Kinetics of Action of Different Photosystem II Herbicides on Thylakoids

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Photosystem II Inhibitors, Binding, Fluorescence, Action Kinetics

The kinetics of inhibition of photosystem II electron transfer by different diuron-like herbicides (ureas, triazines, triazinones, biscarbamates, uraciles) were studied, mainly by chlorophyll fluorescence measurements. Uracil derivatives and cyanazine, a particular triazine, were the slowest acting compounds. The half-times of action were strongly temperature-dependent and were of the order of tens of seconds at 5 °C for urea or triazine inhibitors. The role of different limiting steps in the binding process is discussed.

Introduction

Photosystem II inhibiting herbicides from different chemical families (amides, ureas, triazines, triazinones, uraciles), with the exception of the phenolic herbicides [2], bind competitively to the same site [1]. The binding site of these diuron-like herbicides is located on the D1 protein [3], on the external face of the thylakoid membrane, close to the stroma but in a hydrophobic protein environment.

In isolated thylakoids deprived of the chloroplast envelope, diuron-like herbicides rapidly block the $Q_A \rightarrow Q_B$ electron transfer by displacing the Q_B secondary quinone. However, a slow inhibition has been reported for two triazine inhibitors, procyazine and cyanazine [4]. A slow action of ioxynil, a phenolic herbicide, on thylakoids was also observed, but could be ascribed to the pH-dependent ionization of the acidic OH [5].

Here we have studied the progressive inhibition induced by uracil derivatives (bromacil, terbacil, lenacil) and, to a lesser extent, by some diuron-like inhibitors which also act slowly on electron transfer at temperatures below 10 °C.

Materials and Methods

Chloroplasts were prepared from different species (lettuce, pea, spinach, triazine-susceptible or -resistant lamsquarter) and were disrupted by osmotic shock. The resuspension and measuring medium was 0.1 M sorbitol and 10 mM NaCl buffered either with 20 mM tricine at pH 7.8 or with 20 mM MES at pH 6.5. Usually, 5 mM $MgCl_2$ was added.

The onset of inhibition was followed by fluorescence induction kinetics under a strong actinic light (100 $\mu Em^{-2}s^{-1}$), using an analogic/digital conversion card plugged into an IBM-type microcomputer in which the fluorescence signal was stored and analyzed, as previously described [6].

The inhibition level was measured by the amplitude of the photochemical rise (F_I) related to the variable fluorescence (F_V) or from the complementary area. Inhibitors were assayed at concentrations slightly higher than the half-inhibitory concentration, giving a final F_I/F_V ratio lower than 0.8, in order to avoid a non-linear fluorescence response at higher concentrations. The first measurement was possible 5 s after injection of an inhibitor and 2 s rapid vortex mixing. Inhibition of the Hill reaction ($H_2O \rightarrow$ ferricyanide) was also measured with a Clark oxygen electrode, only for the slowest acting compounds (uracils).

Kinetics of release of bound inhibitors were monitored by fluorescence. Thylakoids at 25 µg/ml were incubated in the presence of nearly saturating concentrations of herbicides, then centrifuged and the pellet was rapidly vortexed in fresh buffer, at a chlorophyll concentration of 5 µg/ml.

Partition coefficients (log P_c) corresponding to octanol/water partition were calculated by Dr. P. M. Carles (Procida-Roussel-Uclaf) from the atomic contributions [7]. The calculated log P_c were generally lower than the experimentally determined log P_E [9–11]. This may be explained by self-associations formed in the octanol phase, which could increase log P_E. Solubilities in water were from ref. [12].

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Results

Although the F_I/F_V ratio is not linearly related to the proportion of centers blocked in the Q_A^- state [13], it allows an apparent half-time (t 1/2) of action to be defined. The complementary area, above the fluorescence induction curve, provides an exact measurement of the Q_A^-/Q_A ratio.

Cyanazine, already reported as a progressive inhibitor [4], was the slowest acting of the compounds here studied (Table). A progressive inhibition was also induced by the three uracils, the slowest one being bromacil (Fig. 1 a), then lenacil, then terbacil. It was checked that bromacil competitively displaced [^{14}C]diuron, confirming that these two compounds bind to identical or overlapping sites [1]. This slow action of uracils was confirmed by measurements of the Hill reaction.

Other classical PS II inhibitors showed a half-time of inhibition between 5 s and 30 s at 15 °C. The fastest acting compound was ametryne, with a t 1/2 close to the minimum measuring time (5 s). The rise of inhibition was strongly temperature-dependent, as demonstrated by the t 1/2 for bromacil between 5 °C and 25 °C (Fig. 1 b). In pea thylakoids at 5 °C, the half-time of action were for bromacil t 1/2 = 36 s, for diuron t 1/2 = 55 s, for ametryne t 1/2 = 40 s.

