Evidence for different binding sites on the 33-kDa protein for DCMU, atrazine and Q_B

Chantal Astier, Alain Boussac and Anne-Lise Etienne

Laboratoire de Photosynthèse, CNRS, BP 1, 91190 Gif-sur-Yvette, France

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Two DCMU-resistant strains of the cyanobacterium *Synechocystis* 6714 were used to analyse the binding sites of DCMU, atrazine and Q_B. DCMU^r-II$_A$ was DCMU and atrazine resistant; it presented an impaired electron flow and its 33-kDa protein was weakly attached to the membrane. DCMU^r-II$_B$, derived from the former, simultaneously regained atrazine sensitivity, normal electron flow and a tight linkage of the 33-kDa protein to the membrane. This mutant shows that loss of DCMU binding does not necessarily affect the binding of either atrazine or Q_B. The role of the 33-kDa protein is discussed.

Cyanobacteria *Mutant* *Herbicide* *Photosynthesis* *Photosystem II*

1. INTRODUCTION

Electron transfer on the acceptor side of Photosystem II occurs through a primary acceptor Q_A [1] (a particular plastoquinone of the general pool [2] closely associated with the chlorophyll center), a secondary acceptor Q_B [3] bound to its apoprotein supposed to be the '32-kDa' protein [4–10] and the plastoquinone pool [11].

Several inhibitors, including DCMU and atrazine, block electron transfer between Q_A and Q_B. They very likely bind to the same 32-kDa protein as Q_B [4–12] as shown by the results of a mild trypsin digestion: the reduced rate of electron flow from Q_A to Q_B and the increased resistance towards DCMU or atrazine are all attributed to a partial digestion of a protein, [4,5,12] of 32–34 kDa [7,8,13,14]. The same sensitivity to trypsin treatment was found for a high-turnover 32 kDa polypeptide [15] showing a photoaffinity binding of azido[^{14}C]atrazine [16]. It was shown recently that a specific attack of lysine residues resulted also in a partial release of inhibition by SN58132, a DCMU-type inhibitor [17].

Radioactivity labelling experiments were used to demonstrate a competition between DCMU and atrazine [6,18–20]. A similar competition was proposed in [11] between DCMU and Q_B. In agreement with this hypothesis, it was shown in [21] that the apparent affinity constant of DCMU was dependent on the redox state of Q_B: high when Q_B was oxidized and loosely attached to its site, low when Q_B was in its semi-reduced state and tightly bound to its protein site [21]. These competitions were first taken as proofs for a unique binding site for the two herbicides and Q_B. This conclusion was also supported by the existence of DCMU-resistant mutants presenting cross-resistance to atrazine [22,23]. These mutants also generally showed impaired electron flow.

However, mutants of higher plants resistant to atrazine but still DCMU sensitive have been characterized by two different groups [24,26]. We have also selected several mutants resistant to one herbicide and sensitive to the other [25]. These findings cannot be easily explained in the framework of a unique binding site.

Abbreviations: DCMU, 3-(3,4 dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; Wt, wild type; PS II, Photosystem II; BSA, bovine serum albumin

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Recent reports concerning the nucleotide sequence of the gene coding for the equivalent of the 33-kDa protein described here point out different modifications, though at a unique site, in 3 mutants with varying phenotypes towards DCMU and/or atrazine resistance. This probe does not have any codons corresponding to lysine residues ([27], McIntosh and Rochaix, personal communication).

The analysis here of a number of properties of two mutants of the cyanobacterium *Synechocystis* (Aphanocapsa) 6714, resistant to DCMU and atrazine or to DCMU alone, has permitted the determination of the relationship between the sites of attachment of these herbicides and Q_B, and the role of the 32 kDa protein in electron transfer. A model is presented.

2. MATERIALS AND METHODS

The wild-type strain of the cyanobacterium *Synechocystis* (Aphanocapsa) 6714 was provided by R.Y. Stanier [27]. The DCMU^r-II, now called DCMU^r-II$_A$, a spontaneous DCMU-resistant mutant, was selected as in [23]. From this mutant a new resistant strain was isolated as follows: the DCMU^r-II$_A$ mutant was grown for many generations in the presence of 10^{-5} M DCMU. Samples were plated on solid minimal medium and isolated colonies replica-plated on the same solid medium containing 5×10^{-6} M DCMU. The fast growing colonies were picked and subcloned. One of them, growing in the presence of 10^{-5} M at the same rate as the wild type without DCMU, was kept and termed DCMU^r-II$_B$.

Growth conditions, measurements of oxygen evolution and of the pigment concentration were as in [23].

Measurement of the decay of fluorescence yield following a short saturating laser flash was performed on the set-up in [21].

