Chloroplast Ultrastructure in Mutant Strains of Chlamydomonas reinhardtii Lacking Components of the Photosynthetic Apparatus

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Abstract. The fine structure of the chloroplast of wild-type and 9 photosynthetic mutant strains of Chlamydomonas reinhardtii is described. The chloroplast phenotypes of the mutant strains are clearly distinct from the wild type in all but 2 cases. Moreover, strains with similar photosynthetic disabilities have structurally similar chloroplasts. These differences are apparently not the result of altered chlorophyll content, nor of photosynthetic inactivity. It is therefore proposed that the structural alterations are in some way related to the mutant strains' inability to synthesize active components of the photosynthetic electron transport chain.

The chloroplast of the unicellular green alga, Chlamydomonas reinhardtii, when viewed with the electron microscope (16, 29, 30, 35), exhibits a phenotype characteristic of other species of Chlamydomonas (8, 21), other members of the Volvocales (19), and many other green algae (17). The basic structural components of the chloroplast are membranes. These are organized into very long, flat vesicles called discs (thylakoids); the discs, in turn, are appressed to one another in such a way as to form an elaborate, anastomosing array of "stacked" membranes. In thin section, then (Fig. 2), the membranes of the wild-type chloroplast are seen either as single discs or, more frequently, in stacks of from 2 to 10 discs. A given disc may leave one stack to join another (Fig. 2, arrow), but the length of such "unstacked" discs is rarely extensive. It is also apparent in Fig. 2 that the stacks are not constant in size over long distances: a stack of 4 soon bifurcates, then acquires a third disc, fuses with an adjacent stack of 2, and so on. Such a "confluent" arrangement of discs is clearly distinctive from the long, regular stacks found in most other divisions of algae (17). It is also very different from the chloroplast membrane system of higher plants, in which short, uniform segments of many stacked discs ("grana") are interconnected by a fretwork of long, single discs.

The components of the photosynthetic electron transport chain are known to be associated with the membranes. However, it is not known what role, if any, the complex and very specific patterning of these membranes plays in the photosynthetic process. In an attempt to answer this question, a study has been made of a number of mutant strains of C. reinhardtii that are unable to carry out photosynthesis. The mutant gene in each strain exhibits Mendelian segregation and the affected component has, in most cases, been identified (see 22 for review). It has thus been possible to examine the effect of the absence or inactivity of a given photosynthetic component on the morphology of the C. reinhardtii chloroplast or, more specifically, on the 3 levels of chloroplast organization: the formation of membrane, the assembly of membrane into discs, and the complex patterning of the anastomosing stacks of discs.

Materials and Methods

Culture of the Organism. Cultures of wild-type (strain 137c, mating-type plus) and mutant strains (ac-1, ac-21, ac-80a, ac-115, ac-141, ac-205, ac-208, F-1, F-34, and F-60) of C. reinhardtii were grown either on tri-acetate-phosphate medium (12), on minimal medium (41), or on minimal medium supplemented with 0.2% sodium acetate. Cultures were maintained at 25° in continuous light of either 2000 or 4000 lux from daylight fluorescent lamps, and they were agitated on rotary shakers. Cells were harvested during the logarithmic phase of growth—36 hr for the wild type and 72 hr for most of the mutant strains.

Electron Microscopy. Fixation and embedding procedures were as previously described (16) except that in some cases, 4 X 10^{-5} M potassium phosphate buffer, pH 7. was substituted for the collidine buffer. The quality of fixation with either buffer can be comparable, but the phosphate buffer is more reliable and therefore preferable. Each strain was fixed at least twice with essentially identical results. A Hitachi HU 11C electron microscope was used throughout the study. The plates for Figs. 2 to 8 were photographed at original magnifications of 42,500×.

