

Light Affects Flagellar Agglutinability in *Chlamydomonas eugametos* by Modification of the Agglutinin Molecules

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ABSTRACT

The effect of light on the sexual competence of a light-sensitive mating type minus strain (mt^-) of *Chlamydomonas eugametos* obtained by crossing a light-sensitive mating type plus strain (mt^+) with a light-insensitive mt^- strain is described. As previously demonstrated for the mt^+ parent, this study of one of the mt^- offspring shows that (a) a light-sensitive mechanism affects flagellar agglutinability in a rapid process that does not require protein synthesis; (b) only the activity of the flagellar agglutinins (glycoproteins responsible for agglutination) is susceptible to light while agglutinins on the cell body surface are not affected by light. We further demonstrate that (a) membrane vesicles naturally released from nonagglutinable dark gametes remain inactive. Extracts of these vesicles also remain inactive even though they contain agglutinin-like components; (b) inactive mt^- agglutinin is present in extracts of flagella from nonagglutinable dark gametes by comparison of its chromatographic, electrophoretic, and immunogenic properties with those of active agglutinin. When purified of all other flagellar proteins, it remains inactive; (c) a monoclonal antibody directed against the sexual agglutination site of the mt^- agglutinin discriminates between active and inactive agglutinins when present in a native state on the flagellar surface, but is unable to discriminate between them when they are denatured in sodium dodecyl sulfate-electrophoresis gels and blotted onto nitrocellulose. Taken collectively these observations suggest that light activation involves the chemical modification of the agglutinins *in situ* on the flagellar surface.

The first step in the sexual reproduction of the unicellular green alga *Chlamydomonas eugametos* is the highly specific mutual adhesion of gametes of opposite mating types (mt^- and mt^+).² This agglutination process takes place between the flagellar surfaces, and initiates other mating reactions such as flagellar tip activation (2) and the transport of membrane components to the flagellar tip (tipping) (6, 10). Subsequently, the gametes activate their mating structures by which they eventually fuse to give rise to *vis-à-vis* pairs.

Förster and Wiese (5) showed that the mating competence of mt^+ cells of *C. eugametos* depends on light. No *vis-à-vis* pairs were formed when nonilluminated mt^+ gametes were mixed with

competent mt^- cells. Earlier, we reported that it is the agglutinability of the mt^+ cells that depends on light (12). When dark-treated, nonagglutinable gametes were illuminated, maximal agglutinability was achieved within 15 min. These cells lost their agglutination activity within 30 min of returning to the dark. Other mating reactions such as flagellar tip activation and tipping (both induced by wheat germ agglutinin) were light-independent.

Agglutination activity is the consequence of two different agglutinins present on the flagella of mt^+ and mt^- gametes, respectively. They have been identified as large glycoproteins, bound extrinsically to the membranes of flagella and cell bodies of all gametes (11, 12, 18, 21). However, only the agglutinin bound to the flagella seems to be influenced by the light, for the same level of agglutination activity can be extracted from the cell bodies of dark gametes as from light gametes (12). Nonetheless, it should be appreciated that as a consequence of the continuous presence of the gametic cell wall during the mating reaction, these agglutinins are not involved in agglutination. It seems that a membrane barrier, present in the transition zone between the flagellum and cell body, separates the plasma membrane into two independent domains (20) and that the light-controlled mechanism only operates in the flagella. What is the nature of this mechanism? Since protein synthesis is not necessary for activation or deactivation, it was postulated that the mechanism might involve the *in situ* modification of agglutinin rather than the rapid turnover of these molecules. To confirm this hypothesis, it was necessary to isolate inactive agglutinin from dark gametes. However, the mt^+ gametes used to study this phenomenon are not suitable, for its agglutinin is relatively labile and present in only limited quantities on the flagella. Since light sensitivity is not sex-linked (12), we constructed a light-sensitive mt^- strain that does not possess these limitations. As an extra advantage, we possess a monoclonal antibody that is specific for the mt^- agglutinin (WL Homan, unpublished data) by which we can monitor its presence independent of its biological activity. In this report we illustrate that just as much inactive agglutinin is present on the flagella of dark gametes as there is active agglutinin on the flagella of light gametes. One may therefore assume that light stimulates a mechanism in the flagella, whereby the agglutinin molecules are modified and activated *in situ*.

MATERIALS AND METHODS

Cell Cultures. Gametes of the *Chlamydomonas eugametos* strains UTEX 9 (mt^+) and UTEX 10 (mt^-) from the Culture Collection of Algae, University of Texas at Austin, were crossed. The zygotes were germinated and resulting tetrad cells were isolated and cloned as described by SCHURING *et al.* (22). UTEX 9, UTEX 10, and the daughter cells were cultivated in Petri dishes on agar-containing medium in a 12 h light/12 h dark

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² Abbreviations: $mt^{+/-}$, mating type plus/minus; GTC, guanidine thiocyanate; Mab, monoclonal antibody; QAE, quaternary aminoethyl, CHI, cycloheximide.

regimen as described by Mesland (15). Cell suspensions were obtained by flooding 2- to 4-week-old cultures with a 10 mM Hepes buffer (pH 7.6) just before the start of the dark period. Cells referred to as dark gametes were put into the dark directly after flooding and harvested at the same time as comparable light-treated cells. Illumination of dark-treated cell suspensions occurred at 20°C, using white fluorescent light with an intensity of 6.8 W · m⁻².