To isolate thylakoids performing oxygen evolution, we modified the procedure in [10]. The cells were grown and harvested as in [10], washed once in Mes buffer (pH 6.5), and resuspended in isolation buffer (0.5 M potassium phosphate, 0.26 M sodium citrate, 10 mM $MgCl_2$; pH 6.5). They were then passed through a French press. BSA was added to the crude extract. Cell disruption and subsequent centrifugations were performed at 4°C. Unbroken cells were removed by centrifugation at 500 $\times$ *g* for 5 min. The supernatant was spun at 17 000 $\times$ *g* for 1 h to sediment the thylakoid membranes. The pellet was carefully resuspended with a paintbrush in the minimum volume of the isolation buffer. Thylakoids were stored at high concentration of chlorophyll (1–5 mg Chl/ml) at −20°C. Slab gels (14% polyacrylamide) were run according to [28] with the modifications in [10].

The binding of DCMU and atrazine was measured by incubating thylakoid membranes in 1 ml of isolation buffer at 50 μg Chl/ml at room temperature for 5 min with various concentrations of either herbicide. [^{14}C]DCMU was obtained from Dupont de Nemours (1 Ci/mol); [^{14}C]atrazine was a gift from Ciba-Geigy (5.5 Ci/mol). The incubation mixtures were then centrifuged to sediment the membranes (in a microfuge at 10 000 $\times$ *g* for 10 min). The supernatants were collected and the radioactivity of the samples measured to determine the amount of free DCMU or atrazine. The amount of bound inhibitor was calculated from the difference between the total radioactivity and that found in the supernatant. The number of sites and the binding constants were computed by linear regression analysis of double-reciprocal data plots as in [18].

3. RESULTS

In the absence of inhibitor, the mutants and the wild type had identical generation times of 7 h in our standard growth conditions [23]. Measured in the presence of increasing DCMU concentrations, DCMU^r-II$_A$ growth started being inhibited at 10^{-5} M DCMU whereas DCMU^r-II$_B$ was still growing at optimal rate. Using a range of concentrations of the two inhibitors, DCMU and atrazine, we have determined the concentrations necessary to decrease by half the growth rate of each strain (table 1A).

Half-inhibition values for oxygen evolution are given in table 1B. The slight discrepancies observed with the values of table 1A might be due to non-specific binding of the herbicide in growing cells. The DCMU^r-II$_B$ strain did not require any adaptation to DCMU, but DCMU^r-II$_A$ showed half inhibition at 7×10^{-6} M for non-adapted cells and 3×10^{-5} M for adapted cells [10].

Table 1

Half-inhibition concentrations of growth rate (A) and oxygen evolution (B) of whole cells

	A		B	
	[DCMU] (M)	[Atrazine] (M)	[DCMU] (M)	[Atrazine] (M)
Wild type	5×10^{-7}	3×10^{-6}	10^{-7}	4×10^{-6}
DCMU^r-II$_A$	3×10^{-5}	$>10^{-4}$	7×10^{-6}-3.5×10^{-5}	–
DCMU^r-II$_B$	3×10^{-4}	5×10^{-6}	5×10^{-5}	5×10^{-6}

The efficiency of electron transfer between Q_A and Q_B can be deduced from the fluorescence decay after a saturating flash, Q_A being a quencher of fluorescence in its oxidized form. In the wild type and the DCMU^r-II$_B$ mutant, fluorescence decay was achieved within some milliseconds, showing efficient electron transfer. In the DCMU^r-II$_A$ mutant the electron transfer between Q_A and Q_B was slowed down as described in [10]. When transfer between Q_A and Q_B is blocked, reoxidation of Q_A^- after a saturating flash takes place via a back reaction with a positive charge stored on the donor side and is achieved in some seconds. In the presence of DCMU for the wild type and atrazine for the DCMU^r-II$_B$ mutant, the fluorescence decay was slowed down and occurred at the same rate. This showed that the back reaction between Q_A and the S states was not modified in the mutant (fig.1).

The sensitivity to the herbicides of PS II electron transfer was also measured on isolated thylakoids. The rate of DCPIP photoreduction (Hill activity) was measured at different concentrations of DCMU or atrazine. As shown in table 2 the thylakoids showed the same behavior as the whole cells.

The polypeptide compositions of these different types of thylakoids were analysed on SDS–polyacrylamide gels (fig.2). We [10] have shown that for the DCMU^r-II$_A$ strain a 33-kDa polypeptide was missing under certain conditions, as compared to the polypeptide profile of the wild type. The resistance to inhibitors could be correlated with the loss of that polypeptide. In contrast no difference was observed between the polypeptide profiles of the DCMU^r-II$_B$ and the wild-type thylakoids, with a well defined 33-kDa protein band. The resistance to DCMU of this mutant had then to be explained by a different mechanism and it was important to check whether DCMU could still bind to the thylakoid membranes.

