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Dichlorophenylurea-insensitive Reduction of Silicomolybdic Acid by Chloroplast Photosystem II

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ABSTRACT

The photoreduction of silicomolybdate and other heteropoly ions by chloroplasts is insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Both water and diphenylcarbazide can be used as electron source for the reduction. Three different assays for silicomolybdate reduction are described including oxygen evolution, formation of a reduced heteropoly blue silicomolybdate, or an indirect assay for reduced silicomolybdate by redox indicators, such as ferricyanide or cytochrome c. The effects of detergents and tris washing are consistent with silicomolybdate reduction through photosystem II before the DCMU site. The effects of orthophenanthroline and bathophenanthroline indicate chelator-sensitive sites in photosystem II before the site of DCMU action.

The effect of SM[1] in the reduction of Cyt *c* by aldehyde oxidase was described by Glenn and Crane (5). Giaquinta *et al.* (3) have reported briefly SM reduction by chloroplasts, measured as O_2 evolution in the presence of DCMU. No photophosphorylation by PS II occurred in this reaction. This study involves a more detailed account of the effects of silicomolybdic acid on electron transport in whole chloroplasts and PS II particles. Optimum conditions for SM reduction are reported along with treatments that abolish it.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) leaves were obtained from the local market. Chloroplasts were isolated according to the process described by Jagendorf and Avron (6) in 0.4 M sucrose containing 0.05 M NaCl. PS I and II reactions were assayed as previously reported by Brand *et al.* (2). The reaction medium for following silicomolybdic acid reduction spectrophotometrically at 750 nm contained in a 3-ml volume: chloroplasts (50 μg Chl), 150 μmoles of tris-Mes (pH 7), 30 μmoles of $MgCl_2$, 12 μmoles of NH_4Cl, and 0.2 mg of SM (optimum concentration) from a solution of 10 mg/ml dissolved in H_2O and filtered before use. DCMU (0.03 μmole) or 1.5 μmoles of diphenylcarbazide were added where indicated. The reaction mixtures were illuminated for 1 min with white light. Rates of SM reduction at 750 nm were calculated using a millimolar extinction coefficient of 8 $mM^{-1} \cdot cm^{-1}$, obtained by ferricyanide titration of the isolated compound. The 750-nm wavelength to monitor SM reduction in chloroplasts was chosen to avoid interference by Chl absorbance at 680 nm.

Silicomolybdic acid reduction was also measured as Cyt *c* reduction at 550 nm (5). The reaction mixture contained in 3 ml: 0.15 mg of Cyt *c* (type III from horse heart), 0.3 mg of polylysine (mol wt 30,000 or 70,000) or 5 μmoles of dibromothymoquinone in addition to chloroplasts and buffer. Reaction rates were calculated using a millimolar extinction coefficient, $e = 19\ nM^{-1} \cdot cm^{-1}$, for reduced-oxidized Cyt *c*. Longer periods of illumination were unsuitable because chemical reduction of SM by DPC occurred.

PS I and II fractionation with digitonin was carried out according to Boardman (1). Chloroplast washes with SM contained 1 g of SM dissolved in 100 ml of grinding and suspension medium (0.4 M sucrose with 0.05 M NaCl), filtered before use. Chloroplasts containing 1 mg Chl/50 ml wash solution were recentrifuged to collect the chloroplast pellet. Tris washes of chloroplasts were performed in a like manner using 0.8 M tris (pH 8) (9).

The spectrum of heteropoly blue formed by chloroplasts in the presence of SM was taken with a Cary recording spectrophotometer. The sample for the spectrum was collected from several SM reductions by chloroplasts in the light, concentrated, and filtered free of particulate matter before use.

Silicomolybdic acid was obtained from K and K Chemicals; polylysine and Cyt *c* were obtained from Sigma.

RESULTS AND DISCUSSION

Silicomolybdic acid reduction in chloroplasts can be measured in three ways: (*a*) as direct reduction from either H_2O or DPC following an increase in absorbance at 750 nm in saturating white light as a result of heteropoly blue formation (5); (*b*) as O_2 evolution in the presence of DCMU; and (*c*) as nonenzymic reduction of Cyt *c* by reduced SM measured at 550 nm.

