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Covalent binding of 3-azido-monuron to thylakoids of DCMU-sensitive and -resistant strains of *Chlamydomonas reinhardtii*

Arminio Boschetti *, Mathias Tellenbach and Annegret Gerber

Institut für Biochemie, Universität Bern, Freiestr. 3, CH-3012 Bern (Switzerland)

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Six different mutants of *Chlamydomonas reinhardtii* resistant to DCMU (*N*-(3,4-dichlorophenyl)-*N'*-dimethylurea; Diuron) have been isolated and are described. Five of them, including one of the so-called secondary mutants which are resistant only as whole cells, show uniparental inheritance of the resistance. Thylakoids isolated from all mutants as well as from the parent strain were covalently photolabelled with the radioactive DCMU analogue 3-[^{14}C]azido-monuron (*N*-(3-azido-4-chlorophenyl)-*N'*-dimethylurea). In the parent strain, and in the secondary mutants, two polypeptides of 32 kDa and 41 kDa were specifically labelled. In the primary mutants, where the Photosystem II activity of isolated thylakoids was resistant to DCMU, the 32 kDa protein was not labelled. The photolabelled 32 kDa protein was identified as the 'rapidly labelled 32 kDa herbicide-binding polypeptide' by comparison of its proteolytic fingerprints with those of [^{35}S]methionine-labelled, in organello synthesized 32 kDa protein. The 3-azido-monuron was found to be covalently bound to a small proteolytic fragment of about 8 kDa.

Introduction

In recent years, much evidence has accumulated showing that the rapidly labelled 32 kDa protein (herbicide-binding protein, Q_B protein) is the site of action of most Photosystem II herbicides [1]. Through competitive binding studies with different herbicides, it became obvious that atrazine, DCMU, metribuzin [2], dinoseb [3] and other herbicides, as well as plastoquinone analogues [4,5], are bound to the same site on isolated thylakoid membranes. Furthermore, after photoaffinity labelling of thylakoids with radioactive azido-atrazine [6] and an azido-triazinone [7] the only labelled polypeptide was the 32 kDa protein. Finally, the analysis of the DNA sequence of cloned genes for the 32 kDa protein (*psb A* gene) from atrazine-resistant and -sensitive higher plants such as spinach, *Amaranthus, Solanum nigrum* and *Nicotiana* [1,8], as well as from an atrazine/DCMU-double-resistant strain of *Chlamydomonas* [9], showed that the mutation to resistance is accompanied by a single exchange of serine-264 (numbering according to Zurawsky et al. [10]) by glycine and alanine, respectively, in the 32 kDa protein. Similarly, in *Chlamydomonas* mutants resistant solely to DCMU or to atrazine mutations were found at valine-219 and phenylalanine-255, respectively, on the 32 kDa protein [11].

However, some difficulties still exist in understanding the details of the mechanism of action of these herbicides. In *Chlamydomonas* at least two distinct classes of DCMU-resistant mutants can be

* To whom correspondence should be addressed.

Abbreviations: DCMU, *N*-(3,4-dichlorophenyl)-*N*-dimethylurea; 3-azido-monuron, *N*-(3-azido-4-chlorophenyl)-*N'*-dimethylurea.

found [12]. In the so-called primary mutants, the resistance can be traced to a lower binding affinity of DCMU to isolated thylakoid membranes [13]. In the secondary mutants the resistance is lost once the cells are broken. In the latter mutants the photosynthetic electron transport is not resistant and the site of action is still obscure.

Another difficulty arises from the fact that, although atrazine and DCMU displace each other competitively from their binding sites [2], the loss of binding affinity for one of these herbicides does not necessarily result in a resistance to the other compound. This applies for atrazine-resistant weed species isolated in the field [14], as well as for DCMU-resistant strains of *Chlamydomonas* [12,15]. Therefore, overlapping rather than fully identical binding sites for Photosystem II herbicides have been postulated [14].

