Inhibition of Photosystem II by Ioxynil in Wild Type and Resistant Mutant of *Synechocystis* 6714

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A *Synechocystis* 6714 mutant resistant to the phenol-type herbicide ioxynil was isolated and characterized. Ioxynil was shown to inhibit both the donor and the acceptor sides of photosystem II, but at different concentrations. The mutation found in the *psb*A gene (encoding the D_1 protein) at codon 266 (asparagine to threonine) [G. Ajlani, I. Meyer, C. Vernotte, and C. Astier, FEBS Lett. **246**, 207–210 (1989)] gives a ten-fold resistance of the acceptor side to ioxynil without any modification of the sensitivity of the donor side. Electron transfer between the primary and the secondary acceptor of photosystem II was identical in the mutant and the wild type. The mutant remains sensitive to atrazine and is even more sensitive to DCMU than the wild type.

Introduction

A variety of herbicides of agricultural importance inhibit the functioning of photosystem II (PS II). Several observations have led to the distinction between "urea-triazine" type inhibitors (DCMU, atrazine ...) and "phenol" type inhibitors (nitro- and halogen-substituted phenols such as dinoseb, ioxynil ...) [1]. All inhibit the photosynthetic electron transfer chain at the reducing side of PS II. They block electron flow between Q_A and Q_B, the primary and secondary quinone acceptors of PS II. They also displace each other from the membrane, hence the concept of overlapping binding sites on a common binding domain on the D_1 protein [2]. Significant differences nonetheless exist in the functional and chemical behaviour of these two families of herbicides, as summarized in [3] and [1].

A detailed model for the molecular topology of the plastoquinones Q_A and Q_B and the herbicide niche on the D_1 and D_2 polypeptides has been presented by Trebst and Draber 1986 [4]. The quinones bind to the protein *via* two hydrogen bridges (among other interactions) one to Histidine 215 and the other to a peptide bond close to serine 264. According to the properties of the two families of inhibitors (cross-resistance of mutants, charge of the inhibitors, and loss or not of inhibitory potency in Tris-treated thylakoids), Trebst proposed that "classical" urea/triazine type inhibitors would principally interact with serine 264 (serine family) and that phenol-type inhibitors would be directed towards histidine 215 (histidine family).

These assumptions are based upon knowledge of the details of the molecular topology of the amino acids in the binding niche and of the description of many mutants resistant to herbicides. But only mutants resistant to urea/triazine type inhibitors have been well characterized so far. This is owing to the fact that phenol-type inhibitors are more complex in that they have multiple binding sites (shown by binding analysis of radioactive herbicide) [5, 6]; their azido derivatives bind to several PS II polypeptides [7–10]; they present a lag time in the inhibition of the PS II activity of thylakoids [2, 10, 11] and furthermore, they have two types of inhibition other than that on the acceptor side, one on the donor side of PS II and one of the uncoupler type.

Inhibition of the donor side of PS II was characterized by van Assche 1981 [12], Pfister and Schreiber 1984 [13], Mathis and Rutherford 1984 [14] and Rutherford *et al.* 1984 [15]. Phenol type inhibitors (like dinoseb or ioxynil) inhibit H_2O to silicomolybdate (reaction insensitive to DCMU) [12]. They produce, like hydroxylamine, inhibition of Q_A reoxydation by recombination with positive

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; atrazine, 2-chloro-4-ethylamino-6-isopropyl-amino-*s*-triazine; bromoxynil, 3,5-dibromo-4-hydroxybenzonitrile; D_1, 32 kDa polypeptide subunit of the photosystem II reaction center (*psb*A gene product); ioxynil, 3,5-diiodo-4-hydroxybenzonitrile; Q_A and Q_B, primary and secondary quinone acceptor of photosystem II; S_2, state of the oxygen evolving complex with 2 positive charges stored.

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charges carried by the oxygen evolving complex in S_2 state [13]. They transform cytochrome b_{559} from the reduced high-potential form to the oxidized low potential form, cause EPR signal II slow to disappear, and induce the formation of a carotenoid radical cation in PS II upon flash excitation, either in normal or in Tris-washed thylakoids. Then they have some of the characteristics of ADRY inhibitors [14, 15].