Binding of DCMU and atrazine to thylakoid membranes was studied using radioactively labeled herbicides; fig.3 shows double-reciprocal plots of the amount of inhibitor bound per mg Chl versus the amount of free inhibitor. No specific binding of DCMU was obtained for DCMU^r-II$_B$ thylakoids

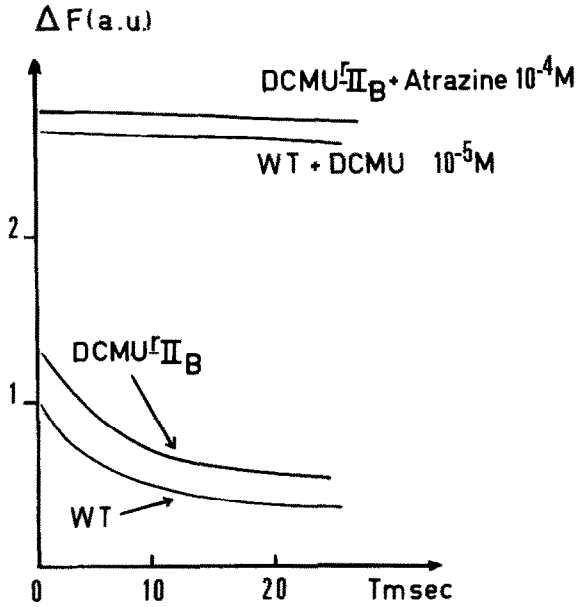

Fig. 1. Fluorescence decay after a saturating laser flash (duration 1 μs). The fluorescence yield was monitored by synchronous detection of the fluorescence excited by a train of weak detecting light pulses from an He-Cd laser. The kinetics are the average of 16 accumulations. The photomultiplier was not protected during the flash but an analog gate was used to ground the PM. The blindness time after the flash is 15 ms. Top curves were recorded in the presence of herbicides. Bottom curves are the fluorescence decays of uninhibited cells.

Table 2

Half-inhibition concentrations of Hill reaction (DCPIP reduction)

	[DCMU] (M)	[Atrazine] (M)
Wild type	8×10^{-8}	3×10^{-7}
DCMU^r-II$_A$	10^{-5}	–
DCMU^r-II$_B$	2.5×10^{-5}	4×10^{-7}

whereas normal atrazine binding was observed. In wild-type membranes, about 4.8 nmol DCMU was bound per mg Chl, i.e. about one binding site per 205 chlorophyll molecules. The DCMU binding constant was about 8.6×10^{-8} M. Atrazine could bind at about 5.4 nmol per mg Chl, corresponding to one binding site per 185 chlorophyll molecules. The atrazine binding constants for the wild type and DCMU^r-II$_B$ mutant were identical at 3×10^{-7} M.

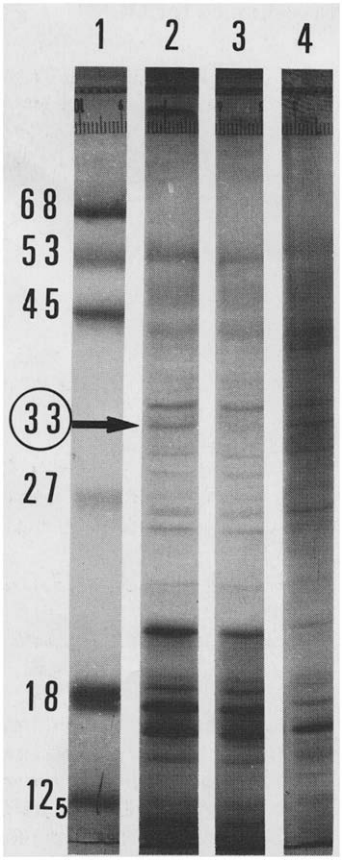

Fig. 2. SDS-polyacrylamide gel separation of the polypeptides from WT (2), DCMU^r-II$_A$ (3), DCMU^r-II$_B$ (4) and molecular mass standards (1). The numbers on the left correspond to the molecular mass in kDa.

