Differential Sensitivity of 32 kDa-D1 Protein Degradation and Photosynthetic Electron Flow to Photosystem II Herbicides

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Degradation of the 32 kDa-D1 protein, a photosystem II reaction centre component, was studied as a function of linear electron flow in visible light in the presence of various photosystem II herbicides. Under these conditions, herbicide specific effects on protein degradation were clearly evident. 32 kDa-D1 protein degradation and electron flow between Q_a and Q_b proved to be only partially correlated. We conclude that inhibition of protein degradation by PS II herbicides in visible light is not simply correlated to displacement of Q_b.

Introduction

Photosystem II (PS II) of oxygenic phototrophs is a highly structured protein-pigment complex consisting of a water splitting system, a light-harvesting chlorophyll protein complex and a reaction centre [1, 2]. The core of the PS II reaction centre consists of the 32 kDa-D1, D2 and cytochrome b_{559} proteins [3, 4]. Based on the resolved crystal structure of the functionally-analogous reaction centre of photosynthetic bacteria [5], and molecular characterization of herbicide resistant mutants [2], a structure for the PS II reaction centre has been proposed [6, 7]. The 32 kDa-D1 and D2 proteins each span the membrane five times and are, respectively, the apoproteins of Q_b and Q_a [8]. Various classes of PS II herbicides interact with the 32 kDa-D1 protein at overlapping but different sites [7, 9−11]. These herbicides interrupt electron flow from PS II to the plastoquinone pool by seemingly-competitive displacement of Q_b from the protein [12−16].

The 32 kDa-D1 protein is unstable in the light, turnover occurring at low light intensities with rates exceeding those for other proteins [17−19]. A cleavage site for light-dependent degradation of the protein has been proposed in the loop between helices 4 and 5, somewhere between Arg 238 and Ile 248 [20]. This site is in close proximity to the Q_b/herbicide binding niche [6, 7]. Degradation

Chemical names of herbicides used: Diuron (DCMU), 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Dinoseb, 2-(1-methylpropyl)-4,6-dinitrophenol; Bromoxynil, 4-hydroxy-3,5-dibromobenzonitrile.

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is promoted by UV (250−400 nm), visible (400−700 nm) and far-red (700−730 nm) irradiation. The visible and far-red light photosensitizers are most likely chlorophyll and other bulk photosynthetic pigments [21]. In the UV region, plastoquinone in one or more of its forms has been proposed as a photosensitizer [21]. The occurrence of rapid 32 kDa-D1 protein degradation at UV-B wavelengths (280−320 nm), where photosynthesis is not supported, indicates that electron flow is not essential for degradation. However, the question remains whether these two processes are normally coupled in visible light, where the same photosensitizer (chlorophyll) is postulated to trigger them both. In this regard, we note that the PS II herbicide diuron, which blocks electron flow, also inhibits light-dependent degradation of the 32 kDa-D1 protein [17]. Little is known about the mechanism by which diuron inhibits degradation.

In this work, we applied different PS II inhibitors to study whether inhibition of electron flow to the plastoquinone pool is correlated to inhibition of 32 kDa-D1 protein degradation.

Materials and Methods

Spirodela oligorrhiza (Kurtz) Helgm was grown phototropically under cool white fluorescent lamps. Radiolabeling with [^{35}S]methionine was carried out as previously described [22]. Plants were radiolabeled for 3 h under 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light and chased in the presence of inhibitors for up to 20 h under 6 $\mu E \cdot m^{-2} \cdot s^{-1}$ white light. Membrane proteins were prepared by SDS-PAGE and visualized by fluorography [22]. The rate of 32 kDa-D1 protein degradation was quantified by microdensitometry [20]. Herbicides (chemical pu-

rity, 99%) were obtained from Riedel-de Haën AG, F.R.G. (diuron), or Chem Service, P.A., U.S.A. (bromoxynil, dinoseb).

