Characteristics of Thermoluminescence Bands of *Euglena* Cells Belonging to 2 Lines Presenting Different Degrees of Diuron-Resistance

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We have analysed the thermoluminescence (TL) properties of two lines of *Euglena* exhibiting two degrees of resistance to diuron, by a factor of 100 (ZR 25) and 1000 (ZR 250) respectively, as compared to wild type line (Z). In addition, the two ZR lines developped an identical resistance to atrazine since the I_{50} for this herbicide in each line was 75 times larger than in wild type. Special TL characteristics were evidenced in the two lines. Bands after 2 flashes (or more) showed a shift of the peak maximum towards low temperature, the shift being the largest in the most DCMU-resistant cells. Similar results were obtained with isolated thylakoids, except that the TL bands appeared at a temperature higher than in corresponding cells. Oscillations in the amplitude of the bands in a flash sequence were largely damped in cells (and thylakoids), particularly in the most DCMU-resistant lines. The results are interpreted as indicating accumulation of $Q_A^- Q_B$ after flashes due to a decrease of the equilibrium constant for the reaction $Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$ accompanying the DCMU resistance.

Introduction

In several higher plants, especially weeds, lines have been characterized which are resistant to s-triazines: herbicides acting on the reaction center of PSII. Resistance is the consequence of a single mutation at the level of the Q_B niche on the D_1 protein of the reaction center (Trebst, 1987) due to the substitution of the serine residue at the 264 position, by glycine (Hirschberg and McIntosch, 1983), asparagine (Pay *et al.,* 1988) or threonine (Smeda *et al.,* 1993). This substitution results in a decreased binding affinity for s-triazines by a factor of 100–1000, depending on the nature of the substituted amino acid. With such "atrazine-resistant" plants, for instance *Amaranthus hybridus*, it was shown that resistance to phenylurea herbicides was very low (Pfister and Arntzen, 1979). However, in algae and cyanobacteria, substitution of serine by glycine (or other amino acids) at the 264 position in D1 leads to a resistance to a wide spectrum of herbicides including both phenyl ureas and s-triazines (Horowitz *et al.,* 1989; Bowyer *et al.,* 1991). Phenolic herbicides remain very active in these mutants.

Induction of diuron (DCMU) resistance has been observed in strain Z of *Euglena* when cultured in photoorganotrophic conditions in the presence of 33 mM lactate and 25 or 250 μM DCMU (Laval-Martin *et al.,* 1977; Calvayrac *et al.,* 1979a; Calvayrac *et al.,* 1979b). It has been characterized as a dynamic process leading to a resistance to 25 μM DCMU within 10 weeks (ZR 25 line), and to 250 μM DCMU within 6 months (ZR 250 line). This acquired resistance to 25 and 250 μM DCMU persists since 1979 (Calvayrac *et al.,* 1979 a,b) whatever the conditions of the culture are: autotrophic or heterotrophic, in the presence or absence of DCMU, under light or in darkness (unpublished results). In spite of a mutation detected in the D_1 (32–34 kDa) protein (Karabin *et al.,* 1984) the exact nature of the mutation(s) responsible of these resistances is still unknown.

Abbreviations:: DCMU, 3-(3–4-dichlorophenyl)-1,1-dimethylurea; D_1, a 32 kDa protein component of the PS II reaction center, psbA gene product; FR, far-red illumination; I_{50}, concentration of herbicide allowing for a 50% inhibition of oxygen evolution; MV, methylviologen; PAR, photosynthetically active radiation; pBQ, parabenzoquinone; PQ, plastoquinone; PS II, photosystem II; Q_A, primary quinonic electron acceptor; Q_B, secondary quinonic electron acceptor; TL, thermoluminescence.

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Considering previous studies performed with atrazine-resistant higher plants, a thermoluminescence study has been undertaken to 1) compare the operating conditions of PS II between the two lines of *Euglena* resistant to DCMU, and to 2) link these results with the already known TL signals characteristics of the wild type strain, Z (Farineau and Laval-Martin, 1992).

Material and Methods

Organisms and culture conditions

Euglena gracilis Z. Klebs and DCMU-resistant lines ZR 25 and ZR 250 were grown at 21°C under a photon flux density of 50 μmol quanta m^{-2} s^{-1} photosynthetically active radiation (PAR) (daylight fluorescent tubes, Philips). Cells were inoculated at 2 X 10^4 cells ml^{-1} in a purely mineral autotrophic medium (pH 6.9) defined by Cramer and Myers (1952), supplemented with B_1 and B_{12} vitamins. Cells from the culture were sampled at the end of the exponential phase of growth and maintained in culture in these strictly autotrophic conditions. Aliquots of the cell suspension were centrifuged (1000×*g*/10 min). The pellets were resuspended in the same medium to a final chlorophyll concentration of 30 μM and dark-adapted for at least one hour before TL measurements.