A third problem concerns the exact molecular geometry of the binding site. Photoaffinity binding of radioactive azido-*i*-dinoseb [16] to thylakoid membranes of spinach did not preferentially label the 32 kDa protein, but labelled also a polypeptide of 41 kDa. This points to the possibility that binding sites for the inhibiting structure of the herbicide and for the covalently binding azido group may not be located on the same protein.

In order to obtain further information about the binding site(s) and the inhibitory action of DCMU we found it necessary to synthesize the radioactively labelled azido derivative of DCMU, 3-azido-monuron. In this paper we study the covalent binding of this compound to the thylakoid membranes of a DCMU-sensitive strain of *Chlamydomonas* and of the six DCMU-resistant mutants which we have isolated previously. The aim was to further our insight into the molecular nature of DCMU-resistance by comparing the labelled proteins in the sensitive strain and in the primary and secondary DCMU-resistant mutants.

Materials and Methods

Strains, culture conditions and crossing

Chlamydomonas reinhardtii 137c *arg-2*$^-$*mt* + (Cambridge Culture Collection) was the parent strain for mutant induction. Culture conditions, induction and selection of DCMU-resistant (*dr*) mutants were as described earlier [15]. As tester strains in tetrade analysis we used *C. reinhardtii pab-1*$^-$*mt* − and *C. reinhardtii thi-2*$^-$*mt* − (strains CC-600 and CC-24, respectively, from Chlamydomonas Genetic Center, Duke University) as indicated in Table I.

For tetrade analysis all strains were grown for 5 days on agar containing the medium I of Sager and Granick [17] supplemented with arginine-HCl (100 mg/l), *p*-aminobenzoic acid (0.5 mg/l) or thiamine-HCl (1 mg/l), and yeast extract (4 g/l, Difco). Then the cells were transferred to 35 ml of liquid medium of the same composition, but containing only 1/10 of the normal nitrogen content and no yeast extract. After 3 days incubation on a shaker, cells were harvested and resuspended in distilled water (10^8 cells/ml). After 3–4 h, when they had developed full motility, different mating types were mixed. After 2 h fusion had occurred and 0.2 ml of the zygote suspension was plated on the above agar medium containing no nitrogen source. The plates were incubated in the light for 42 h and then in the dark for 5–14 days. Isolation of the zygotes and tetrade analysis on complete, fresh agar medium was as described by Ebersold and Levine [18].

Radioactive material

[^{14}C]Atrazine. This, with a spec. act. of 5.86 Ci/mol, was a gift from Ciba-Geigy (Basel).

[^{14}C]DCMU (N-(3,4,dichlorophenyl)-N'-di[^{14}C]methylurea). 1 mCi (15 mg) of di[^{14}C]methylamine hydrochloride (Amersham, U.K.) in 1.15 ml water and 40 mg 3,4-dichlorophenylisocyanate (Fluka, Switzerland) in 2 ml toluene and 0.08 ml 2 M NaOH were shaken at room temperature for 15 h. After addition of 3 ml $CHCl_3$, three extractions with water, evaporation of the solvent and recrystallization from chlorobenzene/ligroin (1 : 1), 44 mg of the product were obtained (m.p. 153–154°C; spec. act. 5.8 Ci/mol).

3-Azido-monuron (N-(3-azido-4-chlorophenyl)-N'-dimethylurea). 4-Chloro-3-nitroaniline (Fluka, Switzerland) was acetylated with acetic anhydride according to standard procedures. To 35 g (0.15 mol) of the resulting 4-chloro-3-nitroacetanilide (m.p. 143°C) in 300 ml boiling ethanol, 102 g (0.6 mol) $Na_2S_2O_4$ in 150 ml water were added and the mixture was refluxed for 1 h. After evaporation of ethanol, acidification with HCl and filtration, the