Oxygen evolution was measured *in vivo* by photoacoustic spectroscopy [23]. The measuring light ($15 \, \mu E \cdot m^{-2} \cdot s^{-1}$) was modulated at a frequency of 15 Hz. Signals were processed by a lock-in amplifier (SR 530, Stanford Research Systems, U.S.A.) using vectorial analysis [24]. Photochemical quenching of fluorescence (Q-quenching), representing the fraction of Q_a oxidized, was calculated according to Schreiber [26]. Steady-state chlorophyll fluorescence was studied using modulated measuring light (660 nm) of low intensity ($0.1 \, \mu E \cdot m^{-2} \cdot s^{-1}$) from a pulse fluorometer (PAM 101, Fa. Walz, Effeltrich, F.R.G.) operated at 1.6 kHz pulse frequency [25]. The measuring light and the actinic light (660 nm, $6 \, \mu E \cdot m^{-2} \cdot s^{-1}$) were combined on the leaf using fibre optics. Chlorophyll fluorescence-induction kinetics were studied using the same pulse fluorometer as above, coupled to a storage oscilloscope (7623 A, Tektronix, U.S.A.). The intensities of the modulated light (100 kHz, 660 nm) and the actinic light (660 nm) were $0.07 \, \mu E \cdot m^{-2} \cdot s^{-1}$ and $40 \, \mu E \cdot m^{-2} \cdot s^{-1}$, respectively. The degree of inhibition of electron transport between Q_a and Q_b was calculated from the decrease of the complementary area above the induction curves, which was normalized to maximal fluorescence [27].

Results and Discussion

The effects of bromoxynil, dinoseb and diuron on oxygen evolution, Q-quenching of fluorescence, fluorescence induction area and 32 kDa-D 1 protein degradation are shown in Fig. 1. The rates of electron flow and protein degradation remained constant during the experiment. Thus, under the conditions used, the herbicides did not noticeably damage the photosynthetic apparatus. Using these data, the inhibition of PS II electron flow (oxygen evolution or Q-quenching measurements) or the inhibition of electron flow between Q_a and Q_b (fluorescence-induction area measurements) was plotted *vs.* inhibition of 32 kDa-D 1 degradation. The derived plots are shown in Fig. 2.

In the presence of diuron, a linear correlation was found between inhibition (up to 90%) of photosynthetic electron flow and 32 kDa-D 1 protein degradation. However, the diuron regression lines

are displaced from the origin. This shows that at concentrations where Q-quenching, oxygen evolution, and electron flow between Q_a and Q_b were
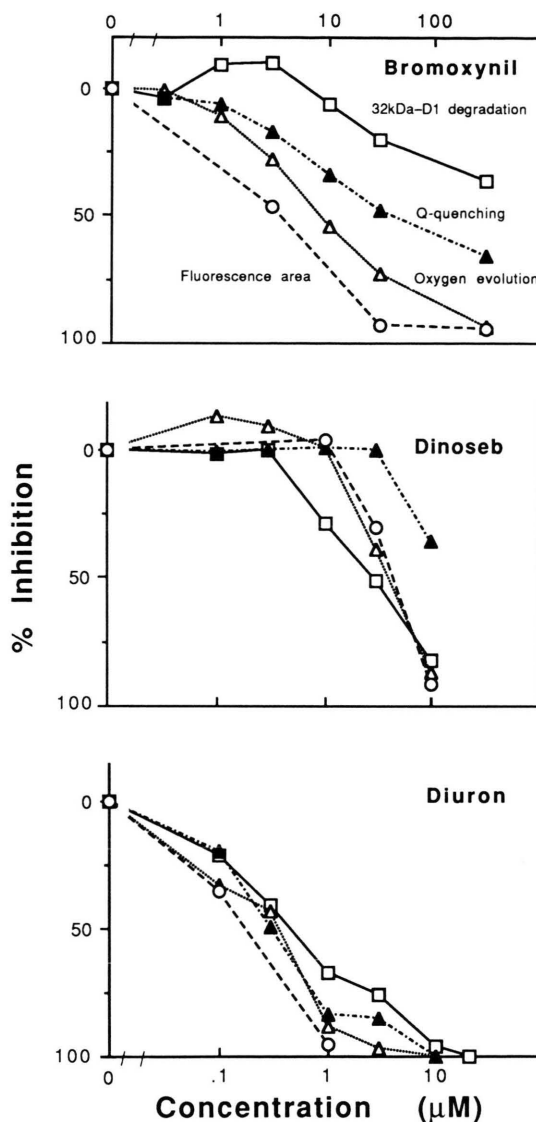