Isolation of thylakoids

Thylakoids were isolated at 4°C from pelleted Z and ZR cells resuspended in a medium composed of 0.35 M sorbitol, 10 mM $MgCl_2$ and 10 mM N - [2 - Hydroxyethyl] piperazine - N′ - [2 - ethanesulfonic acid] - NaOH (pH 7.5) and sonicated for 15 s using a Biosonik III apparatus (Brownwill Scientific Rochester NY 14 603 USA) set on full intensity. Unbroken cells were eliminated by centrifugation (300×*g*/10 min) and thylakoids collected from the second pellet (5000×*g*/10 min) in the medium mentioned above, supplemented with 5 mM KCl.

Measurement of electron transport activity and herbicide inhibition

Electron transport was measured polarographically as O_2 evolution rates using a Clark-type electrode (Hansatech) at a controlled temperature of 22°C. The suspension medium was the culture medium (supplemented with 5 mM KCl) for cells and the resuspension buffer previously described for thylakoids adjusted to a chlorophyll concentration of 30 μM. Under a photon flux density (PAR) of 400 μmol quanta $m^{-2} \cdot s^{-1}$ and in the presence of 1 mM pBQ, rates of O_2 evolution were in the range 30–60 μmol mg $Chl^{-1} \cdot h^{-1}$ for cells and 60–90 μmol · mg $Chl^{-1} \cdot h^{-1}$ for thylakoids. In case of isolated thylakoids, the rates of O_2 evolution stayed linear during the first two minutes of illuminution.

Herbicide inhibition of O_2 evolution was assayed in the same cuvette electrode. Stock solutions of atrazine and DCMU in ethanol were prepared at adequate concentrations in order to assure a final ethanol concentration of the suspension in the range of 0.2–0.5%. Cells or thylakoids suspensions containing 30 μM chlorophyll were preincubated in the presence of pBQ and herbicide for 2 minutes in the dark before light was turned on for the determinion of O_2 evolution rates. Controls indicating 100% (uninhibited) rates contained 0.2–0.5% ethanol without herbicide. I_{50} for a given herbicide (DCMU or atrazine) were determined from regression curve drawn through 5 data points corresponding to different herbicide concentrations (each of them representing the mean of 2–3 repetitions).

Thermoluminescence assays

The TL device has been described in our previous publication (Farineau and Laval-Martin, 1992). It includes a photomultiplier connected to a photon counting system to determine luminescence intensity which was then indicated as counts in the ordinate of the different curves in the Figures 1 and 2. TL glow curves were recorded in the range from -40 to +80°C with a heating rate of 0.5°C/s. Charging of TL bands was performed by firing flashes spaced by 0.5 s at -10°C. Each of the glow curve presented in figures corresponded to Fourrier-filtering of the data points for one experiment and the peak maximum was determined using the computed first derivative (not shown in the figures).

Results

Effect of differents herbicides on photosynthetic electron transfer rate in intact cells and isolated thylakoids

The effect of various concentrations of herbicides (DCMU, atrazine) on photosynthetic elec-

tron transfer rates in susceptible and DCMU-resistant lines of *Euglena*, was investigated for both intact cells and thylakoids isolated from these cells. As indicated in Table I, DCMU resistance of the cells is higher in ZR 250 line than in ZR 25, exhibiting an I_{50} for electron transfer rates of about 250 and 25 μM, respectively, as expected (Laval-Martin *et al.*, 1977; Calvayrac *et al.*, 1979a), whereas the I_{50} value for the control cells (Z line, DCMU susceptible) was 0.2 μM. In the thylakoids isolated from resistant lines, the I_{50} for DCMU was still higher as compared to the low value of 0.08 μM for control thylakoids (isolated from wild type cells). However, for both resistant and susceptible lines, the I_{50} found for thylakoids were 5 to 10 times lower than those for intact cells, suggesting the difficult diffusion of the herbicide through the pellicule limiting the cell, and/or a possible detoxifying effect.

Susceptibility of *Euglena* cells to atrazine was investigated. Cells of the two ZR lines appeared largely and identically resistant to this herbicide. The determined I_{50} were found higher, by factors of 75 times for cells and 100 times for thylakoids of resistant lines, than in the corresponding susceptible controls. Thus ZR lines are both DCMU and atrazine-resistant.

