Modification of Photosystem II by phenylglyoxal

Evidence for the involvement of arginine in atrazine binding to chloroplasts

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Shipman [(1981) J. Theor. Biol. 90, 123–148] has recently noted that herbicides from various chemical classes which inhibit electron transport through Photosystem II have a flat polar component in the range 3–5 debye, and he suggests that this component of the herbicide interacts with a strong electric field across the protein binding site in the thylakoid. This polar portion of the binding site could be a salt bridge, the cationic end of which may be an arginine residue. Phenylglyoxal is a protein-modifying reagent with specificity toward arginine residues. Pretreatment of chloroplast membranes with 50 mM phenylglyoxal for 30 min at 30°C completely abolished the ability to bind specifically [^{14}C]atrazine. Pretreatment with 50 mM phenylglyoxal also completely abolished the subsequent ability of the chloroplasts to carry out photosynthetic electron transport. These data provide strong evidence for the involvement of arginine residues in the binding and action of Photosystem II herbicides.

Photosystem II *Herbicide binding site* *Atrazine* *Arginine modification* *Diuron* *Phenylglyoxal*

1. INTRODUCTION

Many of the world's commercially important herbicides act by inhibiting photosynthetic electron transport. Biophysical studies have indicated that many of these molecules, including triazines and ureas, block Photosystem II-dependent Hill reactions by preventing reduction of the second electron carrier, called 'B', on the reducing side of Photosystem II (PS II). Biochemical properties of PS II herbicide action are much less understood. The development of a direct herbicide binding assay [1] now permits structure-activity studies on the nature of the binding itself rather than only on its physiological consequence, the inhibition of a functional PS II. The use of the binding assay along with an azido analogue of atrazine has recently provided structural information on the binding site: photoaffinity labeling of the atrazine receptor indicates that a polypeptide of 32 kDa is associated with B [2]. Information on the functional biochemistry of the binding site is still sparse.

Classical quantitative structure-activity studies have indicated that within each class of PS II herbicide there is usually a linear relationship between the octanol/water partition coefficient of the molecule and the log of the reciprocal of the herbicide concentration required to inhibit photosynthetic electron transport by 50% [3]. Shipman [4] has observed that PS II herbicides from various classes have a flat polar component with a dipole moment in the range 3–5 debye as well as hydrophobic substitution which increases the lipid solubility of the molecule. He suggests that:

> "The primary contributor to herbicide binding is a strong electrostatic interaction between the dipole moment of the herbicide and a locally

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strong protein-generated electric field across the binding site for B in the PS II protein."

Two possibilities were noted for the polar portion of the binding site, α-helices and salt bridges, and an arginine residue was suggested as a possible candidate for the cationic end of such a salt bridge. Arginine modification experiments were suggested to test the involvement of arginine in electron transport through B [4].

The experiments reported here are a direct test of the hypotheses of Shipman [4]. Reagents such as the α-diketones 1,2-cyclohexanedione [5] and phenylglyoxal [6,7] have been shown to react with and modify arginine side chains on proteins. These two reagents are evaluated here for their ability to interfere with photosynthetic electron transport and to prevent binding of the herbicides atrazine and diuron.

2. MATERIALS AND METHODS

Chloroplast membranes were prepared from 7–8-day-old Alaska peas (*Pisum sativum* L., cv. Alaska) as in [2]. Chloroplasts were resuspended at a final chlorophyll concentration of 500 μg/ml in 0.1 M Bicine buffer (pH 7.8), containing 0.2 M sorbitol, 10 mM NaCl, and 10 mM $MgCl_2$, and mixed with an equal volume of either phenylglyoxal (Aldrich) or 1,2-cyclohexanedione (Aldrich) in the same buffer to the final concentration indicated. The mixtures were incubated at 30°C for the times indicated, and then excess reagent was removed by passing 1-ml aliquots through a prepacked, disposable gel filtration column of Sephadex G-25M (PD-10, Pharmacia). The excluded volume of the column (5 ml) was diluted to 10 ml with the same Bicine resuspension buffer. The control sample was treated in the same way, with buffer added instead of the arginine-modifying reagent. Samples were kept on ice at all times except during the 30°C incubation.

Atrazine binding experiments were carried out as in [2] using 0.2 μM [^{14}C]atrazine + 10 μM non-radioactive atrazine. 'Specific' binding is the binding of low concentrations of radioactive ligand that is abolished by higher concentrations of non-radioactive ligand. Diuron binding was measured in a similar fashion using [^{14}C]diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) obtained from California Bionuclear Corp. (Sun Valley CA) with a specific activity of 12.2 mCi/mmol. The PS II-mediated reduction of dichlorophenolindophenol (DCPIP) was measured at 580 nm using a Hewlett-Packard Model 8450A UV/VIS spectrophotometer equipped with a spectrophotometer-controlled actinic source.