aqueous phase, combined with a hot HCl extract of the residue of the filtration, was neutralized and extracted three times with ethyl acetate. Evaporation and recrystallization from ligroin/ethyl acetate gave 15 g of 3-amino-4-chloroacetanilide (m.p. 164°C). To 13 g (0.07 mol) of this compound in 150 ml water and 16 ml 37% HCl at 0°C, 4.8 g (0.07 mol) $NaNO_2$ in 50 ml water were added slowly with stirring, followed by 7.9 g of NaN_3 in 50 ml water. After 1 h at 0°C and 1 h at room temperature, the 3-azido-4-chloroacetanilide was recovered by filtration, washed with water and recrystallized from ethanol/water. The yield was 12.4 g; m.p. 153°C (decomp.). Hydrolyzation of 11 g of the acetanilide by boiling in 4% KOH and steam-distillation yielded 9 g of 3-azido-4-chloroaniline of m.p. 83–85°C. 4.2 g of this aniline (0.025 mol) were solubilized in 50 ml 20% phosgene in toluene (Fluka, Switzerland) at 0°C. After 4 h stirring, the solution was slowly heated to 70°C, with continuous addition of gaseous phosgene. After standing overnight, nitrogen was bubbled through the solution at 40°C for 2 h, then evaporated under reduced pressure. The residue was extracted three times with hot ligroin, yielding 4.3 g 4-azido-3-chlorophenylisocyanate (m.p. 57–58°C), which can be stored in toluene in brown sealed ampoules under nitrogen. Using this compound, three different preparations of *N*-(3-azido-4-chlorophenyl)-*N'*-dimethylurea were synthesized according to the above-described synthesis of [^{14}C]DCMU: unlabelled for I_{50} measurements; labelled to a spec. act. of 4.8 Ci/mol for binding studies; and labelled to a spec. act. of 57 Ci/mol for the photoaffinity labelling.

Photoaffinity labelling

Cells from photoautotrophic cultures harvested in the exponential phase were washed with 25 mM Tricine-NaOH pH 7.8, 40 mM KCl, 5 mM $MgCl_2$. After cell breakage by ultrasonication and sedimentation for 10 min at $10000 \times g$, the crude thylakoid membranes were washed and resuspended in the above buffer to 0.3 mg/ml chlorophyll. Of this suspension 0.8 ml was transferred to a cuvette and supplemented with 0.8 nmol 3-[^{14}C]azido-monuron (57 Ci/mol) in 16 μl ethanol, to give a final concentration of 10^{-6} M. The mixture was stirred in the cold-room and illuminated with light of 254 nm from a UV lamp used for chromatographic work. After 5, 10 and 30 min, 250-μl aliquots were withdrawn, immediately centrifuged for 10 min at $15000 \times g$ and the sediments were dissolved in 50 μl of a solution containing 4 M urea, 2% SDS, 20 mM Tris-HCl (pH 7.6), 14 mM β-mercaptoethanol. The samples were heated for 5 min at 90°C, centrifuged as above and electrophoresed on a 7.5 to 15% polyacrylamide gradient gel containing SDS. Conditions for electrophoresis, staining and fluorography of the gel were as described earlier [19].

Other methods

The preparation of thylakoids, the measurements of O_2 evolution, of photosystem II activity and of the herbicide binding were as described earlier [15]. For measuring the displacement of [^{14}C]DCMU from the membranes the mixture of the binding assay contained in addition 50 μM unlabelled 3-azido-monuron.

Partial proteolytic digestion and fingerprint analysis of the fragments from polypeptide bands were described by Michel et al. [20]. The incorporation of [^{35}S]methionine into the rapidly labelled 32 kDa polypeptide by intact chloroplasts from *Chlamydomonas* was done as previously [19].

Results

Characteristics of the DCMU-resistant strains

In this study, six DCMU-resistant mutants isolated in our laboratory were used. For five of these mutants tetrade analysis revealed uniparental inheritance of the resistant phenotype, whereas one mutant, *dr*-409, showed biparental inheritance with variable ratios of sensitive and resistant daughter cells (Table I). The mutants *dr*-406 and *dr*-409 are so-called secondary mutants (see below). In contrast to the findings of Galloway and Mets [12], whose secondary mutants are of the mendelian type, our *dr*-406 shows exclusively uniparental inheritance.

