

Predicted effects on herbicide binding of amino acid substitutions in the D1 protein of photosystem II

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The effects of an amino acid substitution in the D1 protein of photosystem II on its binding to different herbicides are here successfully predicted. This is done by assuming additivity in the contributions to the binding energy of the amino acids at positions 255 and 264 of this protein which are part of the herbicide binding niche. Additivity in D1 protein-herbicide interactions is demonstrated, thus indicating an apparent lack of interactions between the amino acids at these two positions.

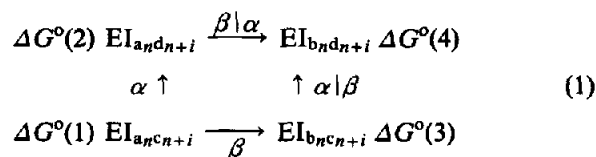
Protein, D1; Herbicide resistance; Photosystem II; Protein engineering; Site-specific mutagenesis

1. INTRODUCTION

There are two quinone intermediates in the light-driven electron transfer process in photosystem II (PS II) from H₂O to plastoquinone. These are the primary and secondary electron acceptors, Q_A and Q_B, respectively. D1 is a 32 kDa apoprotein of Q_B [1,2] that forms part of the reaction center of PS II [3]. PS II herbicides inhibit electron transfer from Q_A to Q_B by binding to the D1 protein, thereby causing displacement of the quinone Q_B [4,5]. Competition between the herbicides and Q_B for the same binding site has been demonstrated [5,6]. Different amino acid substitutions in the D1 protein have been previously found to reduce herbicide binding, thereby conferring herbicide-resistance [7,8]. Using site-directed mutagenesis it is possible to replace any amino acid in a protein. As the number of possible substitutions is extremely large, a method for predicting the effect of a specific mutation, from data available on other mutations, is of great value. A simple way of achieving

this goal is here described and applied to the case of the D1 protein.

A thermodynamic cycle (for the alternative binding of four homologous protein inhibitor molecules to a proteinase) that consists of two parallel and identical changes was proposed [9] as follows:



In this diagram a, b, c and d represent different amino acids of the inhibitor, and the subscripts *n* and *n+i* denote their position in the homologous sequences, all other amino acids being identical. α and β indicate the amino acid replacements $c_{n+i} \rightarrow d_{n+i}$ and $a_n \rightarrow b_n$, respectively. $\alpha|\beta$ and $\beta|\alpha$ indicate the temporal order of the replacements. $\Delta G^{\circ}(1)$, $\Delta G^{\circ}(2)$... $\Delta G^{\circ}(4)$ stand for the free energies of binding of the respective inhibitor to the proteinase. A measure for non-additivity is given by:

$$\begin{aligned} \Delta G^{\circ}(I) &= \Delta G^{\circ}(\alpha|\beta) - \Delta G^{\circ}(\alpha) \\ &= \Delta G^{\circ}(\beta|\alpha) - \Delta G^{\circ}(\beta) \end{aligned} \quad (2)$$

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where $\Delta G^{\circ}(I)$ is the coupling or interaction free energy. Additivity implies that the free energy change due to a substitution at one site does not depend on which amino acid is occupying the second site. By applying the principle of free energy conservation and assuming intermolecular additivity [9] one obtains:

$$\frac{K_2}{K_1} = \frac{K_4}{K_3} \quad (3)$$

where K_1, K_2, \dots, K_4 are the binding constants of the respective inhibitors to the enzyme. From eqn 3 one may calculate any one of the four binding constants provided the remaining three in that equation are known.

2. MATERIALS AND METHODS

2.1. Strains and growth conditions

Cell cultures of *Synechococcus* PCC7942 (Pasteur culture collection no. 7942) were grown in BG11 medium at 35°C as described by Williams and Szalay [10]. For selection of herbicide resistant mutants, cells were plated on solid BG11 medium containing 1.5% agar and the appropriate concentration of herbicides. Isolation of the atrazine resistant mutants Di1 and D5 has been described [11,12].