Isolation of Flagella and Isoagglutinin. Flagella were amputated by the pH-shock technique described by Witman *et al.* (23). Flagella and cell bodies were separated by centrifugation on a cushion of 25% sucrose (1000g, 15 min). The flagella, present on the top of the cushion, were purified by repeating the separation procedure once. Subsequently, the flagella were pelleted by centrifugation at 12,000g for 20 min. Isoagglutinin was isolated as described by Homan *et al.* (9) except for the use of a Tris buffer that was replaced by a 10 mM Hepes buffer, pH 7.6. For the isolation of flagella from nonilluminated cells, the pH-shock procedure was performed in the dark. The isolation of membrane vesicles naturally released by gametes into the medium (isoagglutinin) (9) from dark-treated cell cultures was performed in darkness until the cells were removed from suspension by centrifugation (1000g, 15 min). The agglutinability of amputated flagella and isoagglutinin was not affected by light.

Extraction of Biologically Active Material. *Mt*⁻ cell bodies, flagella and isoagglutinin were extracted in 3 M GTC for 30 min at room temperature and centrifuged at 50,000g for 30 min. The glycoproteins in the supernatant were separated from GTC by gel filtration in water using a G-25 sephadex column (Pharmacia). Any insoluble material was removed by centrifugation at 50,000g for 15 min.

Biological Assays. The agglutination activity of *mt*⁻ cells was quantitated by determining the highest dilutions of a standard suspension of *mt*⁺ flagella that still evoked isoagglutination of *mt*⁻ gametes (12). The agglutinability of flagella and isoagglutinin was assayed by determining the highest dilution of these particles that still caused isoagglutination of gametes of the opposite mating type. Biological activity in GTC-extracts was detected after dialysis adding *mt*⁺ gametes (9). In the presence of *mt*⁻ agglutinin, *mt*⁺ gametes accumulate at the water/air interface and without aggregating, lie there twitching in a manner resembling the movement of sexually agglutinating gametes.

Monoclonal Antibody. The monoclonal antibody Mab 66.3 has been used in this study as a specific label for the *mt*⁻ agglutinin. Although its characteristics will be presented in detail by W. L. Homan (unpublished data), a summary is given here to justify its acclaimed specificity. The hybridoma clone was first selected because the supernatant completely blocked the biological activity of isolated *mt*⁻ agglutinin bound to Sepharose beads. The purified antibody is even able to block the agglutinability of intact *mt*⁻ gametes. *mt*⁺ agglutinability is not affected. The specificity of Mab 66.3 for extracted *mt*⁻ protein was tested by immunoblotting after separation in SDS-electrophoresis gels, by affinity chromatography using Mab 66.3 bound to protein A-Sepharose, and by immunoprecipitation. Using all those techniques, the affinity for the *mt*⁻ agglutinin was obvious. Only when using the affinity chromatography technique did it appear to bind another component. However, since this component was not detected in the original cell extract, we conclude that it is either a degradation product from the *mt*⁻ agglutinin or a component that is present in such low concentrations that it could only be detected after concentration on the column. It was never detected in any of the other techniques used. When Mab 66.3 was tested for binding to intact cells in the immunofluorescence test, it only labeled the flagella of gametes and not those of vegetative cells which do not synthesise the agglutinin. In this article, we shall demonstrate that Mab 66.3 binds to the flagella

of some strains in the light, when they are agglutinable, but not to the same cells in the dark, when they are nonagglutinable. Last, during sexual agglutination when flagellar agglutinability increases, the binding of Mab 66.3 increases in parallel (R Demets, A Tomson, unpublished data). Thus we feel justified in claiming that it may be used as a specific label for the *mt*⁻ agglutinin.

Immunofluorescence. After fixation in 1.25% glutaraldehyde, cells were washed 3 times in phosphate-buffered saline (pH 7.2) (PBS) and subsequently incubated for 15 min with a 20 times diluted solution of the Mab 66.3 in PBS. Excess of antibody was removed by washing 3 times with PBS. Flagella-bound Mab 66.3 was labeled with 1:100 diluted goat-anti-mouse IgG conjugated with fluorescein isothiocyanate (H and L chain, Tago Inc., Burlingame, CA). The cells were examined under a Zeiss (Oberkochen, FRG) fluorescence microscope. When binding of Mab 66.3 on the flagella of living cells was examined, cells were fixed in glutaraldehyde after a 15-min incubation with Mab 66.3.

Anion Exchange Chromatography. GTC extracted material from flagella and isoagglutinin was chromatographed over a QAE Sephadex A-25 column (φ 16 mm, length 55 mm, Pharmacia) in 10 mM histidine buffer pH 6.0, containing 50 mM NaCl. After a washing step with 15 ml of the same buffer, the components that bound to the resin were stepwise eluted with 200 mM and 400 mM NaCl in the same buffer.

Gel Electrophoresis and Immunoblotting. Lyophilized GTC-extracted material was dissolved in sample buffer and the components separated by SDS-PAGE according to Laemmli (13) in 1 mm thick 8 × 8 cm slab gels containing a 2.2 to 20% acrylamide gradient. Approximately the first 5 mm of the gel consisted of 2.2% acrylamide. Immunoblotting was performed as described by Homan *et al.* (8).