3. RESULTS AND DISCUSSION

One of the first experiments is shown in table 1. The control sample did show some loss of specific binding after 1 h at 30°C. Treatment with 50 mM cyclohexanedione slightly reduced the activity of the preparation at 0°C, but no further reduction, relative to the control, occurred with incubation at 30°C. Phenylglyoxal at 50 mM also caused a slight reduction of activity at 0°C, but incubation of the mixture at 30°C for 1 or 2 h completely abolished specific atrazine binding. Since the effect of phenylglyoxal treatment was so much more dramatic than that of cyclohexanedione, further experiments were carried out only with phenylglyoxal.

The time course for the inactivation of atrazine binding by 50 mM phenylglyoxal is shown in fig.1. Binding activity was completely abolished after 30 min incubation, and the reaction had a half-life of about 4.3 min. The concentration dependence of the reaction is shown in fig.2. For a 1 h incubation at 30°C, 50 mM phenylglyoxal was required for complete inactivation. Significant inactivation occurred at 1 mM, and half the atrazine binding activity was abolished at a phenylglyoxal concentration slightly greater than 10 mM. Phenylglyoxal also caused similar inactivation of specific [^{14}C]diuron binding to thylakoid membranes (fig.3), although the half-life for the reaction was slightly longer at about 6.4 min.

It is conceivable that a reagent could modify atrazine binding without having a gross effect on photosynthetic electron transport (analogously to triazine-resistant mutants [8]). Fig.4 shows that this is not the case. Pretreatment of chloroplasts with 50 mM phenylglyoxal for 30 min at 30°C followed by removal of excess reagent completely abolished the ability of the preparation to carry out the PS II-mediated reduction of DCPIP.

Our data provide strong evidence for the involvement of arginine residues in the binding and action of PS II herbicides. Thus the hypothesis in

Table 1

Effect of arginine-modifying reagents on [^{14}C]atrazine binding to pea chloroplasts

Reagent	Binding assay	Incubation conditions		
		0°C	30°C, 1 h	30°C, 2 h
Control	[^{14}C]Atrazine binding (dpm; ± SE)	2933 ± 12	2304 ± 80	2314 ± 12
	+ 10 μM [^{12}C]atrazine	198 ± 2	174 ± 8	256 ± 43
	Specific binding	2735 ± 12	2130 ± 80	2058 ± 45
	% of control	100	78	75
1,2-Cyclohexanedione	[^{14}C]Atrazine binding	2666 ± 38	2228 ± 6	1944 ± 32
	+ 10 μM [^{12}C]atrazine	194 ± 6	173 ± 5	180 ± 4
	Specific binding	2472 ± 38	2055 ± 8	1764 ± 32
	% of control	90	75	64
Phenylglyoxal	[^{14}C]Atrazine binding	2576 ± 60	112 ± 7	109 ± 3
	+ 10 μM [^{12}C]atrazine	180 ± 8	112 ± 4	112 ± 4
	Specific binding	2396 ± 60	0 ± 8	13 ± 5
	% of control	88	0	0

Pea chloroplasts were incubated with 50 mM 1,2-cyclohexanedione or 50 mM phenylglyoxal as indicated. Excess reagent was removed by gel filtration on Sephadex G-25, and the chloroplasts were resuspended at a chlorophyll concentration of 50 μg/ml. [^{14}C]Atrazine was added at 0.2 μM, along with solvent (methanol) or 10 μM non-radioactive atrazine. All assays were carried out in duplicate at 0–4°C, and values are given as means ± SE

[4] is supported. Direct proof would require isolation and identification of the covalent adduct formed between phenylglyoxal and the guanidino group of arginine [6]. Demonstration of specificity and reversibility of phenylglyoxal inactivation would strengthen the argument, although this will be difficult because the reagent would be expected to modify arginines not directly involved in herbicide binding as well as those at the active site.

Reports of effects of protein-modifying reagents on photosynthetic electron transport are few. Authors in [9] used the lipophilic thiol compound

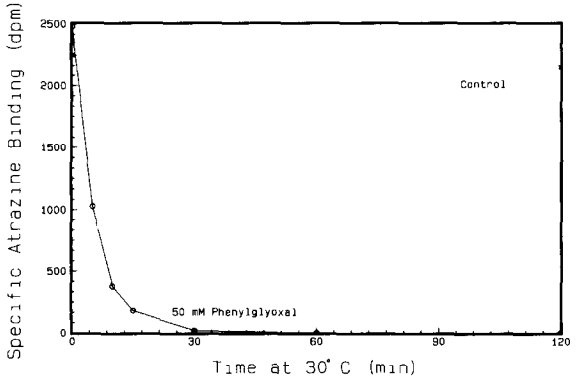

Fig.1. Effect of phenylglyoxal on atrazine binding. Pea chloroplasts were incubated at 30°C with 50 mM phenylglyoxal for the times indicated. Excess reagent was removed and binding assays were carried out as in table 1. Specific binding is the amount of [^{14}C]atrazine displaced by 10 μM non-radioactive atrazine.

