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A new mutation in the gene coding for the herbicide-binding protein in *Chlamydomonas*

Udo Johanningmeier, Ulrich Bodner and Günter F. Wildner

Ruhr-Universität Bochum, Lehrstuhl Biochemie der Pflanzen, Postfach 102148, 4630 Bochum 1, FRG

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Sequence analysis of herbicide-resistant *Chlamydomonas* mutants revealed a new mutation in the *psb*A gene coding for the herbicide-binding (D1) protein. A point mutation at codon 251 leading to an amino acid substitution from alanine in wild-type cells to valine in the mutant was identified by sequencing total cellular RNA. The folding pattern of D1 predicts Ala 251 to be part of the Q_B-binding niche.

Herbicide-binding protein; Herbicide resistance; Point mutation; Amino acid substitution; RNA sequencing; (*Chlamydomonas*)

1. INTRODUCTION

The 32 kDa Q_B-binding or D1 protein is the target for inhibitors blocking photosynthetic electron transport in photosystem (PS) II. The *psb*A gene coding for D1 has been sequenced in several plants and algae. By DNA sequence analysis one especially interesting aspect of the herbicide-binding protein, namely herbicide resistance, could be correlated with single point mutations leading to certain amino acid substitutions in D1. Particularly, a point mutation at amino acid position 264 has been detected in two atrazine-resistant higher plants [1,2] and an algal mutant [3] first. In addition, Erickson et al. [4,5] recently described three other amino acid substitutions at positions 219, 255 and 275 in *Chlamydomonas* cells having differential cross resistances towards different herbicides.

The architecture of the herbicide-binding niche is of considerable importance for understanding primary events in PS II and the modelling of herbicides. By analogy to the reaction center of *Rhodopseudomonas viridis* which has been crystallized and analysed by X-ray crystallography and the homology in amino acid sequence [6,7] it was proposed that two polypeptides in PS II, D1 and D2, comprise the reaction center in higher eucaryotes [6,8] and participate in chlorophyll, pheophytin, iron, Q_A and Q_B binding.

The binding site of the secondary acceptor Q_B is thought to be part of or identical to the herbicide-binding site. There is evidence that inhibitors displace Q_B and – depending on the structure – occupy different but overlapping parts of the Q_B-binding niche, thus preventing electron flow from Q_A to the plastoquinone pool [9,10]. The data obtained by mutant analysis strongly support this concept since all amino acid substitutions in D1 known to date are clustered within a sequence stretch which is likely to be involved in Q_B binding (fig.1). The model for Q_A and Q_B binding and of the folding of the D1 and D2 polypeptides was based on the amino acid changes identified in herbicide-tolerant mutants.

We have previously described *Chlamydomonas* mutants showing different cross-resistances towards inhibitors like DCMU, atrazine and metribuzin [11]. We report here on a new amino acid change in one of these mutants. By RNA and

Correspondence address: U. Johanningmeier, Ruhr-Universität Bochum, Biochemie der Pflanzen, Postfach 102148, 4630 Bochum 1, FRG

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DNA sequence analysis we could identify an amino acid substitution at position 251 from alanine in wild-type cells to valine in the *Chlamydomonas* mutant MZ2. The folding pattern of D1 predicts Ala 251 to be part of the Q_B-binding niche.

2. MATERIALS AND METHODS

2.1. *Preparation of RNA*

C. reinhardii cultures were grown in TAP medium [12] to a density of approx. 1.5×10^6 cells/ml and harvested at $3000 \times g$ for 5 min. The pellet was washed with sterile water and resuspended in 3 ml TL buffer (100 mM Tris-HCl, pH 8.5, 400 mM LiCl, 10 mM EGTA, 5 mM EDTA, 1 mM aurintricarboxylic acid) per 4×10^8 cells. Cells were lysed by adding SDS (2%) and proteinase K (50 μg/ml) and subsequent incubation for 10 min on ice. After two phenol/chloroform extractions the nucleic acids were precipitated, washed with 70% ethanol and resuspended in sterile water. DNA was removed by precipitating the RNA with 2 M LiCl.