Table I. Effect of two herbicides, DCMU and atrazine, on photosynthetic electron transfer rates in cells and thylakoids of different *Euglena* lines: Z control and ZR 25 and ZR 250 DCMU-resistant. Rates of oxygen evolution ($\mu mol \cdot mg^{-1}$ chl $\cdot h^{-1}$) were determined using an Hansatech electrode in the presence of 1 mM pBQ as electron acceptor and increasing herbicide concentrations in order to determine the I_{50} for the two herbicides as explained in Methods. ZR 25 and ZR 250 are defined in text; Z is a control susceptible to DCMU. L: lines; M: material and H: herbicides.

M \ L	H	*Euglena* lines Z	ZR 25	ZR 250
		I_{50} [μM]		
Cells	DCMU	0.2 ± 0.05	19 ± 2	200 ± 15
	Atrazine	4 ± 1	300 ± 27	300 ± 40
Thylakoids	DCMU	0.08 ± 0.02	4 ± 1	20 ± 3
	Atrazine	0.50 ± 0.1	50 ± 5	50 ± 3

Thermoluminescence glow curves induced by a flash sequence

TL glow curves induced by a flash sequence were studied both in the intact cells of the two ZR lines and in the corresponding isolated thylakoids (Fig. 1).

Cells

Cells were dark-adapted for at least 1 hour before the beginning of the experiment. Depending on the number of flashes applied, the evolution of the signal-shape of glow curves in ZR 25 cells, resembled that already presented in a previous paper for wild type Z cells (Farineau and Laval-Martin, 1992). After 1 flash, TL band exhibited a low amplitude and a flat shape and differed from the bands observed after 2 or more flashes, which exhibited single bands with the highest amplitude (Fig. 1). The maxima of these bands were nevertheless located at about 12°C (after 2 flashes) and 9–10°C after 5 flashes and more. Clear oscillations in the band amplitude were observed after sequences comprizing from one to eight flashes; the amplitude was totally damped after 7 flashes.

In order to oxidise the pool of redox carriers between the two photosystems, a pretreatment by 5 seconds illumination with far-red light in the presence of 100 μM methylviologen was applied 1 minute before delivering the flash(es). Such a pretreatment had only a very slight effect on ZR cells resulting in a shift by 2–3°C of the band maximum (Fig 1). Moreover, it did not induce any change in the shape of the TL bands (not shown).

In ZR 250 cells, TL bands were flat and presented maxima at low temperatures of 6°C (after 2 flashes) and nearly 0°C after five flashes. Amplitude of the band after 1 flash was relatively high. Oscillations in the amplitude of bands depending on the number of flashes delivered could hardly be seen. The far-red plus MV treatment was without effect.

Thylakoids

The TL bands induced by a flash sequence presented maxima at 23°C for ZR 25 line and 14°C for ZR 250 line (Fig.1), i.e. they were shifted by about 10 and 20°C, respectively, as compared to the corresponding bands determined for control Z

