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HERBICIDE BINDING AT PHOTOSYSTEM II

A NEW AZIDO-TRIAZINONE PHOTOAFFINITY LABEL

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Azido-triazinone (3-dimethylamino-4-methyl-6-(3′-azidophenyl)-1,2,4-triazin-5-one) was found to be an efficient inhibitor of Photosystem II electron transport. This compound has an I_{50} value of 69 nM (extrapolated to zero chlorophyll concentration), a high-affinity binding constant of 12.6 nM, and a number of binding sites of 1.9 nmol/mg chlorophyll. This corresponds to 550–580 molecules of chlorophyll per bound inhibitor; i.e., one molecule inhibitor per electron transport chain. In isolated spinach thylakoids, [^{14}C]azido-triazinone upon ultraviolet illumination covalently binds almost exclusively to a 34 kDa protein. Covalent binding is prevented in the presence of other Photosystem II inhibitors. The protein labeled by azido-triazinone is identical to the 34 kDa herbicide-binding protein which is tagged by another photoaffinity label azido-atrazine (2-azido-4-(ethylamino)-6-(isopropylamino)-*s*-triazine).

Introduction

In the current concept for the mode of action of herbicidal ureas, triazines, triazinones, etc. ('DCMU-type herbicides') as inhibitors of photosynthetic electron transport, these compounds are assumed to bind reversibly to a 34 kDa protein of the Photosystem II complex. The binding of the herbicide is thought to prevent binding of plastoquinone from the pool and thus inhibition of photosynthetic electron flow is accomplished. The identification of the 34 kDa protein as herbicidal target relies solely on photoaffinity labeling experiments with one single compound: azido-atrazine. In isolated thylakoids, azido-atrazine almost exclusively labels a protein in the 34 kDa range [1,2]. Furthermore, it was shown that azido-atrazine does not bind any longer to this protein in thylakoids isolated from atrazine-resistant weeds [3]. The idea of the 32–34 kDa herbicide-binding protein has met criticism by Gressel [4] due to the fact that photoaffinity labeling experiments in general are not unambiguous. It is feasible that a photoaffinity label does not bind to the target protein, but to a neighboring protein instead.

Especially, Gressel's criticism is based on the fact that in the azido-atrazine molecule the azido-

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Abbreviations: Atrazine, 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine; azido-atrazine, 2-azido-4-(ethylamino)-6-(isopropylamino)-*s*-triazine; azido-triazinone, 3-dimethylamino-4-methyl-6-(3′-azidophenyl)-1,2,4-triazin-5-one; Chl, chlorophyll; DAD, diaminodurol; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2,4,4′-trinitro-2′-iodo-3′-methyl-6′-isopropyl-diphenylether; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; metribuzin, 4-amino-6-*tert*-butyl-3-(methylthio)-1,2,4-triazin-5-(4H)-one.

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group and the structural element generally recognized for herbicidal activity lie on opposite parts of the molecule. Therefore, the possibility might exist that the 34 kDa protein as tagged by azido-atrazine is not the real herbicide-binding protein [4]. This notion is further corroborated by Gressel's observation that Spirodela plants with most of their 34 kDa protein depleted are not more tolerant toward atrazine than those plants containing this protein [4].

For further investigation, we have synthesized a radioactively labeled azido-triazinone, which belongs to the same chemical class among which potent herbicides like metribuzin and metamitron have been found. The results indicate that in fact azido-triazinone and azido-atrazine bind to an identical protein. Furthermore, the photoaffinity labeling experiments presented confirm the idea of one common binding area for all 'DCMU-type' herbicides.

Materials and Methods

Synthesis of [^{14}C]azido-triazinone

3-Di[^{14}C]methylamino-4-methyl-6-(3'-azidophenyl)-1,2,4-triazin-5-one. 360 μg (4.41 μmol) di-[^{14}C]methylamine hydrochloride, supplied in 1 ml aqueous solution (250 μCi; Spec. act. 58 mCi/mmol, Amersham Buchler, Braunschweig, F.R.G.) was lyophilized. The residue was taken up in 150 μl dimethylformamide which contained 1.37 mg (5 μmol) 3-methylthio-4-methyl-6-(3'-azidophenyl)-1,2,4-triazin-5-one. The solution was transferred into a small vial, 10 μl dimethylformamide containing 6.1 μg (50 nmol) 4-dimethylaminopyridine as a catalyst and finally 50 μl 1,8-diazabicyclo(5,4,0)undec-7-ene as a base were added. The vial was sealed and kept at 40°C for 3 days. The vial was opened, the contents were transferred into 2 ml 0.1 N NaOH and extracted three times with ether. The combined ether extracts were dried over $MgSO_4$, the ether was evaporated and the residue was chromatographed on silica-gel-precoated plastic sheets 60 F-254 (Merck AG, Darmstadt, F.R.G.) with ethyl acetate as the solvent. The zone corresponding to the [^{14}C]azido-triazinone (R_f value 0.39) was eluted with methanol. The concentration was estimated from the absorption at 336 nm ($\varepsilon = 13003\ M^{-1} \cdot cm^{-1}$). The compound was obtained in a yield of 646 nmol (14.6%) and specific activity of 51 mCi/mmol.