RESULTS

Light Activation of Agglutinability in an *mt*⁻ Strain of *C. eugametos*. The light sensitivity of sexual competence in *C. eugametos* gametes has been described for the *mt*⁺ strain UTEX 9, but this strain is unsuitable for isolating the agglutinins from the flagella because of the limited amounts present. We have therefore constructed a light-sensitive *mt*⁻ strain (henceforth designed as 5.39.4) obtained by crossing UTEX 9 with the light insensitive *mt*⁻ strain UTEX 10 (22). The kinetics of activation and deactivation of flagellar agglutinability in this strain is illustrated in Figure 1. Compared with the original *mt*⁺ strain, the attainment of maximum agglutinability usually took twice as long, whereas the deactivation in the dark usually went faster. However, these are not mating type-linked differences but simply variations between strains, for we have isolated *mt*⁺ strains which exhibit similar characteristics and also *mt*⁻ strains that more closely resemble the UTEX 9 parent. A more pronounced difference is seen in their sensitivity to CHI. In UTEX 9, both light-activation and dark-inactivation took place independent of CHI-treatment (12), but treatment of 5.39.4 with CHI prevented the activation of agglutinability in the light, whereas deactivation was unaffected (Fig. 2). This difference in CHI-sensitivity is again not sex-linked but a common variation between strains (Table I). We propose that this effect is not due to an inhibition of the synthesis of the *mt*⁻ agglutinin. This stems from the fact that the iron chelator α,α-bipyridyl, which, like CHI, completely inhibits the synthesis of active agglutinin during gametogenesis of all *mt*⁻ strains (20), had no effect on the light-activation process (Fig. 2). A possible explanation is that in some strains the proteins involved in activation are subject to a rapid turnover. When gametes were treated for decreasing lengths of time with CHI, before illumination, it became obvious that also a short pretreatment had a profound effect, even though the inhibition was not absolute (Fig. 3). This could indicate an extremely rapid turnover

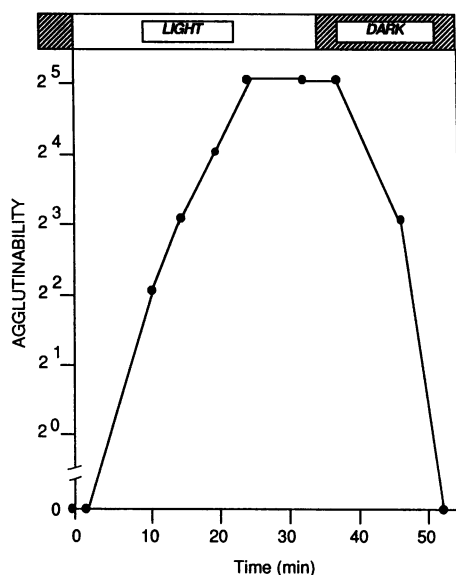


FIG. 1. Kinetics of the light-activation and dark-inactivation of flagellar agglutinability in *C. eugametos*, *mt*⁻. Agglutinability is measured using a twofold dilution series of *mt*⁺ flagella and expressed as a titer, which is the highest dilution that still causes *mt*⁻ gametes to isoagglutinate. □ = light-period, ▨ = dark-period.

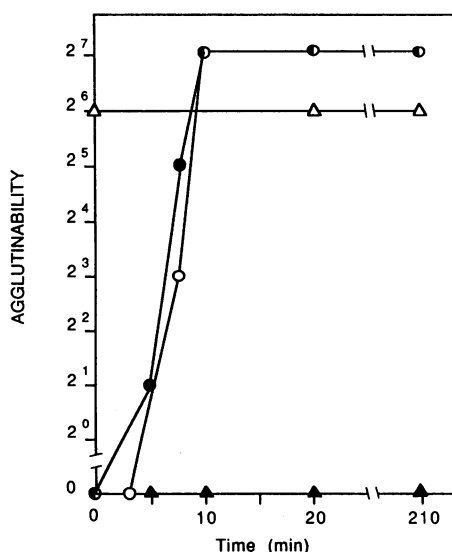


FIG. 2. Effect of cycloheximide and α, α -bipyridyl on the light induced activation of flagellar agglutinability. Nonilluminated cells were preincubated for 1 h with 5 μ g/ml CHI (▲—▲) or 0.3 mM α, α -bipyridyl (●—●). Control cells without inhibitors: (○—○). Illumination was started at $t = 0$ min. CHI did not affect flagellar agglutinability when added to light-treated cells (△—△).

of components involved in activation, or alternatively a more direct effect of CHI on the activation process. Since the protein synthesis inhibitor Emetine also resulted in a comparable effect (data not presented), we prefer the former explanation. Cells that were not activated in the light and treated with CHI maintained their agglutinability for several hours (Fig. 2). Nonetheless, their light activation mechanism was inoperative, for when they were inactivated in the dark, they could not be reactivated while maintained in the presence of CHI. Thus once light activation has occurred, the mechanism need not be retained in an operative state; inactivation does not immediately follow exposure of light-activated gametes to CHI.

Table I. Characterization of Some Strains Isolated from Zygotes 5.39 and 17.17, Obtained by Crossing of *C. eugametos* UTEX 9 and 10

The mating type was established by presenting cells with gametes of the parent strains. Light sensitivity was determined by testing dark-treated cells for agglutinability before and after a light period. The sensitivity of the light activation for CHI was tested by preincubating dark-treated cells with 5 μ g/ml CHI for 1 h. At different time intervals after the start of illumination, the cells were tested for agglutinability by mixing them with gametes of the other mating type. The production of isoagglutinin activity in the dark and in light was determined as follows. One h after flooding two agar plates in the dark, the cell suspensions were mixed and divided into two equal parts of which one was placed in continuous light. After 48 h, the cells were removed by two centrifugation steps (10 min at 1000g and 20 min at 10,000g). The isoagglutinin in the supernatant was spun down at 50,000g for 30 min. This crude isoagglutinin material was suspended in 0.25 ml HEPES buffer (pH 7.6) and the isoagglutination activity was assayed as described in "Materials and Methods."

Tetrad Products	Mating Type	Light Sensitivity	CHI Sensitivity	Isoagglutinin Production	
				Dark	Light
5.39.1	+	-	ND ^a	2 ⁷	2 ⁸
5.39.2	-	+	+	<2 ⁰	2 ³
5.39.3	+	-	ND	2 ⁰	2 ¹
5.39.4	-	+	+	2 ⁰	2 ⁸
17.17.1	-	-	ND	2 ¹⁰	2 ⁹
17.17.2	+	+	+	<2 ⁰	2 ⁵
17.17.3	-	+	-	<2 ⁰	2 ⁴
17.17.4	+	-	ND	2 ⁶	2 ⁵

^a Not determined.

