, silicomolybdate; MV, methyl viologen; 1799,α,α′-bis(hexa(fluoroacetonyl)-acetone; S_6,5-chloro-3-(*p*-chlorophenyl)-4-chlorosalicylanilide; S_{13},5-chloro-3-(*t*-butyl)-2′-chloro-4′-nitrosalicylanilide; FCCP, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone.

Uncoupler added	Concentration (μM)	PS II ($H_2O \rightarrow$ SM (+DCMU))				PS I and PS II $H_2O \rightarrow$ MV (azide)	
		pH 6		pH 8		pH 7	
		O_2	% of control	O_2	% of control	O_2	% of control
NH_4Cl	0	130	—	164	—	181	—
	2000	181	+39	164	0	784	+333
	5000	169	+30	158	−4	558	+208
Gramicidin d	0	62	—	62	—	146	—
	6	56	−10	34	−45	558	+282
	12	34	−45	11	−82	1009	+591
Nigericin	0	136	—	175	—	181	—
	2	192	+41	158	−10	519	+187
	5	209	+54	141	−19	451	+149
FCCP	0	107	—	124	—	226	—
	2	113	+6	68	−45	603	+167
	5	118	+10	45	−64	671	+197
Desaspidin	0	68	—	73	—	203	—
	2	51	−25	23	−68	829	+308
	5	0	−100	0	−100	739	+264
1799	0	85	—	96	—	203	—
	50	118	+39	68	−29	739	+264
	100	113	+33	56	−42	986	+386
	150	107	+26	17	−82	951	+368
S_6	0	130	—	164	—	124	—
	5	164	+26	118	−28	546	+340
	10	146	+12	62	−62	463	+273
S_{13}	0	124	—	136	—	271	—
	2	136	+10	90	−34	727	+168
	5	124	0	56	−59	761	+181

Other studies have shown that two different reaction centers may exist in PS II [21]. Joliot and Joliot [22] have shown that the Q site may be subdivided into Q_1 and Q_2. If the high and low pH DCMU-insensitive sites arise from Q_1 and Q_2, they reflect the natural organization of PS II electron transport.

DCMU-insensitive silicomolybdate reduction in PS II may be coupled to photophosphorylation, as shown by Giaquinta et al. [4,5], Berg and Izawa [7] and Rosa and Hall [8]. This study also shows (Table II) that the pH 6 DCMU-insensitive site is stimulated up to 50% by the addition of uncouplers such as NH_4Cl, nigericin, carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP), α,α'-bis(hexafluoroacetonyl)acetone (1799), 5-chloro-3-(*p*-chlorophenyl)-4′-chlorosalicylanilide (S_6) and 5-chloro-3-(*t*-butyl)-2′-chloro-4′-nitrosalicylanilide (S_{13}) [23]. Gramicidin D and desaspidin, however, are not good uncouplers for the DCMU-insensitive silicomolybdate sites at pH 6 or 8 because they inhibit these reactions at concentrations which stimulate electron transport on the $H_2O \rightarrow$ methyl viologen pathway. Further studies are underway to explain the differences between various uncouplers shown in Table II.

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