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Stacking Profiles. Chloroplast structure was analyzed, in part, by a modification of the method described by Teichler-Zallen (42). Electron micrographs of randomly-sectioned cells were collected by systematically photographing, at magnifications of 14,000 or 23,000 X, every cell to appear in a given grid square. Four random lines were drawn across each micrograph, and the number of chloroplast discs in every transversely-sectioned (i.e. scorable) stack crossed by a line was counted. At least 18 different cells and from 500 to 1000 discs were scored for each strain. The total number (N) of stacks containing a given number (n) of discs was then calculated for each strain. These numbers are most meaningfully expressed by plotting the percent probability of finding a given chloroplast disc in a stack of a given size, i.e. \( \frac{nN}{\Sigma nN} \), against the number of discs per stack (n). Such plots are referred to as stacking profiles.

Chlorophyll Determinations. Total chlorophyll and chlorophylls a and b were determined by a modification (1) of the method of Mackinney (25). Cells were counted with the aid of a hemacytometer.

Photosynthetic Capacity. Diagnostic tests were performed in order to be certain that the mutant strains posses-ed the photosynthetic properties previously ascribed to them. The procedures for these tests have been described elsewhere (4,23,24) except for measurements of photosynthetic oxygen evolution, which were obtained with a Yellow Springs Instrument Company Clark-type oxygen electrode.

Results

The Wild Type. The fine structure of the wild-type chloroplast of C. reinhardi, shown in Fig. 2 and described in the Introduction, is depicted by the first 2 stacking profiles of Fig. 1. It is seen that the discs have a greater tendency to form stacks of a large size (n > 5) when the cells are grown auto- trophically on minimal medium than when grown mixotrophically in the presence of acetate. Larger stacks also tend to dominate the chloroplasts of cells from stationary-phase cultures (not shown). Otherwise, the shapes of the 2 curves are similar, with a peak at n = 3.

At the outset of this study, it was important to determine whether the wild-type profiles were altered if wild-type cells were cultured under conditions which did not permit photosynthesis. If alterations occurred, then clearly any similar alterations which might be found in the chloroplasts of photosynthetic mutants could be most readily attributed to the fact that the mutant chloroplasts were not actively engaged in photosynthesis at the time they were fixed, i.e. that one was merely observing a “dormant” chloroplast.

To test this possibility, wild-type cells were grown in the light in the presence of 10 \( \mu \text{M} \) 3.(3,4-dichlorophenyl)1,1-dimethyl urea (DCMU). At the time of fixation, the cells exhibited no photosynthetic oxygen evolution. The stacking profile of DCMU-inhibited wild-type chloroplasts (Fig. 1), however, is not significantly different from the profiles of actively-photosynthesizing wild-type chloroplasts, except that the DCMU-treated cells tend to form somewhat larger stacks, a phenomenon that has been noted in other organisms (18,37).

Chlorophyll Variation. The total chlorophyll content of log-phase cells from wild-type and certain mutant strains is given in Table I. The wild-type value fluctuates with the age of the culture, growth conditions, and probably many other variables. The chlorophyll content of the photosynthetic mutant strains fluctuates similarly, but the range tends to be somewhat lower, i.e. they are somewhat chlorophyll-deficient compared to wild type. It should be noted, however, that the chlorophyll content of cells of the wild-type strain has increased over the years that it has been kept in stock in this laboratory. Moreover, the wild-type cells are generally larger, on the average, than those of most mutant strains under comparable growth conditions. Thus, it might rather be said that wild type is pigment-excessive compared to the other strains.

Table I. Representative Values for Chlorophyll/Cell and Chlorophyll a:b in Wild-type and Mutant Strains of C. reinhardi

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total chlorophyll ( \mu g/10^6 \text{ cells} )</th>
<th>chl a : chl b</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3.0 - 4.6</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>F-60</td>
<td>2.9</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>ac-21</td>
<td>2.8</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>ac-80a</td>
<td>3.9</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>F-1</td>
<td>3.3</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>ac-206</td>
<td>2.3</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>ac-208</td>
<td>3.3</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>ac-115</td>
<td>3.7</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>ac-141</td>
<td>2.6</td>
<td>2.6</td>
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</tr>
</tbody>
</table>

