Dynamics of Concanavalin A Binding Sites on *Chlamydomonas moewusii* Flagellar Membranes

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(Algae-Symposium, Göttingen, September 1980)

Introduction

*Chlamydomonas* is a single-celled, biflagellated, photosynthetic alga that can be grown axenically in a simple salts solution in the light. Many species that exist in culture have two mating types, usually designated (+) and (−). Both mating types of *Chlamydomonas* can be maintained separately in an actively growing, motile state (vegetative cells) in liquid media. The mating types have no affinity for each other at this stage. Upon transfer to a medium deficient in nitrogen and low in other salts (induction medium) the vegetative cells differentiate into gametes without morphological change. Flagellar membrane differentiation is the main detectable difference between vegetative cells and gametes. Differentiation can be tested by mixing cells with previously differentiated gametes of the opposite type to observe clumping or agglutination of cells which is caused by adhesion of flagellar membranes. In the case of *C. moewusii*, a fusing pair of opposite gametes is released eventually from the clump with their flagellar membranes unattached to each other and lacking an affinity for other free-swimming gametes (Lewin 1956, Trainor 1959, Wiese 1969). A summary diagram of the *Chlamydomonas moewusii* sexual cycle is presented in Figure 1.

The isoagglutination of *Chlamydomonas* gametes by concanavalin A (Con A) was first demonstrated by Wiese and Shoemaker (1970). This tetravalent lectin attached to the flagellar membranes to cause a common bond between cells. We later reported (McLean and Brown 1974) that although tetravalent Con A could isoagglutinate gametes and prevent their interaction with opposite gametes, the monovalent form could do neither. Treatment of gametes with trypsin would eliminate their mating adhesiveness but not their ability to

KEY WORDS: *Chlamydomonas* — concanavalin A — membrane motility — colchicine — cell recognition — fertilization.
Chlamydomonas moewusii Sexual Cycle

Fig. 1

Agglutinate in the presence of Con A. This suggested that the adhesive mating sites and the Con A binding sites were not the same.

Subsequent reports have demonstrated the presence of multiple sites associated with the flagellar membrane of Chlamydomonas (Claes 1975, Solter et al. 1977, Forest and Goodenough 1977, Adair and Goodenough 1978, Adair et al. 1979, Monk et al. 1979, Bloodgood 1979).

The work of Bloodgood (1977, 1979) and co-workers (1979) is one of the more interesting breakthroughs in the area of flagellar membrane research and its effect on our understanding of the Chlamydomonas mating reaction. They reported on the movement of membrane binding sites observed by the attachment of polystyrene microspheres, in a bidirectional fashion on the flagella of Chlamydomonas. This movement, which was parallel to the long axis, was observed on both gametes and vegetative cells. It is rapid and observable without time-lapse photography. Two different binding sites may pass one another while moving in opposite directions (Bloodgood 1977).

The flagellar surface motility can be reversibly inhibited by high sodium or potassium, lowered temperature, or lowered calcium concentration. Inhibition of protein synthesis results in loss of microsphere binding and surface motility (Bloodgood 1979). Binding sites can be removed from flagella by pronase treatment, but are replaced in the presence of new protein synthesis. Bloodgood (1979) further reported that there is a normal turnover of membrane proteins including those involved in marker adhesion and motility. The turnover is thought to occur by the release of membrane vesicles into the medium (McLean et al. 1974, Bergman et al. 1975, Snell 1976). Recently, Snell and Moore (1980) reported that rapid turnover of adhesive sites oc-
curred in response to cellular adhesion while turnover was slower in the absence of it.

Other evidence of binding site movement has been reported in *Chlamydomonas* by Goodenough and Jurivich (1978). This movement seems to be a result of gametic differentiation since only gametes were observed to move antibody toward the distal end or tip of the flagella when presented with antisera against gametic or vegetative flagella of *Chlamydomonas reinhardtii*. The resulting agglutination of gametes occurs at the flagellar tips whereas adhesion of vegetative cells to one another by antisera can be seen along the entire length of the flagella.

