Non-Mendelian Inheritance of 3-(3,4-Dichlorophenyl)-1,1-dimethylurea-Resistant Thylakoid Membrane Properties in *Chlamydomonas*¹

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ABSTRACT

A uniparentally inherited 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-resistant mutant of *Chlamydomonas reinhardtii*, Dr2, which has a resistance mechanism of the type defined as 'primary,' has been isolated. *In vitro* Hill reactions catalyzed by isolated thylakoid membranes reveal a reduced apparent affinity of the thylakoids for DCMU. These changes in membrane properties quantitatively account for the resistance of mutant Dr2 to herbicide inhibition of growth. The properties of this mutant show that all of the Hill reaction-inhibiting DCMU binding sites are under identical genetic control. Mutant Dr2 is a useful new uniparental genetic marker, since it has a novel phenotype and it may be possible to identify its altered gene product. The low cross-resistance of Dr2 to atrazine suggests that there may be considerable flexibility in exploiting induced herbicide resistance of crop plants for improving herbicide specificity.

Four mendelian mutants in at least three loci all have resistance mechanisms in the class we define as 'secondary.' They are as sensitive as wild type to *in vivo* inhibition of the Hill reaction, and must acquire resistance *in vivo* by preventing the active form of the herbicide from reaching the sensitive site.

There are several chemically distinct classes of herbicides which inhibit photosynthesis by blocking electron transport away from the reduced PSII acceptor, Q (20). DCMU, or diuron, is a potent member of the urea class, and atrazine is a potent and widely used triazine. In addition to having nearly identical physiological effects, these various herbicides show competitive binding to the same site on the thylakoid membrane (29). All of the herbicides share the common structural feature of a sp2 hybrid orbital carbon bonded to a nitrogen atom (9). At the same time, studies of substituent effects on the binding of different chemical classes show that one class may have important contacts with the binding site which are in a different spatial domain from some of the contacts which are important to a different class. Thus, it seems that each chemical class of these herbicides shares a portion of its binding site with all of the others, but that a portion is specific for that class (2).

The chemical composition of the binding site has been investigated with both biochemical and genetic methods. An azido derivative of atrazine which retains competitive binding to this site becomes covalently bonded to a 32,000 D thylakoid membrane polypeptide when the complex is UV irradiated (6). The reaction of this protein is dependent upon specific binding at the site, since it cannot be labeled in thylakoids from an atrazine-resistant biotype of *Amaranthus* which do not bind atrazine (23). Thus, this 32,000 D polypeptide is either part of or close to the binding site, but it is not known whether this part is shared with other herbicides or whether it interacts uniquely with triazines. Mattoe *et al.* (16) have shown that a 32,000 D polypeptide of *Spirodea* thylakoids, which is ordinarily quite sensitive to trypsin treatment of the membranes, is rendered resistant to digestion upon DCMU binding. If this protein is homologous with the azido-atrazine labeled protein, then it is apparent that it has important interactions with both triazines and ureas. Oettmeier *et al.* (21) found that an azido derivative of dinoseb (a dinitrophenol) acted as a photoaffinity label for a spinach thylakoid membrane polypeptide in the 40,000 D range. If this protein is not homologous to the atrazine-labeled protein, then one must conclude that the binding sites for the different classes of herbicides reach into the region of at least two proteins. It will be of interest to determine if herbicide resistance can arise from mutations in more than one thylakoid protein gene.

We have initiated a study of resistance to DCMU in *Chlamydomonas reinhardtii* with two main objectives (a) By using genetic variants, we hope to identify the molecular components of the sensitive site on the thylakoid membranes. (b) In the process, we hope to obtain a new chloroplast gene marker in this species which could be used in mapping studies of the chloroplast genome and whose gene product might be identifiable.