2.2. Molecular cloning and sequencing

Methods for isolating DNA from *Synechococcus* PCC7942 and for transformation of cyanobacterial cells were according to Williams and Szalay [10]. Restriction enzyme digestion, Southern hybridization and cloning, followed conventional protocols [13]. The *E. coli* strain MV1190 was used as host for the plasmid vectors pBR328 and pUC118.

2.3. Site-directed mutagenesis of *psbAI*

In order to change the phenylalanine residue at position 255 of D1 to tyrosine, a 1.5 kb *SalI-BamHI* DNA fragment, containing ~85% of the coding region of the *psbAI* gene [11,12], was subcloned in the plasmid pUC118. Single stranded DNA was prepared and served as template for oligonucleotide-directed mutagenesis that was carried out by the method of Nakamaye and Eckstein [14]. The linear 1.5 kb fragment carrying the new mutation was transfected to wild type cells and herbicide resistant transformants were selected on plates containing 5 μ M atrazine. *psbAI* of the mutant has been sequenced in order to verify the change in codon 255. The new mutation was designated Tyr5 [15].

2.4. Measurement of PS II-dependent electron transport

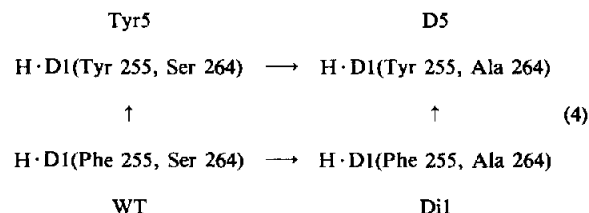
Cells from 0.5 l suspension culture of *Synechococcus* PCC7942 were harvested by centrifugation and washed once in distilled water. The cells were resuspended in 5 ml of Mes buffer: 50 mM Mes, pH 6.5, 5 mM CaCl_2 , 100 mM sucrose, 1 mM α -aminocaproic acid (Sigma) and 1 mM benzamidine, at 4°C.

The suspension was sonicated three times for 15 s each and centrifuged at $2500 \times g$ for 2 min. The supernatant was loaded on top of 2 ml of 60% (w/v) sucrose and centrifuged for 15 min at 4°C in a Ti50 rotor at 40000 rpm. The green pellet was resuspended in 200 μ l of Mes buffer. These membranes could be stored at -80°C. PS II dependent electron transfer from H_2O to 2,6-dichlorophenolindophenol (DCPIP) was analysed in these membranes by measuring the rate of photochemical reduction of DCPIP in a dual wavelength spectrophotometer (Aminco-Chance). I_{50} was determined as the concentration of herbicide that inhibited the rate of DCPIP reduction by 50%. All herbicides used in this study were chemically pure and were supplied by various companies which manufacture them or by Chem Service, West Chester, PA, USA. Two optical isomers of cyanoacrylates (ethoxyethyl-3-alkyl-2-cyano-3- α -methylbenzylamino acrylate) were kindly provided by Dr John Phillips, CSIRO, Australia.

3. RESULTS

A single mutation at position 264 (Di1) and a double mutation at positions 255 and 264 (D5) of the D1 protein in the cyanobacterium *Synechococcus* PCC7942 have been described [11,12]. Amino acids in these positions in D1 of both cyanobacteria and higher plants are known to contribute to the binding of various herbicides [8,16]. A third mutation at position 255 (Tyr5) was subsequently designed and genetically engineered in the same organism so that the wild type and the three mutants would give rise to a thermodynamic cycle that consists of two parallel and identical substitutions: Phe 255 \rightarrow Tyr 255; Ser 264 \rightarrow Ala 264. Such a cycle can provide information about interactions that may exist between the amino acids in positions 255 and 264.