Although Con A has continued to be used as a tool in the study of other cell systems, its use has been more limited in this cell system. We present here a further study of Con A isoagglutination, distribution and movements of binding sites on the flagellar membrane, and a discussion of what this means to the *Chlamydomonas* mating reaction.

### Materials and Methods

#### Culture Maintenance

The (+) and (−) strains of *Chlamydomonas moewusii* (Utex 96 and 97) are maintained in the vegetative state in modified Bristol's medium (Bold 1949) under continuous illumination of 400 ft-c. intensity and Bristol's agar plates under a 16—8 h light-dark cycle. The two mating types are induced to undergo gametogenesis in a one-to-one solution of soil extract (Starr 1964) and induction medium (McLean and Brown 1974) and dark incubation for 14—15 h followed by a 30 min exposure to light. Flagellated vegetative cells are obtained by dark incubation in Bristol's medium supplemented with 0.1% NH₄Cl (Wiese 1965).

#### Sexual Agglutination and Lectin Isoagglutination Assays

Cell suspensions are adjusted to $1 \times 10^7$ cells ml$^{-1}$ using the Coulter Counter Model ZF (settings-amplification = 1, threshold = 5; aperture-current = 1/2). Equal volumes of cell suspensions and soil extract-induction medium as a control or 0.01 M colchicine are combined and incubated for 1 h at room temperature in the light. Following incubation, 2—4 ml of cells now at a concentration of $5 \times 10^6$ cells ml$^{-1}$ are pipetted into $12 \times 100$ mm test tubes or a 1 ml aliquot is added to $9 \times 75$ mm test tubes. Either test tube size is adequate for the experiment. For mating studies, equal volumes of both mating types are mixed; for lectin studies the incubation volume consists of single mating types and appropriate concentrations of lectin. The test tubes are inverted and then left undisturbed for the appropriate time period during which clustered cells settle out. Following incubation, 0.1 ml samples are removed just under the meniscus and counted in 9.9 ml of Isoton II (Coulter Electronics). This procedure selects for the single cells that remain in suspension. Controls of individual mating types are included in order to discount any natural settling of cells in agglutination calculations. Calculations of sexual agglutination also are corrected for total pairing in the vessel and for pairs in the 0.1 ml sample from the top. The procedures for our calculations are as follows:
In all cases, background Isoton counts are subtracted from Coulter Counter (CC) values.

% sexual agglutination
\[
a = \text{total cell } \# \text{ from CC}; \\
b = \% \text{pairing determined by hemocytometer}; \\
a \times b = \text{total } \# \text{ pairs}; \\
x = a - (a \times b) = \text{total } \# \text{ cells that could agglutinate at any one time}; \\
c = \text{average of CC counts from settling controls (96 and 97 separate)}; \\
(1 - \frac{c}{a}) \times 100 = \% \text{cells that naturally settle}; \\
z = c \times (1 - \frac{c}{a}) = \# \text{cells that settle naturally}; \\
r = x - z = \# \text{cells that may agglutinate}; \\
d = \text{CC count from experimental (96 and 97, mixed)}; \\
e = \% \text{pairing in 0.1 ml top sample determined by hemocytometer}; \\
d \times e = \text{total } \# \text{pairs in 0.1 ml top sample}; \\
y = d - (d \times e) = \text{total } \# \text{cells not agglutinating}; \\
r - y \times 100 = \% \text{agglutination} \\
a
\]

% lectin isoagglutination
\[
a = \text{total cell } \# \text{ from CC}; \\
c = \text{counts from settling control (96 or 97)}; \\
d = \text{count from experimental (96 or 97 + lectin)}; \\
c - d \times 100 = \% \text{isoagglutination} \\
a
\]

Concanavalin A and Ferritin-Con A Labeling

A concentration curve for the effect of Concanavalin A (Con A) on agglutination showed saturation at 150–200 \( \mu \text{g ml}^{-1} \) of the lectin. All experiments were run with the lectin at 200 \( \mu \text{g ml}^{-1} \).

