The interaction of silicomolybdate with the photosystem II herbicide-binding site

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Received 25 July 1986

Silicomolybdate and silicotungstate are unique among photosystem II electron acceptors in that they catalyze photosystem II-dependent electron transfer in thylakoid membranes that is resistant to inhibition by diuron. On this basis it is generally accepted that these compounds interact with the photosystem II electron transfer sequence prior to the site of diuron inhibition thereby creating an even more abbreviated partial reaction than is found with other photosystem II electron acceptors. The data presented here demonstrate that low concentrations of the silicomolybdate anion (10–30 μM) inhibit the binding of [^{14}C]diuron to spinach thylakoid membranes. This effect of silicomolybdate is reversible and is a function of the redox state of silicomolybdate. Reduction of silicomolybdate leads to a loss of its ability to prevent herbicide binding. Thus, there is no reason to believe that silicomolybdate intercepts electrons from photosystem II prior to the site of diuron intervention or at a site different from any other photosystem II electron acceptor.

Photosystem II *Diuron* *Q_b protein* *Silicomolybdate* *Thylakoid membrane* *Herbicide*

1. INTRODUCTION

Advances in the understanding of the photosynthetic electron transfer chain and its coupling to photophosphorylation have been greatly facilitated by the ability to dissect the system into many partial reactions. This has been possible due to the development and characterization of a variety of inhibitors, electron donors, and electron acceptors. These partial reactions of electron transfer have also made an important contribution to understanding the response of, or damage to, the photosynthetic apparatus imposed by environmental stresses such as drought, high light, or temperature extremes. Thus the demonstration by Girault and Galmiche [1] and Giaquinta et al. [2] of diuron-insensitive oxygen evolution in the presence of silicotungstate and silicomolybdate (SiMo), was appropriately greeted with enthusiasm. Although the reaction rates were low and the reaction lifetime was short (≈1 min), SiMo emerged as the more utilized of the two because it supported higher rates of photosystem II-dependent electron transfer.

Although originally used in the presence of excess ferricyanide, Barr et al. [3] and Zilinskas and Govindjee [4] demonstrated that SiMo alone supported diuron-insensitive oxygen evolution. Although Giaquinta et al. [2] were not able to observe ATP synthesis coupled to the diuron-insensitive SiMo reduction, Izawa and Berg [5] demonstrated that photophosphorylation was coupled to SiMo reduction if certain oxygenated organic solvents, that stabilize SiMo in aqueous media, were included in the reaction mixture. The phosphorylation efficiency of the diuron-insensitive SiMo reduction was then observed to approach the range of values found for other photosystem II electron acceptors.

Electron transfer on the acceptor side of

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00145793/86/$3.50

photosystem II involves two quinones, Q_a and Q_b, as well as an iron atom that may facilitate electron tranfer between the quinones [6]. The tightly bound plastoquinone designated Q_a is the first stable electron acceptor of the reaction center. Plastoquinone from the membrane's bulk pool fills the Q_b site but is associated only relatively weakly in the quinone or quinol forms. In recent years it has been established that herbicides such as diuron compete with plastoquinone for binding to the Q_b site and in this way inhibit the oxidation of Q_a^- [7–9]. Exogenous quinones, often employed as photosystem II electron acceptors, have also been shown to compete with herbicides for binding at the Q_b site [10].

Primarily on the basis of oxygen evolution that persists in the presence of concentrations of diuron which fully inhibit all other known acceptors, SiMo and silicotungstate were proposed to be photosystem II electron acceptors that intercepted electrons prior to the site of diuron inhibition. Girault and Galmiche [1] originally presented evidence that suggested that silicotungstate did not impair the binding of diuron to its inhibitory site. They proposed that silicotungstate modified the chloroplast membranes such that Q_a was now accessible to exogenous oxidants such as ferricyanide [1]. Ben-Hayyim and Neumann [11] came to the same conclusion regarding membrane modification by SiMo. On the other hand, Böger [12] presented evidence that SiMo displaces atrazine from these membranes and that this was the origin of its insensitivity to photosystem II herbicides. SiMo reduction has been employed frequently as an assay for the portion of the photosystem II electron transfer sequence prior to Q_b, the site of diuron inhibition [13–19]. Thus, identifying the actual site of reduction of SiMo by the acceptor side of photosystem II is important as it leads to entirely different assessments of the significance of SiMo catalyzed partial reaction. This paper addresses the resolution of this disagreement. Our results demonstrate the validity of the suggestion of Böger; insensitivity to diuron is a result of SiMo's ability to replace diuron at the herbicide binding site. SiMo binds to chloroplast membranes directly affecting the herbicide-binding site in a reversible redox-dependent manner. This behavior is sufficient to explain the widely reported and accepted observation of diuron-insensitive SiMo reduction. Thus, SiMo cannot be employed to distinguish electron transfer that is presumed to be prior to and independent of the Q_b site.