It is reasonable to assume that altered membrane properties could arise from mutations in chloroplast genes. Many thylakoid membrane polypeptides are known to be synthesized within the chloroplast, and we adopt the working hypothesis that they are also coded there. Indeed, atrazine resistance in higher plants has proven to be a maternally inherited character (5, 15). This genetic behavior is consistent with a chloroplast location of the mutation. On the other hand, many thylakoid proteins are synthesized in the cytoplasm and presumably coded in the nucleus. It was interesting to determine if thylakoid alterations producing DCMU resistance can be produced by mendelian or non-mendelian mutations.

MATERIALS AND METHODS

Cultures of *Chlamydomonas reinhardtii* were grown on Trisphosphate minimal medium (1.5% agar) as described by Surzycki (27) at 25°C and 4000 lux. Cells to be harvested for thylakoid membrane preparations were grown in liquid minimal medium aerated with 5% CO₂. Mutagenesis was carried out in 10 mM acetate liquid medium in the dark as described previously (26). Cells were pre-grown in 1 mM 5-fluorodeoxyuridine and then treated with ethylmethane sulfonate. The cells actually used in these experiments were mu-

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tagenized by R. Spreitzer and were from the same batch screened for acetate-requiring mutants in the previously described experiment 12 (26). DCMU-resistant (Dr) mutants were selected by plating 2 to 3 × 10^5 cells/plate on minimal medium containing 3 μM DCMU at 4,000 lux. Several independent mutants were selected and maintained in the absence of DCMU for further study. Strains GB221, mt* and GB222, mt*, both of which contain uniparental erythromycin and streptomycin resistance, were kindly supplied by E. Harris, Duke University. They were used in crosses with the mutants to determine the inheritance pattern of DCMU resistance. Resulting tetrads were scored for erythromycin, streptomycin, and DCMU resistance or sensitivity by replica plating to media containing 100 μg/ml erythromycin, 200 μg/ml streptomycin, or 3 μM DCMU, respectively.

DCMU resistance levels of the mutants and wild type were compared by examining growth over a range of DCMU concentrations from 200 μM down to 0.1 μM. Three growth conditions were used: phototrophic (minimal medium, 4,000 lux); heterotrophic (acetate medium, dark); and mixotrophic (acetate medium, 4,000 lux). Each strain was tested by spotting a single drop of 2 to 3 × 10^5 cells/ml on each plate of herbicide-containing medium. Resistance levels were scored by determining the DCMU concentration which completely inhibited growth.

Thylakoid membranes were prepared as described by Levine and Gorman (13). The preparative procedure was slightly altered with modifications from Lien et al. (14) for the disruption of cells with the YEDA press and additional purification of the thylakoid membranes by differential centrifugation.

Hill reactions using 0.1 mM DCIP* as the electron acceptor were used to test PSII activity and DCMU sensitivity of purified thylakoids. DCIP reduction was followed spectrophotometrically at 590 nm. The actinic light source (247 μE/m^2/s) was passed through a 640 nm long pass filter from Ditric Optics. The spectrophotometer photomultiplier tube was protected from actinic light by a 590 nm narrow pass interference filter. Each reaction mixture contained 4 μg Chl/ml. Stocks of DCMU or atrazine dissolved in 95% ethanol were added to give the appropriate herbicide concentration at no more than 1% ethanol. Control reactions were performed with 1% ethanol. To analyze these data, we assume that the reversible interaction of DCMU with the membrane is governed by a simple binding in which all sites have equal affinity for the herbicide. The dissociation constant (K_d) is given by K_d = [E][D]/[E][D]. [D] is the concentration of DCMU in solution, [E] is the concentration of DCMU binding sites which are occupied, and [E] is the concentration of unoccupied sites.) Estimates of the number of Chl molecules per DCMU binding site range from 300 to 1,000 (11, 29). In our reactions, the total concentration of binding sites ([E] + [ED]) is therefore in the range of 0.5 to 1.5 × 10^10 M. Over the range of DCMU concentrations we have used, [D] is very close to the concentration of DCMU added to the reaction mixture. We relate the equilibrium equation to kinetic parameters by assuming that the measured rate of the Hill reaction (V) is proportional to the concentration of unbound sites (i.e. all sites have equivalent activity). Thus, V = A[E], where A is a proportionality constant. At zero DCMU concentration, the measured rate (V_o) is proportional to the total concentration of binding sites. Thus, V_o = A[(E) + (ED)]. Using these two relationships, the equilibrium equation may be reworked to obtain V_o/V = (1/ K_d)[D] + 1. A plot of V_o/V versus [D] should yield a straight line with a slope of 1/K_d and an intercept of one. When V_o/V = 2 (50% inhibition), K_d = [D]. The operationally defined I_50 (concentration of DCMU which gives 50% inhibition) is therefore equivalent to the dissociation constant in this model for DCMU action. The I_50 values which we report were calculated from the slopes of these plots.