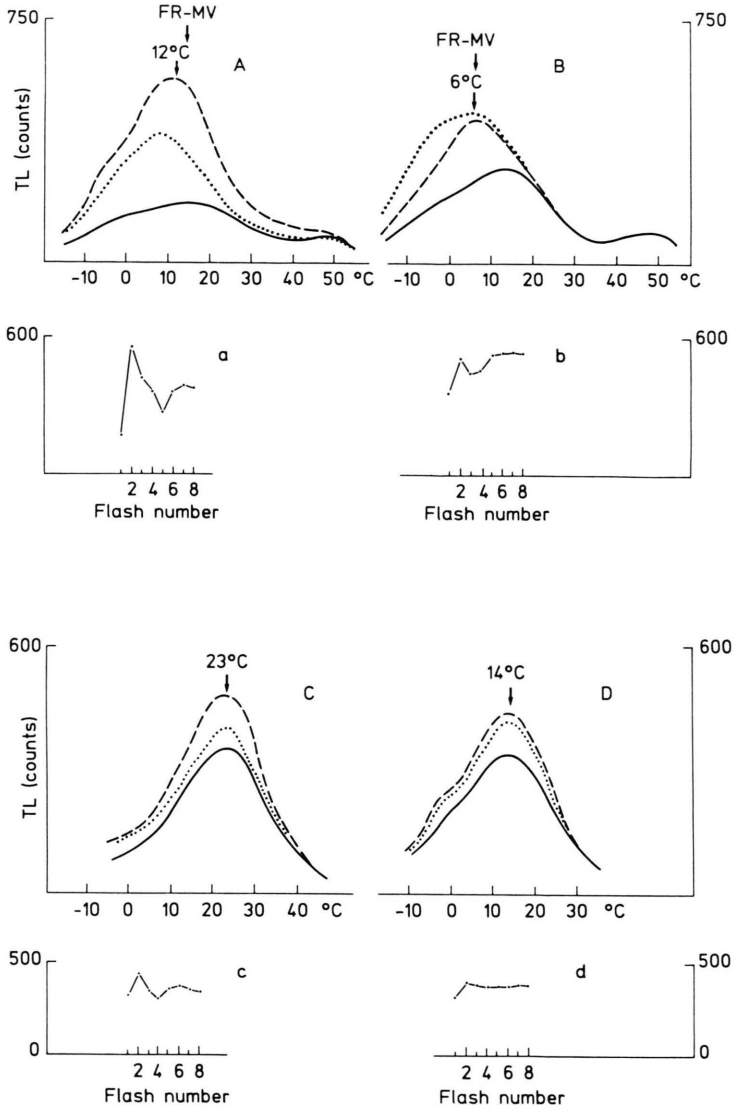

Fig. 1. (A-D) Thermoluminescence glow curves of *Euglena*, dark-adapted for at least 1 hour before the charging of bands by saturating flashes (one to 5) fired at −10 °C at 2 Hz frequency. One flash, ——, two flashes, – – – and five flashes, ·····. (a-d) variation in amplitude of TL bands (the luminescence photons are detected as counts, represented in ordinate, see Methods) as a function of the number of flashes (from one to eight) used to charge the bands. A and C, respectively cells and thylakoids of ZR 25 line; B and D, cells and thylakoids of ZR 250 line. The "arrow FR-MV" indicates the temperature at which is peaking the TL band induced by 2 flashes delivered one minute after a pretreatment consisting in 5 s far-red (FR) illumination in the presence of 100 μM methylviologen.

line (Farineau and Laval-Martin, 1992 and Fig. 2). Some oscillations in the band amplitudes were observed in ZR 25 line, whereas they were nearly absent in ZR 250 line. The amplitude of oscillations was lower for thylakoids than for intact cells as it has already been observed in Z lines (Farineau and Laval- Martin, 1992). This is probably due to a PS II alteration induced by the sonication procedure used for thylakoid preparation.

With the addition to thylakoids of concentrations of DCMU giving 100% inhibition of PSII activity, nearly identical TL curves, both in shape and in position of the peak maximum, could be observed (Fig. 2).

Discussion

Resistance of lines to two herbicides

Whole cells of the two ZR lines exhibit a DCMU-resistance by factors of 1000 for ZR 250 and 100 for ZR 25. By comparison, the corresponding thylakoids exhibited resistances to concentrations 5–10 times lower. Nevertheless and interestingly, the ratios of resistance between Z

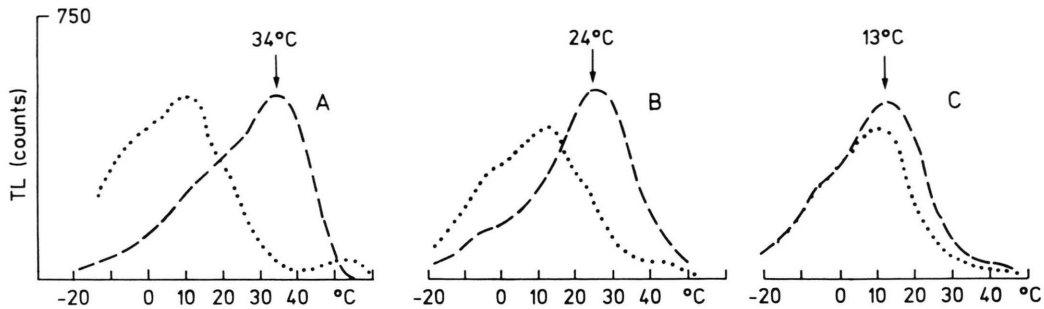

Fig. 2. Thermoluminescence glow curves of thylakoids of susceptible line (Z, A) and 2 resistant lines (ZR 25, B and ZR 250, C) induced by 2 fl. Control: – – –, no addition; DCMU added: ·····, at concentrations 10 μM (A), 100 μM (B) and 500 μM (C). Luminescence photons are detected as counts represented in ordinate.

and ZR 25, on the one hand, and ZR 25 and ZR 250, on the other hand, were preserved (Table I). This indicates that the two degrees of resistance observed *in vivo* correspond to some authentic differences originating in the properties of PS II. It is interesting to note that DCMU-resistant lines were obtained by cultivating cells in photoautotrophic conditions in media containing one of the two concentrations of the herbicide: 25 and 250 μM DCMU. It would be of importance to know if lines with different degrees of resistance - and especially to much higher concentrations, could yet be obtained or not.