Table I shows the electron transport rates from water or DPC with SM as the electron acceptor. Highest rates were obtained by the polarographic assay measuring O_2 evolution with a Clark-type electrode. Absorbance measurements at 750 nm from H_2O of DPC and nonenzymic Cyt *c* reduction by reduced SM at 550 nm in the presence of polylysine or dibromothymoquinone to prevent PS I reactions were more difficult to measure accurately because of turbidity problems. Rates of SM reduction by chloroplasts in presence of DCMU were only slightly inhibited. Chloroplasts washed several times with 0.8 M tris gave reduced rates with SM, thus indicating that the O_2 evolved in the presence of SM was from chloroplast H_2O oxidation.

Figure 1 compares the electron transport rates from $H_2O \rightarrow$ ferricyanide with or without SM and DCMU as a function of

[1] Abbreviations: SM: silicomolybdic acid; DPC: diphenylcarbazide; PS I, PS II: photosystem I and II.

pH. The O_2 evolution rate from $H_2O \rightarrow$ ferricyanide at pH 8 was triple the rate at pH 6. The O_2 evolution rate by ferricyanide in the presence of SM was higher and nearly tripled at pH 8. DCMU abolished this increase. Thus, the PS II-induced SM reduction, measured as O_2 evolution, was virtually pH independent between pH 6 and 8.

With either water or DPC as an electron donor, SM reduction is inhibited less than 10% by DCMU in the O_2 evolution assay (Table I). Orthophenanthroline, a chelator for iron, inhibits electron transfer from the primary oxidant to ferricyanide or quinones at a site near DCMU action (8). The reduction of SM by water shows up to 100% inhibition by bathocuproine, about 50% by bathophenanthroline, and about 25% by orthophenanthroline (Fig. 2). The effect of the more lipophilic chelators may be on sites before the traditional orthophenanthroline-DCMU sites because SM accepts electrons before the DCMU block. The weak orthophenanthroline effect on SM reduction is a contrast to the strong inhibition this chelator shows for indophenol reduction (10).

Figure 3 shows the absorbance spectrum of the blue heteropoly ion formed in chloroplasts by the reduction of SM. Maximum absorbance of chloroplast heteropoly blue is at 680 nm with a shoulder between 800 and 850 nm. It is different from the heteropoly blue spectrum obtained by Glenn and Crane (5) from reduction of SM by aldehyde oxidase or by ascorbate. Theoretically, various combinations of the 12 molybdenum atoms of an SM complex may be reduced separately leading to differences in the absorbance spectrum.

Reduction of other heteropolyanions is shown in Table III. The highest reaction rates were obtained with SM or phosphomolybdate, with slower rates for the tungstate analogs. Molybdate ions were ineffective.

Fractionation of chloroplasts with digitonin shows that all

Table I. *Reduction of Silicomolybdic Acid by Chloroplast Photosystem II*

Chloroplasts	Reductant	Method of Assay	Inhibitor	Electron Transport Rate[1]
Untreated	H_2O	O_2 evolution	None	343
Untreated	H_2O	O_2 evolution	DCMU	335
Untreated	H_2O	O_2 evolution with $K_3Fe(CN)_6$	None	200[2]
Untreated	H_2O	O_2 evolution with $K_3Fe(CN)_6$	DCMU	148[2]
Untreated	H_2O	Absorbancy at 750 nm	None	113
Untreated	H_2O	Absorbancy at 750 nm	DCMU	88
Untreated	H_2O	Cyt *c* reduction	None	120
Untreated	H_2O	Cyt *c* reduction	DCMU	80
Untreated	DPC	Absorbancy at 750 nm	None	222
Untreated	DPC	Absorbancy at 750 nm	DCMU	189
Untreated	DPC	Cyt *c* reduction	None	100
Untreated	DPC	Cyt *c* reduction	DCMU	56
Boiled	H_2O	O_2 evolution	None	0
Tris-treated	H_2O	O_2 evolution with $K_3Fe(CN)_6$	None	0

[1] Microequivalents O_2 evolved mg Chl·hr or μmoles acceptor reduced mg Chl·hr.