Fig. 1. Inhibition of photosynthetic activities and protein degradation as a function of herbicide concentration. 32 kDa-D 1 protein degradation (□), Q-quenching of fluorescence (▲), oxygen evolution (△) and fluorescence area above induction curves (○) were determined as indicated in Materials and Methods. All values were normalized to 0% inhibition in the absence of herbicides. Values represent averaged data from several experiments: 32 kDa-D 1 degradation, $n = 8$, $\overline{s.e.} = 8.5\%$; Q-quenching, $n = 6$, $\overline{s.e.} = 1.4\%$; Oxygen evolution, $n = 6$, $\overline{s.e.} = 5.8\%$; Fluorescence area, $n = 3$, $\overline{s.e.} = 4.7\%$.
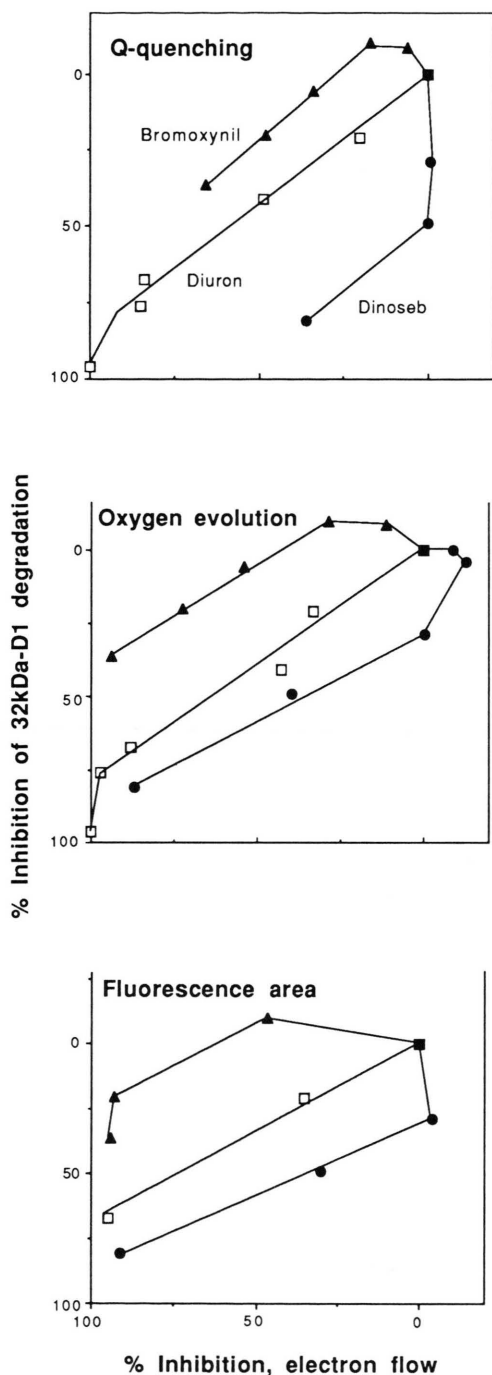
Fig. 2. Inhibition of linear electron flow *versus* inhibition of 32 kDa-D 1 protein degradation. Electron flow was measured as Q-quenching, oxygen evolution and fluorescence area above induction curves as described in Materials and Methods. The plots were derived from the data in Fig. 1. Bromoxynil (▲); Diuron (□); Dinoseb (●).

abolished, degradation of the 32 kDa-D 1 protein still proceeded at appreciable rates. Only at higher herbicide concentrations was degradation fully blocked. Bromoxynil, on the other hand, inhibited 32 kDa-D 1 protein degradation with a considerably lower efficiency than it did photosynthetic electron flow (measured by any of the three methods). Thus, at herbicide concentrations which reduced electron flow to less than 50% of non-inhibited control, protein degradation was not measurably impeded. Only at higher bromoxynil concentrations was 32 kDa-D 1 degradation also affected. In contrast, dinoseb inhibited degradation more strongly than it did photosynthetic electron flow. A concentration reducing the rate of protein degradation by about 50%, hardly affected oxygen evolution, Q-quenching or electron flow between Q_a and Q_b. Only higher inhibitor concentrations affected photosynthetic electron flow and degradation proportionally.

Although effects of herbicide impurities at the highest concentrations used cannot be excluded, the derived plots in Fig. 2 suggest that under photosynthetically active radiation, correlations between 32 kDa-D 1 protein degradation and electron flow do exist, but are complex. We conclude that inhibition of protein degradation by PS II herbicides in visible light is not simply correlated to displacement of plastoquinone, although the two processes are related.