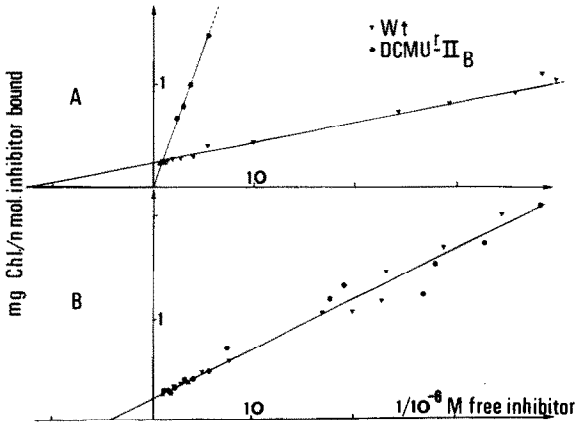

Fig. 3. Double-reciprocal plots of [^{14}C]DCMU (A) and [^{14}C]atrazine (B) binding to wild type (▼) and DCMU^r-II$_B$ (●) membranes.

The different values obtained for the DCMU and atrazine binding constants are in agreement with the differences observed for the half-inhibitory concentrations of the Hill reaction. The number of binding sites per chlorophyll molecule for the two inhibitors can be considered as being very similar.

4. DISCUSSION

The resistant phenotype of mutant DCMU^r-II$_A$ requires an adaptation in the presence of DCMU to be effective. This adaptation can be achieved in the absence of a source of energy and of de novo protein synthesis [23]. It probably corresponds to the release of the 33-kDa protein from the thylakoid membranes. Since the thylakoids presenting the higher resistance to DCMU were those in which the protein was absent, we postulated that a conformational change [10] was responsible for the solubilization of this protein, this loss inducing a lower rate of electron transfer between Q_A and Q_B. The 33-kDa protein can be easily released from the membranes of non-adapted DCMU^r-II$_A$ cells [10] with a resulting increase of the resistance towards DCMU. However, it is impossible to

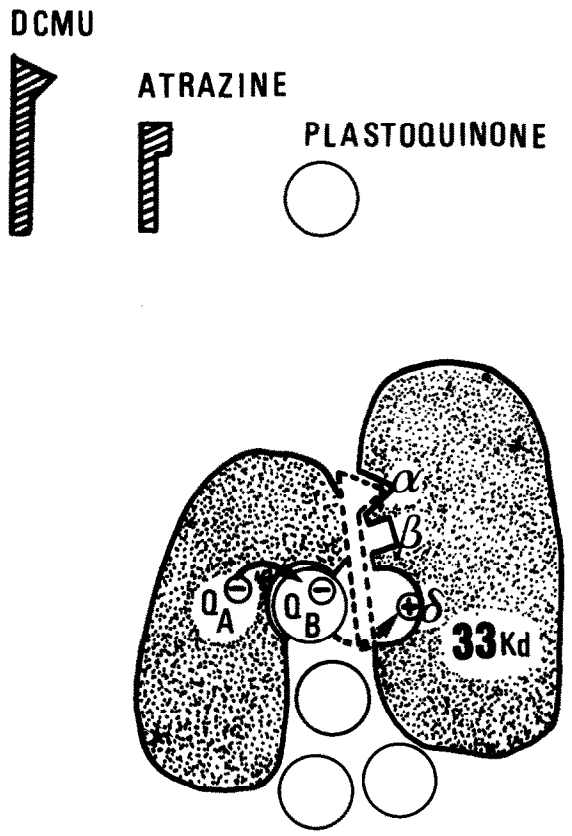

Fig. 4. Simplified model of the role of the 33-kDa protein in the transfer between Q_A and Q_B. 'Steric hindrance model' of the two inhibitor action.

know at this stage whether the modification resulting from the mutation affects the 33-kDa protein itself or a surrounding one to which it would be bound.

The $DCMU^r$-II_B mutant which showed a selective advantage over the $DCMU^r$-II_A one, had regained a stable 33-kDa protein and a normal electron transfer capacity between Q_A and Q_B. Its resistance can best be explained by a specific loss of the binding capacity at the DCMU site. This modification affects neither the binding and the inhibiting action of atrazine, nor the binding of Q_B^- in the absence of inhibitors. The two herbicides are known to act at the same level. However our results show that the site where they act is distinct from that where they each bind. Their inhibitory action could be due to either steric hindrance with, or a conformational change of the Q_B site. It remains valid if the Q_B site is located on another protein as is now proposed by some groups.

The results obtained with the two mutants lead to the model given in fig.4, the dashed line indicating the possibility of two distinct polypeptides for herbicide and Q_B binding.

Since only Q_B^- is tightly bound to its site, we postulate that the binding is due to an electrostatic effect, the active site bearing a positive charge. The binding of uncharged molecules such as DCMU and atrazine must follow a different mechanism.

Further information on the chemical nature (including the amino acid sequence) of the binding sites for the herbicides and Q_B^-, in both the wild type and the mutants, is still needed to complete the understanding of the mechanisms involved.

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