2. MATERIALS AND METHODS

2.1. *Chloroplast isolation*

Chloroplast thylakoid membrane isolation from commercial spinach (*Spinacia oleracea* L.) and the determination of chlorophyll concentration were performed as described [20].

2.2. *Radioactive herbicide binding*

[*methyl*-^{14}C]Diuron (spec. act. 57 Ci/mol) was obtained from Amersham and dissolved in methanol. Chloroplasts containing 50 μM chlorophyll in 1.0 ml were incubated at room temperature for 10 min with varying concentrations of [^{14}C]diuron and additional compounds as indicated. After centrifugation to separate the herbicide bound to thylakoid membranes from the free herbicide, an aliquot (0.85 ml) of the supernatant was removed and its concentration of free herbicide was determined by scintillation counting. Best-fit estimates of the binding constant and the number of binding sites were obtained by nonlinear regression analysis using the computer program LIGAND [21]. This program does the fitting in the more statistically appropriate bound vs total coordinate system although the data have been plotted in the more traditional bound vs free coordinate system.

2.3. *Silicomolybdate*

Sodium silicomolybdate was obtained from Dr C.J. Arntzen. A solution of 50 mg/ml was dissolved in water and centrifuged briefly to remove insoluble material. The concentration of the SiMo solution was determined from the molar absorptivity at 300 nm [22]. Stannous chloride, employed as a chemical reductant for SiMo, was dissolved in concentrated HCl. This stock solution (1.2 M) was diluted with water as appropriate.

3. RESULTS

The data in fig.1 illustrate clearly that SiMo interferes with the association of [^{14}C]diuron with the photosystem II herbicide-binding site. This effect occurs at concentrations of SiMo lower than

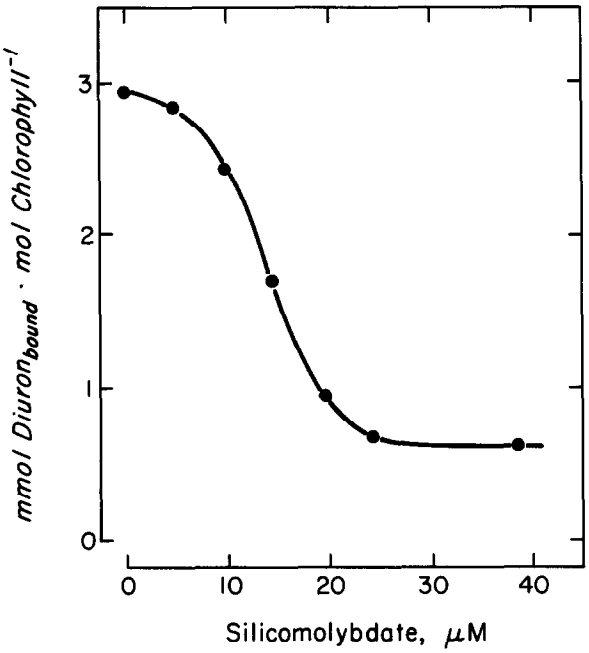

Fig.1. Displacement of [^{14}C]diuron from the photosystem II herbicide-binding site by silicomolybdate. Diuron binding was determined from a reaction mixture consisting of 0.4 M sorbitol, 50 mM Mes-KOH (pH 6.5), 20 mM KCl, 2 mM $MgCl_2$, 5% (v/v) glycerol, and chloroplasts containing 50 μM chlorophyll. Glycerol has been shown to protect the chloroplasts from deleterious effects of SiMo [5]. In the absence of SiMo the [[^{14}C]diuron]$_{free}$ was 170 nM.

those normally used for measurements of steady-state electron transfer with SiMo functioning as the terminal electron acceptor. Similar results were also obtained with silicotungstate (not shown). The data in fig.1 also suggest that there may be a portion of the diuron-binding sites which are inaccessible to the highest concentrations of SiMo employed in this experiment. An examination of the binding parameters for [^{14}C]diuron in the presence of SiMo indicate an apparent non-competitive interaction. That is, the presence of SiMo results in a loss of diuron-binding sites without a significant change in the affinity of the remaining sites for diuron (fig.2 and table 1). Table 1 shows the presence of 15.3 μM SiMo has a negligible effect on the apparent dissociation constant but the number of binding sites is reduced from 3.41 to 1.16 mmol/mol chl. In contrast, a