Other chemicals

Azido-atrazine, [^{14}C]azido-atrazine, and 2-iodo-4-nitro-6-isobutylphenol were synthesized as described recently [2,5]. [^{14}C]Metribuzin had a specific activity of 25.7 mCi/mmol.

Biochemical methods

Chloroplasts from spinach were prepared according to Ref. 6 and stored in liquid nitrogen until use.

Photosynthetic DCIP-reduction was followed spectrophotometrically at 600 nm in a Zeiss PMQII spectrophotometer, equipped for cross-illumination with actinic light (filter RG 645, Schott, Mainz, F.R.G.), intensity 0.1 W cm^{-2}. The reaction mixture contained in a volume of 2 ml 30 mM Hepes (pH 7.0), 3 mM $MgCl_2$, 3 mM DCIP, 7 μg gramicidine, and varying amounts of chloroplasts.

Photosynthetic NADP-reduction was determined as in Ref. 7.

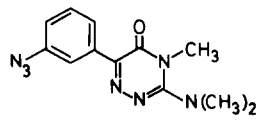
Binding of radioactively labeled inhibitors was determined as in Ref. 8, method B, and radioactivity was counted for the supernatant and pelleted chloroplasts as well.

For photoaffinity labeling experiments, samples were illuminated for 10 min in a nitrogen atmosphere with a mercury lamp at 0°C. Gel electrophoresis (LDS, 10–15%) and assay for radioactivity after cutting the gel into pieces were performed as in Ref. 9. The following marker proteins (Bio-Rad) were used: lysozyme, 14.4; soybean trypsin inhibitor, 21.5; carbonic anhydrase, 31, ovalbumin, 45; bovine serum albumin, 66.2; phosphorylase *b*, 92.5 kDa.

Results and Discussion

Inhibitor and binding properties of azido-triazinone

1,2,4-Triazin-5-ones are powerful inhibitors of



Azido-triazinone

Structure 1.

TABLE I

INHIBITION DATA FOR AZIDO-TRIAZINONE

For conditions, see Materials and Methods.

DCIP reduction (in the presence of 1 μM DNP-INT)		
μg Chl	μmol reduced/ mg Chl per h	I_{50} value (nM)
	Control	
20	57.7	137
15	55.4	120
10	55.4	102
5	46.2	88
0	–	69 (extrapolated)

photosynthetic electron transport at the acceptor side of Photosystem II [10]. The same is also true for azido-triazinone.

In Table I, Chl dependency of its I_{50} values for Photosystem II DCIP-reduction (in the presence of DNP-INT [11]) is listed. I_{50} values decrease with decreasing Chl concentration and an I_{50} value of 69 nM (corresponding to a pI_{50} value of 7.16) for zero Chl concentration [12] could be extrapolated.

Photosystem I dependent photoreductions are not inhibited by azido-triazinone. In a NADP-reduction experiment, the original rate of NADP-reduction (100.3 μmol reduced/mg Chl per h) is inhibited to 1.2 μmol reduced/mg Chl per h in the presence of 1 μM azido-triazinone. However, in the presence of the Photosystem I donor system 0.1 mM DAD, 1 mM sodium ascorbate, the rate of NADP-reduction goes up to 106.1 μmol reduced/mg Chl per h again.

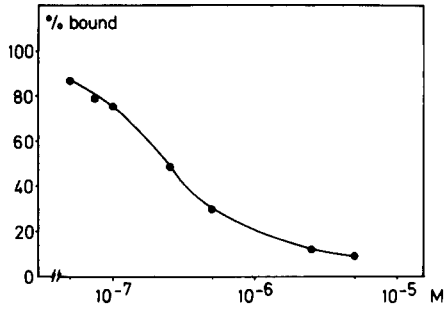


Fig. 1. Displacement of [^{14}C]metribuzin ($1 \cdot 10^{-7}$ M) by azido-triazinone from the thylakoid membrane.