Furthermore, phenol-type inhibitors present a third type of inhibitory effect, uncoupling of phosphorylation, attributed to their protonophoric action and collapsing of ΔpH by alteration of the integrity of the thylakoid membrane. This has been well characterized by Moreland and Novitsky 1987 [16].

All three types of inhibition have to be correlated with the multiple binding site and affinity studies by Laasch *et al.* [17, 5] and Oettmeier *et al.* [7, 8, 6].

The complexity of phenolic inhibitors like ioxynil is also enhanced by the effects of pH. Thiel et Böger [11] showed that pH influences time dependence of ioxynil binding to spinach thylakoids: binding sites seem to be only accessible to the uncharged molecules (ioxynil pK = 3.96) and affinity seems also to be modified by the charge of the receptor around the binding sites.

In order to better define the inhibitory functions of ioxynil and its interaction with the D_1 protein, we chose to isolate mutants resistant to phenol-type herbicides. We first characterized ioxynil inhibition in *Synechocystis* 6714 cells to define selection conditions for acceptor side mutants. In the present report, we describe a *psb*A gene mutant that has acquired 10-fold resistance to ioxynil on the acceptor side, without modification of sensitivity on the donor side of PS II.

Materials and Methods

The strain *Synechocystis* PCC 6714 was of the American type culture collection no. ATCC 27178.

Growth conditions

The minimal medium for growth was that defined by Herdman *et al.* [19] with twice the concentration of nitrate. For the solid medium, 1.5% agar autoclaved separately was added. Standard photosynthetic growth was achieved by incubation in a Gallenkamp rotatory shaker under constant agitation at 34 °C under 2500 lux in a CO_2 enriched atmosphere. The generation time was 6 h.

PS II activity assays

Fluorescence under continuous illumination was measured as described in [20]. The fluorescence, excited with a tungsten lamp through 4−96 + 5−59 Corning filters, was detected in the red region through a 2−64 Corning filter and a Wratten 90 filter. The recording was with a multichannel analyzer. The cell suspension contained about 1 μg Chl/ml. PS II activity of thylakoids, prepared as in [21], was measured with dichlorophenol-indophenol as an electron acceptor, at pH 6.8, from absorption changes at 580 nm.

Fluorescence decay after excitation by one saturating flash was monitored by detection of the fluorescence excited by a train of non-actinic flashes as in [22]. For thermoluminescence measurements, cell suspensions at ~ 400 μg/ml were dark adapted then cooled to −5 °C; a saturating flash was given followed by immersion in liquid nitrogen. Temperature from −80 °C to 40 °C was increased at a rate of 0.5 °C/sec, and thermoluminescence measured with a photomultiplier.

Results

Characterization of inhibition by ioxynil of Synechocystis 6714 wild type cells

To determine inhibition by ioxynil on the acceptor side, *i.e.*, the inhibition of Q_A to Q_B electron transfer, we measured fluorescence inductions (Fig. 1a). It is known that chlorophyll fluorescence yield is controlled by the redox state of Q_A. In our conditions, in the absence of herbicide, the fluorescence did not rise much above the initial F_O level because, at 440 nm, photosystem I is preferentially excited and very few centers are in the Q_A^- state. Addition of herbicide produced an increase of variable fluorescence quasi proportional to the number of PS II centers blocked by the herbicide.

To determine inhibition by ioxynil on the donor side, we utilized the fact that ioxynil blocks reoxydation of Q_A^- by recombination with positive charge on the donor side. Therefore (Fig. 1b) DCMU was added in all samples (to block Q_A^- to Q_B electron transfer), plus various concentrations of ioxynil or 1 mM NH_2OH. Hydroxylamine is known to completely block Q_A^- reoxydation by recombination and is utilized to define 100% inhibition. A first illumination produced the reduction of all Q_A. Then 20 sec darkness allowed centers that are not inhibited on the

