PHOTOAFFINITY LABELLING OF THE PHOTOSYSTEM II HERBICIDE BINDING PROTEIN

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1. Introduction

More than half of all currently utilized herbicides act as inhibitors of photosynthetic electron transport. Of major importance are the chemical groups of ureas, amides, triazines, trazinones, pyridazinones and carbamates (DCMU-type herbicides) (reviewed [1,2]). These herbicides all have a structural element N–C=X in common, where X signifies N or O [3]. Their site of action is located at the reducing site of photosystem II (PS II), where they bind reversibly and non-covalently to the so-called B-protein. This binding leads to a conformational change of the B-protein, which in consequence lowers the midpoint potential of a quinone bound to the B-protein in respect to the midpoint potential of the primary acceptor of PS II [4]. Thus, electron flow between the two photosystems is inhibited. We have studied the binding properties at the thylakoid membrane of phenolic herbicides as represented by a radioactively labelled 2,4-dinitro-6-isobutyl-phenol (*i*-Dinoseb), an isomer of the well known herbicide Dinoseb [5,6]. We have concluded that the binding sites for phenolic herbicides and DCMU-type herbicides are not identical, though they are located at the same protein component [5,6]. *i*-Dinoseb could be easily converted into a photoaffinity label for the PS II herbicide binding protein. We report here on the synthesis of this compound and its binding to the thylakoid membrane.

2. Materials and methods

2.1. *4-Nitro-2-amino-6-[2',3'-^{3}H]isobutyl-phenol*

i-Dinoseb [6] 48 mg (200 μmol) with spec. act. 490 mCi/mmol were dissolved in 0.5 ml H_2O and 0.5 ml concentrated NH_3. This solution was heated to 100°C and 0.5 ml 40% $(NH_4)_2S$ were added. The reaction mixture was kept at 100°C for 45 min. After cooling to room temperature, concentrated HCl was added until the colour of the reaction mixture turned to yellow. An additional 0.5 ml concentrated HCl was added and the reaction mixture was kept at 100°C for another 10 min. After removal of precipitated sulfur by filtration, the aqueous phase was extracted 4 times by diethylether. The diethylether phase was dried over $MgSO_4$ and diethylether evaporated in the vacuum.

2.2. *4-Nitro-2-azido-6-[2',3'-^{3}H]isobutyl-phenol*

The residue from the above was dissolved in 1.5 ml 35% fluoroboric acid and cooled to 0°C. $NaNO_2$ (50 mg) in 0.3 ml H_2O was added and the reaction mixture was stirred for 20 min and after addition of 300 mg NaN_3 in 1 ml H_2O for 15 h at 0°C. It was extracted 3 times with diethylether, the diethylether dried over $MgSO_4$ and concentrated in the vacuum to ~2 ml. From this stock solution aliquots were chromatographed on silica gel pre-coated plastic sheets (Polygram SIL G/UV_{254}, thickness 0.25 mm; Macherey-Nagel GmbH, Düren) with benzene as the solvent. The zone, corresponding to the azide (R_F 0.61) was cut out and eluted with methanol.

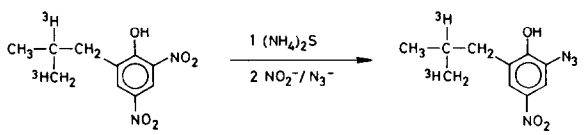
The concentration of the azide was determined from its A_{437} maximum (ϵ = 14 600 $M^{-1} \cdot cm^{-1}$) in 0.1 M phosphate buffer (pH 8.0). The labelled compound showed no difference in chemical and biochemical behaviour as compared with the unlabelled compound, whose identity was checked by elementary analysis and NMR spectroscopy. The radiochemical yield was 33%.

Chloroplasts were prepared from spinach leaves following [7]. For the photoaffinity labelling experiments they were additionally washed 3 times with 5 mM tricine buffer (pH 8.4). The pI_{50}-value of 4-nitro-2-azido-6-isobutylphenol in photosynthetic NADP-reduction was determined as in [8]. The binding of 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol to chloroplasts was determined as in [6].

