A New Ioxynil-Resistant Mutant in *Synechocystis* PCC 6714: Hypothesis on the Interaction of Ioxynil with the D 1 Protein

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A new *Synechocystis* 6714 mutant, $IoxII_A$, resistant to the phenol-type herbicide ioxynil was isolated and characterized. The mutation found in the *psb*A gene (encoding the D 1 photosystem II protein) is at the same codon 266 as for the first ioxynil-resistant mutant $IoxI_A$ previously selected [G. Ajlani, I. Meyer, C. Vernotte, and C. Astier, FEBS Lett. **246,** 207–210 (1989)]. In $IoxII_A$, the change of Asn 266 to Asp gives a 3 × resistance, whereas in $IoxI_A$, the change of the same amino acid to Thr gives a 10 × resistance. The effect of these different amino acid substitutions on the ioxynil resistance phenotype has allowed us to construct molecular models and calculate the hydrogen-bonding energies between the hydroxyl group of ioxynil and the respective amino acids at position 266.

Introduction

Phenolic herbicides like nitro- and halogen-substituted phenols constitute a unique class among the herbicides blocking photosystem II (PS II). Unlike other PS II inhibitors they do not have the common structural element $-N-C- = X$ (where X = O or N) and they have a different charge distribution (for a review see [1]). They are known to have multiple binding sites. They block electron transfer between Q_A and Q_B, the quinonic electron acceptors of the PS II as the "classical" urea/triazine type herbicides. They have also two other types of inhibition, one on the donor side of the PS II and one of the uncoupler type.

Characterization of PS II inhibition by ioxynil in the *Synechocystis* PCC 6714 strain allowed us to determine the conditions to select acceptor side resistant mutants [2, 3], the I_{50} of ioxynil on the donor side being 20-fold higher than that on the acceptor side [2]. A first mutant, 10-fold resistant to ioxynil on the acceptor side has already been described. The mutation was localized in its *psb*A_1 gene encoding the D 1 protein of the PS II core and results in a substitution from asparagine to threonine at position 266 (codon AAC to ACC) [3]. In this paper we describe a new ioxynil-resistant mutant having a different substitution on the same codon.

Furthermore, other mutants resistant to "classical" herbicides were previously characterized [4]. Two of them, selected to be resistant to atrazine exhibited cross resistance to ioxynil. AzI which is 10-fold resistant to atrazine, was found sensitive to DCMU and 3-fold resistant to ioxynil and a point mutation in its *psb*A_1 gene was found at codon 211 (Phe → Ser). AzV was derived from AzI and has acquired a higher resistance to atrazine (70-fold), it is still sensitive to DCMU but exhibits an increased resistance to ioxynil (15-fold). This double mutant, in addition to the substitution found in AzI at position 211 (Phe → Ser), has accumulated a second change at codon 251 (Ala → Val).

Based on the effect of these different amino acid substitutions in the D 1 protein on the ioxynil resistance phenotype, we propose an hypothesis for the interaction of ioxynil with amino acids at position 266. Models were constructed in order to study this hypothesis by quantum mechanics using the semi-empirical method AM 1 [5].

Materials and Methods

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

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Growth conditions

The minimal medium for growth was that defined by Herdman *et al.* [6] 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 3500 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 [7]. The fluorescence, excited with a tungsten lamp through 4–96 and 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 [8], was measured with dichlorophenol indophenol as an electron acceptor, at pH 6.8, from absorption changes at 580 nm.

Molecular modelling

Model amino acids as well as ioxynil were constructed using the SYBYL package facilities [9, 10] and fully minimized by AM 1 [5].

Thr was modelled by $CH_3-CHOH-CH_3$,
Asn by $CH_3-CH_2-CONH_2$ and
Asp by CH_3-CH_2-COOH or $CH_3-CH_2-COO^-$.

Charges were calculated on fully optimized structures. Formation of ioxynil-amino acid complexes was then analyzed and hydrogen-bonding energies deduced from the "super molecule" model (energy of interaction = heat of formation of the fully optimized complex − sum of the molecular heats of formation of each molecule).