Cells were treated with lectin or lectin conjugate either before or after fixation depending on the experiment. Fixation involved treatment of cells with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. Following a 3 × buffer rinse, the cells were post-fixed with 2% osmium-1.8% potassium ferrocyanide (Karnovsky 1971) for 2 h. This was followed by another buffer rinse, dehydration and embedding in Spurr (1969) medium or Epon-Araldite.

Con A and ferritin-Con A were obtained from Miles Labs.

An alternate procedure which proved equally as effective involved fixation for 45 min at room temperature in 0.1% glutaraldehyde and 0.5% osmium-0.45% potassium ferrocyanide. The cells were then rinsed in 0.1 M phosphate buffer (pH 7.2) and incubated with ferritin-Con A for 15 min. Following 3 × rinse the cells were collected on a 3 \( \mu \text{m} \) Unipore polycarbonate filter (Bio-Rad). The cells were then dehydrated by passing an acetone series through the filter. The filter was then removed and placed in one part Epon-
Araldite and 2 parts acetone for 1 h. The filter was then placed for 15 min each in two thirds plastic and 100% plastic (2×). The filters were cut and placed in molds for curing for 1.5 h at 99 C. This procedure is modified after RITTENBURG et al. (1979) and requires approximately 4.5 h.

Freeze-fracture

Cells were fixed with 2% glutaraldehyde for 5 min, rinsed in buffer and treated with 5% glycerol prior to freezing.

Ruthenium Red (RR) Labeling

Cells were exposed to RR (Polysciences) that was purified by the extraction method of LUFT (1971). The fixation procedure was the same as outlined above with the following exceptions: RR at 2 g l⁻¹ final concentration was present in glutaraldehyde and osmium (only) and 0.1 M cacodylate (pH 7.3) was the buffer.

Results

The use of osmium-ferrocyanide in the fixative produced an enhanced image of the fuzzy flagellar coat (Figs. 2 and 3). The thickness seemed about the same on both mating types and on vegetative cells and gametes.

The inclusion of RR in the fixation schedule revealed particles with increased electron density on the flagellar surface. The particles can be seen in both longitudinal and cross sections (Figs. 4 and 5). The uptake of RR demonstrates the polyanionic nature of the sites and suggests carbohydrate, probably in the form of glycoprotein.

Ferritin-Con A could isoagglutinate gametes the same as native Con A. If live cells were labeled with ferritin-Con A, nearly all the label would be lost during incubation and handling. Cells prefixed with glutaraldehyde and osmium-ferrocyanide bound and retained the lectin-conjugate. Glutaraldehyde fixation alone was not sufficient to stabilize the membrane coat. Figures 6 and 7 can be seen to have a patchy or clustered distribution of ferritin-Con A particles that is similar to the site distribution revealed by RR.Particles averaged a distance of 25 nm away from the outer electron dense layer of the unit membrane. If α-methylmannoside (0.2 M) is included during incubation nearly all the ferritin-Con A is prevented from binding (Fig. 8). Native ferritin does not bind to prefixed cells under identical conditions (Fig. 9). Ferritin-Con A did not bind to cell walls except for mother cell walls and walls discarded in the medium.

The binding of ferritin-Con A particles to flagella of gametic and vegetative cells was compared (Fig. 10). Gametic flagella had significantly more particles bound than vegetative flagella. The increase was proportional in both mating types. The difference of particles bound between mating types was insignificant.

At this point we asked the question whether the degree of ferritin-Con A binding was correlated with the isoagglutinability of the different cell types in the presence of Con A. Table 1 shows the isoagglutination percentage of each cell type as determined by our settling technique and use of the Coulter Counter. There appears to be no correlation between the degree of ferritin-Con A binding and agglutination by Con A.
Figs. 2 and 3. Longitudinal and cross-sections, respectively, of gametic flagella fixed in glutaraldehyde and osmium-ferrocyanide. Stained ($\times 72,500, \times 89,900$).

Figs. 4 and 5. Longitudinal and cross sections, respectively of gametic flagella fixed in the presence of ruthenium red. Note the densely staining particles on the membrane surface ($\times 98,600, \times 68,400$).