Similar kinetics were obtained with thylakoids from different plant species, above 10 °C. Some

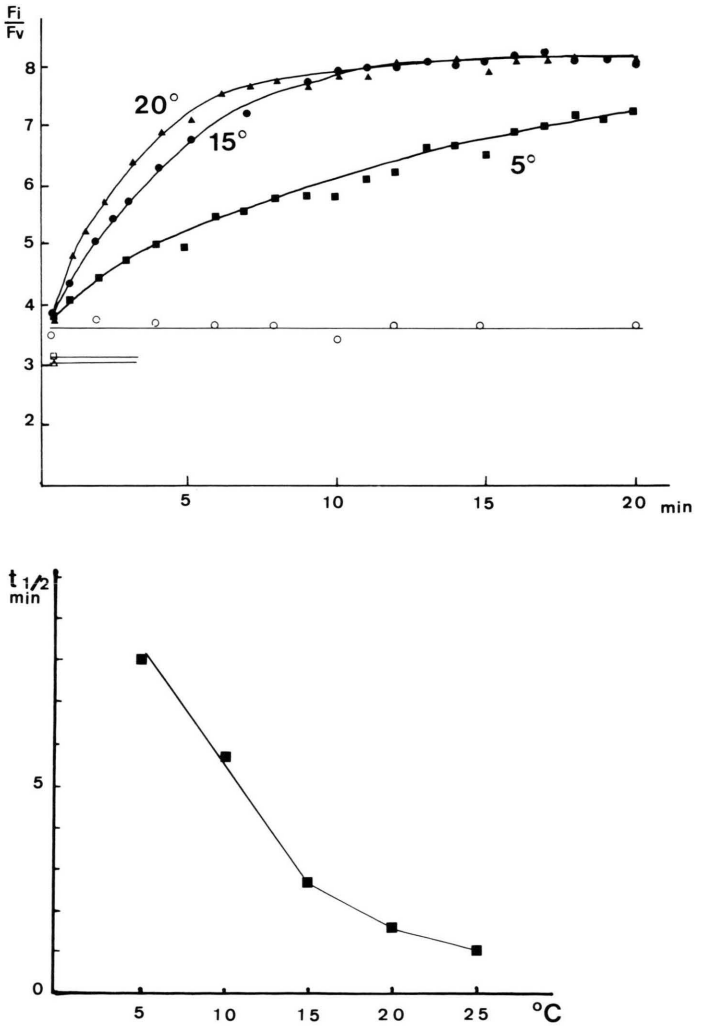

Fig. 1. Progressive inhibition by 0.1 μM bromacil at pH 7.8 on lettuce thylakoids. a) The inhibition is followed by the increase of the fluorescence induction level (amplitude of the photochemical phase: F_I) related to the amplitude of variable fluorescence. b) From this kinetic, a half-time of action could be estimated for 0.1 μM bromacil.

Table I. Inhibition parameters of different photosystem II herbicides. Half-time of inhibition for different diuron-like herbicides on lettuce thylakoids at 15 °C. t 1/2 were estimated from F_I/F_V fluorescence increase at different times after injection of the herbicide. Concentrations were determined in order that the F_I/F_V final values fall in the 0.60–0.85 range [5]. Chlorophyll concentration: 5 μg/ml, pH 7.8.

Herbicides	Conc. [nM]	t 1/2 [s]	MW	log P_c	log P_E	Water solubility
Uracils						
Bromacil	100	170	261	1.31	2.11	815
Terbacil	100	60	217	1.40		710
Lenacil	100	85	234	1.50		6
Amides/Ureas						
Diuron	50	17	233	2.31	2.60	42
Phenmedipham	100	30	300	3.01	4.80	<10
Triazines						
Atrazine	100	25	216	1.30	2.05	33
Terbutryne	10	30	241	2.31	2.56	58
Ametryne	10	<10	227	2.31		193
Cyanazine	100	710	241	0.91		171
Triazinones						
Metribuzin	100	20	214	2.58	1.70	1200
Ametridione	25	35				

variation could be observed below 10 °C (data not shown).

Kinetics of action were usually determined at pH 7.8 in the presence of 5 mM $MgCl_2$, which induces a large variable fluorescence by favoring the stacking of thylakoids. Since this stacking could hinder the access of inhibitors towards appressed regions, we checked that the absence of $MgCl_2$ did not significantly affect the slow binding character of inhibitors. However, a slight decrease (20 to 30%) of the t 1/2 could be noticed, which can be ascribed to the modified fluorescence properties of thylakoids. The pH of the medium (pH 7.8 or 6.5) did not influence significantly the t 1/2.

On triazine-resistant thylakoids from *Chenopodium album*, bromacil did not show the same slow inhibition as in susceptible thylakoids (Fig. 2). The I_{50} values at equilibrium were 0.06 M for S and 4 M for R, giving a R/S ratio of 67.