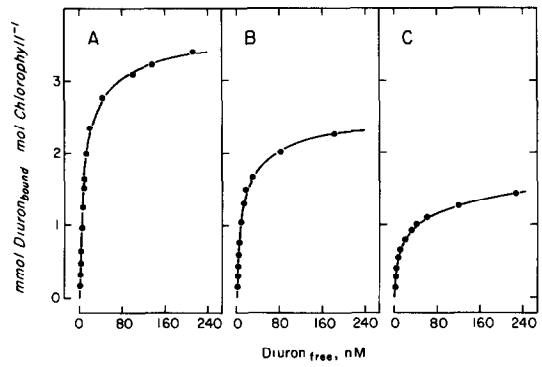

Fig.2. Saturable binding of [^{14}C]diuron to chloroplast thylakoid membranes in the absence and presence of silicomolybdate. Experimental conditions as in fig.1.

competitive interaction would be characterized by a decreased binding affinity without a change in the number of binding sites.

The ability of SiMo to prevent the binding of [^{14}C]diuron to thylakoid membranes is dependent upon its redox state. In fig.3 the addition of stannous chloride, an effective reductant of SiMo [22], leads to a restoration of the full complement of [^{14}C]diuron binding in the presence of SiMo. Ascorbate is also capable of reversing the SiMo-induced loss of [^{14}C]diuron-binding sites (table 2). When conditions are made more oxidizing upon the addition of ferricyanide, SiMo becomes even slightly more effective at displacing [^{14}C]diuron (table 2). The demonstration of redox-dependent reversibility may help to explain the conflicting

Table 1

[^{14}C]Diuron dissociation constant and number of binding sites as a function of silicomolybdate concentration

[SiMo] (μM)	K_d (nM)	Binding sites (mmol/mol chl)	Nonspecific binding
0	10.8 ± 0.8	3.41 ± 0.09	0.031 ± 0.024
9.6	10.9 ± 1.3	2.24 ± 0.11	0.040 ± 0.030
15.3	9.5 ± 0.8	1.16 ± 0.04	0.072 ± 0.011

The parameter estimates and approximate standard deviations were obtained from nonlinear regression analysis of the data shown in fig.2. Nonspecific binding is the ratio of nonspecifically bound to free ligand

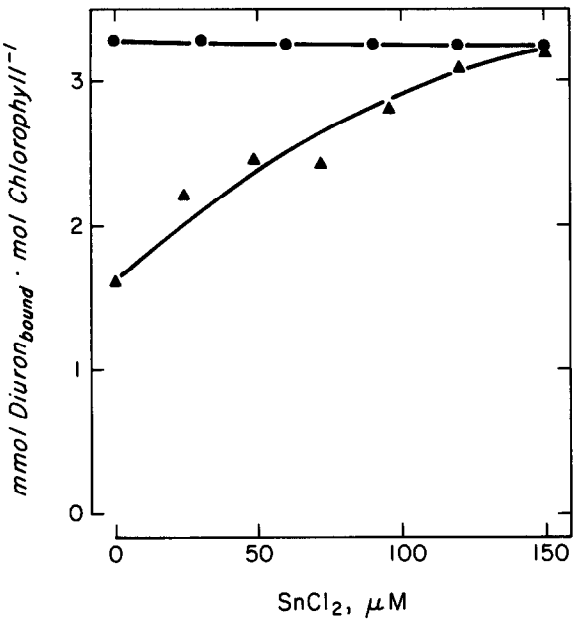

Fig.3. Reduction of silicomolybdate by stannous chloride permits rebinding of [^{14}C]diuron. Experimental conditions as in fig.1.

reports regarding the diuron sensitivity of various electron transfer reactions in the presence of SiMo. In addition the observation by Berg and Izawa that bovine serum albumin inhibits the ability of SiMo to act as a photosystem II electron acceptor [23] corresponds to the ability of bovine serum albumin to prevent SiMo from displacing [^{14}C]diuron from the photosystem II herbicide-binding site (table 2).