2.2. *RNA and DNA sequencing*

Approx. 80 μg RNA was coprecipitated with 5×10^6 cpm of a (5'-^{32}P)-labeled oligonucleotide. The precipitate was dissolved in 12.5 μl annealing buffer (50 mM Tris-HCl, pH 8.2, 60 mM NaCl, 10 mM DTT) and placed into 200 ml water of 68°C. After slowly cooling down to about 35°C the annealing mixture was placed on ice and 4.5 μl 5 × RTB (250 mM Tris-HCl, pH 8.2, 30 mM MgCl, 500 mM NaCl, 50 mM DTT), 5 μl dNTP mixture (2 mM) and 1 μl AMV reverse transcriptase (25 U/μl, Boehringer Mannheim) were added. 4 μl of this solution were then added to 1 μl of each of the four ddNTPs (800 μM) and incubated at 45°C for 20 min. For a chase reaction 1 μl of the dNTP mixture was added and the reaction carried out for another 15 min. Finally 1 μl RNase A (10 μg/μl) was added and incubated for additional 15 min. The reaction was terminated by adding 21 μl stop mixture (98% deionized formamide, 10 mM EDTA, 0.1% xylene cyanol and bromphenol blue). The samples were boiled for 3 min and immediately placed on ice. An aliquot of each sample was loaded on 8% polyacrylamide-urea sequencing gels and electrophoresed at 65 W. The gels were exposed to Kodak XAR-5 X-ray film in combination with an intensifying screen at −80°C.

Plasmid pAT153 containing a 10 kbp *Bam*HI/*Bgl*II fragment of *Chlamydomonas* MZ2 chloroplast DNA was sequenced by using the oligomer described in fig.1. Sequencing of the plasmid DNA was carried out according to Zagursky et al. [13].

3. RESULTS AND DISCUSSION

The *psb*A gene coding for the D1 protein in *C. reinhardii* is located within the inverted repeat of the chloroplast genome and thus present in two copies, both of which have been sequenced [14]. Determining the nucleotide sequence of herbicide-resistant mutants by classical methods involving cloning and sequence analysis revealed that both copies carried the same base change. For screening a large number of mutants this technique is time-consuming. It can be replaced by sequencing the corresponding mRNA directly using end-labeled synthetic oligonucleotides as primers for dideoxy-sequencing reactions. This approach obviates the need to clone and sequence two *psb*A copies.

Synthetic DNA primers were designed to hybridize specifically to mRNA regions coding for that part of the D1 protein being most likely involved in herbicide binding (fig.1). As could be seen by Northern blot analysis the primers used for sequencing reactions all hybridized to a 1.2 kb mRNA species in wild-type *Chlamydomonas* RNA as well as in MZ2 RNA (not shown). Annealing radiolabeled oligonucleotides with total RNA isolated from the *Chlamydomonas* mutant MZ2 and extending the primer with reverse transcriptase in the presence of dideoxynucleotides revealed a single base transition from C to T (fig.2). This mutation gives rise to an amino acid change at position 251 from an alanine in the wild type to valine in MZ2. In addition, RNA sequencing also confirmed the predicted splice site [14] between exons 4 and 5.

To verify the base change on a DNA level we determined part of the DNA sequence of a plasmid containing the *psb*A gene of MZ2. Using the rapid sequencing method described by Zagursky et al. [13] the mutation could be confirmed for one of the two *psb*A copies (fig.3).