In any case, it was necessary to determine whether variations in chlorophyll content affect chloroplast fine structure. This was done in 2 ways. Samples of a wild-type culture were taken at 12 and at 36 hr after inoculation and fixed for electron microscopy. The chlorophyll contents of the 2 samples were, respectively, 2.2 and 4.7 \( \mu g \) chlorophyll/10^6 cells, yet the chloroplast structures of cells from the 2 samples were comparable (not shown). A more definitive answer was obtained with the use of ac-1, a markedly pigment-deficient but photosynthetically-active strain of C. reinhardi. The chlorophyll content of ac-1 ranges between 0.8 and 1.3 \( \mu g \) chlorophyll/10^6 cells, which is about 25% of wild type and much lower than any of the photosynthetic mutant strains (table I). The fine structure of the...
Fig. 1. Stacking profiles of wild-type and mutant strains of C. reinhardii. A plot of \(100 \frac{nN}{\Sigma nN}\) vs. \(n\) is given for each strain, where \(n\) = number of discs in a stack and \(N\) = total number of stacks of size \(n\).
Fig. 2. Wild-type chloroplast, showing stacks of from 2 to 5 discs. At arrow, single disc is seen "switching" from 1 stack to another. St., starch; S, stroma containing chloroplast ribosomes; CE, chloroplast envelope. 71,000×.

Fig. 3. ac-1 chloroplast, exhibiting wild-type pattern of stacks containing 2 to 4 discs. 71,000×.

Fig. 4. ac-80a chloroplast, showing several long, wide stacks of discs. 71,000×.

Fig. 5. F-1 chloroplast, exhibiting long, wide stacks of discs. 71,000×.
Fig. 6. ac-206 chloroplast with long stacks of 2 discs predominating. 71,000×.

Fig. 7. ac-115 chloroplast, with long, single discs predominating. Large stacks beneath chloroplast envelope are indicated by arrows. 71,000×.

Fig. 8. ac-141 chloroplast, showing long, single discs. 71,000×.
ac-1 chloroplast is shown in Fig. 3, and its stacking profile in Fig. 1. Stacks of \( n \geq 4 \) were not encountered, but the normal distribution of stacks and the characteristic anastomosing pattern of wild type is found in \( ac-1 \). It is thus considered unlikely that chlorophyll variations can account for alterations found in the appearance of other mutant strains.

**The Photosynthetic Mutants: General Features.** It is apparent in Fig. 3 that the 4-fold pigment deficiency found in \( ac-1 \) does not affect the formation of either chloroplast membranes or chloroplast discs. This same statement applies to the 9 photosynthetic mutant strains that have been studied. Figs. 4 to 8 illustrate the chloroplasts of 5 of these strains, and the appearance of the membrane and the flattened disc is indistinguishable from that of wild-type cells. Some variation is apparent in the width of the intradisc space, but the size of this space varies considerably from one fixation to the next in any strain, including wild type. The total amount of membrane in the mutant chloroplasts appears comparable to the wild type, although such a statement is difficult to quantify. Certainly the membrane content is not markedly reduced in any of the strains. Finally, the structure of the pyrenoid and chloroplast envelope, the levels of the chloroplast ribosomes, and the appearance of all cytoplasmic components are unaffected by the mutations. The only manifestation of the mutations detectable in glutaraldehyde-fixed thin sections, then, is an altered stacking pattern of the chloroplast discs seen in some, but not all, of the mutant strains.

**F-60 and ac-21.** That an altered stacking pattern is not an obligate phenotype of a photosynthetic mutant is seen in 2 strains, F-60 and ac-21. F-60 is capable of photosynthetic electron transport, but it lacks an active 1,5-phosphoribulokinase (B. Moll, unpublished observations), and thus fixes almost no CO\(_2\). Since the kinase is a soluble component of the chloroplast stroma, it is perhaps not unexpected that the stacking profile of F-60 (Fig. 1) resembles the wild-type profile.