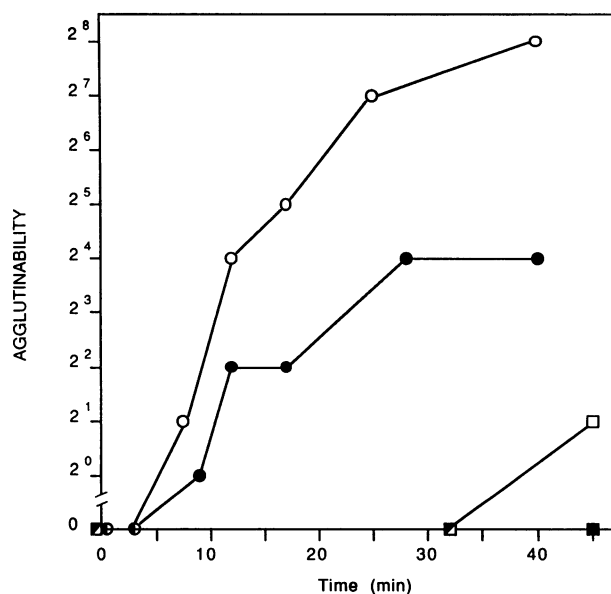


FIG. 3. Effect of different preincubation periods with CHI on the light-induced activation of flagellar agglutinability. Nonilluminated cells were preincubated with 5 μ g/ml CHI, which was added 30 min (■—■), 15 min (□—□), and 0 min (●—●) before the illumination was started at $t = 0$ min. Control cells without CHI (○—○). Control cells in continuous light were not affected by CHI.

Although it is the flagella that are involved in sexual agglutination, most of the agglutinin in a cell has been shown to be present on the outside of the cell body (21). We recently demonstrated that a membrane barrier separates the flagellar membrane from that of the cell body (20) and that glycoproteins are

therefore not able to diffuse from one membrane domain to the other. An illustration of this is that only those agglutinins confined to the flagellar surface of UTEX 9 are subject to a light-controlled activation system. Having produced a new light-sensitive *mt⁻* strain, we had to test whether in this strain the light sensitivity was also restricted to the flagella. Thus GTC extracts of both membranes from light- as well as from dark-treated gametes were obtained and tested for biological activity. The results are summarized in Table II. The vast majority of *mt⁻* agglutinin from this strain is present on the cell body surface and its activity is not influenced by light. In contrast, the activity of the fraction at the flagellar surface is strictly controlled by a light-sensitive mechanism as we have shown before (12).

Isoagglutinin Production in Light and Dark. Most *Chlamydomonas* gametes naturally release membrane vesicles into the culture medium which, when added to gametes of the opposite mating type, evoke isoagglutination. These vesicles, referred to as isoagglutinin, are thought to originate from the flagellar membrane by budding (1, 14, 16). The question was raised whether isoagglutinins produced in the dark, show isoagglutinative activity, or in other words, whether the flagellar membrane becomes agglutinable when naturally released from any restraints imposed by the living cell in the dark. In order to answer this question, we first had to be sure that isoagglutinins were indeed released from the flagella and not from the cell body where, as already explained, the light-sensitive mechanism does not operate. In Figure 4, the glycoprotein composition of isoagglutinins is compared with that of flagella and the cell body membrane. Isoagglutinins contain nearly all of the typical flagellar components but none of the major cell body glycoproteins. This is considered convincing evidence that isoagglutinins do not originate from the cell body surface. We therefore studied the release of isoagglutinin into the medium from 5.39.4, exposed to light or kept in the dark. The results are presented in Figure 5. Active isoagglutinins were only found in cultures in the light. Production of isoagglutinin in general is not a light-dependent process for all light-insensitive strains yield as much in the dark as in the light (Table I). It is only the light-sensitive strains that do not release active isoagglutinin in the dark. The conclusion is that they are only produced from agglutinable flagella. This was also illustrated when gametes of 5.39.4 were transferred from the dark to the light. They became agglutinable within 10 min, and within 2 h, they had produced a considerable quantity of active isoagglutinin (Fig. 5), but when the light activation was blocked by CHI treatment, the production of active isoagglutinin was similarly prevented.

Next, we investigated whether in the dark inactive cells produce inactive isoagglutinin. To this end, particulate material was isolated from both dark- and light-grown cultures containing

Table II. Influence of Light on Isoagglutination Activity of Isolated Flagella and GTC Extracts of Strain 5.39.4

A nonilluminated cell suspension with 2×10^9 cells was divided into two equal parts of which one part was illuminated for 1 h. Subsequently, the flagella were amputated by a pH shock (23) and separated from the cell bodies on a sucrose cushion. A small portion of the flagella was assayed for isoagglutination activity as described in "Materials and Methods." The rest of the flagella and the cell bodies were extracted in 3 M GTC. After removal of GTC from the supernatant by gel filtration, the extracts were assayed for biological activity by making a series of twofold dilutions of the extracts and determining the highest dilution that still evokes twitch activity in *mt⁺* gametes (9).