Results

Isolation and characterization of ioxynil-resistant mutant

The selection procedure was described in [2]. A new mutant called $IoxII_A$ was selected following exactly the same procedure from which we had selected the $IoxI_A$ mutant previously described [2–3]. Sensitivity to ioxynil of whole cells and thylakoids of $IoxII_A$ and cross resistance to other types of herbicides were analyzed to determine the phenotype of this new mutant. Results are presented in Table I. This $IoxII_A$ mutant is 3-fold resistant to ioxynil compared to the wild type. Its donor side sensitivity to ioxynil is unchanged. It is still sensitive to DCMU and to atrazine.

Molecular characterization

To clone the $IoxII_A$ *psb*A_1 gene and determine its sequence, a genomic library was constructed in the vector λEMBL$_3$ and probed with the wild type gene previously cloned [4]. From the DNA of positive recombinant phage, a 0.7 Kb *Kpn*I fragment was subcloned in bluescript plasmid and sequenced. A point mutation at codon 266 in the *psb*A_1 coding locus (AAC to GAC) was found. This substitution results in an amino acid change from asparagine to aspartic acid in the Q_B-binding domain of the D1 protein (Fig. 1).

Quantum chemistry models

Charges calculated by AM1 for amino acid models and ioxynil are reported in Fig. 2. Each amino acid is represented by its side chain. Numbers give the values of the calculated charges of the various atoms, only those exceeding ± 0.15 are given.

Since the hydroxyl group of ioxynil may act as an hydrogen bond donor, we performed semi-empirical quantum mechanical calculations of ioxynil binding with these models of amino acid side chains.

Formation of ioxynil-amino acid complexes were studied in two cases:

- in the first case, the initial position of ioxynil was determined in order to form an ideal hydro-

Table I. Herbicide sensitivity (I_{50}) of wild type and $IoxII_A$. In whole cells, I_{50} (M) is the concentration needed to block half of the variable fluorescence; in thylakoids, I_{50} represents the concentration which blocks half of the Hill activity.

Inhibitor	Whole cells		Thylakoids	
	WT	$IoxII_A$	WT	$IoxII_A$
Ioxynil	8.0×10^{-6}	3.0×10^{-5}	4.0×10^{-7}	1×10^{-6}
DCMU	1.3×10^{-7}	8.0×10^{-8}	1.2×10^{-7}	8×10^{-8}
Atrazine	3.0×10^{-6}	2.5×10^{-6}	7.0×10^{-7}	7×10^{-7}

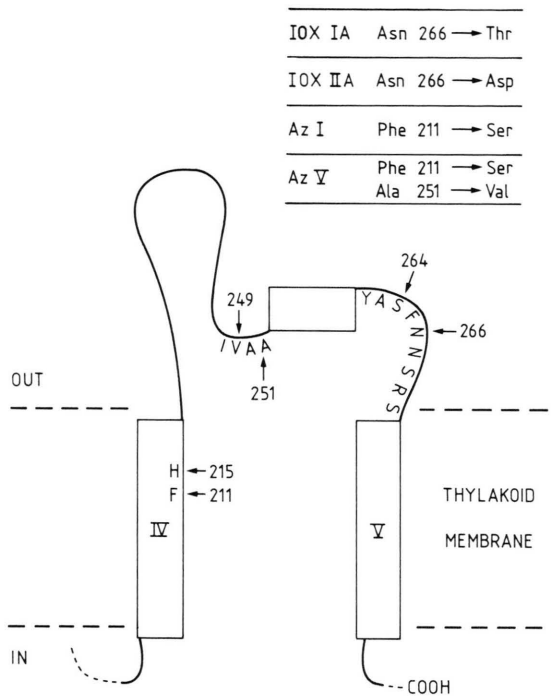

Fig. 1. Scheme based upon the model of Trebst and Draber [19] for the herbicide-binding environment in the D1 protein. The presumed helices are represented by boxes. Only that region of D1 is shown which covers the segment from the beginning of the 4th membrane-spanning helix to the COOH end of the polypeptide. Arrow at 249 denotes amino acid residue involved in fixation of azidoioxynil [20]. Other arrows, except at 215, denote amino acids involved in the herbicide resistance of *Synechocystis* 6714 mutants listed in the insert.

gen bond geometry between its hydroxyl as donor and Asn carbonyl as acceptor. The complex geometry with the two other amino acids is defined by the same position of C_α;

- in the second case, ioxynil was positioned so that two hydrogen bonds could be formed with Asn. The ioxynil hydroxyl group is donor to the carbonyl group of Asn as in the first case and acceptor for the NH_2 group.