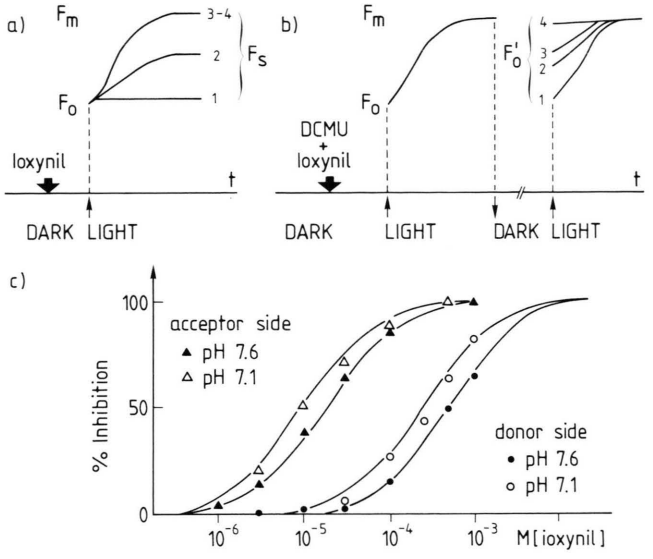

Fig. 1. (a) and (b): Schematic representations of fluorescence measurements to estimate the inhibition by ioxynil on the acceptor (a) and donor (b) sides of photosystem II. In (a) various concentrations of ioxynil [1–3] or a saturating concentration of DCMU (10^{-5} M) [4] were added in dark adapted samples 30 sec before fluorescence recording. Inhibition is given by

$$\frac{F_s\,(\text{Iox}) - F_s\,(o)}{F_s\,(\text{DCMU}) - F_s\,(o)}.$$

In (b) DCMU (10^{-5} M) was added in all the samples, and at the same time, either various concentrations of ioxynil [1–3] or 1 mM NH_2OH [4] were added. Two illuminations were done separated by 20 sec darkness. Inhibition is calculated as

$$\frac{F'_o\,(\text{Iox}) - F'_o\,(o)}{F'_o\,(NH_2OH) - F'_o\,(o)}$$

(c): inhibition by ioxynil of the donor and acceptor sides of PS II of wild type cells at 2 pHs. For more details see Materials and Methods and Results.

donor side to be reoxidized by back reaction ($t\,1/2 \sim 1$ to 3 sec), but inhibited centers remained in the Q_A^- state.

Fig. 1c gives the results of these measurements at 2 different pH values. First, it shows that inhibition of the donor side occurs at higher concentration than that needed to inhibit the acceptor side (25 ×). Second, it shows that the increase of pH from 7.1 to 7.6 shifts both curves by a factor of about 2 to higher concentrations of ioxynil. At higher pH, ioxynil was even less efficient and at pH 9.0 no inhibition at all occurred (data not shown). As the pK of ioxynil is 3.96, the proportion of protonated uncharged molecules decreases when pH is increased. It has been shown [23, 18] that in cyanobacteria, both cytoplasmic and intrathylakoid pH values are maintained quasi constant over a range of external pH of 6.5 to 8 or 9. The pH effect we observed probably results from the fact that only uncharged molecules are able to penetrate into the cells rather than a direct influence of pH on the binding site(s).

No lag time for action of ioxynil on the cells has been found in the limits of the time of mixing of our apparatus (~ 5 sec), in either the dark adapted or in the preilluminated samples.

Isolation of mutants

As the growth medium was not buffered and the pH during growth varied between 8.6 and 7.7, we also measured the inhibition by ioxynil in the growth conditions and found an I_{50} of 5.10^{-8} M. We chose the concentration of 10^{-4} M ioxynil to select mutants because, according to Fig. 1c, at this concentration, the donor side would not be inhibited at all. At this concentration, the wild type strain presented residual growth and specific procedures have been used to isolate resistant mutants. Series of cultures and subcultures were done in the presence of 10^{-4} M ioxynil until subcultures growing well were obtained. Then samples of these cell suspension were plated in selective solid medium and various clones were isolated and tested. One of them, IoxI, was used for the present study.