Figs. 6 and 7. Longitudinal and cross sections, respectively, of gametic flagella that were prefixed and treated with ferritin-Con A. The ferritin grains have a clustered distribution. Unstained ($\times 75,400, \times 62,900$).

Fig. 8. Inclusion of $\alpha$-methylmannoside in the incubation with ferritin-Con A eliminates most label binding. Unstained ($\times 52,200$).

Fig. 9. Native ferritin does not bind significantly to flagella. Unstained ($\times 84,100$).
Fig. 10. Quantification of FCA binding to prefixed flagellar membranes shows a greater density of binding sites on gametic flagella as compared with vegetative flagella. 96V = (+) vegetative cell; 97V = (−) vegetative cell; 96G = (+) gamete; 97G = (−) gamete.

Table 1
Isoagglutination of cell types in the presence of concanavalin A

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Isoagglutination, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>(+) gamete</td>
<td>25 ± 5.2</td>
</tr>
<tr>
<td>(+) vegetative cell</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>(−) gamete</td>
<td>22 ± 1.6</td>
</tr>
<tr>
<td>(−) vegetative cell</td>
<td>27 ± 3</td>
</tr>
</tbody>
</table>

Cells were treated with 200 μg ml⁻¹ of Con A for 15 min and isoagglutination determined by the Coulter Counter method. Colchicine-treated cells were incubated for 1 h with 0.005 M colchicine prior to use. The numbers represent the percent of cells isoagglutinating with one standard error.

Since it was suggested that colchicine inhibited flagellar binding site migration during sexual interactions (GOODENOUGH et al. 1980) we decided to examine further the effects of colchicine during both sexual and lectin interactions. Opposite gametes were pretreated with 0.005 M colchicine for 1 h prior to utilization. Colchicine had only minor effects on cell response to Con A. After opposite gametes were mixed, agglutination and pairing were measured during a 2 h period.

Although agglutination occurred in both control and colchicine-treated cell mixtures, pairing was significantly inhibited in colchicine-treated cells (Fig. 11). Agglutination also dissipated during the incubation period as colchicine-treated gametes failed to pair.
Fig. 11. Effect of colchicine on sexual agglutination and pairing during a 2 h period. A. After cells are mixed, agglutination (×) and pairing (▲) increase rapidly. Agglutination tapers off as those cells become pairs. B. Pairing never exceeds 5% during the 2 h period. Agglutination dissipates as gametes fail to pair. (●) represents the total of agglutination and pairing, or the cells involved in the sexual process.

The cause of unsuccessful pairing was investigated. Colchicine-treated cells were examined by light microscopy first. Pseudo-pairs were noticed in which cells were not aligned by their anterior ends. The flagella were adhering but never completed the tip-to-tip locking that would have resulted in proper positioning for the cytoplasmic bridge to form (Fig. 12). Cells from control and colchicine treatments were fixed and examined by electron microscopy to determine if plasma papilla outgrowth had occurred. Figures 13 and 14 represent unactivated and activated plasma papilla respectively. The plasma papilla forms the cytoplasmic bridge between cells to initiate gametic fusion. Table 2 shows that outgrowth of the plasma papilla of colchicine-treated cells was severely inhibited, suggesting that activation of the growth of that structure never occurred.

Since site migration on flagellar membranes has been shown to be necessary for subsequent events in the sexual process (Goodenough et al. 1980, Mesland et al. 1980), we studied the changes in Con A binding site distribution on unmixed gametes, gametes mixed for 30 sec, gametes mixed for 30 min (including many pairs), and colchicine-treated gametes mixed for 4 min. Unmixed gametes have a random distribution of ferritin-Con A along the proximal and distal ends of their flagella (Table 3). Flagellar sections that could not be identified
Fig. 12. Pseudo-pairs are observed in colchicine-treated populations. They lack the flagellar tip-to-tip orientation of normal pairs and rarely form the cytoplasmic bridge. The pseudo-pairs eventually break their adhesion.

