An Amino Acid Substitution in the Q_B-Protein Causes Herbicide Resistance without Impairing Electron Transport from Q_A to Q_B

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Dedicated to Prof. Dr. Achim Trebst on the occasion of his 60th birthday

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Structure and function of the Q_B-protein of a metribuzin resistant mutant of *Chlamydomonas reinhardii* were analyzed. The amino acid residue Leu-275 of the wild type protein is changed to Phe as was determined by RNA-sequence analysis. This mutation caused a 20-fold and 5-fold resistance to metribuzin and DCMU, respectively. No resistance to atrazine was observed. The kinetics of the electron transport from Q_A to Q_B was similar to that of the wild type ($t_{1/2} = 0.4$ ms).

Introduction

The 32 kDa Q_B-protein (*psbA*-gene product) is an integral part of the photosystem II reaction center [1–3]. It has binding sites for the secondary plastoquinone Q_B and for several electron transfer inhibitors (for a review see ref. [4]). The competitive displacement of Q_B by those inhibitors causes interruption of electron flow from photosystem II to the plastoquinone pool [5–7]. Overlapping binding sites of the inhibitors have been proposed which share the binding domain of the Q_B-molecule [8, 9]. Analysis of several mutants of photosynthetic bacteria [10–13], blue-green algae [14–16], green algae [17–21, 40] and higher plants [22–24] support this model by demonstrating that mutagenic induction of herbicide resistance is paralleled by impaired electron flow between Q_A and Q_B.

The isolation of herbicide resistant mutants with unimpaired electron flow and the identification of the mutation provides valuable information concerning the Q_B-binding site [19, 25]. The folding of the membrane protein was predicted by comparing the amino acid sequence of the Q_B-protein with that of the L-subunit in the bacterial reaction center and its three-dimensional structure [2, 3]. The resulting model of the three-dimensional structure of the Q_B-protein can be refined by the analysis of a series of mutants with variant herbicide resistance. Comparing electron transfer rates between Q_A and Q_B with inhibitor sensitivity and relating these data to specific amino acid substitutions in the mutants extend our view of the topography of the Q_B-protein and its structural requirements for binding plastoquinone and inhibitor molecules.

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Materials and Methods

A new mutant was isolated by the following procedure: the *Chlamydomonas reinhardii* strain 137+ was grown under mixotrophic conditions (5% CO_2 in air, 10 mM acetate, 9.5 klx and 20 °C [27]). The cell suspension was diluted to 5×10^4 cells/ml, supplemented with 1 mM 5-fluoro-2′-desoxyuridine, and grown to the stationary phase. 24 h later cells were harvested, washed with 0.1 M phosphate buffer, pH 6.9, and incubated in light (9.5 klx) with 0.27 M methanesulfonic acid ethylester for 1 h. After mutagenesis the cells were washed several times and plated on minimal medium plates supplemented with 10^{-5} M metribuzin and kept in light (6 klx) at 20 °C for two weeks.

Thylakoids were isolated as described earlier [18]. The pI_{50}-values were determined from the effect of

inhibitors on the rate of electron transport from water to dichlorophenol-indophenol under uncoupled conditions [18].

Absorbance changes were monitored at 335 nm (band width 4 nm FWHM) with a photomultiplier (EMI 9818QB) and stored at 17 μs/address in a signal processor (TRACOR TN1500). Electrical band width was d.c. to 10 kHz. The sample was illuminated with additional far-red light of 716 nm with an intensity of 35 W/m^2 to oxidize the acceptor pool of photosystem II. The traces are the average of 3000 signals induced at a repetition rate of 1.4 Hz. Excitation of the algal suspension was $t_{10-90} = 6$ μs by short flashes of red (610–740 nm) light (Schott filter RG 610/3 mm plus Balzers filter Calflex C). Suspensions of intact cells contained 20 μg chlorophyll/ml, 0.1 M Tris-HCl buffer pH 7.5, 10 mM $MgCl_2$.

Total cellular RNAs from *Chlamydomonas* wild type strain and mutant MZ4 were sequenced as described previously [30]. The method is a modification of a procedure initially described by Zimmern and Kaesberg [34] and Hamlyn *et al.* [35] and has been applied to RNA of various sources [36–39]. In brief, a 5′-end-labeled, synthetic oligonucleotide complementary to non-coding strand nucleotides 844–860 [33] was annealed to RNA. Labeled cDNA fragments were produced by reverse transcriptase in the presence of dideoxynucleotides and separated on 8% polyacrylamide-urea gels.