* Abbreviation: DCIP, 1,6-dichlorophenolindophenol.

Growth rates were estimated from measurements of colony diameters. Cells were plated at less than 100 cells/plate and incubated at 25°C, 4,000 lux. The diameters of about 20 colonies were measured every day using a calibrated ocular micrometer in a dissecting microscope. Under these conditions, there is a lag period followed by a linear increase in diameter which continues for at least 5 d. The slope of this linear portion (estimated by linear regression) was taken as the growth rate. For wild type and Dr2, the rates in the absence of herbicide were 109 and 101 μm/d, respectively.

RESULTS

Wild type cells are far more sensitive to DCMU under phototrophic growth conditions than under either heterotrophic or mixotrophic conditions (Table I). We selected mutants which could grow phototrophically on 3 μM DCMU in the hope of obtaining mutants specifically altered in the influence of DCMU on photosynthesis. The frequency of cells which can form colonies under these conditions is extremely low (about 5 × 10^7 per viable mutagenized cell) compared with other mutant phenotypes selected from the same mutagenized cell population (26). This suggests that only very specific types of mutations can lead to DCMU resistance. The five mutants which we have studied in detail (Dr2, Dr4, Dr8, Dr9, Dr10) are all more resistant than wild type under phototrophic conditions, but are similar to wild type in their sensitivity on acetate medium (Table I). For all of them, DCMU is still a selective inhibitor of phototrophic growth.

To characterize the mechanism of resistance more fully, we studied DCMU inhibition of the Hill reaction catalyzed by thylakoid membrane preparations. A plot of inhibition of the Hill reaction versus DCMU is shown in Figure 1. A linear transformation of these data was used to estimate accurately the DCMU concentration required to achieve 50% inhibition (Fig. 1, inset). This number is equivalent to an apparent membrane-DCMU dissociation constant (see "Materials and Methods"). The I_50 for Dr2 is considerably higher than that for wild type, but the other mutants are all indistinguishable from wild type (Table II). The linearity of this plot for Dr2 implies that all of the DCMU-sensitive sites are equivalently modified. The altered I_50 of Dr2 is sufficient to explain its resistance to DCMU inhibition of growth. Both show a 10- to 20-fold difference from wild type (Table I; Fig. 1). Also, the percent inhibition of colony growth rate at a range of DCMU concentrations is quite comparable to the percent inhibition of the Hill reaction for both wild type and Dr2 (Fig. 2). This suggests that the growth rate is directly related to the number of active PSII centers and that there is no significant concentration gradient of DCMU between the inside of the chloroplast and the medium in these two strains. The growth resistance of Dr4, Dr8,
The relative rates of H₂O to DCIP Hill reactions catalyzed by thylakoid membranes of wild type (○) and mutant Dr2 (Δ) at various DCMU concentrations. The rate at zero DCMU concentration is taken as 100%. The inset graph shows the control rate divided by the rate observed at a particular DCMU concentration. The inset table shows the calculated I₅₀ values.