The effects of some herbicides on our DCMU-mutants, and on the parent strain from which they have been derived, are given in Tables II and III. None of the mutants is significantly resistant to atrazine. In comparing the I_{50} values for the inhibition of O_2 evolution of intact cells and of the

TABLE I

TETRADE ANALYSIS OF CROSSES OF THE DCMU-RESISTANT MUTANTS (*dr*) OF MATING TYPE+(MATERNAL) WITH A DCMU-SENSITIVE TESTER STRAIN OF MATING TYPE −

Only those tetrades were analysed where the nuclear markers arginine auxotrophy (*arg*$^-$) and *p*-aminobenzoic acid (*pab*$^-$) or thiamine (*thi*$^-$) auxotrophy showed segregation of the non-parental ditype (*arg*$^-$ *pab*$^-$; *arg*$^+$ *pab*$^+$) or of the tetratype (*arg*$^-$ *pab*$^-$; *arg*$^-$ *pab*$^-$; *arg*$^+$ *pab*$^+$). In no case was a paternal inheritance of DCMU sensitivity observed.

Crosses	Number of tetrades (octades) analysed			
	Nuclear markers segregating as		Inheritance of DCMU resistance	
	ditype	tetratype	maternal	non-maternal
dr-460 *arg*$^-$ *mt* + × *pab*$^-$ *mt* −	2	4	6	0
dr-409 *arg*$^-$ *mt* + × *pab*$^-$ *mt* −	4	5	2	7
dr-412 *arg*$^-$ *mt* + × *thi*$^-$ *mt* −	7		6	1
dr-416 *arg*$^-$ *mt* + × *pab*$^-$ *mt* −	1	6	7	0
dr-420 *arg*$^-$ *mt* + × *pab*$^-$ *mt* −	8	15	23	0
dr-423 *arg*$^-$ *mt* + × *pab*$^-$ *mt* −	2	6	8	0

Photosystem II activity (H_2O to dichlorophenylindophenol) of crude thylakoid preparations by DCMU, it is apparent that *dr*-406 and *dr*-409 belong to the so-called secondary mutants, according to Galloway and Mets [12]. These two mutants are resistant only as whole cells, but contain a sensitive Photosystem II. In contrast, mutants *dr*-412, *dr*-416 and *dr*-420, whose isolated thylakoids show Photosystem II activity also resistant to DCMU, are designated primary mutants. This classification agrees well with the difference observed in the dissociation constants for DCMU (Table III). However, the mutant *dr*-423 may be considered of the secondary type with respect to the I_{50} value, but of the primary type with respect to the dissociation constant and the labelling by 3-azido-monuron (Fig. 2). This discrepancy might be due to an instability of this mutant.

3-Azido-monuron as DCMU analogue

The inhibitory action of 3-azido-monuron was measured only with the mutant *dr*-416 and the parent strain. In both the mutant and parent strains the I_{50} values of 3-azido-monuron for O_2 evolution and Photosystem II activity are higher by a factor of about 20–100 as compared to the DCMU inhibition, showing 3-azido-monuron to be a much less effective inhibitor than DCMU (Table II).

TABLE II

HALF-INHIBITORY CONCENTRATIONS (I_{50}) OF DIFFERENT HERBICIDES FOR THE O_2 EVOLUTION OF INTACT CELLS AND FOR THE PHOTOSYSTEM II ACTIVITY (H_2O TO DCPIP) OF ISOLATED, CRUDE THYLAKOIDS

ps, parent strain from which the DCMU-resistant mutants (*dr*) have been derived. *R*/*S*, ratio of I_{50} of resistant to I_{50} of parent strain.