The ratio of I_{50} values for resistant (MZ2) and

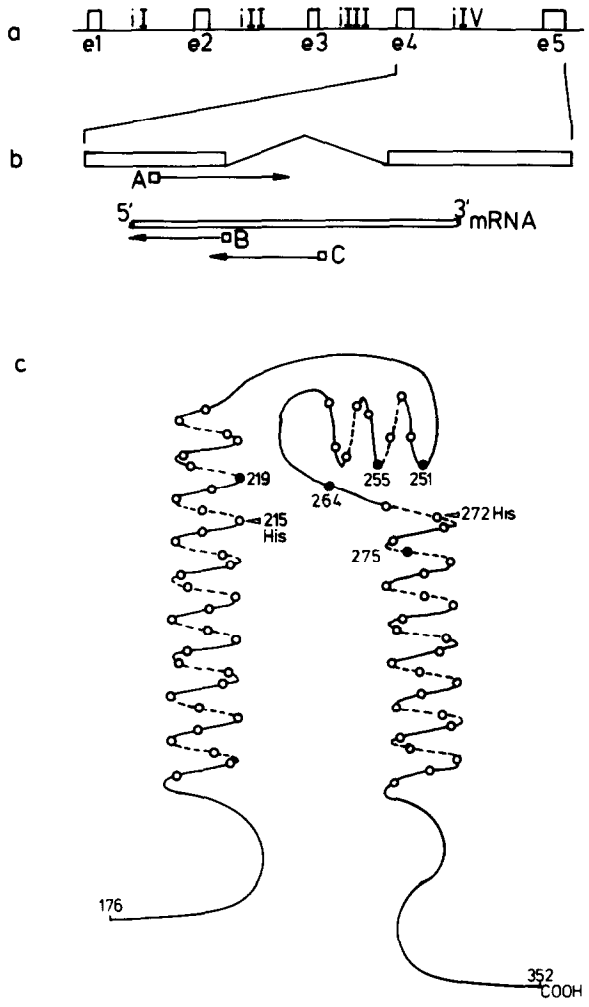

Fig.1. (a) Structure of the *psb*A gene in *C. reinhardii* consisting of five exons (e1–5) and four introns (iI–IV) according to [14]. (b) Expanded view of exons 4 and 5 together with the corresponding part of the spliced *psb*A mRNA. A indicates the position of the primer used for DNA sequencing. Its sequence was chosen to be complementary to the coding strand nucleotides 633–647 [14]. Primers B and C are complementary to the noncoding strand nucleotides 672–685 and 844–860 and were used for RNA sequencing. (c) Part of the D1 protein encoded by exons 4 and 5. Amino acid residues known to be changed in herbicide-resistant mutants are indicated by filled circles. His 215 and 272 are involved in Fe binding. The folding pattern is based on the model proposed by Trebst [8]. Amino acid residues 176–279 were analysed by RNA and in part DNA sequence analysis in the *Chlamydomonas* mutant MZ2.

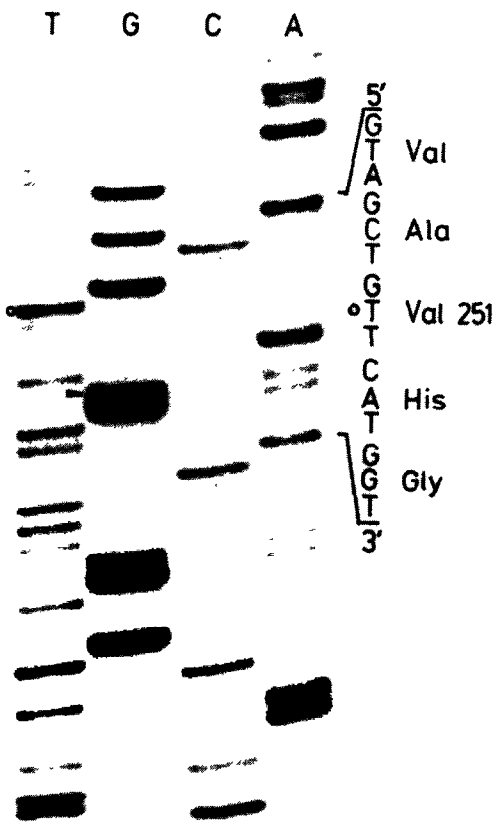

Fig.2. RNA sequencing gel demonstrating the base change in codon 251 leading from an alanine in wild-type *Chlamydomonas* to valine in MZ2. The open circle indicates the mutated base and the arrow the first base in exon 5. A, G, C and T indicate the nucleotide in the RNA, i.e. the nucleotide complementary to the ddNTP added. Primer C in fig.1 was used for sequencing reactions.

susceptible thylakoids were shown to be 1000, 25 and 5 for metribuzin, atrazine and DCMU, respectively. Interestingly, MZ2 is also less susceptible to ioxynil compared with the wild type, whereas all other mutants are more susceptible to this substance [11]. Two other mutants, MZ1 and MZ3 [11], have amino acid substitutions at position 264 (unpublished). Compared to those mutants MZ2 is 25–40-times and 4–5-times less resistant towards DCMU and atrazine, respectively. From these and published data [3,4] one could assume that changes in position 264 have the most disturbing effect on certain inhibitors in the herbicide-binding niche while changes in position 219, 251, 255 and 275