Thermoluminescence characteristics

A characteristic feature of TL glow curves described in atrazine resistant weeds (Demeter *et al.*, 1985) could be here evidenced in *Euglena* material consisting in a shift of band maxima towards low temperatures both in cells and thylakoids. Concerning the position of the peak maximum of TL bands of atrazine-resistant higher plants belonging to various species, some diversity exists (Demeter *et al.*, 1985). Thus, thylakoids of ZR 25 line, behave similarly to thylakoids of the atrazine - resistant *Solanum nigrum* line, whereas ZR 250 line presented special characteristics already observed in *Amaranthus retroflexus;* unhapilly, the degree of atrazine resistance in the different higher plants thylakoids was not determined which prohibits further comparison.

Moreover, decrease in amplitude of oscillations of TL bands in a flash regime, especially in ZR 250 cells, was observed. A comparable damping in the oscillations of the oxygen emission under a flash sequence has been previously observed in herbicide-resistant species and attributed to an increased percentage of misses (Holt *et al.*, 1981; Vermaas *et al.*, 1984, Gleiter *et al.*, 1992).

In the two ZR lines here studied, which exhibit an identical atrazine-resistance, two levels of DCMU - resistance were characterized which resulted in: 1) shifts of band maxima much higher in ZR 250 line than in the ZR 25 one, and 2) oscillations damped but still easily observable in ZR 25, while so much damped that they became hardly detectable in ZR 250 (i.e. the percentage of misses would be higher in ZR 250 than in ZR 25). In the ZR 250 line, the TL bands induced by n flashes present features rather similar to those observed in herbicide blocked material (see the effect of 2 fl in the presence and absence of DCMU in Fig. 2, C) in which charge recombinations between the acceptor side (Q_A^-) and the donor side (S_2/S_3) of PSII would occur intensively and be responsible of large misses in the functioning of PS II.

In herbicide-resistant thylakoids, the shift of maxima towards lower temperatures is generally ascribed to a decrease in the value of the midpoint potential for the Q_B^-/Q_B couple, while that of Q_A^-/Q_A would be unchanged (Vermaas and Arntzen, 1983; Demeter *et al.*, 1985). These changes result in a large decrease in the value of the apparent equilibrium constant for the reaction:

$$Q_A^- Q_B \rightleftharpoons Q_A Q_B^-$$

which would contribute to maintain a large amount of $Q_A^- Q_B$ under illumination (Perewoska *et al.*, 1994). The constant, instead of being of

about 20 in susceptible chloroplasts, would then be as low as 1 to 5 in resistant material (Ort *et al.*, 1983; Vermaas *et al.*, 1984; Demeter *et al.*, 1985). According to such a hypothesis and per comparison to the ZR 25 line, the lowest value of this constant might be encountered in ZR 250 cells.

Maxima of TL bands were shifted by 11°C (ZR 25) and 8°C (ZR 250) in cells compared to the respective thylakoids. A shift of comparable amplitude had already been observed in Z cells (12°C) and seems to be characteristic of *Euglena* when comparing this species to other algae or cyanobacteria: for the comparison, see glow curves for dark-adapted material in Ohad *et al.*, (1990) and Gleiter *et al.*, (1992). It was ascribed to some poising of the chain of redox carriers due to metabolic activity able to maintain both the acceptor and donor sides of PS II in reduced states (Farineau and Laval- Martin, 1992). In such cells, an "oxidising" pretreatment (far-red illumination plus methylviologen) allowed recovery *in vivo* of bands of the B type nearly similar to those observed with isolated thylakoids; the shift in position of the corresponding maxima was only 0–3°C (see Fig. 3, in Farineau and Laval- Martin, 1992). However, and at the difference of the results previously obtained for cells of Z line grown autotrophically, the same pretreatment had nearly no effect on ZR lines and an important shift between position of peak maxima for bands *in vivo* and *in vitro* persisted: about 8°C. The limited effect of far-red light on TL properties in the ZR lines is difficult to explain. In fact, the light-treatment could have multiple effects *in vivo* and would not lead only to reoxidation of the PQ pool. Some light-dependent cellular processes (such as phosphorylation of proteins) which could have an important role in determination of the TL properties of thylakoids could be inactivated after mutations in D1 protein.

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