Strain	I_{50} of intact cells (μM) O_2 evolution				I_{50} of thylakoids (μM) Photosystem II					
	DCMU	*R*/*S*	3-azido-monuron	*R*/*S*	DCMU	*R*/*S*	3-azido-monuron	*R*/*S*	atrazine	*R*/*S*
ps	0.4	–	16	–	$2 \cdot 10^{-2}$	–	1–2	–	12	–
dr-406	6	15			$1.6 \cdot 10^{-2}$	0.8				
dr-409	10	25			$2.0 \cdot 10^{-2}$	1.0				
dr-412	5.6	14			$16 \cdot 10^{-2}$	8.0			30	2.5
dr-416	6.3	16	100	6	$30 \cdot 10^{-2}$	15	7.5–17	8	12	1.0
dr-420	8.0	20			$50 \cdot 10^{-2}$	25			12	1.0
dr-423	5.0	12.5			$3.2 \cdot 10^{-2}$	1.6				

TABLE III

BINDING OF DIFFERENT RADIOACTIVE HERBICIDES TO ISOLATED CRUDE THYLAKOIDS OF THE PARENT STRAIN (*ps*) AND OF THE DCMU-RESISTANT MUTANTS (*dr*)

The values were measured and calculated according to the method of Tischer and Strotmann [2]. For DCMU mean values of 2–6 independent experiments, for atrazine of two experiments are given. For 3-azidomonoron all results from three individual experiments are listed.

Strain	Dissociation constant K_b (μM)			Number of binding sites (nmol/mg chlorophyll)		
	[^{14}C]DCMU	3-[^{14}C]azidomonuron	[^{14}C]atrazine	[^{14}C]DCMU	[^{14}C]azidomonuron	[^{14}C]atrazine
ps	$2.34 \cdot 10^{-2}$	0.28	0.23	2.03	10.0	2.65
		0.21				
		0.23				
dr-406	$2.41 \cdot 10^{-2}$		0.18	2.04		2.7
dr-409	$2.24 \cdot 10^{-2}$		0.29	2.45		4.0
dr-412	$12.93 \cdot 10^{-2}$		0.39	1.64		2.35
dr-416	$13.26 \cdot 10^{-2}$	0.61	0.23	2.50	13.33	3.03
		0.62			6.66	
		0.62			5.71	
dr-420	$23.07 \cdot 10^{-2}$		0.53	2.18		3.22
dr-423	$9.51 \cdot 10^{-2}$		0.43	2.00		3.2

Nevertheless, in the mutant, both the O_2 evolution of intact cells and the Photosystem II activity of isolated thylakoids are resistant to the same degree against 3-azido-monuron, as shown by the R/S values of 6 and 8 ($R/S = I_{50}$ of resistant/I_{50} of sensitive strain). The respective R/S values for DCMU inhibition are also almost equal for both reactions ($R/S = 16$ and 15) and are of the same order of magnitude as the R/S values for 3-azido-monuron. These results may indicate a similar mode of action of the two inhibitors.

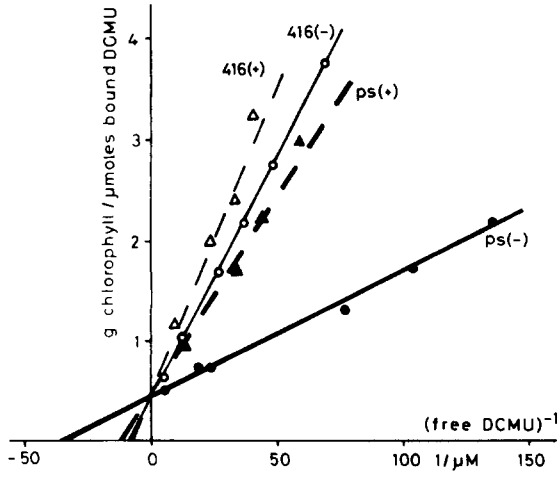

Fig. 1. Competitive displacement of DCMU by 3-azido-monuron from its binding sites on thylakoids of the parent strain (*ps*) and of the DCMU-resistant mutant *dr*-416 (416). Thylakoids were incubated with different amounts of [^{14}C]DCMU in the presence (+) and the absence (−) of 50 μM unlabelled 3-azido-monuron (20 μg/ml chlorophyll).