[2] At pH 8.

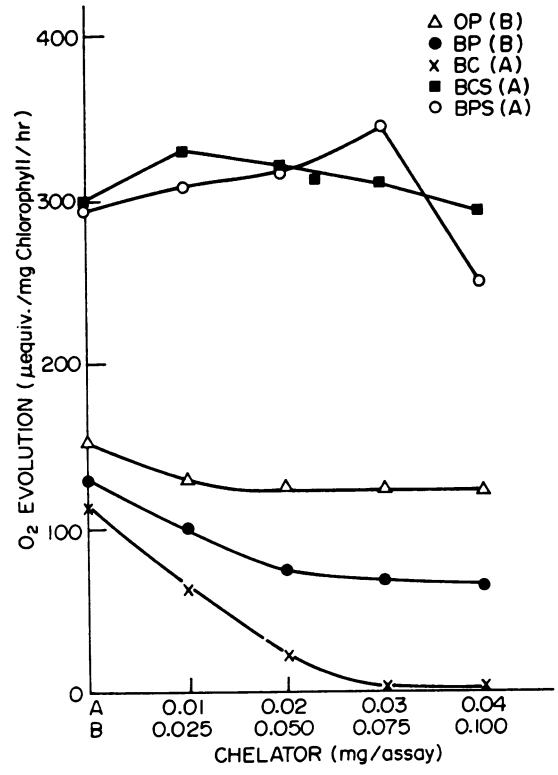

FIG. 2. Effect of chelators on SM reduction by chloroplasts. Inhibition of the O_2 evolution rate is shown with the more lipophilic chelators, bathocuproine and bathophenanthroline, while orthophenanthroline inhibits only about 20%. Bathophenanthroline sulfonate and bathocuproine sulfonate did not show significant inhibition but a slight stimulation of O_2 evolution. Scale A shows bathocuproine, bathocuproine sulfonate, and bathophenanthroline sulfonate concentrations; scale B, ortho- and bathophenanthroline concentrations. The O_2 evolution rates are lower because lipophilic chelators were added in ethanol.

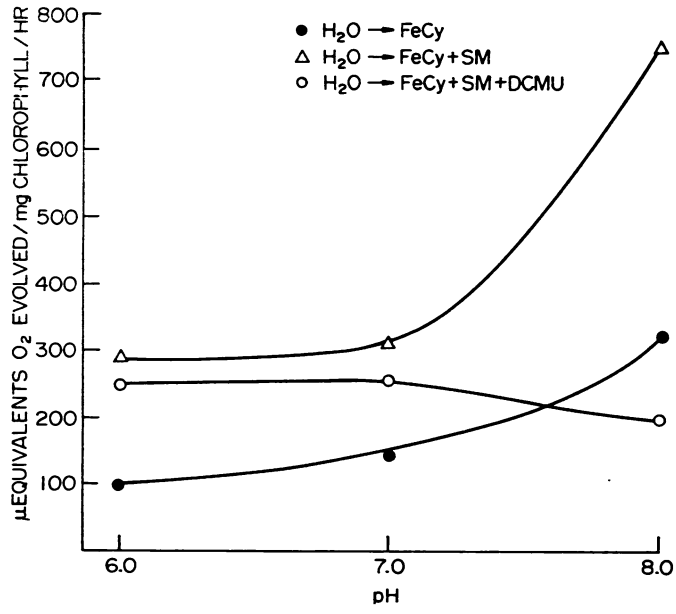

FIG. 1. Effect of pH on SM reduction by chloroplast PS II. The effect of pH on the reduction of ferricyanide is used for comparison.

SM reduction activity is in the heavy PS II fraction and none in the light PS I fraction (Table II).

Table IV shows how SM reduction by chloroplasts is affected by various washes. Washing chloroplasts once with 0.8 M tris abolished the $H_2O \rightarrow$ indophenol reaction, reduced the rate of the DPC→indophenol reaction, and inactivated the DPC→SM reaction by nearly 50%. Several tris washes totally inactivated SM reduction by PS II (Table I).