Cell Suspension	Dark	Light
Isolated flagella	<2 ⁰	2 ⁴
Extracts of flagella	<2 ⁰	2 ⁴
Extracts of cell bodies	2 ⁸	2 ⁹

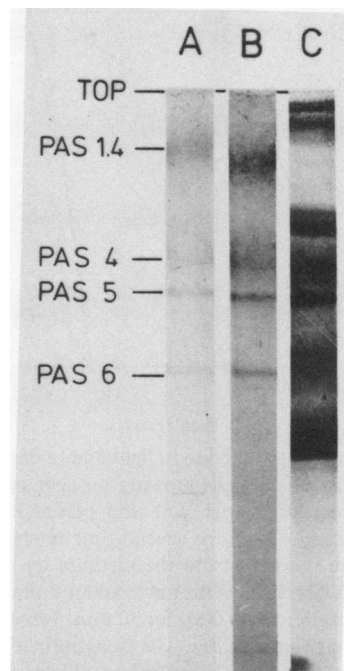


FIG. 4. SDS-PAGE of GTC extracted material from cell bodies, flagella, and isoagglutinin. Flagella, isoagglutinin, and 5% of the cell bodies were isolated from an illuminated cell suspension, containing 10^{10} cells, as described in "Materials and Methods." The GTC extracts were lyophilized to dryness and resolved in 100 μ l sample buffer. A, Flagella extract, 5 μ l; B, isoagglutinin extract, 10 μ l; C, cell body extract, 10 μ l. The gels were stained for glycoconjugates with periodic acid Schiff reagent (24) and dried onto Whatman chromatography paper No. 3. The various bands are indicated by numbers in accordance with Musgrave *et al.* (19). PAS 5 is due to residual contamination of cell wall material.

approximately 2×10^{10} cells. While the optical density of both preparations was the same, only that from the illuminated culture was biologically active (titer 2^8 compared with inactive). When GTC extracts were made of the two different samples, that from the dark culture was inactive, that from the light culture had a titer of 2^5 in the twitch assay. The two extracts were subjected to SDS-PAGE. Just as much glycoprotein-staining material was present in the extract of particles obtained from dark cells as in that of the particles obtained from light cells. The pattern of glycoprotein bands was similar to the high mol wt region of that shown in Figure 4. Thus it is clear that dark-grown gametes release inactive membrane vesicles from their flagella and that the state of activity or inactivity is maintained after release. It was not possible to activate these vesicles by illumination, yet as far as one can judge from the stained gels, the agglutinin band seemed to be equally present in both types of particles (Fig. 6A). Thus the inactivity of flagellar membranes in the dark seems not due to the complete absence of agglutinins or a temporary restraint imposed by the intact membrane, but rather to a chemical modification, and when the agglutinin is isolated from the living cell, the state of activity (inactive or active) is maintained.

Inactive Agglutinins. In order to reinforce the conclusion stated above, that the light-activation/dark-inactivation involves the chemical modification of flagellar agglutinins, it was considered important to demonstrate that inactive agglutinins are present on the flagella of dark grown gametes. Accordingly, flagella were isolated from nonagglutinable dark gametes as well as from agglutinable light gametes and GTC-extracts were made from them. The *mt⁻* agglutinin was then partially purified from other high mol wt species by anion exchange chromatography using a

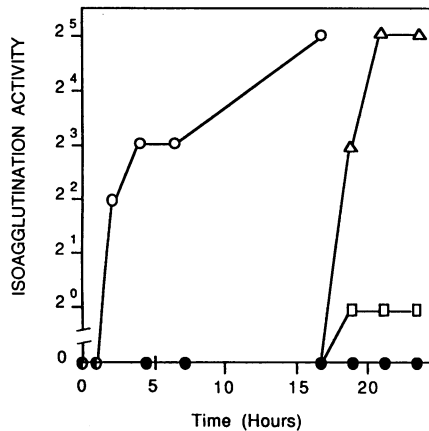


FIG. 5. Isoagglutinin production in light and in darkness by the light-sensitive mt^- strain 5.39.4. Cells growing on agar plates were flooded with 10 mM Hepes buffer (pH 7.6) and placed either in darkness (●—●) or in light (○—○). At several time intervals, the cells from one agar plate were removed from the medium by two centrifugation steps (10 min at 1000g and 20 min at 10,000g), the isoagglutinin was pelleted by centrifugation at 50,000g for 30 min. Subsequently, the pellet was taken in 0.25 ml Hepes buffer. The isoagglutination activity in this suspension was determined as described in "Materials and Methods." After 17 h, a batch of the nonilluminated cells, without isoagglutination activity, was split into three parts. One part was kept in the dark (●—●), another part was illuminated (△—△) and the third part was illuminated in the presence of 5 μ g/ml CHI which was added 1 h before the start of the illumination (□—□). Subsequently, the isoagglutination activity in the medium was determined.

QAE-Sephedex A25 column, eluted stepwise with increasing salt concentrations. Most of the agglutinin activity in the extract of flagella from illuminated gametes eluted in the 200 mM NaCl fraction. This fraction was subjected to SDS-PAGE next to the equivalent fraction from nonilluminated gametes. The photograph in Figure 6B shows that a typical agglutinin band was present in both fractions and that such a band was relatively absent from neighbouring fractions that contained less biological activity. The identity of agglutinin in extracts of dark flagella was further confirmed by immunoblotting using a Mab (66.3) that binds specifically to agglutinin among the mt^- flagellar components (WL Homan, unpublished data). Figure 6C shows that the Mab 66.3 bound just as effectively to an agglutinin band from dark-flagella as to that from light-flagella. We therefore feel justified in stating that during activation/deactivation, agglutinins are neither fed into, nor lost from the flagellar membrane but are modified and demodified while remaining on the outer flagellar surface. To test whether the mt^- agglutinin from dark flagella was indeed inactive when completely free of all other flagellar proteins, it was purified by gel filtration in the presence of SDS as described by Musgrave *et al.* (18). It remained inactive while the retention time was equivalent to that of purified active agglutinin. Therefore the modification by light does not involve the formation of a complex with other high mol wt components.