The dissociation constants for 3-azido-monuron are 10–20-times larger than those for DCMU (Table III). Probably due to its chemical reactivity, at higher inhibitor concentrations unspecific binding begins to interfere and prevent the evaluation of the number of binding sites. In contrast with [^{14}C]atrazine, having dissociation constants of the same order of magnitude, low unspecific binding is observed. Nevertheless, when binding of [^{14}C]-DCMU was measured in the presence of non-radioactive 3-azido-monuron, the latter compound displaced DCMU competitively and specifically from thylakoids of the parent strain, but not of the mutant, where only unspecific binding occurs (Fig. 1). This indicates that with respect to the binding specificity, 3-azido-monuron is an analogue of DCMU.

Photoaffinity binding of 3-azido-monuron to thylakoids of DCMU-sensitive and -resistant strains

We then tried to photolabel the thylakoids isolated from the mutants covalently with 3-[^{14}C]azido-monuron of the highest possible specific radioactivity that we could prepare. Using con-

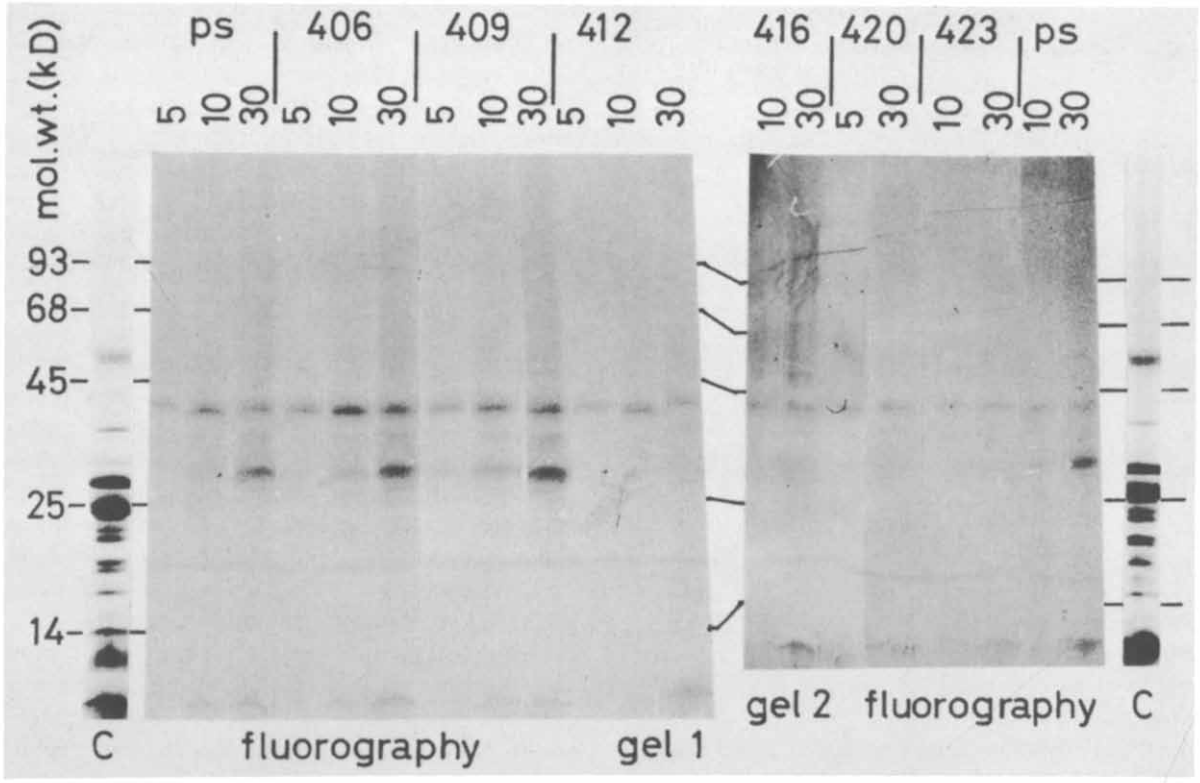

Fig. 2. Fluorograph of thylakoid proteins photolabelled with 3-[^{14}C]azido-monuron. Crude preparations of thylakoid membranes from the parent strain (*ps*) and the six DCMU-resistant mutants (*dr*-406, -409, -412, -416, -420, -423) were incubated under UV illumination with 10^{-8} M 3-[^{14}C]azido-monuron. At different time intervals (5, 10, 30 min, as indicated) samples were withdrawn, solubilized and the proteins were separated on SDS-polyacrylamide gels. The patters stained with Coomassie brilliant blue R250 (C) were all very similar.