The kinetics of release of bound inhibitor from thylakoids rapidly diluted in a herbicide-free medium did not show such differences between compounds. For lettuce thylakoids at 15 °C, the half-time of release was 6 mn for bromacil, 4 mn for diuron, 1.5 mn for atrazine. This agrees with previous reports of a slow turnover of these inhibitors on their binding site [14, 15].

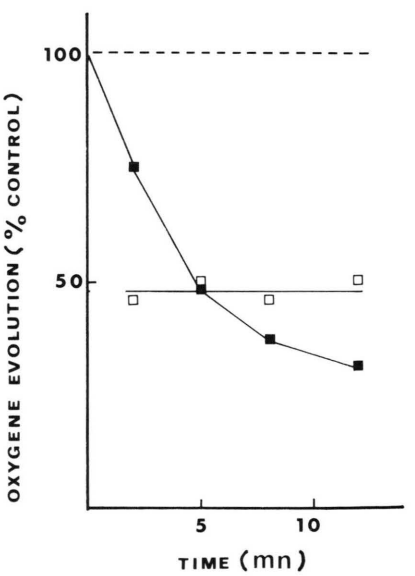

Fig. 2. Time dependence of oxygen evolution inhibition by bromacil in triazine-resistant or -susceptible thylakoids of *Chenopodium album*. Open symbols: resistant chloroplasts, bromacil 4 μM; closed symbols: susceptible chloroplasts, bromacil 0.1 μM. Temperature: 15 °C.

Discussion

Herbicides could enter the Q_B pocket *via* the membrane lipids or, more unlikely, through the external part of the D 1 protein close to the stroma.

Different limiting steps in the binding process could explain the delay in inhibition:

a) Penetration into the membrane, which can be hindered by the dehydration of the inhibitor molecule. However, there is no direct relation (Table) between the half-time of action (t 1/2) and the partition coefficients (log P) or the solubility in water. Furthermore, the same phenomenon should exist at the membrane/water interface of triazine-resistant thylakoids, which does not occur as demonstrated by the fast action of uracils on this kind of material. This indicates that desolvation is not likely to be the limiting step in the kinetics of inhibition.

b) Translocation of inhibitors within the lipid phase. However, diffusion times of hydrophobic compounds within lipidic membranes are in the millisecond range [16], particularly in the very fluid thylakoid lipids. This does not preclude the possibility that particular lipids associated with proteins could constitute a barrier to diffusion.

c) Hindrances encountered within a protein channel leading to the site. This phenomenon has been theoretically studied in the case of oxygen or carbon monoxide binding in myoglobin [17]. However, a rough comparison shows that a bulky compound such as phenmedipham acts faster than the more compact uracil molecules. Furthermore, crystallographic structures of bacterial reaction centers, as far as they represent the situation in PS II, show a Q_B pocket largely exposed to the membraneous lipid environment.

d) Conformational rearrangements of the protein within the binding site and/or of the inhibitor molecule which might be a prerequisite to allow all the binding interactions to be formed. This has been studied in the case of slow acting enzyme inhibitors [18–20]. These compounds act usually as transition-state analogs and a slow conformational change of the enzyme-inhibitor complex occurs [19]. The time range for such phenomena to occur is of the same order as the kinetics that we describe. In the case of photosystem II, the transition state would correspond to the Q_B^- state. In this state, a semiplastoquinone anion radical is bound on the site which might undergo a change of conformation during the $Q_B \rightarrow Q_B^-$ reduction. Contradictory evidence exists concerning the extent of structural changes induced within the site by the binding of an inhibitor, in *R. sphaeroides* (Paddock *et al.*, this issue) or *R. viridis* (Sinning *et al.*, this issue).

Resistance to triazines is induced by a single mutation ($Ser_{264} \rightarrow Gly$) located in the Q_B pocket. The rapid action of bromacil on triazine-resistant chloroplasts also suggests that its fitting into the site becomes easier, further supporting the idea that this step is the limiting one in the wild type. Similar behavior was observed with certain slow acting cyanoacrylate derivatives (J. N. Phillips, personal communication).

The requirement of conformational changes in inhibitor molecules for their fitting into the site has also to be considered. Different rotamers exist for triazine, in which energy minima correspond to the NH-alkyl in the plane of the triazine ring [21]. However, the same calculations were carried out for cyanazine and the rotational energy barriers (12 kcal/mol) are similar to those of atrazine. This excludes the hypothesis of a slow rotation of the NH-alkyl as an explanation of the slow binding. The existence of minor tautomeric forms of uracils have been demonstrated by spectroscopy in an organic solvent [22], and one of these tautomers could be the active form. Time needed to displace the equilibrium as the active form binds to the site may then explain the slowness of the process observed.

The binding kinetics of structurally different inhibitors could provide some clue about the mechanism of their binding into the Q_B site.

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