A thermodynamic cycle similar to that in scheme 1 for the alternative binding of a herbicide to four variants of the D1 protein is given as follows:



In this scheme H and D1 stands for herbicide and the D1 protein, respectively. WT, Di1, Tyr5 and D5 designate the D1 protein of the wild type, the two single mutants and the double mutant respectively. Eqn 3 when applied to scheme 4 may be

written as follows:

$$\frac{K_{D5}}{K_{D1}} = \frac{K_{Tyr5}}{K_{WT}} \quad (5)$$

Predictions, using eqn 5, are here made for I_{50} concentrations of different herbicides with respect to the D1 protein of the Tyr5 mutant and are compared with actual experimental results. I_{50} values of different PS II herbicides, as determined by in vitro assays under identical conditions, correspond to their binding constants [17,18].

Table 1

I_{50} values of different herbicides for the wild type (WT) and mutants D1 and D5

Herbicide	I_{50} [M]		
	WT 255 Phe 264 Ser	D1 255 Phe 264 Ala	D5 255 Tyr 264 Ala
Ureas			
Fenuron	3.0×10^{-6}	3.0×10^{-3}	6.0×10^{-4}
Monuron	8.0×10^{-7}	2.0×10^{-4}	2.0×10^{-4}
Diuron	3.0×10^{-8}	3.0×10^{-6}	5.0×10^{-6}
Linuron	1.5×10^{-7}	1.5×10^{-5}	1.5×10^{-5}
Fluomethuron	2.0×10^{-6}	2.5×10^{-5}	3.0×10^{-4}
Tebuthiuron	4.0×10^{-6}	1.0×10^{-4}	1.0×10^{-5}
Ethidimuron	2.0×10^{-6}	5.0×10^{-5}	3.0×10^{-5}
s-Triazines			
Simazine	9.0×10^{-8}	6.0×10^{-6}	1.5×10^{-5}
Atrazine	3.0×10^{-7}	1.0×10^{-5}	6.0×10^{-5}
Propazine	6.0×10^{-7}	2.0×10^{-6}	7.0×10^{-5}
Ametryne	5.0×10^{-8}	1.5×10^{-6}	2.0×10^{-5}
Prometryne	9.0×10^{-8}	3.0×10^{-7}	8.0×10^{-6}
Terbutryne	1.5×10^{-8}	2.5×10^{-7}	1.0×10^{-5}
Triazinones			
Metribuzin	2.0×10^{-7}	1.0×10^{-3}	4.0×10^{-4}
Metamitron	2.5×10^{-5}	6.0×10^{-4}	5.0×10^{-5}
Uracyls			
Bromacil	3.0×10^{-7}	4.5×10^{-5}	5.0×10^{-6}
Cyanoacrylates			
JLH964 (S)	6.0×10^{-8}	7.0×10^{-7}	1.5×10^{-5}
JLH965 (R)	6.0×10^{-6}	1.5×10^{-5}	8.0×10^{-5}
Biscarbamates			
Phenmedipham	2.0×10^{-9}	2.5×10^{-7}	4.0×10^{-5}
Phenolics			
Bromoxynil	1.0×10^{-5}	8.0×10^{-6}	8.0×10^{-6}
Ioxynil	4.0×10^{-7}	1.5×10^{-7}	2.0×10^{-7}
Dinoseb	1.0×10^{-6}	1.0×10^{-7}	8.0×10^{-8}

PS II dependent electron transfer from H_2O to DCPIP was measured in isolated thylakoid membranes. Concentration of herbicide that inhibits the rate of electron transfer by 50% (I_{50}), was determined for each herbicide. Common names of herbicides are used here

Table 1 shows the different resistance profiles towards various herbicides of the single mutant D1 (264 Ser \rightarrow Ala), the double mutant D5 (255 Phe \rightarrow Tyr; 264 Ser \rightarrow Ala) and the wild-type. A third mutant Tyr5 (255 Phe \rightarrow Tyr) was then designed and genetically engineered so that a cycle with two parallel and identical substitutions as described above would be formed. Predictions of I_{50} values for the different herbicides with respect to this mutant were made by treating the I_{50} values of the wild type and mutants D1 and D5 given in table 1 as binding constants, according to eqn 5. These calculated values were compared with the actual I_{50} values that were measured. Mutant Tyr5 exhibited resistance against s-triazines, but not against the urea inhibitors or bromacil. For example, it is $25 \times$ more resistant to atrazine than the wild type strain (I_{50} mutant/ I_{50} wild type). The phenotype of Tyr5 is identical to mutant Ar207 of *Chlamydomonas reinhardtii* in which the same mutation in the D1 protein has been found [19].