Complexes were then fully optimized by AM1. Results are presented in Table II. In the first case, ΔE is slightly higher for Asn (3.42 kcal·mol^{-1}) than for protonated Asp (2.76 kcal·mol^{-1}). As

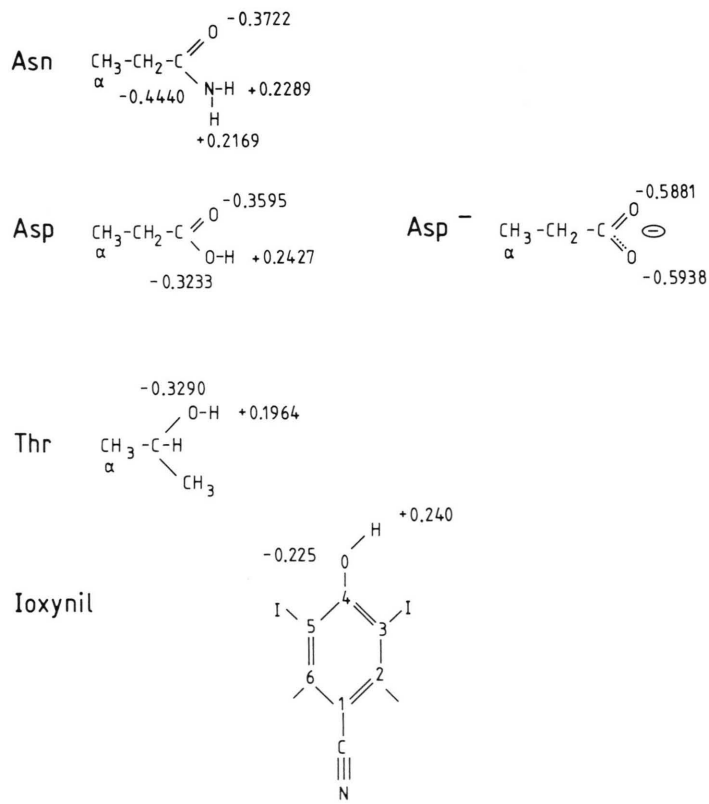

Fig. 2. Calculated charges for the amino acid models and ioxynil. Each amino acid is represented by its side chain. Models were constructed as described in Materials and Methods. Numbers give the values of the calculated charges when they exceed ± 0.15.

Table II. Hydrogen bonding energies and distances between ioxynil and models of amino acid side chains calculated as described in Materials and Methods. ΔE is the calculated energy of interaction in kcal·mol^{-1}; d(O···H) is the distance (Å) between the hydrogen of ioxynil hydroxyl and the acceptor oxygen of each amino acid; d(H···O) is the distance (Å) between the oxygen of ioxynil hydroxyl and the hydrogen of NH_2 for Asn and of OH for Asp.

Cases		Asn	Asp	Thr
Case 1	ΔE	3.42	2.76	0.39
	d(O···H)	2.1	2.2	3.1
Case 2	ΔE	5.45	4.38	1.49
	d(O···H)	2.1	2.1	2.2
	d(H···O)	2.2	2.3	–

long as a distance of 3 Å may be considered as a correct hydrogen bond distance, ΔE revealed a very weak bond with Thr (0.39 kcal·mol^{-1}).

In the second case, ΔE was still higher for Asn (5.45 kcal·mol^{-1}) than for protonated Asp (4.38 kcal·mol^{-1}). A reasonable bond was obtained with Thr (1.49 kcal·mol^{-1}) but ΔE was still very low compared to Asn and Asp.