Characterization of the mutant

a) *Sensitivity of whole cells and thylakoids to ioxynil and other herbicides.* Inhibition by ioxynil of the acceptor and donor sides of IoxI was measured as described for the wild type. Fig. 2 shows that for the acceptor side, IoxI presents an $I_{50} \times 10$ compared to the wild type, *i.e.*, 8×10^{-5} M compared to 8×10^{-6} M at pH 7.1. In contrast, no difference was found for the donor side ($I_{50} = 2 \times 10^{-4}$ M as for wild type). These results show that the two inhibitor sites are clearly distinct because it is possible to modify one site and not the other. Table I shows the resistance of IoxI to other herbicides, and that in all cases the resistances to herbicides are retained in isolated thy-

Table I. Resistance to various herbicides of IoxI mutant. *R/S* is the ratio of the I_{50} of IoxI to that of wild type, measured by fluorescence as in Fig. 1 a for whole cells and by Hill reaction (H_2O to dichlorophenol-indophenol) for thylakoids.

R/S	Ioxynil	Bromoxynil	DCMU	Atrazine	Metribuzine
Whole cells	10	>5	0.6	0.8	7
Thylakoids	9	15	0.7	1	n.d.

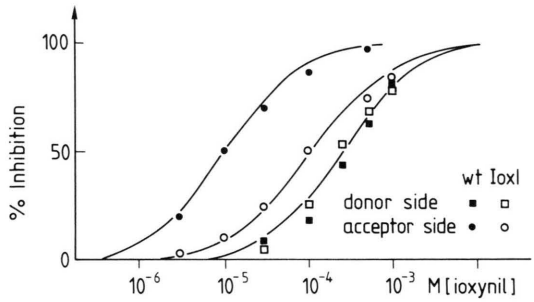

Fig. 2. Inhibition by ioxynil of the donor and acceptor sides of PS II of wild type and IoxI mutant at pH 7.1. Same conditions as Fig. 1.

lakoids (*i.e.*, that the mutant IoxI is not a detoxification mutant or a permeation mutant). As expected, IoxI is also resistant to bromoxynil, another phenolic inhibitor. It remains sensitive to atrazine, and is even more sensitive to DCMU than the wild type. But, it is clearly resistant to metribuzine, which is a "classical" type inhibitor.

b) *Characterization of electron transfer in photosystem II.* The herbicides which block electron transfer between Q_A and Q_B are bound in the same domain of D_1 as the plastoquinone Q_B. It has been shown in several herbicide resistant mutants, that the modifications of D_1, which lead to decreased affinity of the herbicide for D_1, also perturbs the electron transfer between Q_A and Q_B [24, 22, 25, 26]. In the IoxI mutant, no modifications have been found. The kinetics of reoxydation of Q_A^- after one saturating flash, measured by fluorescence, was quasi unchanged in the mutant (Fig. 3).

Conclusion

Our results clearly show that the inhibitions by ioxynil on the acceptor side and the donor side of PS II are related to two different sites of action. D_1 is supposed to span the thylakoidal membrane and to participate in the binding of manganese and extrinsic polypeptides involved in oxygen evolution. Then, it would have been possible that the binding of ioxynil in the Q_B niche produces a change of conformation of D_1 and perturbs the donor side of PS II just as Tris treatment (that removes the three peripheral polypeptides of PS II oxygen evolution) leads to a decrease in inhibitory potency of the classical inhibitors triazine, triazinone and ureas, but not of phenol, quinolone and pyridone inhibitors [1]. But the fact that in the IoxI the I_{50} for inhibition of the donor side is not modified compared to the wild type whereas the I_{50} for electron transfer between Q_A to Q_B is tenfold that of the wild type, is in favor of the existence of two distinct sites of action.