Most of the predictions (20 out of 22) are in agreement with the experimental results. A plot of

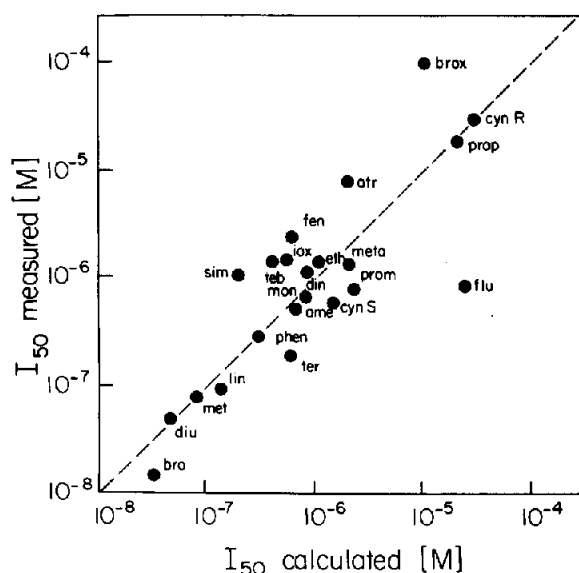


Fig.1. Correlation between experimental and predicted I_{50} values of the different herbicides with respect to the Tyr5 mutant. The average experimental error in log I_{50} is about ± 0.15 . Abbreviations for herbicides are according to the first three or four letters of their common name as given in table 1 with the exception of cyn S, cyn R for the two isomers of cyanoacrylates JLH964(S) and JLH965(R) and brox for bromoxynil.

the experimental vs predicted values is given in fig.1. In the case of a perfect fit all of the points would be expected to fall on the dashed line with a slope of 1 given in this figure. As may be seen, 11 out of the 22 points do indeed fall on this line or very close to it. Most of the predictions (16 out of 22) are well within the limits of experimental error. Four additional predictions, for the herbicides atrazine, fenuron, tebuthiuron and simazine, are very close to being within experimental error. Only two predictions, for the herbicides bromoxynil and fluomethuron, clearly do not fit the experimental results.

4. DISCUSSION

Additivity in the contributions to the binding of herbicides of amino acids in positions 255 and 264 of the D1 protein is here clearly demonstrated, reflecting an apparent lack of interactions between the amino acids in these two positions. Previously, such additivity was demonstrated in the case of the interaction of proteinase inhibitors with proteinases [20] and was assumed to be due to the rigid structure of those inhibitors. Here it is shown for a membrane protein. For such proteins detailed structural information usually does not exist and is to date rather difficult to obtain. Application of the above described method can therefore yield information which, in the case of membrane proteins, is otherwise hard to obtain. For example, the apparent lack of interactions between the amino acid at position 255 and the residue at position 264 of the D1 protein is demonstrated by the fact that they contribute to the binding of herbicides in an additive manner. The plot in fig.1 is also of value in detecting herbicides that deviate from the general trend. Non-additivity seems to exist in the case of the interaction of the D1 protein with only two herbicides, fluomethuron and bromoxynil, out of the 22 tested. These herbicides probably interact with the D1 protein slightly differently than the others although no chemical differences, for example in hydrophobicity, polarity or side-chain branching, between them and the other herbicides are readily apparent. The mode of interaction of

these herbicides thus warrants further investigation. Finally, the method applied here should prove to be of use in designing new mutants with desired herbicide resistance profiles. It should also serve as a paradigm for site-directed mutagenesis experiments in other systems as well.

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