Effect of Light on Antigenicity of Agglutinin. We have seen that Mab 66.3 binds to inactive as well as to active agglutinin on nitrocellulose filters. This Mab blocks the agglutinability of active agglutinin. This implies that it binds at, or close to the sexual binding site. Although it does not appear to be able to discriminate between active and inactive agglutinin once they are denatured in SDS and blotted, it can readily distinguish between them when they are in a native state at the flagellar surface. When dark and light gametes were fixed with glutaraldehyde and tested in the indirect immunofluorescence assay for antigenicity using Mab 66.3, only the flagella of illuminated cells gave a weak

positive reaction (Fig. 7B). Considering that the agglutinins are minor flagellar components, a strong reaction was not to be expected. Dark-exposed 5.39.4 cells did not react with this Mab (Fig. 7A), but dark gametes of light-insensitive strains (*e.g.* UTEX 10) reacted just as strongly as their light-grown counterparts. In other words, there is a strong correlation between the binding of the antibody and the activity of the agglutinin. Living cells treated with Mab 66.3 directly labeled with fluorescein isothiocyanate exhibited the same binding characteristics. However, binding to flagella of illuminated gametes resulted in the antigens being transported to the flagellar tips, where their presence was seen as two fluorescent spots (10) (Fig. 7C). Dark-grown gametes never exhibited tipping, presumably because they were unable to bind the antibody. It was not because the tipping mechanism is inoperative in the dark, for the same cells were able to transport the lectin wheat germ agglutinin to their tips as effectively as light-grown cells (12). One must conclude then that when the mt^- agglutinin is in its native active state, the antigenic site for Mab 66.3 is exposed, but when it is in its native inactive state the antigenic site is cryptic. If the molecule is denatured, the antigenic site becomes exposed.

DISCUSSION

The kinetics of light-activation/dark-inactivation in the mt^- strain 5.39.4 are essentially the same as those described for the original mt^- strain UTEX 9 (12), from which it was derived. The mechanism in both strains is expressed only in the flagella and does not involve the turnover of agglutinins. This may be concluded from the fact that the mechanism in 5.39.4 is insensitive to α, α -dipyridyl-treatment which blocks agglutinin synthesis in this and other mt^- strains (20), and from the fact that the mechanism in several strains is independent of protein synthesis. Instead, activation/deactivation involves the regulation of the activity of agglutinins on the flagellar surface, for they can be extracted as inactive species from the flagella of dark-grown cells. Since the state of activity or inactivity is not affected by purification of the agglutinin in SDS, we may conclude that inactivation is not a restraint imposed on the agglutinin by association with another flagellar protein, rather it is a covalent modification which is irreversible once the cell is killed or the agglutinin is extracted. In some strains, including 5.39.4, light activation is inhibited when protein synthesis is prevented during activation. This implies that one of the components involved in triggering activation or in the mechanism of activation has a very rapid turnover. However, once the agglutinin is activated, protein synthesis is not needed to maintain the activated state, for 5.39.4 can be treated with cycloheximide for several hours in the light, without agglutinability being seriously reduced. Thus inactivation does not automatically follow when the activation system is switched off. It seems likely therefore, that activation and inactivation are two independent processes that are separately triggered, the one in the light and the other in the dark. What do these processes involve? Clearly the mechanism is operative with respect to mt^+ and mt^- agglutinin molecules which have two different binding sites. The modification is subtle because the electrophoretic and chromatographic properties of the active and inactive agglutinins are alike. Similarly, while the native inactivated mt^- agglutinin cannot bind the Mab 66.3 (in contrast to the active agglutinin) it can do so when the molecule is denatured after SDS-electrophoresis and blotting. These results suggest that a minor modification of the molecule affects its activity, *e.g.* (de)phosphorylation or (de)sulfation. This would imply that particular ectoenzymes such as kinases and phosphatases are operative at the outer surface. Such enzymes have been demonstrated by Ehrlich *et al.* (4). We are presently trying to detect their presence.