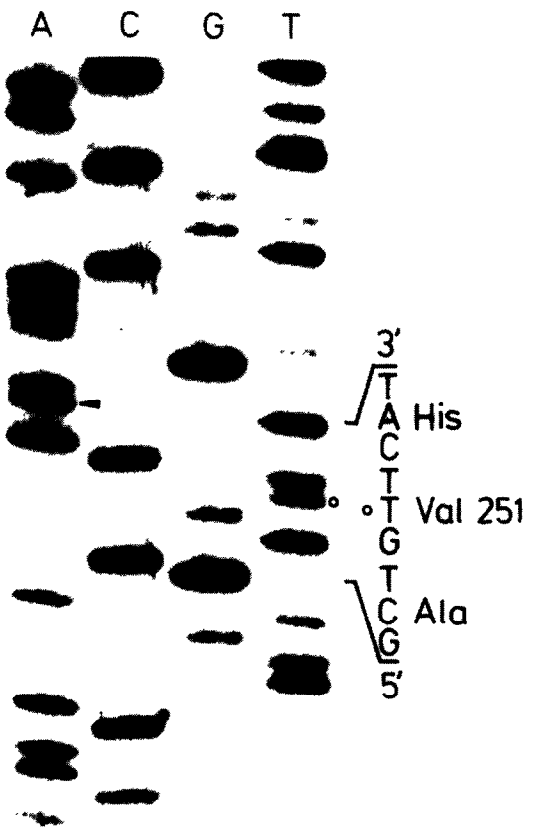

Fig.3. DNA sequencing gel confirming the base change detected by RNA sequencing as shown in fig.2. Plasmid DNA containing the MZ2 *psb*A gene was sequenced using primer A as described in fig.1. Open circles indicate the mutated base and the small arrow points to the first base of intron IV.

have a less pronounced influence. Characterization of more mutants with different cross-resistances will be necessary to estimate the relative importance of single amino acids in binding of different herbicides. The technique of sequencing the *psb*A transcript directly rather than both DNA copies will help in detecting those mutants.

It now seems likely that the amino acids known to be changed in the mutants have a short-range effect on herbicide binding. An additional long-range disturbance, however, might play a role in MZ2 due to the observed substitution. This mutant has an as yet incompletely characterized defect on the water-splitting side. Assuming a direct contact of D1 with proteins on the donor side a slight change in D1 folding could confer this defect.

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REFERENCES

[1] Hirschberg, J. and McIntosh, L. (1983) Science 222, 1346–1349.
[2] Goloubinoff, P., Edelman, M. and Hallick, R.B. (1984) Nucleic Acids Res. 12, 9489–9496.
[3] Erickson, J.M., Rahire, M., Bennoun, P., Delepelaire, P., Diner, B. and Rochaix, J.-D. (1984) Proc. Natl. Acad. Sci. USA 81, 3617–3621.
[4] Erickson, J.M., Rahire, M., Rochaix, J.-D. and Mets, L. (1985) Science 228, 204–207.
[5] Erickson, J.M. and Rochaix, J.D. (1985) in: Abstracts First Int. Congress of Plant Molecular Biology (Galau, G.A. ed.) p.54/OR-25-02, The University of Georgia Center for Continuing Education for the Int. Soc. for Plant Mol. Biol., Athens.
[6] Deisenhofer, J., Epp, O., Miki, K. and Michel, H. (1985) Nature 318, 618–624.
[7] Michel, H., Weyer, K.A., Gruenberg, H., Dunger, I., Oesterhelt, D. and Lottspeich, F. (1986) EMBO J. 5, 1149–1158.
[8] Trebst, A. (1985) Z. Naturforsch. 41c, 240–245.
[9] Velthuys, B.R. (1981) FEBS Lett. 126, 277–281.
[10] Oettmeier, W. and Soll, H.J. (1983) Biochim. Biophys. Acta 724, 287–290.
[11] Pucheu, N., Oettmeier, W., Heisterkamp, U., Masson, K. and Wildner, G.F. (1984) Z. Naturforsch. 39c, 437–439.
[12] Amrhein, N. and Filner, P. (1973) Proc. Natl. Acad. Sci. USA 70, 1099–1103.
[13] Zagursky, R.J., Baumeister, K., Lomax, N. and Berman, M.L. (1985) Gene Anal. Tech. 2, 89–94.
[14] Erickson, J.M., Rahire, M. and Rochaix, J.-D. (1984) EMBO J. 3, 1753–1762.