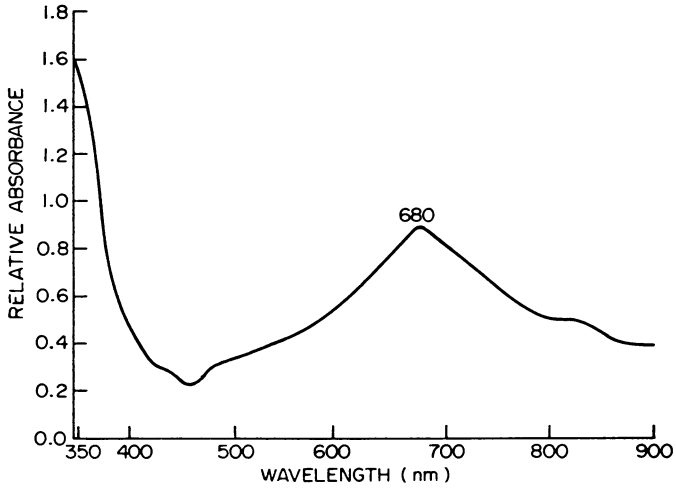

FIG. 3. Absorbance spectrum of heteropoly blue formed by the reduction of SM in chloroplasts. Maximum absorbance is at 680 nm with a shoulder between 880 to 850 nm.

Table II. *Reduction of Silicomolybdic Acid by Photosystem I and II Particles*

Fraction[1]	Chl *a/b*	$H_2O \rightarrow$SM O_2 Evolution Rate		$H_2O \rightarrow$ Ferricyanide
		−DCMU	+DCMU	
	ratio	*μeq/mg Chl·hr*		*μmoles/mg Chl·hr*
Control chloroplasts	2.86	290	286	245
D-1		86	93	71
D-10	2.36	82	86	126
D-50		0	0	0
D-144	4.82	0	0	0

[1] Designation according to Boardman's digitonin fractionation (1).

Table III. *Reduction of Heteropolyanions by Photosystem II of Spinach Chloroplasts*

Acceptor	Concn	O_2 Evolution Rate	
		−DCMU	+DCMU
	mg/assay	*μeq/mg Chl·hr*	
Silicomolybdic acid	0.2[2]	313	299
Silicotungstate	0.4	208	134
Phosphomolybdate	1.0	208	149
Phosphotungstate	0.2	76	76
Molybdate	2.5	0	
Ferricyanide	0.8	351	0

[1] A blank rate of about 15% which occurs in absence of chloroplasts is subtracted from all silicomolybdic acid assays.

[2] Amount added to give optimum rate.

Table IV. *Effect of Various Washes on Spinach Chloroplasts*

Treatment	Reaction	Rate
		μmoles acceptor reduced/mg Chl·hr
None	$H_2O \rightarrow$DCPIP	198
Tris wash	$H_2O \rightarrow$DCPIP	0
Silicomolybdic acid wash	$H_2O \rightarrow$DCPIP	0
None	DPC→DCPIP	240
Tris wash	DPC→DCPIP	90
Silicomolybdic acid wash	DPC→DCPIP	0
None	DPC→SM	116
Tris wash	DPC→SM	68
Silicomolybdic acid wash	DPC→SM	58

As data presented in this communication show, the reduction of SM by PS II in spinach chloroplasts is not inhibited by DCMU. This indicates that SM can accept electrons at a site close to the primary oxidant for photosystem II (3). Other heteropoly molybdate and tungstate ions also show a similar effect.

The only other acceptor which has been shown to act before the DCMU site is Hg^{2+} ions (7). Previously Girault and Galmiche (4) reported that silicotungstate was able to cause DCMU-insensitive ferricyanide reduction. We believe that the correspondence between heteropoly blue formation and O_2 evolution in absence of other acceptors clearly shows that heteropolyanions are reduced by PS II and that the effect is not a perturbation of the membrane to allow DCMU-insensitive ferricyanide reduction. The greater lipid solubility of heteropoly anions compared to ferricyanide may account for their ability to react before the DCMU site.

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