Results and Discussion

Cross-resistancies to inhibitors were tested to characterize a selection of mutants. Mutant MZ4 described here (Table I) showed highest resistance to metribuzin with a 20-fold higher pI_{50} as compared with the susceptible cells (R/S = 20), but only a minor tolerance to DCMU (R/S = 5) and none to atrazine. This differentiation between the binding of metribuzin and of atrazine agrees well with results of cross-resistance analysis with other mutants (V219I, A251V, F255Y and S264A [25, 27] indicating allotopic differences in the binding niche.

Both plastoquinone and the inhibitors bind to the same area of the Q_B-protein. Therefore, it was of particular interest to analyse the electron transport between Q_A and Q_B in MZ4. This aspect was investigated by flash-photometry measuring the absorbance changes at 335 nm. At this wave-length the major contribution is due to the dismutation reaction of the semiquinones $Q_A^- + Q_B^- \rightarrow Q_A + Q_B^{2-}$; the extinction coefficient of the superimposing absorbance changes of the S-state transitions is less than 30% [28] of that of the semiquinone of 10.4 mM^{-1} cm^{-1} [29]. The half-time of the rapid decay of the flash-induced absorbance change shown in Fig. 1 was analyzed by a *semi*-logarithmic plot. It follows a first-order time course with a half-time of 0.4 ms in the wild type cells and 0.43 ms in the mutant MZ4. This difference is within the accuracy of the experiment. The signal amplitudes were not corrected for particle flattening and were found to change during the growing cycle in contrast to the half-time. For wild type cells we compared the kinetics in whole cells with those in isolated thylakoids and found no difference (data not shown).

The agreement of the kinetics in wild type and MZ4 cells shows that the mutation in MZ4 did not alter the electron transfer reaction between Q_A and Q_B. Therefore, those data indicate that binding of Q_B to the Q_B-protein is not affected in the mutant. This is in striking contrast to our measurements of the absorbance changes in the MZ1 mutant. MZ1 with an amino acid change at position 264 from Ser to Ala

Table I. Cross-resistance of the *Chlamydomonas reinhardii* mutant MZ4.

Inhibitor	pI_{50} (WT)	pI_{50} (MZ4)	R/S[1]
Metribuzin[a]	7.2	5.9	20
DCMU[b]	7.7	7	5
Benzthiazuron[c]	6.2	5.6	4
Phenmedipham[d]	7.8	7.6	1.5
Atrazine[e]	6.7	6.7	1
Cyanoacrylate[f]	7.9	8.2	0.5
i-Dinoseb[g]	5.8	5.3	3
J 820[h]	6.7	6.4	2
BNT[i]	7.3	7.1	1.5
Ioxynil[k]	6.1	6.8	0.2

[1] R/S, ratio of I_{50} of the mutant over I_{50} of the wild type; inhibitors: [a], 4-amino-6-(*t*-butyl)-4-methylthio-1,2,4-triazine-5-one; [b], 3-(3,4-dichlorophenyl)-1,1-dimethylurea; [c], 1-(benzothiazol-2-yl)-3-methylurea; [d], methyl *m*-hydroxycarbanilate *m*-methylcarbanilate; [e], 2-chloro-4-(ethylamino)-6-(*iso*-propylamino)-*s*-triazine; [f], L-isomer of 2-cyano-3-methylbenzylamino-3-ethylacrylate ethoxythylester; [g], 2,4-dinitro-6-*sec*-butylphenol; [h], tetrabromo-4-hydroxy-pyridine; [i], bromonitrothymol; [k], 4-hydroxy-3,5-diiodobenzonitrile. The pI_{50}-values were determined graphically from rates obtained in the uncoupled reaction of H_2O to dichlorophenol-indophenol (DCIP) with isolated thylakoids. The assay contained 3.3 μg Chl/ml. Control rates were 78 μmol DCIP/mg/h.