Table II. Mutant and Wild-Type I₅₀ Values from Hill Reactions

<table>
<thead>
<tr>
<th>Strain</th>
<th>I₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.10</td>
</tr>
<tr>
<td>Dr2</td>
<td>1.72</td>
</tr>
<tr>
<td>Dr4</td>
<td>0.09</td>
</tr>
<tr>
<td>Dr8</td>
<td>0.08</td>
</tr>
<tr>
<td>Dr9</td>
<td>0.08</td>
</tr>
<tr>
<td>Dr10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Dr9, and Dr10 cannot be explained by a change in the thylakoid membrane and must arise by some other mechanism.

Inasmuch as Dr2 has an altered DCMU-sensitive site on the thylakoid membranes, it was of interest to check for cross-resistance to atrazine. As seen in Figure 3, the I₅₀ for atrazine inhibition of the Hill reaction is higher for Dr2 than for wild type. Again, the sensitive sites seem to be altered as a single homogeneous class. The relative resistance of Dr2 to atrazine (I₅₀-Dr2/I₅₀-wt) is

2, whereas the relative resistance to DCMU is 17. Thus, the Dr2 mutation has a significant, but comparatively minor effect on atrazine cross-resistance of the membranes.

All of these mutants have shown stable inheritance of their DCMU resistance character during maintenance for over 2 years in the absence of herbicide, and they all transmit the character through meiosis in crosses. Dr4, Dr8, Dr9, and Dr10 all show 2:2 segregation of resistant and sensitive meiotic progeny in complete tetrads. Crosses among themselves indicate that they represent mutations in at least three different nuclear genes. Dr2, on the other hand, shows the characteristic features of uniparental gene inheritance (7). Most of the tetrads from a given cross show 4:0 transmission of the Dr or Drs allele present in the mt" parent. A few tetrads (1−5% in different crosses) show biparental transmission of both alleles through meiosis followed by segregation during subsequent mitotic divisions. Standard uniparental genetic markers (streptomycin resistance, Sr, erythromycin resistance, Er) present in the crosses also show biparental inheritance in these same tetrads. From biparental tetrads, we have recovered strains with recombinant combinations of Dr, Er, and Sr markers. The quantitative analysis of these recombinant types will be presented in detail elsewhere. Extremely rarely, we have seen tetrads which show 4:0 transmission of the Dr or Drs allele present in the mt" parent. We have never seen a tetrad which shows 2:2 segregation of Dr2. Because chloroplast DNA shows uniparental inheritance in Chlamydomonas (8, 19), Dr2 (along with other uniparental genetic markers) is a candidate for a chloroplast gene mutation.

We have studied complete tetrads from crosses of Dr2, mt" × GB221, mt" and GB222, mt" × Dr2, mt" and found that the Hill reaction resistance and growth resistance are co-inherited in a uniparental pattern (Table III). These results support the conclusion that the growth resistance arises from the increased membrane resistance to DCMU. They also exclude the possibility that the membrane alteration in Dr2 is determined by a mendelian gene.

**DISCUSSION**

**Biochemical Mechanism of DCMU Resistance.** The resistance mechanisms we have found can be described as either 'primary' or 'secondary' depending upon whether or not they have an alteration in the interaction of the herbicide with its site of action. The specific primary mechanism in the case of Dr2 involves a reduction in the apparent affinity of the thylakoid binding site for DCMU. The effect of herbicide binding is still the same as it is in wild type: bound sites are completely inactive in electron transport. The primary mechanism of atrazine resistance which has been seen in several weed biotypes also involves a reduction in the
Table III. Inheritance of Resistance in Tetrads