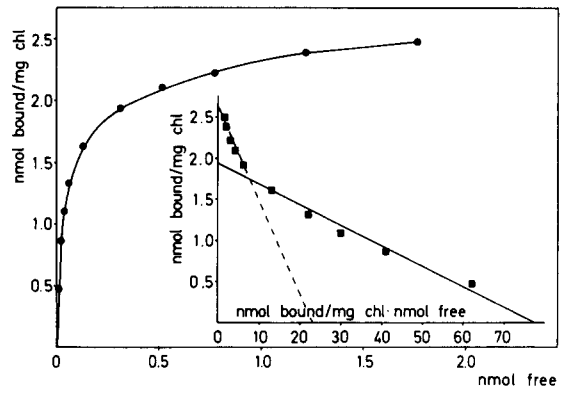


Fig. 2. Binding curve of [^{14}C]azido-triazinone to isolated spinach thylakoids. Inset: Woolf-Augustinsson-Hofstee plot of binding data.

Fig. 1 shows a displacement experiment with azido-triazinone. [^{14}C]Metribuzin is easily displaced from the thylakoid membrane by azido-triazinone (approx. 90% at 5 μM; Fig. 1).

The binding curve of azido-triazinone is presented in Fig. 2. It has the same characteristics as reported for other Photosystem II inhibitors [12,8,9]. To distinguish between high-affinity (specific) and low-affinity (unspecific) binding, as inset in Fig. 2 a Woolf-Augustinsson-Hofstee plot is shown (this type of plot was chosen because it discriminates better between both types of binding than the commonly used Lineweaver-Burk plot [12]). Clearly, the two types of binding can be distinguished. The corresponding binding parameters are listed in Table II. We note that the low-affinity binding is about 5-times less than the high-affinity binding. According to Tischer and Strotmann [12], K_b and I_{50} values extrapolated to zero Chl concentration should be identical. Indeed, both

TABLE II

BINDING PARAMETERS FOR AZIDO-TRIAZINONE

For conditions, see Materials and Methods.

	K_b (nM)	x_t (nmol/ mg Chl)	Number of Chl molecules per bound inhibitor
High affinity	12.6	1.96	567
Low affinity	55.4	2.61	425

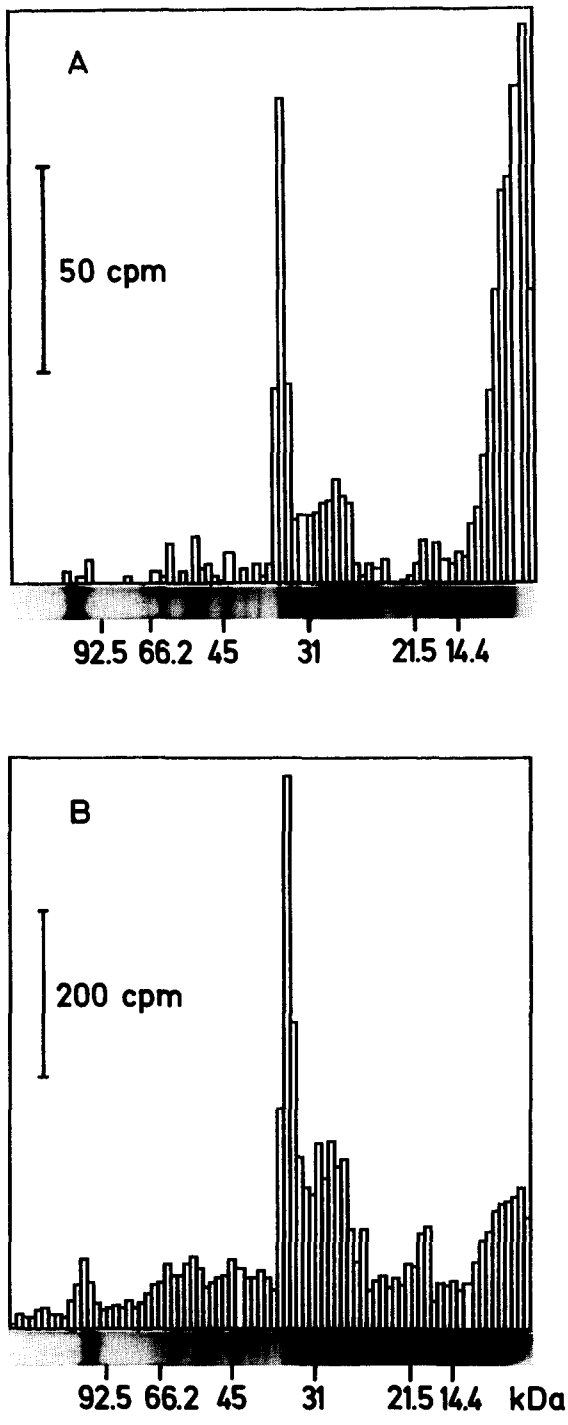


Fig. 3. Photographs of LDS-polyacrylamide gel electrophoresis gels (10–15%) and radioactivity distribution of thylakoids labeled with [^{14}C]azido-triazinone (A, 2.5 nmol/mg Chl; B, 20 nmol/mg Chl).