The monoclonal antibody used in this study, Mab 66.3, did

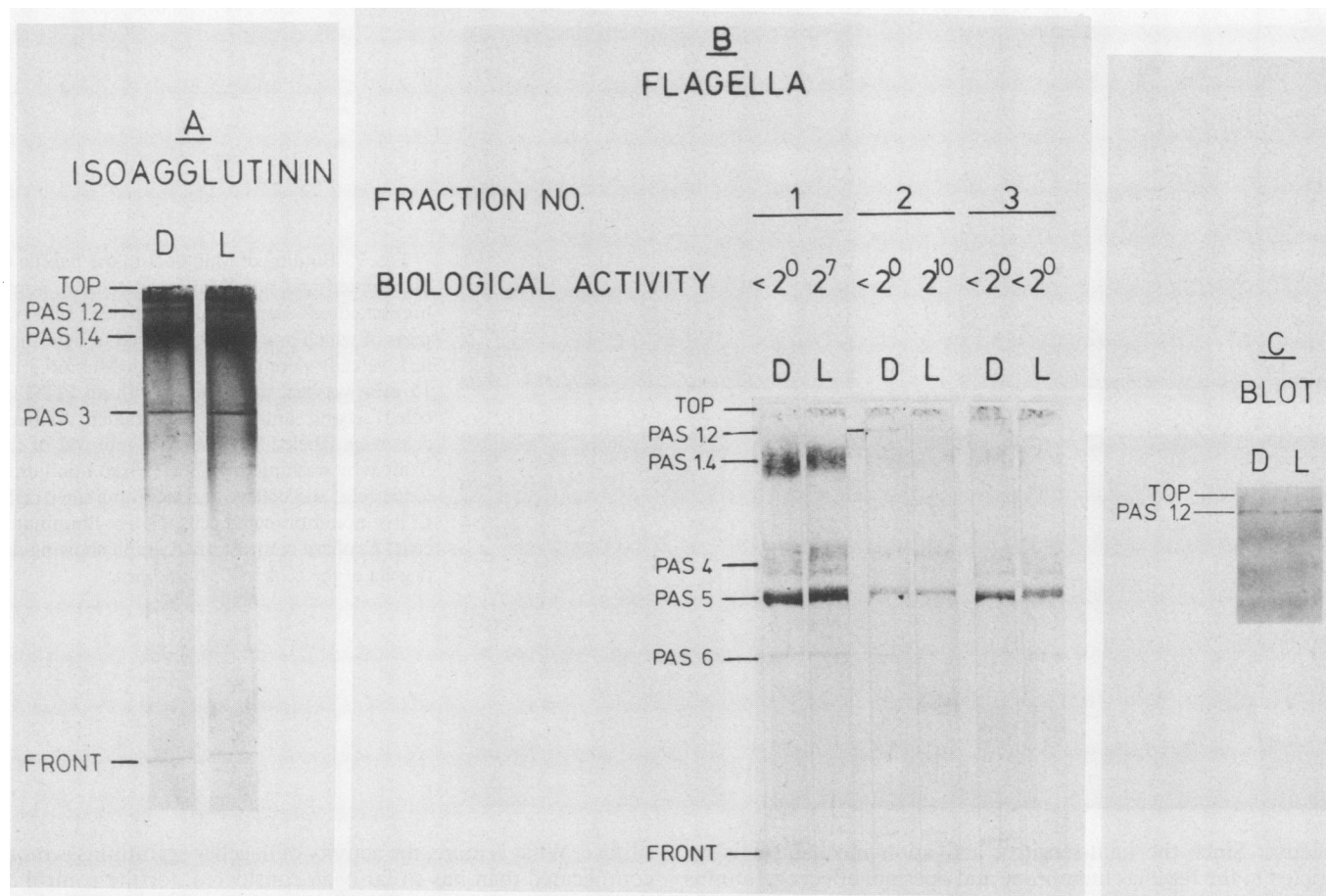


FIG. 6. SDS-PAGE of GTC-extracted material from isoagglutinin and flagella showing the mt^- agglutinin. Two cell suspensions were obtained by flooding two series of 100 agar plates with 10 mM Hepes buffer (pH 7.6). Then one suspension was placed in the dark and the other one was exposed to the same dark-light regime as vegetatively growing cells. After 40 h, *i.e.* 2 h after the start of the light period, the cells were separated from isoagglutinin particles by centrifugation at 1000g for 15 min. Subsequently, the isoagglutinins and the flagella were isolated and extracted in GTC as described in "Materials and Methods." The agglutinin in the extracts from isoagglutinin and flagella was partially separated from other components by anion exchange chromatography using a QAE Sephadex A-25 column (see "Materials and Methods"). Nonbound material, the 200 mM fraction and the 400 mM fraction were desalted, lyophilized to dryness, and resuspended in 250 μ l water. After determination of the biological activity, the fractions were lyophilized, taken in 80 μ l sample buffer and subjected to gel electrophoresis. *A*, Isoagglutinin extracts, 16 μ l: 200 mM fractions; *B*, flagellar extracts, 8 μ l (1-non-QAE-bound material, 2–200 mM fractions, 3–400 mM fractions). Gel *A* was silver stained according to Morrissey *et al.* (17). Gel *B* was stained for glycoproteins with periodic acid Schiff reagent (24) and photographed after the gels were dried onto Whatman chromatography paper No. 3. PAS 1.2 has been identified as the mt^- agglutinin (18). *C*, Immunoblots showing the immunoreactivity of active and inactive agglutinin to Mab 66.3. The 200 mM fractions from flagellar extracts of dark- and light-treated cells were subjected to SDS-PAGE as described above using 3 μ l sample buffer. The components in the upper part of the gel were transferred to nitrocellulose paper in 190 mM glycine, 20% (v/v) methanol and 25 mM TRIS-HCl buffer (pH 8.3). After a blocking step in 4% BSA, the nitrocellulose was incubated with Mab 66.3. Specific binding of Mab 66.3 was visualized by use of a second antibody conjugated with horseradish peroxidase (8). D = dark, L = light.

not bind to the flagella of cells in the dark. This is an interesting result because the Mab was selected for its ability to block the activity of isolated mt^- agglutinin. In the meantime Fab fragments have been demonstrated to block the agglutinability of living mt^- gametes, without affecting that of mt^+ gametes (WL Homan, unpublished data). Thus there was already good evidence for believing that Mab 66.3 binds to, or close to, the sexual adhesion site of the mt^- agglutinin. The new finding, that Mab-binding to the flagella of 5.39.4 is strictly correlated with flagellar agglutinability in the light, provides particularly convincing evidence for this contention. However, the antigenic epitope does not seem to include the site modified during inactivation, for when the inactive agglutinin is denatured, the epitope becomes available to bind Mab 66.3. Thus agglutinin-inactivation in the dark, while possibly a minor modification of an amino acid or sugar residue, seems to result in a change of conformation whereby the antigenic epitope becomes cryptic and the sexual

adhesion site inaccessible. The modification of the agglutination activity may therefore not involve regions directly involved in adhesion, but a site in the vicinity that affects the accessibility of the adhesion site. The idea that the adhesion site is not destroyed in the dark is strongly supported by the fact that low concentrations of glutaraldehyde can partially activate nonagglutinable gametes (data to be presented elsewhere).