Table 2

Effect of reductants, oxidants, and bovine serum albumin on the ability of silicomolybdate to prevent [^{14}C]diuron binding to thylakoid membranes

Addition	[^{14}C]Diuron-binding sites (mmol/mol chl)	
	14.4 μM SiMo	– SiMo
None	1.98	3.20
$SnCl_2$	2.84	3.14
$K_3Fe(CN)_6$	1.58	3.19
Ascorbic acid	2.57	3.12
Bovine serum albumin	2.66	3.11

The various compounds were all present at 100 μM. All other experimental conditions as in fig.1

Bovine serum albumin probably binds SiMo thereby reducing the concentration of SiMo free in solution. Both the effect of reductants and bovine serum albumin indicate the reversible nature of the effect of SiMo on the herbicide-binding site.

4. DISCUSSION

There has been considerable interest of late regarding the molecular details of the photosystem II polypeptide complex. This work has included both investigations into fundamental biochemical and biophysical questions as well as more applied problems concerning the condition of the photosystem II complex subsequent to a period of environmental stress. For example, there have been several reports concerning the mechanism of inactivation of photosystem II by periods of high-intensity illumination. One of the primary concerns is whether the photoinhibitory treatment induces a lesion at the donor side (e.g. [24]), reaction center (e.g. [14]), or acceptor side (e.g. [13,15]) of the complex. In these studies diuron-insensitive electron transfer to SiMo has often been employed as a measure of photosystem II integrity up to but not including the Q_b site [13–16]. The results of our experiments, as well as the report by Böger [12], indicate that diuron-insensitive SiMo reduction will provide the same information as any other photosystem II acceptor regarding the functional activities maintained after an inhibitory treatment. Diuron-insensitive SiMo reduction has also been used as a diagnostic measurement of photosystem II electron transfer independent of the Q_b site in a study of the binding site involved in bicarbonate-stimulated oxygen evolution [17] as well as the functional location of the diuron-binding site itself in cyanobacteria [18,19]. In view of the findings reported here a re-examination of the earlier data and conclusions drawn therefrom is appropriate.

It is clear from the data shown in figs 1 and 2 that the interaction of SiMo with photosystem II prevents diuron from binding to the Q_b site. Although this conclusion was not reached by most of the previous investigators working with SiMo, many of their observations are nevertheless consistent with this model. For example, there are several reports on order-of-addition effects. Diuron-insensitive SiMo reduction yields a higher rate of electron transfer if the SiMo is added prior to the

diuron [4]. However, if the diuron is added prior to the SiMo the rate of diuron-insensitive SiMo reduction increases with time of incubation [23]. In addition, if diuron is added prior to SiMo there is a 5–20 s lag before diuron-insensitive oxygen evolution is observed [5]. All of these reports are consistent with the notion of diuron and SiMo competing for a common binding site.

However, although SiMo prevents diuron binding the data in fig.2 indicate that increasing concentrations of [^{14}C]diuron are not able to displace any of the SiMo from the thylakoid membranes, suggesting the absence of competition. It is possible that this apparently noncompetitive behavior could be masking true competition if the binding affinity of the membranes for SiMo were much greater than their affinity for diuron (11 nM). For instance, if the binding constant for SiMo were 1 nM or less, then under our experimental conditions (≈0.17 μM herbicide-binding sites) the total concentration of SiMo needed to saturate the herbicide-binding sites would be considerably less than 1 μM, far less than the amount of SiMo which we found to be required. However, these estimates assume a negligible amount of nonspecific binding of SiMo to thylakoid membranes. Girault and Galmiche [1] reported a very substantial amount of relatively high-affinity saturable binding of silico[^{183}W]-tungstate to thylakoid membranes (≈400 mmol/mol chl) [1]. This amount of silicotungstate binding is over 100-times greater than the number of herbicide-binding sites and therefore largely unrelated to the displacement of [^{14}C]diuron. However, we found no evidence indicating a high level of nonspecific binding of SiMo suggesting that the inhibitory mechanism is noncompetitive.

Another possible explanation for the apparent lack of competition between SiMo and [^{14}C]diuron might be that equilibrium binding was not achieved during the 10 min incubation period provided. Although 10 min is adequate for the equilibrium binding of [^{14}C]diuron at room temperature it is possible that SiMo may have a very much longer residency time at the Q_b site. The results of binding experiments with significantly longer incubation periods may be confounded by the deleterious effects of SiMo on chloroplast membranes.

In summary, although our understanding of the molecular details of the interactions among SiMo, diuron and photosystem II remains incomplete, our data combined with the low steady-state rates of electron transfer and well-established side effects of SiMo leave little reason to support the use of SiMo as an effective tool in the investigation of photosystem II.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation Grant PCM-8314461 awarded to Dr Donald R. Ort. The author thanks Dr Ort for a critical evaluation of this manuscript.

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