Fig. 13. The unactivated plasma papilla of a (+) gamete (× 34,000).
Fig. 14. Activated plasma papilla of gamete mixed with opposite gametes. Note the dissolution of the cell wall directly above it and the dense nature of the papilla itself (× 52,200).
Fig. 15. Freeze-fracture of a flagellar tip of (+) gamete. Note rows of intramembranous particles on the inner fracture face (× 39,600).
Fig. 16. Negative stain preparation of (+) gametes. The phosphotungstic acid was cleared away from linear areas that run longitudinal to the long axis. This could be the result of membrane particle movement during drying of live cells on the grid (× 118,900).
Table 2
An analysis of sectioned material shows the lack of plasma papilla activation in colchicine-treated gametes

<table>
<thead>
<tr>
<th>Treatment</th>
<th># cells</th>
<th>Plasma papilla noted</th>
<th>Plasma papilla activated</th>
<th>% activation</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>300</td>
<td>48</td>
<td>44</td>
<td>92</td>
<td>—</td>
</tr>
<tr>
<td>0.005 M colchicine</td>
<td>300</td>
<td>38</td>
<td>1</td>
<td>1.2</td>
<td>98.7</td>
</tr>
</tbody>
</table>

Opposite gametes were mixed for 20 min and then prepared for electron microscopy. Experimental cells were pretreated in 0.005 M colchicine for 1 h before mixing. Percentages were calculated based upon the numbers of activated papillae and total papillae observed in sectioned material.

Table 3
Ferritin-Con A concentrations at proximal and distal ends of gametic flagella

<table>
<thead>
<tr>
<th>Gamete</th>
<th>Gametes</th>
<th>Coldchicine-treated gametes mixed 4 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)</td>
<td>(—)</td>
<td>mixed 30 sec</td>
</tr>
<tr>
<td>Distal end</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.9</td>
<td>5.0</td>
<td>7.7</td>
</tr>
<tr>
<td>3.8</td>
<td>3.8</td>
<td>7.7</td>
</tr>
<tr>
<td>4.8</td>
<td>0.7</td>
<td>24.6</td>
</tr>
<tr>
<td>2.0</td>
<td>12.0</td>
<td>6.0</td>
</tr>
<tr>
<td>3.0</td>
<td></td>
<td>23.0</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>18.0</td>
<td>1.0</td>
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</table>

Proximal end

Numbers of ferritin grains per 0.33 μm contiguous segment were counted on negatives of flagella that were subjected to the above conditions. Each number represents the mean of counts from 3—6 flagella. Flagellar length averaged 12—15 μm.

As tips or bases were not used. Gametes mixed for 30 sec showed a higher concentration of ferritin-Con A grains appearing at the tip or distal end. This was quite pronounced in cells mixed for 30 min. Coldchicine-treated cells were indistinguishable from control cells indicating that coldchicine prevented migration of the Con A binding sites.

Visualization of components that are suspect for participation in site migration is provided (Figs. 15 and 16). Figure 15 is the flagellar tip of a whole mount that was negatively stained. The cleared lines running longitudinally may represent migrating binding sites that were still active while the live cells
were drying on the grid. As the sites moved they cleared the phosphotungstic acid from their paths. This activity was noticed more at tips although it was occasionally observed on median areas. In Figure 16, the inner fracture face on the flagellar tip of a (+) gamete shows a high concentration of intramembranous particles arranged in rows that run parallel to the longitudinal axis.

Discussion

The presence of Con A binding sites on the flagellar membrane surface has been visually demonstrated. The sites are more dense on gametes than vegetative cells and occur in patches or clusters along the membrane. Our procedure required that cells be fixed in glutaraldehyde and osmium-ferrocyanide before ferritin-Con A grains were retained by the cells. Although the clustered appearance of ferritin grains may have resulted from prefixation, others have shown in a variety of cell types that prefixation resulted in a uniform distribution in those cases (Grinnell et al. 1976). Several studies have been done with prefixed cells (Nicolson et al. 1977, Virtanen et al. 1978, DePetrис 1978, Yokoyama et al. 1980). In fact, DePetrис (1976) warned against making interpretations of Con A site distribution on live cells since a lectin-induced redistribution usually occurs. Therefore, our approach with prefixed cells shows the distribution before perturbation by the lectin. This was especially important for the studies involving mixed gametes since fixation before labeling with ferritin-Con A enabled us to measure redistribution caused by the interaction of opposite gametes without interference from a lectin response.