The ac-21 strain, on the other hand, is blocked in photosynthetic electron transport as a consequence of the absence or inactivity of an unidentified component (23, 24), yet its stacking profile (Fig. 1) is also similar to wild type. The ac-21 curve has a peak at \( n = 2 \), whereas the wild-type and most controls have a peak at \( n = 3 \); the shapes of the curves are comparable, however, and the stacking-profile assay is not sensitive enough to designate such differences as meaningful, even though they may well be. Similarly, it is clear in the first column of Fig. 1 that the profiles of mixotrophic wild type and F-60 cells are particularly well "matched", as are those of autotrophic wild type and DCMU-treated cells: however, no significance is attached to this observation. This point is stressed to avoid any misinterpretation of the intent of the stacking profiles. They represent an attempt to summarize and quantitate hundrends of electron micrographs in only very striking differences, differences that are in fact obvious by comparing the electron micrographs themselves, are regarded as meaningful.

**ac-80a and F-1.** Stacking patterns which differ markedly from the wild type are found in 2 mutant strains, ac-80a and F-1. Both strains lack an active P700 (4,10), the reaction-center chlorophyll of Photosystem I, and both are deficient in chlorophyll \( a \), although not in total chlorophyll (Table 1). The 2 affected genes are not linked, and the mutation at the ac-80a locus is more "leaky" than that at the F-1 locus. Nonetheless, the stacking profiles of the 2 strains (Fig. 1) are similar: both are "hyperstacked" compared to wild type, with a peak at \( n = 5 \), and with more than half the discs located in stacks of \( n \geq 5 \). Moreover, the discs tend to remain in a given large stack for relatively long distances, giving a banded rather than a confluent pattern to the chloroplast (Fig. 4, ac-80a, and Fig. 5, F-1). This kind of image, superficially reminiscent of the chloroplast of higher plants, is specific to the 2 P700-less strains.

**ac-206 and ac-208.** A second distinct phenotypic grouping includes the strains ac-206 and ac-208. The great majority of discs in these strains are found in stacks of \( n = 2 \) (Fig. 1). Fig. 6 illustrates this phenomenon in ac-206, and it is clear that here again the branching, anastomosing pattern of the wild type has been replaced by a preponderance of long, straight, 2-disc bands. An identical picture is presented by ac-208 (not shown).

The 2 strains are not identical in their photosynthetic disabilities: ac-206 lacks active cytochrome 553, whereas ac-208 lacks active plastocyanin (15, 24). These 2 proteins, however, have similar redox potentials and molecular weights in C. reinhardtii (13,14), and are thought to lie close together in the photosynthetic electron transport chain (15,24). Thus the marked similarity of the chloroplast structure in ac-206 and ac-208 is of interest.

**ac-115, ac-141 and F-34.** It is seen in Fig. 1 that the percent single discs (\( n = 1 \)) in the chloroplasts of ac-206 and ac-208 has increased over the wild-type level. This tendency to "unstack" characterizes the third grouping of photosynthetic mutants, which includes ac-115, ac-141, and F-34. This is evident both in their stacking profiles (Fig. 1) and in electron micrographs (Fig. 7, ac-115, and Fig. 8, ac-141), where long, single discs dominate the chloroplast. Nevertheless, it is important to emphasize that membrane fusion does occur in these strains, often in wide bands near the chloroplast surface (Fig. 7, arrows).

These 3 strains all lack an active cytochrome 559 (4, 24) and are incapable of any Hill activity. Their chloroplast structure in no way resembles DCMU-poisoned wild-type cells, however (Fig. 1), even though DCMU also produces a complete inhibition of Hill activity.

The ac-115 and ac-141 strains have been studied previously and are successfully represented in a single, un-
linked gene, yet their biochemical disabilities are complex. In addition to lacking cytochrome 550 activity, each appears to lack an active Q (20), the quencher of fluorescence of Photosystem II (6), and to be quinone-deficient (40). It now appears that the chloroplast ultrastructure is drastically altered in these strains. The possibility thus arises that the primary effect of the ac-115 and ac-141 mutations is to alter some unidentified component which serves to assemble, or properly orient, Photosystem II, and that the several photosynthetic disabilities observed in these strains are the secondary consequence of such disorganization. We are currently studying this possibility.