When Asp was considered as deprotonated, two bonds were formed in both cases. The two oxygens of COO^- were engaged in hydrogen bonds with the ioxynil hydroxyl. The ΔE had a value of 17 kcal·mol^{-1}.

Discussion

Point mutations within the *psb*A gene which induce resistance towards phenolic herbicides provide information about the binding niche of these inhibitors in the D 1 protein.

The new mutation in the $IoxII_A$ mutant is located at the same codon 266 as in the previously described $IoxI_A$ [3]. In $IoxI_A$, Asn 266 was substituted by Thr, leading to a tenfold resistance on the acceptor side of PS II while in $IoxII_A$ the same Asn 266 is replaced by Asp, leading to only threefold resistance. Both mutants have an unchanged sensitivity to DCMU and atrazine.

DCMU and atrazine belong to the "DCMU-type" inhibitor family and are likely to engage different types of interactions with the Q_B-binding niche than those of phenolic type inhibitors [1]. Mutation of Ser 264 to Gly or Ala induces resistance to "DCMU-type" inhibitors in all PS II organisms (for example [3, 11–13]). In purple bacteria, the homologous mutation of Ser 223 to Ala (*R. viridis* [14]) or Pro (*R. sphaeroides* [15]) also induces resistance to triazines. The X-ray structure analysis of terbutryn-resistant reaction centre of *R. viridis* owning this mutation shows no significant modification of the Q_B niche backbone [16]. This mutation is believed to abolish one of the two hydrogen bonds which are possible between terbutryne and the protein [17, 18]. The same conservation of the structure may be supposed to occur in PS II D 1 protein mutated at position 264. Ioxynil binding does not seem to be clearly affected in these mutants [2], although the Asn 266, playing a role in ioxynil binding [2, 3] is close to this position. Likewise the unchanged activity of DCMU and atrazine in ioxynil-resistant strains mutated at position 266 supports the idea of a preserved overall structure of the site.

A steric effect may not be responsible for the ioxynil resistance in $IoxI_A$ and $IoxII_A$ since the more bulky of the amino acids concerned would be Asn in the wild type. Steric hindrance of Asn and Asp are similar, since only an NH_2 is replaced by an OH group and Thr has the shortest side chain of the three amino acids (Fig. 2). On the other hand, the nature of these substitutions is likely to modify the electrostatic properties of the site.

Various hypotheses for the orientation of ioxynil in the Q_B pocket have been presented. According to Trebst's model [19], phenolic inhibitors might be able to interact *via* an electronegative group (C ≡ N in ioxynil) directed towards His 215 (Fig. 1). Azido-ioxynil binds to Val 249 [20] the azido group being positioned on the carbon 2 of the benzonitrile ring (see Fig. 2). The mutation of Ala 251 to Val which is present in *Chlamydomonas reinhardtii* [21, 22] leads to the highest resistance to ioxynil described so far. The *Synechocystis* AzV mutant (15 × resistant to ioxynil) carries the same mutation which is believed to induce steric hindrance to ioxynil binding [2] since Val has only one methyl more than Ala. The AzV mutant carries a second mutation at position 211, replacing Phe by Ser. It has been selected from the AzI mutant which has this single mutation and presents a threefold resistance to ioxynil. The role of this Phe cannot be clearly established but it may be involved in stacking interactions of the phenol ring in the lower part of the site (see Fig. 1).

In agreement with the previous hypotheses our data are in accordance with an interaction of the phenolic OH group of ioxynil with the side chain of the amino acid at position 266. Changes in this type of interaction with the different amino acids present in the mutants may be at the origin for resistance.

Comparing charges calculated by AM 1 [5] (Fig. 2) on the eventual acceptor atom in each model of amino acid side chain, we obtain a progression (Asn−C=O = −0.3722 < Asp−C=O = −0.3595 < Thr−OH = −0.3290) which is comparable to that of the resistance factor R/S (Asn = 1 < Asp = 3 < Thr = 10) if we consider Asp as protonated.

Table II gives the hydrogen bond energies and distances between the −OH of ioxynil and the various amino acid side chains. In both cases studied, hydrogen bond energies obtained confirmed the progression observed with charges. Such results are in agreement with the hypothesis of hydrogen-bonding interaction between the hydroxyl of ioxynil and side chains of amino acids in position 266.