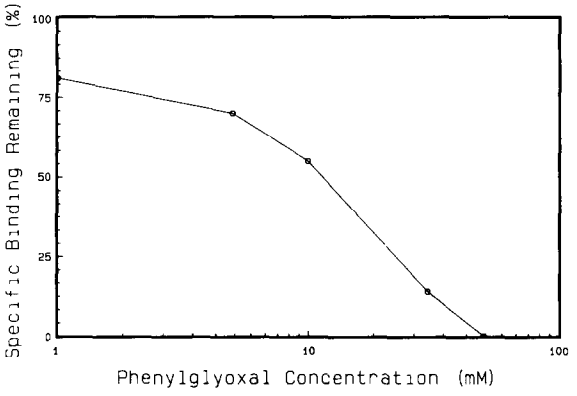

Fig.2. Effect of various concentrations of phenylglyoxal on atrazine binding. Pea chloroplasts were incubated at 30°C for 1 h with the indicated concentrations of the reagent. All other details were as in fig.1.

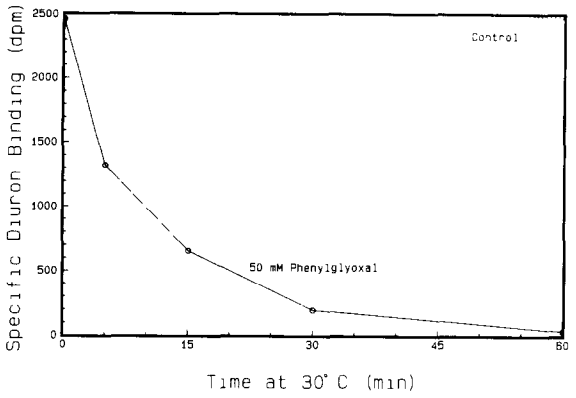

Fig.3. Effect of phenylglyoxal on diuron binding. Specific binding is the amount of 0.2 μM [^{14}C]diuron displaced by 10 μM non-radioactive diuron. All other details were as in fig.1.

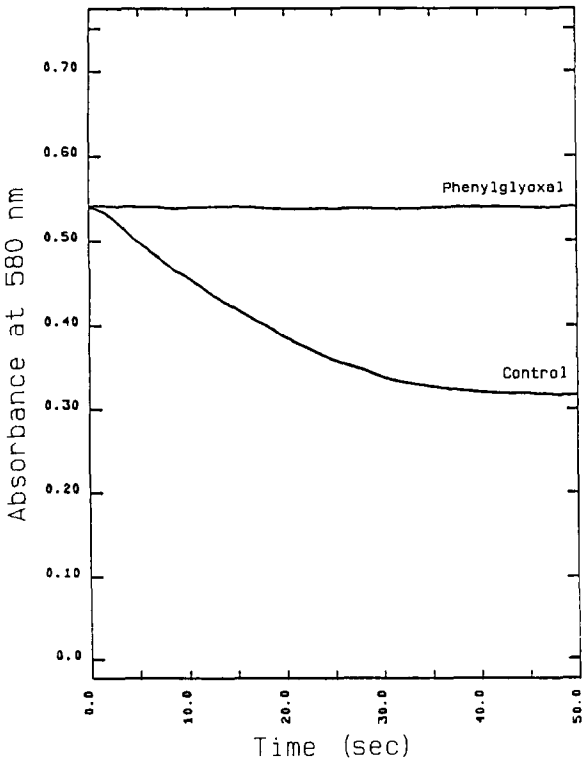

Fig.4. Effect of phenylglyoxal on photosynthetic electron transport. Pea chloroplasts were incubated at 30°C with 50 mM phenylglyoxal for 30 min. Excess reagent was removed, and the chloroplasts were diluted to a final concentration of 5 μg chlorophyll per ml. The PS II-mediated reduction of DCPIP was measured as described in the text. The sample was scanned every 2 s under continuous actinic illumination with light filtered through a red Corning 2-58 cutoff filter. Assays were conducted at room temperature.

p-nitrothiophenol to inhibit chloroplast activity. This compound, thought to interact with sulfhydryl groups on proteins, inhibited PS II-mediated photoreduction of DCPIP in the light but not in the dark, and treatment with *p*-nitrothio-[^{14}C]phenol labeled chloroplast membrane polypeptides of various molecular sizes. While the present work was in progress, authors in [10] noted modification of photosynthetic electron transport by phenylglyoxal, but no data are given, and no mention was made of effects on herbicide binding.

Previous studies of the PS II herbicide binding site using the photoaffinity label azidoatrazine indicated that a 32 kDa polypeptide is involved in the binding of triazines [2]. The specificity of this protein has clearly been demonstrated; however, the photoaffinity experiments do not indicate if other polypeptides are also constituents of the binding site or whether the 32 kDa peptide has any functional role in electron transport. Identification of the polypeptides containing arginine residues essential for PS II activity should help to resolve these questions. Experiments are currently in progress using [^{14}C]phenylglyoxal to label those peptides essential for the activity of PS II herbicides.

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