**Suppressed F-34.** The cultures fixed for electron microscopy in this study were all tested by an appropriate assay to determine that the cells indeed exhibited the appropriate photosynthetic disability. This precaution is necessary since suppressor mutations occasionally arise which have the effect of restoring at least partial wild-type activity to a mutant strain. A suppressed strain of F-34 was intentionally examined, however, to determine whether the ultrastructural alterations seen in the non-suppressed strain are directly related to its biochemical lesion, or whether they are rather some secondary attribute of an isolated population. The suppressed F-34 had a Hill reaction rate with 26-dichlororphenol that was comparable to wild type and, as seen in Fig. 1, its stacking profile has also become comparable to wild type. In fact, electron micrographs of the suppressed and wild-type cells are difficult to distinguish.

**Discussion**

A sizable literature exists on the fine structure of the chloroplasts from mutant strains of higher plants. In all cases, the mutant tissues were originally recognized by their altered pigmentation, and in many cases, the plant is either inviable or the mutant sectors are supported by green portions of the plant. As von Wettstein and his associates have stressed (47,50), the plastids from such plants usually appear to suffer from a developmental block during the course of transformation from proplasts. The morphological consequences of this block may be a relatively undifferentiated organelle (26, 31,33,46,49), or the resultant plastid may be a very distorted one containing disorganized vesicles (5,31,39,52), swollen discs (5), giant, compressed grana (2,26), large lipid accretions (32,43,49), and so on. In some cases, the chloroplast may begin normal development but be secondarily broken down.

A pattern characteristic of carotenoid-deficient tissues (2,7,44,45) and of plastome mutant strains (50). Clearly the chloroplasts of the photo-synthetic mutant strains of *C. reinhardi* do not suffer from such gross morphogenetic lesions, thus underlining their suitability as experimental organisms for photosynthetic research.

Control experiments reported in this paper establish that variations in chlorophyll levels do not necessarily affect chloroplast fine structure in *C. reinhardi*, a conclusion that has been reached in studies of various other organisms (3,9,18,36,43). The concept persists, however, that the presence of chlorophyll per se is, in part, responsible for the chloroplast membrane arrangements seen with the electron microscope (2,26,28,34,48,50). This impression is often given by organisms that are drastically pigment-deficient, and in such cases, the pigment loss is likely secondary to other lesions which more directly affect membrane organization. At present it seems best to conclude that at least 75% of the chlorophyll of *C. reinhardi* can be lost without affecting chloroplast structure, but that the remaining chlorophyll may serve essential structural roles (48). It is, of course, also likely that any pigment variations affect structure at levels that are not detected using present electron-microscope techniques.

Again within the limitation of the technique, the absence of activity of at least 6 essential components of the photosynthetic electron transport chain does not affect the formation of chloroplast membranes or their organization into flattened discs. A parallel observation has been made in yeast cells that carry nuclear gene mutations affecting the activity of various bound mitochondrial cytochromes: the inner membranes of the mitochondria are apparently assembled normally (51). Thus it would appear that active electron transport components are not obligate structural units of either mitochondrial or chloroplast membranes.

It might be argued that mutant proteins or components are still synthesized by the mutant strains and that these are incorporated into the membrane structure. In other words, the mutation might affect that portion of the molecule responsible for its biochemical activity but not that aspect of its structure required for insertion into the membrane (27). If this is the case, then it must be postulated that this structural conformation is adequate for the formation of normal-looking membranes and discs, but inadequate for the formation of the stacking patterns characteristic of the wild-type chloroplast, for with the exception of ac-21, each strain lacking electron transport activity exhibits distinct stacking profiles. Moreover, strains with similar disabilities exhibit similar patterns. The mutant patterning, then, is apparently a reflection of the mutation, but at a higher organizational level than either membrane or disc formation.

As stated at the outset of the paper, a rationale for any of the distinctive membrane patterns found in the chloroplasts of algae and higher plants has not yet been found. Indeed, the relationship between stacking and photosynthesis is not itself established, as discussed in detail in the accompanying paper (11). However, the observations described in this paper indicate that chlorophyll staining is not totally
naphazard, but rather that it is in some way a property of the structural organization of the chloroplast membranes themselves, and that it undergoes specific and distinctive kinds of alterations when specific classes of nuclear gene mutations have occurred.

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