An interaction with deprotonated Asp would be much stronger and would not fit with resistance to ioxynil observed in $IoxII_A$ mutant. The real pKa of protonable amino acids in an hydrophobic protein such as D1 is difficult to estimate but the hypothesis of a protonated Asp 266 is compatible with the pKa of 9.5 found for Glu 212 in the Q_B site of *Rps. sphaeroides* [23].

As the tridimentional structure of the Q_B pocket is unknown for PS II, influence of other parameters on the ioxynil interaction with position 266 cannot be excluded. Nevertheless, our study underlines the relation which appears between the hydrogen bond capability of each amino acid side chain and the effect of the substitution at position 266 on the resistance to ioxynil.

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[1] W. Oettmeier and A. Trebst, in: The oxygen evolving system of photosynthesis (Y. Inoue, A. R. Crofts, Govindjee, N. Murata, G. Renger, and K. Satoh, eds.), pp. 411–420, Acad. Press, New York 1983.
[2] G. Ajlani, I. Meyer, C. Astier, and C. Vernotte, Z. Naturforsch. **44c,** 979–984 (1989).
[3] G. Ajlani, I. Meyer, C. Vernotte, and C. Astier, FEBS Lett. **246,** 207–210 (1989).
[4] G. Ajlani, D. Kirilovsky, M. Picaud, and C. Astier, Plant Mol. Biol. **13,** 469–480 (1989).
[5] M. J. S. Dewar, E. G. Zoebisch, E. F. Healy, and J. J. P. Steward, J. Amer. Chem. Soc. **107,** 3902–3909 (1985).
[6] M. Herdman, S. F. Delaney, and N. G. Carr, J. Gen. Microbiol. **79,** 233–237 (1979).
[7] C. Vernotte, A. L. Etienne, and J. M. Briantais, Biochim. Biophys. Acta **545,** 519–527 (1979).
[8] C. Astier, S. Styring, B. Maison-Peteri, and A. L. Etienne, Photobiochem. Photobiophys. **11,** 37–47 (1986).
[9] J. G. Vinter, A. Davis, J. Computer-Aided Mol. Design. **1,** 31–51 (1987).
[10] SYBIL R TRIPOS CORPORATION Ltd.
[11] K. Pfister and C. J. Arntzen, Z. Naturforsch. **34c,** 996–1009 (1979).
[12] J. M. Erickson, M. Rahire, P. Bennoun, P. Delepelaire, B. Diner, and J. D. Rochaix, Proc. Natl. Acad. Sci. U.S.A. **81,** 3617–3621 (1984).
[13] S. S. Golden and R. Haselkorn, Science **229,** 1104–1207 (1985).
[14] I. Sinning and H. Michel, Z. Naturforsch. **42c,** 751–754 (1987).
[15] M. L. Paddock, S. Rongey, E. C. Abresh, G. Feher, and M. Y. Okamura, Photosynth. Res. **17,** 75–96 (1988).
[16] I. Sinning, this issue (1989).
[17] H. Michel, O. Epp, and J. Deisenhofer, EMBO J. **5,** 2445–2451 (1986).
[18] I. Sinning, H. Michel, P. Mathis, and A. W. Rutherford, Biochemistry **28,** 5544–5553 (1989).
[19] A. Trebst and W. Draber, Photosynth. Res. **10,** 381–392 (1986).
[20] W. Oettmeier, K. Masson, J. Hohfeld, H. E. Meyer, K. Pfister, and H. P. Fischer, Z. Naturforsch. **44c,** 444–449 (1989).
[21] N. Pucheu, W. Oettmeier, U. Heisterkamp, K. Masson, and G. F. Wildner, Z. Naturforsch. **39c,** 437–439 (1984).
[22] U. Johanningmeier, U. Bodner, and G. F. Wildner, FEBS Lett. **211,** 221–224 (1987).
[23] M. L. Paddock, S. H. Rongey, G. Feher, and M. Y. Okamura, Proc. Natl. Acad. Sci. U.S.A. **86,** 6602–6606 (1989).