No correlation was observed between Con A binding site density and isoagglutination of the different cell types. This agrees with the work of Musgrave et al. (1979) with C. eugametos. However, there is a significant disagreement about the relative level of Con A binding on gametic and vegetative flagella between our work and that of Musgrave and coworkers. Their work showed a higher level on vegetative cells while ours showed a higher level on gametes. Our experience suggests that the significant loss of label from live cells during incubation, perhaps caused by perturbation, redistribution and shedding, may have occurred on gametes more than on vegetative cells. Goodenough et al. (1980) showed that membrane surface motility was more active on gametes than vegetative cells. Another possibility for label loss was the pH shock treatment used by Musgrave and coworkers which could have uncoupled the label.

The development of information concerning Chlamydomonas flagellar membrane proteins has been interesting as new technologies have been applied to the problem. McLean and Brown (1974) first suggested at least 2 different sites on the flagellar membrane, one that bound Con A and one for sexual adhesions. Electrophoretic studies by Bergman et al. (1975) and Snell (1976) with C. reinhardtii showed one major membrane band and possibly a minor component. Monk et al. (1979) found 20 polypeptides by use of the surface iodination technique. Musgrave et al. (1979) observed at least 9 Con A binding components in the C. eugametos membrane. Adair and Goodenough (1978) reported on the presence of glycosylated tubulin in the flagellar membrane of C. reinhardtii. A large molecular weight component has been implicated as the adhesive factor (Adair et al. 1979) but the functions of other components have not been established.
It is obvious from the above that we are working with a variety of surface receptors. In our own results it appears that not all the Con A receptors are of the type to participate in site migration in response to sexual interactions since cells that reacted for 30 min still had some Con A receptors at their bases.

Site migration or tipping under different stimuli is summarized in Figure 17. The stimuli include Con A (Goodenough et al. 1980) and flagellar antibodies (Goodenough and Jurivich 1978) in addition to opposite gametes.

Goodenough and Jurivich (1978) suggested that multivalency and the ability to cross-link is important to produce tipping since monovalent antibodies were ineffective. In our study, sexual interaction between opposite gametes caused Con A site migration, thus linking the lectin-binding sites directly to the sexual process. The fact that colchicine prevented the tip accumulation of Con A binding sites suggests the implication of tubulin components in site migration. It would appear that membrane tubulin represented one type of Con A binding site or participated in the movement of the Con A sites.

**Summary**

The objective of the present study was a closer examination of flagellar surface binding sites, their distribution and movements utilizing ferritin conjugated Concanavalin A (FCA) and colchicine. Initial observations of the flagellar surface stained with ruthenium red revealed particles with increased electron density indicating the polyanionic nature of some sites and their probable carbohydrate composition. FCA that bound to the flagellar surface had a patchy distribution with gametes binding more FCA than vegetative cells. In contrast, isoagglutination percentages with native Con A are similar for both gametes and vegetative cells. When gametes are pretreated with colchicine for 1 h before mixing, agglutination is relatively unaffected. However, pairing is greatly inhibited since no papillar outgrowth occurs in these cells. FCA added to unmixed gametes showed comparable binding to both flagellar base and tips. However,
as the incubation time of mixed gametes increased, a greater amount of FCA bound to the tips of gametes. When gametes were pretreated with colchicine before mixing, FCA binding was indistinguishable from controls at the bases and tips. It would appear that Con A binding sites are somehow linked to the sexual process and move to the flagellar tip in response to gametic contact. Membrane tubulin may represent one type of Con A binding site or participate in the movements of the Con A sites.

Literature


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