In the photoaffinity labelling experiment, 10 nmol 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol in 10 μl methanol were added to chloroplasts corresponding to 2 mg chlorophyll (chl) in 2 ml containing 20 mM tricine buffer (pH 8.0) and 20 mM $MgCl_2$. The reaction mixture was cooled in an ice bath and illuminated under an argon atmosphere for 15 min with white light (light source 2000 W). Chloroplasts were then washed 3 times and solubilized in a medium containing 20 mM tricine buffer (pH 8.0), 20 mM $MgCl_2$, 10% glycerol, 5% β-mercaptoethanol and 1.5% SDS. SDS–polyacrylamide gel electrophoresis was performed in a slab gel apparatus (modified from the design in [9]) using a discontinuous buffer system. A 6% stacking gel and a 11–15% separation gel were used. Electrophoresis was carried out for 17 h at a constant 14 mA. Gels were fixed for 1 h in methanol:water:acetic acid (5:5:1), stained for 1 h in the same solution containing in addition 0.05% Coomassie blue G-250 and destained in the solution used for fixation. The gels were scanned at A_{620} in an Isco gel scanner. Afterwards, gels were cut into 2 mm pieces and the pieces dissolved in 1 ml 17% hydrogen peroxide at 55°C for 48 h. Quickszint 212 (Zinsser, Frankfurt) (10 ml) was then added and radioactivity counted in a Packard Tricarb Liquid Scintillation Spectrometer, model 3385.

3. Results and discussion

The synthesis of the photoaffinity label 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol is presented in scheme 1. The *o*-nitro-group in 2,4-dinitro-6-[2′,3′-^{3}H]-isobutyl-phenol (*i*-Dinoseb) is selectively reduced to



I-DINOSEB

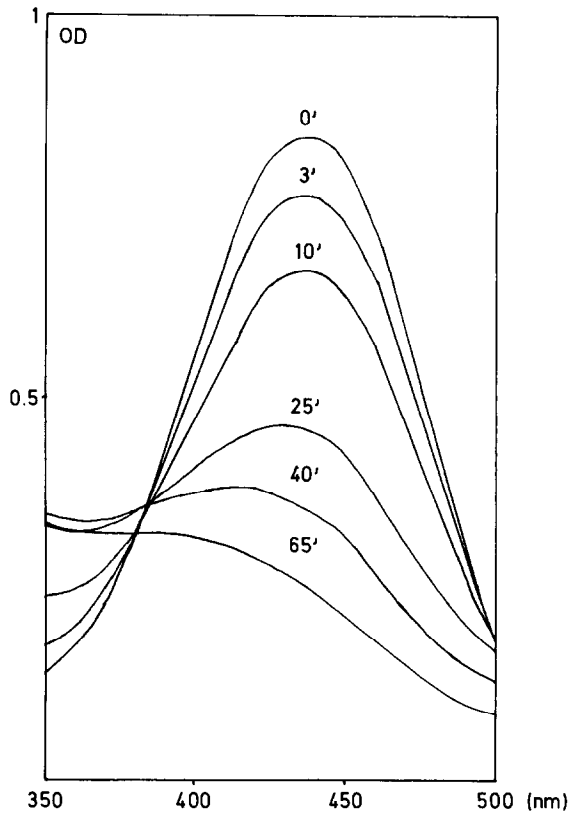


Fig.1. Photolysis of 4-nitro-2-azido-6-isobutyl-phenol (50 μM in 10 mM phosphate buffer (ph 8.0)) by 436 nm light.

the amino-group by ammonium-sulfide by the procedure described in [10]. The aromatic amine was then converted into the diazonium compound and reacted with sodium azide according to [11]. The azide was obtained with spec. act. 350 mCi/mmol.

The photolysis of 4-nitro-2-azido-6-isobutyl-phenol by 436 nm light is demonstrated in fig.1. As can be seen the absorbance at the maximum of 437 nm decreases with increasing time. Photolysis is completed after 65 min as judged from the fact that further illumination does not change the absorption spectrum any more. Using the white light source in section 2, the photolysis was completed within 10–15 min.

4-Nitro-2-azido-6-isobutyl-phenol itself is an effective inhibitor of photosynthetic electron transport. Inhibition of 50% of uncoupled photosynthetic NADP-reduction was achieved at 2 μM (pI_{50}-value 5.7). The binding of 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol is shown in fig.2. Like *i*-Dinoseb [6] it exhibits high affinity (specific) and low affinity (unspecific) bind-