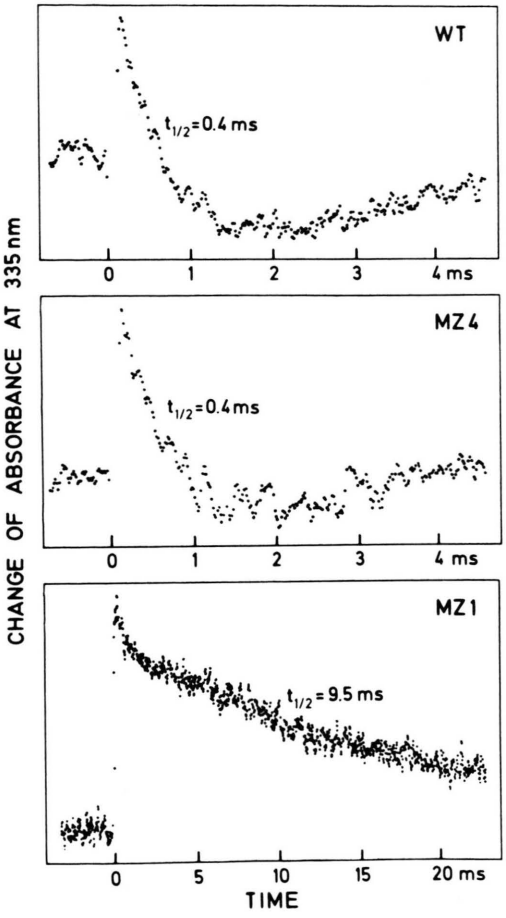

Fig. 1. Absorbance changes in suspensions of intact wild type (wt) and mutant (MZ1, MZ4) cells. Experimental conditions as described in Materials and Methods.

[30] in the Q_B-protein shows a 20-fold slower electron transfer kinetic from Q_A to Q_B. This is in agreement with several resistant biotypes of higher plants [4] and mutants of algae [25]. It has been proposed that the hydroxyl group of Ser-264 is involved in destabilization of the plastoquinol binding at the Q_B-site [31]. A change from Ser to Ala or Gly would increase the binding of the semiquinone in a way that the inhibitor can no longer displace Q_B [32]. As a consequence herbicide resistance is observed.

The molecular basis for herbicide resistance in the MZ4 mutant was determined by RNA-sequence analysis as described earlier [30]. In short, a synthetic, *psbA*-specific DNA oligonucleotide was end-labeled and annealed to total cellular RNA. Labeled cDNA fragments were produced by reverse transcriptase in the presence of dideoxynucleotides. The autoradiograph of the sequencing gel identifies a specific base substitution in mutant MZ4 (Fig. 2, arrow) as compared with the wild type sequence. The transversion from A to C changes amino acid residue 275 from Leu to Phe. No other base changes were observed when about 30% of the *psbA* mRNA comprising helix IV, the loop and part of helix V were sequenced. All mutations known to date have been found within this region. In the mutant and wild type strains screened by sequencing RNA rather than DNA there is premature termination observed at a specific point within codon 276. As determined by inclusion of a primer extension reaction without dideoxynucleotides (Fig. 2, lane B) the termination point is most probably due to RNA secondary struc-

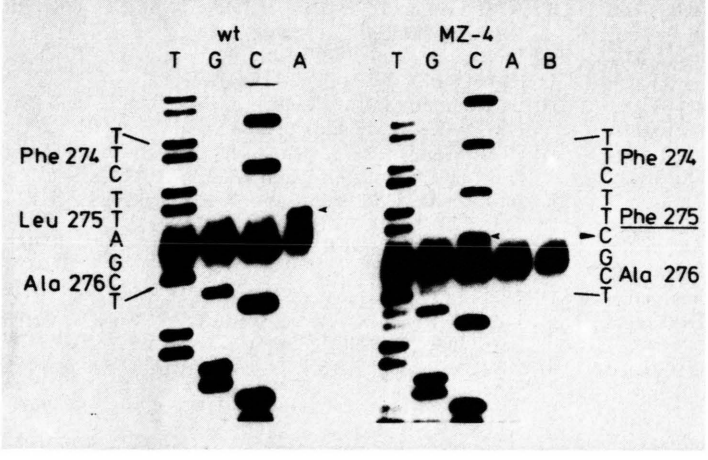

Fig. 2. Autoradiograph of RNA sequencing gels. B indicates a reverse transcriptase reaction without dideoxynucleotides (ddNTPs), A, G, C and T the nucleotides complementary to the ddNTP added. Arrows point to base differences.

ture. Nevertheless, the banding pattern was identified unambiguously using different film exposure times. The base substitution occurs just beyond the termination point and leaves no doubt concerning its identity.

The amino acid residue at position 275 resides at helix V of the Q_B-protein and faces the reaction center core. It is very close to His-272 which is involved in binding of the Fe-atom [31]. The binding niche for electron transport inhibitors reaches from helix IV (Val-219) across the interhelical loop (Ala-251, Phe-255 and Ser-264) to helix V. Our data show that Leu-275 is not involved in the binding of Q_B. This residue should be located in a way which prevents an interference with the Q_B-binding area. This area has been proposed to include the amino acid residues from His-215 to Phe-255 and Ala-263 or Ser-264.

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