centrations of 10^{-4} M, almost all proteins of the membranes become unspecifically labelled, as shown by subsequent SDS-polyacrylamide gel electrophoresis. With a concentration of 10^{-8} M of 3-[^{14}C]azido-monuron, however, specific labelling of two different peptides is observed (Fig. 2). To obtain visible bands on the fluorographs with such a low activity, the dried gels had to be exposed to the X-ray films for about 3 months. The molecular weights of the labelled proteins are 32 000 and about 41 000. In the parent strain and in the secondary mutants *dr*-406 and *dr*-409 both peptides are labelled. Labelling of the 32 kDa protein increases markedly with increased incubation time. In the primary mutants this polypeptide is not labelled, indicating that it has been affected by the mutation and that this alteration may be responsible for the herbicide resistance. The labelling of the 41 kDa protein in the primary mutants is somewhat decreased. The mutant *dr*-423 behaves in this test like a primary mutant.

Identification of the photolabelled 32 kDa protein

To identify the affinity-labelled 32 kDa protein, the radioactive band in the region of 32 kDa was cut out from a polyacrylamide gel, where proteins of the parent strain, photolabelled with 3-[^{14}C]azido-monuron, had been separated as shown in Fig. 2. The gel piece was placed on top of a second gel for one-dimensional fingerprinting after digestion with protease V8 from *Staphylococcus aureus*. As a reference, a piece of a polyacrylamide gel containing [^{35}S]methionine-labelled, in organello synthesized 32 kDa protein [19] was also layered on the same gel and treated in parallel. The fluorograph in Fig. 3 demonstrates that our 32

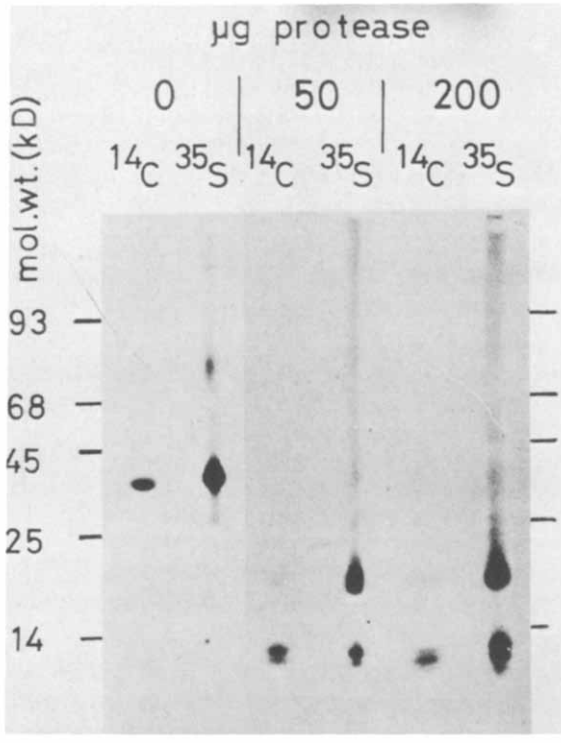

Fig. 3. Fluorograph of proteolytic fingerprints of the 32 kDa protein labelled either by photoaffinity reaction of thylakoids with 3-[^{14}C]azido-monuron (^{14}C) or by light-induced incorporation of [^{35}S]methionine into isolated chloroplasts from *Chlamydomonas* (^{35}S). From a first separation on polyacrylamide gels the protein bands containing the labelled 32 kDa peptides were cut out and the gel pieces positioned on top of this second gel, where prior to electrophoresis they were digsted with different amounts of V8 protease from *Staphylococcus aureus* (0, 50, 200 µg).

kDa protein labelled with 3-azido-monuron was identical with the so-called rapidly labelled 32 kDa thylakoid protein. Without protease, both peptides remain intact, whereas with increasing protease concentrations the typical fingerprints for the rapidly labelled 32 kDa protein are produced from both samples. It is obvious that the 3-azido-monuron is bound to the two smaller fragments. The larger fragment is only very faintly labelled, demonstrating, however, the identity of the in organello synthesized polypeptide with the azido-monuron-labelled 32 kDa protein.