The mutation of IoxI responsible for the resistance was found at codon 266 in the Q_B binding domain of D_1 [27]. It does not modify electron transfer rate in PS II, in contrast to what has been shown for several

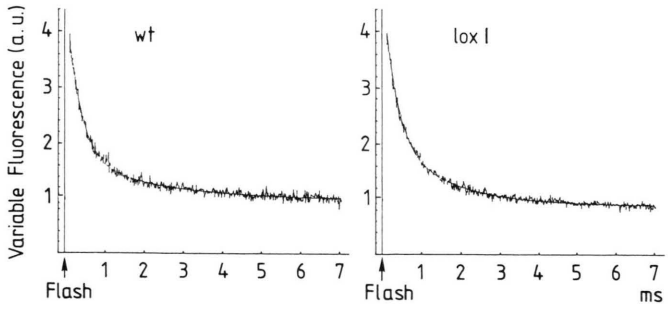

Fig. 3. Variable fluorescence decay curves for dark-adapted cells of wild type and IoxI mutant following an actinic saturating flash. Due to internal gating of the fluorimeter detector, the first signal point was recorded at 112 μs after triggering of the actinic flash.

others herbicide resistant mutants in plants [24], green algae [25], and cyanobacteria [22, 26]. This is confirmed by other biophysical measurements (thermoluminescence and oxygen sequence damping) comparing IoxI and wild type of *Synechocystis* 6714 (Etienne *et al.*, submitted to Biophys. Biochim. Acta).

The IoxI mutant is still sensitive to atrazine and presents a slightly increased sensitivity to DCMU. Conversely, Trebst and Draber [4] have observed several examples in which atrazine or DCMU tolerant cells are equally if not more sensitive to the phenol-type herbicides. In Table II, we have analyzed various mutants of several species, which have been tested for their resistance to ioxynil and which present known points of mutation in the D_1 protein. References are given in Table II.

If we consider all the mutants where serine 264 has been replaced by alanine or glycine, no clear changes in ioxynil sensitivity occur: in *Synechocystis* 6714 D-IIA, there is no change; in *Synechococcus* 7942 Di1, increased sensitivity; and in plants either increased or decreased sensitivity. But among other herbicide resistant mutants, two appear very resistant to ioxynil, the *Synechocystis* 6714 AzV (15×) and the *Chlamydomonas* MZ2 (40 ×). In the latter, alanine 251 is changed to valine. The *Synechocystis* mutant AzV has two point mutations: the same change of alanine 251 to valine and a change of phenylalanine 211 to serine. The mutant from which it is derived is AzI. This mutant presents only the change of residue 211, and is only slightly (3 ×) resistant to ioxynil. It thus seems that the presence of an alanine residue at position 251 is very important for sensitivity to ioxynil. The difference between alanine and valine is a supplementary methyl group for the latter, and not a change of charge as is the case for the 266 change from asparagine to threonine. The presence of valine instead of alanine may produce a steric hindrance to ioxynil binding. Phenylalanine 255 also seems to influence ioxynil binding: change of this residue to tyrosine gives a slight resistance (4×), which surprisingly is suppressed by an additional change of serine 264 to alanine (mutants Tyr5 and D5 of *Synechococcus* 7942). Recently, Oettmeir has shown that azido-ioxynil is bound to valine 249 [10] and that modelization in three dimensions of the Q_B binding domain gives a satisfying proximity between asparagine 266 and valine 249.

All these results lead to the hypothesis that asparagine 266, valine 249, phenylalanine 255 and alanine 251 participate in the binding niche of ioxynil. Cloning and sequencing of the *psb*A gene of other *Synechocystis* ioxynil resistant mutants are in progress in our laboratory and will allow us to specify the interactions of this herbicide with the different amino acids of the D_1 protein.

Acknowledgements

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Table II. Resistance to ioxynil of various mutants from several organisms, selected for resistance to DCMU, atrazine or metribuzine, and their mutations in D_1 protein. *R/S* is the ratio of the I_{50} of the mutant to that of the wild type.

Organisms	Mutants	*R/S*	Mutations	Ref.
Synechocystis 6714	D-II A	1	264 Ser → Ala	[28]
	AzV	10–15	251 Ala → Val 211 Phe → Ser	
	AzI	3	211 Phen → Ser	
Synechococcus 7942	Di1	0.4	264 Ser → Ala	[29]
	D 5	0.5	264 Ser → Ala 255 Phe → Tyr	
	Tyr 5	4	255 Phe → Tyr	
Chlamydomonas	MZ2	40	251 Ala → Val	[30, 31]
Amaranthus hybridus	R	1.6	264 Ser → Gly	[32]
Amaranthus retroflexus	R	0.64	264 Ser → Gly	[33]

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