<table>
<thead>
<tr>
<th>Mating Type</th>
<th>Phototrophic Growth Inhibition Level</th>
<th>I₀</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tetrad A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. mt⁺</td>
<td>3</td>
<td>0.08</td>
</tr>
<tr>
<td>2. mt⁻</td>
<td>2</td>
<td>0.10</td>
</tr>
<tr>
<td>3. mt⁺</td>
<td>2</td>
<td>0.08</td>
</tr>
<tr>
<td>4. mt⁻</td>
<td>3</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Tetrad B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. mt⁺</td>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>2. mt⁺</td>
<td>10</td>
<td>1.5</td>
</tr>
<tr>
<td>3. mt⁺</td>
<td>50</td>
<td>1.6</td>
</tr>
<tr>
<td>4. mt⁻</td>
<td>20</td>
<td>1.6</td>
</tr>
</tbody>
</table>

herbicide affinity of the thylakoid membranes (6, 22, 23) with no change in the effect of binding (2, 22). It is theoretically possible that primary resistance could arise from a change in the effect of herbicide binding rather than from a simple change in affinity, but no actual examples have yet been found.

There are numerous biochemical resistance mechanisms which fall into the secondary class. These include reduced cell or chloroplast permeability and biochemical inactivation of the herbicide. We have not yet determined which of these might be operating in the case of mutants Dr4, Dr8, Dr9, and Dr10. It is interesting, however, that these mutants are unaltered in their sensitivity to DCMU inhibition of heterotrophic growth. This suggests that DCMU has reduced access to the thylakoid binding site, but that its access to the site which inhibits non-photosynthetic growth is unaltered. In a previous study of DCMU-resistant *Chlamydomonas* mutants, McBride et al. (17) also isolated both primary and secondary resistance mutants. We have found that these two biochemical classes may also correspond to two distinct genetic classes (see below).

Recent physiological measurements have been interpreted to indicate the presence of two functionally, and perhaps structurally distinct PSII complexes, referred to as PSII-α and PSII-β (10, 18, 28). Our data place limitations on the applicability of this concept. To measure apparent herbicide binding, we assume that all of the binding sites have equivalent affinities for the herbicide and that all bound herbicide molecules produce equivalent inhibition of electron transport (no cooperativity). Because we obtain straight lines in reciprocal plots (Figs. 1 and 3), these assumptions appear valid for sensitive strains as well as the Dr2 mutant. Thus, the homogeneous population of herbicide binding sites in the wild type is entirely altered by the Dr2 mutation to a new homogeneous class with resistant properties. This result argues that the DCMU-sensitive sites in individual electron transport chains are all equivalent biochemically and that they are all determined in the same way genetically. If there are physiologically distinct PSII centers in the H₂O to DCIP pathway, then they all must contain the same DCMU-sensitive component and respond in the same way to DCMU binding. Any structural differences must reside in other components of the PSII complex.

Mutant Dr2 was selected for resistance to DCMU, and yet shows slight cross-resistance to atrazine. Lien et al. (14) found a similar cross-resistance to another triazine herbicide, simazine, in DCMU-resistant *C. reinhardtii* mutant, Dr-18. Dr2 (and Dr-18; Ref. 14) probably has an alteration in the DCMU-specific portion of the inhibition site which affects the portion shared with triazines only slightly. Several weed biotypes with naturally arising atrazine resistance have a complementary type of change in their thylakoid membranes (1, 22). They show strong resistance to atrazine, but only slight cross-resistance to DCMU. Even though these two herbicides inhibit photosynthetic electron transport at the same point and interfere with each other in binding at their site of action, genetic modification can selectively affect the binding of one and not that of the other. This implies that there could be considerable flexibility in exploiting induced resistance of crop plants for improving herbicide specificity.