Discussion

Our results show that the DCMU analogue 3-azido-monuron binds covalently and specifically to two proteins of the thylakoid membranes of *Chlamydomonas reinhardtii*. The specific binding to two proteins may be explained either by the presence of two independent binding sites on the membrane for the azido compound, or by one binding site formed by two adjacent proteins, both of which are able to react with the azido group equally well. However, the fact that the 41 kDa protein is still labelled in resistant mutants, whereas the 32 kDa protein is not, favors the former explanation. There may be some co-operation between the two binding sites, as indicated by the decreased labelling of 41 kDa protein in primary mutants. The identity of the 41 kDa protein with the polypeptide labelled with radioactive azido-*i*-dinoseb as reported by Oettmeier et al. [16] should be tested by direct comparison of the proteins in one and the same laboratory. The role of this 41 kDa protein in herbicide binding is not clear. In this connection it is noteworthy that, by DNA-sequencing of a fragment of spinach chloroplast DNA, Alt et al. [21] found a '32-like'-protein of a calculated molecular weight of 39465 with domains having 40–60% homology to the rapidly labelled 32 kDa polypeptide.

It is reasonable to assume that the resistance to DCMU in our primary mutants is due to an alteration of the binding site on the 32 kDa protein since (a) the equilibrium-binding of DCMU to the thylakoids of the primary mutants is weaker than in the parent strain (Table III; Ref. 13), (b) the 3-azido-monuron may be considered an analogue of DCMU (Fig. 1), and (c) the 3-azido-monuron does not label the 32 kDa protein in the primary mutants (Fig. 2).

The partial proteolytic digestion of the 32 kDa protein (Fig. 3) shows that the site for covalent binding of the 3-azido-monuron is located on the two smaller fragments of about 8–10 kDa, but not on the larger one of 20 kDa. According to Marder et al. [22], the proteolytic fragments of 8 and 10 kDa resulting from fragmentation by *S. aureus* V8 protease are very similar to the papain-induced fragments of the same size, which contain the carboxy-terminus of the 32 kDa protein. The latter fragments were obtained after proteolysis of an intermediate C-terminal papain-fragment of 12 kDa containing the amino acids 226–340, according to the numbering of Zurawsky et al. [10] and

Erickson et al. [9]. The difference of 4 kDa between the intermediate 12 kDa and the final 8 kDa fragments accounts for about 25 amino acids located towards the N-terminal end of the 12 kDa piece. Therefore, our 3-azido-monuron must be bound to an amino acid between position 250 and 340 (C-terminus). In this region, two different herbicide mutations have been localized by DNA-sequencing [11]. In position 264 of the gene of higher plants and of *Chlamydomonas,* an exchange of serine to glycine or alanine induces atrazine- or atrazine/DCMU-mixed resistance, and in position 255 in *Chlamydomonas* an exchange of phenylalanine to tyrosine gives rise to atrazine- but not DCMU-resistance. Interestingly, the mutational site at position 219, where a replacement of valine by isoleucine has been found in purely DCMU-resistant *Chlamydomonas,* lies outside the labelled 8 kDa framgent. Similarly, the presumed covalent binding site for the azido-atrazine is located near position 215 on the fragment from Pro-141 to Arg-225 [23], rather than on the 8 kDa fragment. These considerations lead us to conclude that the site of action of the herbicides must not coincide with the mutational sites, and that the covalent binding sites of azido-atrazine and of the azido-analogue of DCMU are located at quite different positions on the primary structure of the 32 kDa rapidly labelled protein. Presently, there is no information available concerning the site of mutation on the chloroplast DNA of our mutants. It would be interesting to correlate the observed binding phenomena with specific changes in the amino acid sequence of the 32 kDa protein of our DCMU-resistant mutants.

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