values are of the same order of magnitude; the I_{50} value, however, is better correlated to the low- than to the high-affinity binding constant.

The azido-triazinone binding protein

Isolated thylakoids have been labeled by [^{14}C]azido-triazinone, disrupted by detergent treatment and subjected to gel electrophoresis. The protein and labeling patterns for two different concentrations of azido-triazinone are shown in Fig. 3. As can be seen for both concentrations of azido-triazinone, only one diffuse, poorly staining polypeptide in the 34 kDa region is heavily labeled. If thylakoids are labeled by [^{14}C]azido-triazinone or [^{14}C]azido-atrazine, respectively, and samples run in adjacent lanes of the same gel, radioactivity in both lanes in the 34 kDa region is found in exact the same positions of the gel (data not

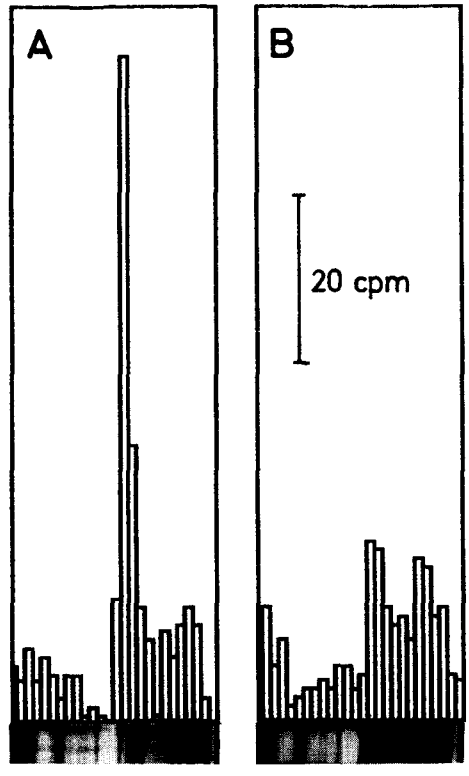


Fig. 4. Photographs of LDS-polyacrylamide gel electrophoresis gels (10–15%) and radioactivity distribution of thylakoids labeled with [^{14}C]azido-atrazine (10 nmol/mg Chl). A, Control; B, in the presence of 50 nmol/mg Chl azido-triazinone. Only the relevant parts of the gels are shown.

shown). Furthermore, the polypeptide labeled by azido-triazinone changes its mobility to a higher R_f value in exactly the same way as does the polypeptide which is labeled by azido-atrazine, if 4 M urea is included in the gel [13] (data not shown). It has to be concluded, therefore, that an identical protein is tagged by either [^{14}C]azido-atrazine or [^{14}C]azido-triazinone. Both compounds differ in their chemical structure and the point of attachment of the azido-group as well. Thus, Gressel's alternative possibility [4] that the azido-atrazine-binding protein is not identical with the herbicide-binding protein can be ruled out.

Competition experiments

For further confirmation of the notion that the 34 kDa protein is the common binding protein for all 'DCMU-type' herbicides, a series of competition experiments has been performed. Fig. 4A shows a labeling pattern of thylakoids after illumination in the presence of [^{14}C]azido-atrazine. As already known from previous work [1–3], the 34 kDa membrane protein gets labeled. In the experiment of Fig. 4B, thylakoids have been illuminated in the presence of inactive azido-triazinone, then [^{14}C]azido-atrazine has been added, and illumination was continued. As can be seen, the amount of radioactivity is greatly diminished as compared to the control. This indicates that indeed azido-atrazine and azido-triazinone compete for binding; if the binding site is occupied already by azido-triazinone, azido-atrazine cannot bind any longer.