Another interesting difference between the several atrazine-resistant weed varieties and *C. reinhardtii* mutant Dr2 is in the properties of electron transport near PSII. The resistant weed biotypes, when compared with the respective sensitive varieties, all show a reduced rate of re-oxidation of the primary PSII acceptor, Q (1, 3, 5). This has been directly measured by studying the rate of restoration of the fluorescence quenching ability of Q following a single saturating laser flash (3, 5). The alteration also dramatically influences the shape of the Chl fluorescence induction transient. Illumination of dark-adapted chloroplasts from the resistant varieties produces a more rapid fluorescence rise from the initial level, F₀, to a higher intermediate level, F₁, as compared with transients observed in sensitive chloroplasts (1). The fluorescence induction transient of mutant Dr2 is indistinguishable from that of wild type (data not shown), which indicates that it has no comparable change in the DCMU rate.

The properties of Dr2 demonstrate that reduced sensitivity of the PSII reaction to herbicides need not be associated with disrupted PSII function. It may be that such disruptions are only associated with changes in the triazine-specific portion of the binding site. On the other hand, the relative resistance of the weeds to atrazine (2) is large (500 to >1000), compared with the modest relative resistance of Dr2 to DCMU (=17). Perhaps the magnitude of the relative resistance reflects the severity of the change in the binding site, and only more drastic changes disrupt PSII function. It is also possible that the atrazine resistance and the altered electron transport of the weeds may be separate consequences of independent genetic alterations. This possibility seems unlikely, since altered electron transport has been observed in several independently collected atrazine resistant weeds of several different species. Also, Darr et al. (5) have demonstrated that both traits are maternally inherited, excluding the possibility that one is nuclear and the other plastidial trait. However, because recombination cannot occur between strictly maternally inherited traits, the possibility of independent mutational origin in two different plastid genes cannot be formally excluded. This problem also arises in interpreting the experiments of Pillai and St. John (24) which demonstrate a difference in the lipid composition of the thylakoid membranes between the atrazine-resistant and sensitive weed biotypes. It will be difficult to determine if the lipid alteration is mechanistically related to the herbicide resistance. Further studies of *Chlamydomonas*, in which chloroplast gene recombination can occur, seem promising as a way of resolving these issues.

**Genetic Basis of DCMU Resistance.** The DCMU resistance of the strains we have examined in this study is stable during vegetative propagation in the absence of herbicide and is transmitted through meiosis in sexual crosses. It clearly has a genetic basis, in contrast to the DCMU resistance which has been observed in Euglena (12). In that case, resistance appears during adaptive growth in DCMU and disappears when the herbicide is removed.

Our results demonstrate that DCMU resistance can arise from mutations in either mendelian or non-mendelian genes. It is interesting that these two genetic classes also differ in their basic resistance mechanisms (see above). It may be that the chloroplast genome controls the structure of the DCMU binding site on the thylakoid membranes while the nuclear genome controls one or more aspects of the access of DCMU to its binding site.
mutants must be studied to determine the generality of this conclusion. The Dr2 mutant which we have described is of considerable value as a new uniparental genetic marker in C. reinhardtii. Because its phenotype involves a property of the thylakoid membrane, we do not anticipate allelic interactions with the available antibiotic resistance markers which affect properties of the chloroplast ribosomes. Since the Dr phenotype is determined independently and can be scored independently, it can be included in multifactor crosses with antibiotic resistance markers in order to refine and extend the uniparental genetic map. Preliminary mapping results show that Dr2 must be substantially outside the cythromycin resistant (er-u-II) and streptomycin resistant (Sr-sm2) markers which define the outer limits of the current maps from Gilliam's laboratory (7).

The properties of Dr2 allow us to focus on the thylakoid membrane in an attempt to identify its mutant gene product. Uniparental mutants which have been studied recently are missing several thylakoid polypeptides (4, 25). For these mutants, it will be difficult to identify the primary lesion. Dr2 is more promising, since it has no observable deficiencies or alterations in mol wt of thylakoid membrane polypeptides (data not shown). Assuming that the DCMU binding site is controlled by a protein, we may be able to identify a single altered protein in the mutant and determine if the alteration is genetically linked to the DCMU-resistant phenotype.

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