The production of isoagglutinin is a well known property of the mt^- strain of *C. eugametos* that was used to establish the identity of the mt^- agglutinin (7, 18). It was assumed that these membrane vesicles originated from the flagellar membrane because their properties reflected those of the flagellar membrane and because Bergman *et al.* (1) obtained electron micrographs which seemed to demonstrate the loss of isoagglutinin from the flagellar membrane of *C. Reinhardtii*. From the present results it is confirmed that isoagglutinins originate from the flagellar membrane. Isoagglutinins which are produced in the dark are

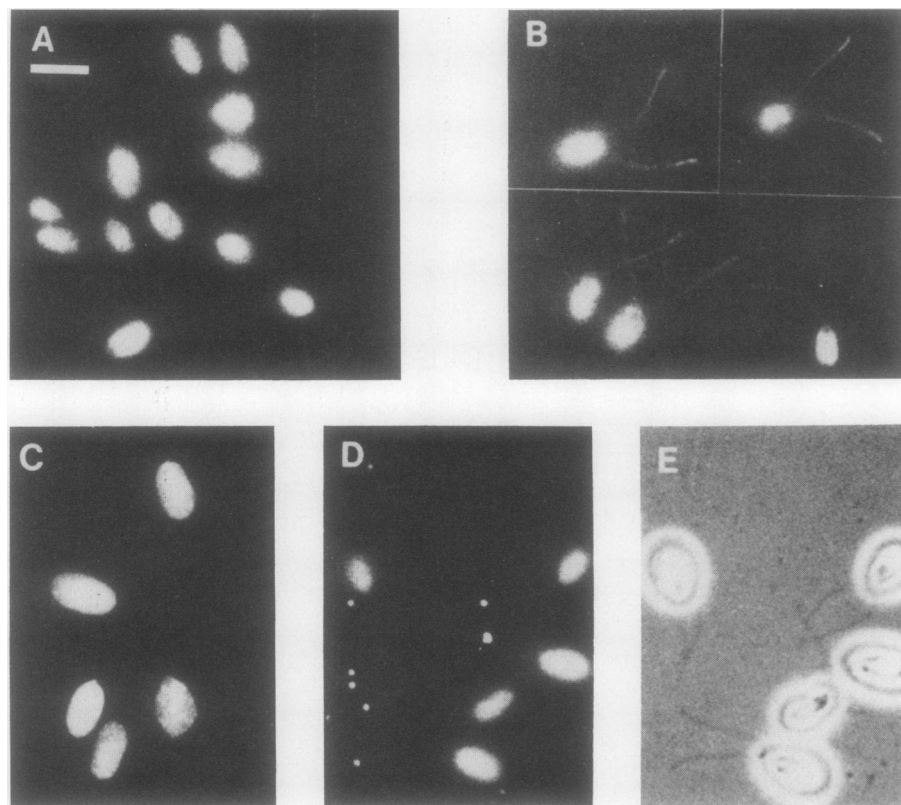


FIG. 7. Binding of Mab 66.3 to the flagella of illuminated and nonilluminated cells. A nonilluminated cell suspension was divided into two parts of which one part was placed in light for 1 h. Live cells were incubated with Mab 66.3 for 15 min, washed, and labeled with an FITC labeled second antibody. Glutaraldehyde fixed cells were labeled likewise after removal of the fixative by washing in PBS. *A*, Fixed nonilluminated cells; *B*, a collage of fixed illuminated cells; *C*, live nonilluminated cells; *D*, live illuminated cells; *E*, phase-contrast micrograph showing the flagella of the cells in *D*. Bar, 5 μm .

inactive. Since the light-sensitive activation mechanism is restricted to the flagellar membrane and does not affect agglutinins on the cell body surface, there can be little doubt that agglutinins bud from the flagellar membrane.

When a function of a plant cell is influenced by the light/dark cycle, it is natural to consider whether it is via photosynthesis, since the source of energy could be limiting in the dark. However, cells kept in the dark are not depleted of energy, for swimming, flagellar growth and glycoprotein synthesis are not affected by the dark. Furthermore, the generation of ATP by oxidative phosphorylation in darkness is 10 times higher than the amount of ATP that can be generated by photosynthesis under the low light intensities (0.2 W/m^2) that are sufficient to induce agglutinability. Thus the generation of ATP is unlikely to play a key role in the light-activation. Other products of photosynthesis are not involved either, since inhibition of photosystem 2 by dichlorophenyl-dimethylurea does not affect the light-activation (R Kooijman, unpublished observations). In conclusion then, while the light receptor may well lie in the cell body and could then involve a signal diffusing to the flagellar membrane, the photosynthetic machinery is not directly involved.

From the evidence presented, we can safely conclude that the light controlled activation/inactivation in *C. eugametos* is an example of post-translational modification of sexual activity. Since the flagella can be readily isolated and the plasma membrane is the only membrane present, this system provides a good model for studying the topography of membrane proteins in general, and particularly for studying their interactions to explain this regulation mechanism. The flagella membrane exhibits other characteristics that makes it an exciting study object. For example, we have recently demonstrated that the agglutinins that become involved in sexual agglutination, are transported to the flagellar tips (10). Therefore there is not only an interesting interplay of components external to the membrane, regulating agglutinin activity, but also internally, where the agglutinins can become attached to the cytoskeleton and redistributed over the

surface. What is more, the activity of flagellar agglutinins is more complicated than has so far been considered, for the control of agglutinability exists at different levels. First of course, there is the mechanism of gametogenesis in which the synthesis of agglutinins is triggered. Second, the density of agglutinin in the membrane is subjected to a circadian rhythm, being appreciably higher at the beginning of the light period than at the end of the day. This has been explained as a rhythm in agglutinin synthesis and incorporation into the flagella, and the only effect of light is in setting the clock that determines the rhythm (3). Third, as we have seen, the complement of flagellar agglutinins is in some strains subject to a light-controlled modification of activity. Lastly, it has been shown that during sexual agglutination, the level of agglutinability rises dramatically (about $10\times$ or 2^{3-4} when expressed as a dilution titer) independent of the original ground state activity (R Demets, A Tomson, unpublished observations). This could be due to different causes, for example the incorporation of new agglutinins into the flagellar membrane, their rearrangement in the membrane, *e.g.*, clustering to form more effective adhesions, or a form of agglutinin activation independent of the light activation described here. We are presently trying to sort out these possibilities.

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