This type of approach was extended to other Photosystem II inhibitors (Fig. 5). As compared to the control (Fig. 5A), addition of a 10-fold excess of DCMU (Fig. 5B) or 2-iodo-4-nitro-6-isobutylphenol (Fig. 5C) prior to addition of azido-triazinone results in a decrease of bound radioactivity within the 34 kDa protein. On the contrary,

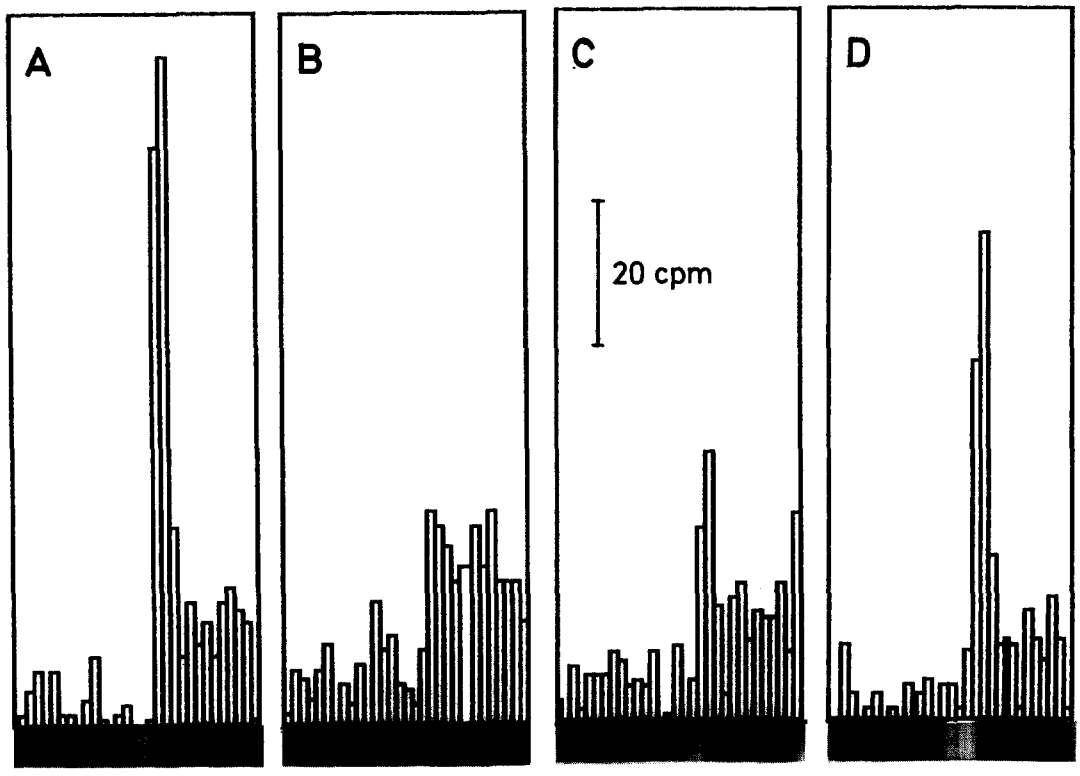


Fig. 5. Photographs of LDS-polyacrylamide gel electrophoresis gels (10–15%) and radioactivity distribution of thylakoids labeled with [^{14}C]azido-triazinone (2.5 nmol/mg Chl). (A) Control, and in the presence of 25 nmol/mg Chl. (B) DCMU. (C) 2-Iodo-4-nitro-6-isobutylphenol. (D) Tetraiodo-1,4-benzoquinone. Only the relevant parts of the gels are shown.

tetraiodo-benzoquinone which is also a powerful Photosystem II inhibitor (I_{50} value 55 nM [14]) is much less effective (Fig. 5D). This is in line with the observation that 5 μM tetraiodo-benzoquinone is required to displace 50% of bound DCMU (at 5 nM) from the thylakoid membrane (Soll and Oettmeier, unpublished data). This would imply that the binding sites for 'DCMU-type' inhibitors and quinones are not identical. A similar conclusion has been reached by Vermaas et al. [15]. These authors have observed that after covalent attachment of an azido-ubiquinone derivative to the thylakoid membrane, binding of either atrazine or the phenolic inhibitor 2,6-diiodo-4-cyanophenol (ioxynil) is diminished. However, the binding constants change whereas the number of binding sites remain constant [15]. One would expect a different behavior, i.e., identical binding constants and different numbers of binding sites, if quinone and inhibitor-binding site were identical. More recently, by use of an azido-plastoquinone photoaffinity label we noted that proteins within the thylakoid membrane labeled by azido-plastoquinone and azido-atrazine, respectively, differ in their R_f values, although their respective molecular weights are within the 30–34 kDa region [16]. One possible interpretation would be that even inhibitor and quinone-binding protein are different. However, further work has to be performed to verify this notion.

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