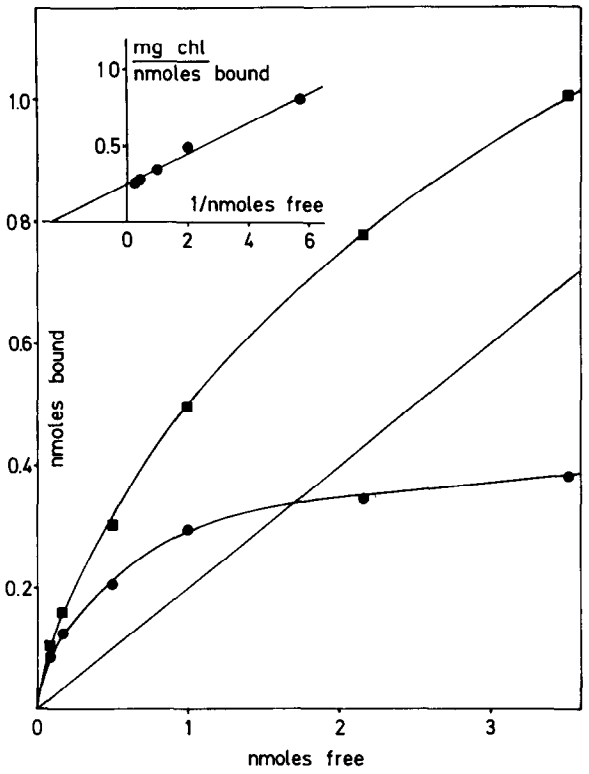


Fig.2. Total binding (▪——▪), low affinity binding (——), and high affinity binding (●——●) of 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol to chloroplasts. The concentration of chloroplasts corresponded to 100 μg chl. Inset: Double reciprocal plot for determination of specific binding constant K_b (abscissa intercept) and no. binding sites x_t (ordinate intercept).

ing. By substracting the unspecific binding (——, in fig.2) the true specific binding was obtained (fig.2). From double reciprocal plots (inset fig.2) according to [12] a binding constant $K_b = 1.9 \times 10^{-7}$ M and a no. binding sites $x_t = 4.03$ nmol/mg chl were calculated. This corresponds to 1 molecule azide/280 molecules chl or ~1 molecule azide/electron transport chain.

In the photoaffinity labelling experiment 5 nmol 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol/mg chl was used. A scan for absorbance and radioactivity of a typical SDS–polyacrylamide electrophoresis gel is shown in fig.3. Due to the unspecific binding of the axide, a high amount of radioactivity is found in lipids and pigments (right side, fig.3). In addition 3 protein components are radioactively labelled: a protein in the 12.5–20 kM_r region; the light-harvesting chl–

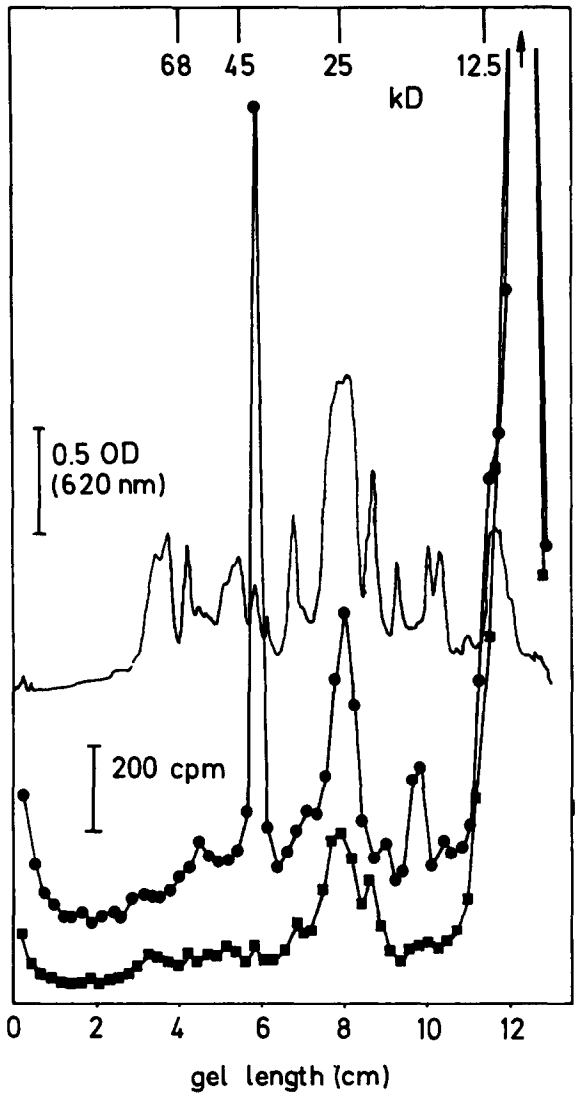


Fig.3. Scan for A_{620} and radioactivity (●——●) of an SDS–polyacrylamide electrophoresis gel of chloroplasts labelled with 5 nmol 4-nitro-2-azido-6-[2′,3′-^{3}H]isobutyl-phenol. (▪——▪) 50 nmol *i*-Dinoseb had been added prior to addition of the azide.

protein complex at 25 kM_r; and the highest amount of radioactivity is found with a protein in the 30–40 kM_r region. This peak completely vanishes (lower trace, fig.3) if chloroplasts are incubated with 50 nmol *i*-Dinoseb/mg chl prior to addition of the photoaffinity label. We conclude, therefore, that this protein corresponds to the photosystem II herbicide binding protein.

Acknowledgements

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