The specific effects of individual herbicides on protein degradation may reflect their different binding sites within the Q_b niche. In this regard, we note the binding of the diuron-type inhibitor, azidomonuron to protein residues Tyr 237 and Tyr 254 [11], and the bromoxynil type inhibitor, azidoioxynil to residue Val 249 [10]. The relationship *in vivo* between linear electron flow and 32 kDa-D 1 protein degradation seems inhibitor specific. Thus, co-analysis of electron flow and degradation, coupled with site directed mutagenesis, may help pinpoint binding sites of various herbicides in the Q_b-region and the mechanism of 32 kDa-D 1 protein degradation.

[1] N. Murata and M. Miyao, TIBS **10,** 122 (1985).

[2] J.-D. Rochaix and J. Erickson, TIBS **13,** 56 (1988).

[3] O. Nanba and K. Satoh, Proc. Natl. Acad. Sci. U.S.A. **84,** 109 (1987).

[4] J. B. Marder, D. J. Chapman, A. Telfer, P. J. Nixon, and J. Barber, Plant Molec. Biol. **9,** 325 (1987).

[5] J. Deisenhofer, O. Epp, K. Miki, R. Huber, and H. Michel, J. Molec. Biol. **180,** 385 (1984).

[6] A. Trebst, Z. Naturforsch. **41 c,** 240 (1986).

[7] A. Trebst, Z. Naturforsch. **42 c,** 742 (1987).

[8] J.-D. Rochaix, M. Dron, M. Rahire, and P. Malnoe, Plant Molec. Biol. **3,** 363 (1984).

[9] K. Pfister, K. E. Steinback, G. Gardner, and C. J. Arntzen, Proc. Natl. Acad. Sci. U.S.A. **78,** 981 (1981).

[10] W. Oettmeier, K. Masson, and J. Höhfeld, Z. Naturforsch. **44,** 444 (1989).

[11] R. Dostatni, H. E. Meyer, and W. Oettmeier, FEBS Lett. **239,** 207 (1988).

[12] B. R. Velthuys, FEBS Lett. **126,** 277 (1981).

[13] C. J. Arntzen, K. Pfister, and K. E. Steinback, in: Herbicide Resistance in Plants (H. M. LeBaron and J. Gressel, eds.), p. 185, John Wiley & Sons, New York 1982.

[14] W. Oettmeier and H. J. Soll, Biochim. Biophys. Acta **724,** 287 (1983).

[15] W. F. J. Vermaas, C. J. Arntzen, L.-Q. Gu, and C.-A. Yu, Biochim. Biophys. Acta **723,** 266 (1983).

[16] W. F. J. Vermaas, G. Renger, and C. J. Arntzen, Z. Naturforsch. **39 c,** 368 (1983).

[17] A. K. Mattoo, H. Hoffman-Falk, J. B. Marder, and M. Edelman, Proc. Natl. Acad. Sci. U.S.A. **81,** 1380 (1984).

[18] V. Gaba, J. B. Marder, B. M. Greenberg, A. K. Mattoo, and M. Edelman, Plant Physiol. **84,** 348 (1987).

[19] K. Gounaris, U. Pick, and J. Barber, FEBS Lett. **211,** 94 (1987).

[20] B. M. Greenberg, V. Gaba, A. K. Mattoo, and M. Edelman, EMBO J. **6,** 2865 (1987).

[21] B. M. Greenberg, V. Gaba, O. Canaani, S. Malkin, A. K. Mattoo, and M. Edelman, Proc. Natl. Acad. Sci. U.S.A. **86,** 6617 (1989).

[22] J. B. Marder, A. K. Mattoo, and M. Edelman, Methods Enzymol. **118,** 384 (1986).

[23] G. Bults, B. A. Horwitz, S. Malkin, and D. Cahen, Biochim. Biophys. Acta **679,** 452 (1982).

[24] P. Poulet, D. Cahen, and S. Malkin, Biochim. Biophys. Acta **724,** 433 (1983).

[25] U. Schreiber, Photosynth. Res. **9,** 261 (1986).

[26] U. Schreiber, U. Schliwa, and W. Bilger, Photosynth. Res. **10,** 51 (1986).

[27] S. Malkin and B. Kok, Biochim. Biophys. Acta **126,** 413 (1966).