AGGREGATION-DEPENDENT TURNOVER OF FLAGELLAR ADHESION MOLECULES IN *CHLAMYDOMONAS* GAMETES

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ABSTRACT

Previous studies on flagellar adhesion in *Chlamydomonas* (Snell, W. and S. Roseman. 1979. *J. Biol. Chem.* **254:**10820–10829.) have shown that as gametes adhere to flagella isolated from gametes of the opposite mating type, the adhesiveness of the added flagella but not of the gametes is lost. The studies reported here show that the addition of protein synthesis inhibitors (cycloheximide [CH] or anisomycin) to the medium of such cell-flagella mixtures causes the cells to lose their adhesiveness. This loss, however, occurs only after the cells have interacted with 4–8 flagella/cell and does not occur if the cells are kept in CH (7 h) without aggregating.

The availability of an impotent (imp) mating type plus (mt^+) mutant (provided by U. W. Goodenough), which adheres but is unable to undergo the fusion that normally follows adhesion, made it possible to determine whether a similar loss of adhesiveness occurs in mixtures of mating type minus (mt^-) and imp mt^+ gametes. In the absence of inhibitor, mt^- and imp mt^+ gametes adhered to each other (without fusing) for several hours; however, in the presence of CH or anisomycin, the gametes began to de-adhere 35 min after mixing, and, by 90 min, 100% of the cells were single again. This effect was reversible, and the rapid turnover of molecules involved in adhesion ocurred only during adhesion inasmuch as gametes pretreated for 4 h with CH were able to aggregate in CH for the same length of time as nonpretreated cells aggregated in CH. By the addition of CH at various times after the mt^- and imp mt^+ gametes were mixed, measurements were made of the "pool size" of the molecules involved in adhesion. The pool reached a minimum after 25 min of aggregation, rapidly increased for the next 25 min, and then leveled off at the premixing level. These results suggest that flagellar adhesion in *Chlamydomonas* causes modification of surface molecules (receptors, ligands), which brings about their inactivation and stimulates their replacement.

KEY WORDS cell-cell interactions · *Chlamydomonas* mating · flagellar adhesion/de-adhesion · receptor turnover

Cell recognition and adhesion are essential processes in eukaryotic fertilization, development, and homeostasis. Because these specific interactions between cells are such complex, multistep phenomena (16, 24), there has not yet been a complete description of the mechanisms of recognition and adhesion. The mating system of the biflagellate alga *Chlamydomonas reinhardtii* has been used as a model system for such studies (see references 7 and 26 for a review).

Mating type plus (mt^+) and mating type minus (mt^-) *C. reinhardtii* cells grown separately, asexually, and in defined medium can be induced to

differentiate into gametes by resuspension in nitrogen-free (N-free) medium (3, 18). When gametes of opposite mating types are mixed, their flagella adhere to each other, and large aggregates of cells are formed. The flagellar interactions then signal two events necessary for the subsequent fusion between individual mt^+ and mt^- gametes within the aggregate: release of a cell wall degrading enzyme (5, 7, 13, 21, 23) and activation of mating structures (7). After the fusion of opposite cell types, there is a rapid loss of flagellar adhesion and the zygote becomes motile.

Although much has been learned about these processes, the basic mechanisms for the initial flagellar adhesion, subsequent signalling, and loss of adhesiveness after fusion have not yet been delineated. To learn more about the entire mating process, it is important to separately study and quantify each event in the sequence. The adhesion step can be investigated by mixing gametes of one mating type with isolated flagella, flagellar membranes, or gamones (mating factors) (7, 26) prepared from gametes of the opposite mating type. In such mixtures, large aggregates of cells form, but no cell fusion occurs because the cells are all of one mating type. Recently, a quantitative assay for *Chlamydomonas* aggregation was described (22). A Coulter counter (Coulter Electronics Inc., Hialeah, Fla.) was used to measure the rate of loss of single cells as they aggregated in suspension. An interesting result of these studies (which confirmed other, possibly related, microscopic observations [6, 13]) was that, in cell-flagella mixtures, flagellar adhesion was quickly followed by deadhesion. That is, gametes aggregated by flagella isolated from gametes of the opposite mating type rapidly disaggregated. By use of the Coulter counter assay and a radioactive flagella binding assay (20, 22), it was shown that the adhesiveness of the added flagella was destroyed, whereas the gametes could still adhere to fresh flagella. Although these results suggest that the adhesiveness of the live gametes was unaffected by their interaction with "dead" flagella, it is possible that adhesive sites on the live cells were lost and then replaced by biosynthesis within the cells.

Our studies were initiated to determine whether protein synthesis might be required for maintenance of adhesiveness. This has been tested in two different ways: (*a*) with cell-flagella mixtures as described above, and (*b*) with cell-cell mixtures in which one gamete is a mutant incapable of the fusion that normally occurs following adhesion (9). The availability of an impotent (imp) mutant made it possible to determine whether the deadhesion seen in cell-flagella mixtures might be demonstrated in cell-cell mixtures. The results indicate that flagellar adhesion in *C. reinhardtii* causes modification of flagellar surface adhesion molecules, which brings about their inactivation and stimulates their replacement.

MATERIALS AND METHODS

Cultures

Stock cultures of *C. reinhardtii* strains 21gr (mt^+), and 6145c (mt^-), originally obtained from Dr. Ruth Sager, Sydney Farber Cancer Center, Boston, Mass., and an impotent (imp) mt^+ mutant (imp-1), generously provided by Dr. U. W. Goodenough, Washington University, St. Louis, Mo., were grown axenically on 1.5% agar slants supplemented with 2 g/liter of sodium acetate and 4 g/liter of yeast extract in medium I (see below) at 12°C in continuous light as previously described (19, 22). Axenic liquid cultures were inoculated from agar slants and grown in 250-ml Erlenmeyer flasks in 150-ml quantities of medium I; 4-liter cultures were grown in 5-liter diphtheria toxin bottles in medium II (see below). Liquid cultures were grown at 25°C with continuous aeration on a cycle of 12 h of light and 12 h of dark.

Materials

Cycloheximide (3[2(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide) and Trizma (Tris base) were obtained from Sigma Chemical Co., St. Louis, Mo. Anisomysin was a gift from Pfizer Chemicals Div., Pfizer Inc., Groton, Conn. The K_2HPO_4 and KH_2PO_4 were analytical reagent grade obtained from Mallinckrodt Inc., St. Louis, Mo. All other chemicals were reagent grade.

Medium I (after Sager and Granick, 18), pH 6.8, consists of the following components (per liter): 1 mg of H_2BO_3, 1 mg of $ZnSO_4$, 0.4 mg of $MnSO_4 \cdot 4H_2O$, 0.2 mg of $CoCl_2 \cdot 6H_2O$, 0.2 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.04 mg of $CuSO_4$ (these trace elements were stored at 4°C as a 10-fold concentrated stock solution), 0.5 g of sodium citrate dihydrate, 0.01 g of $FeCl_2 \cdot 6H_2O$, 0.04 g of $CaCl_2$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of NH_4NO_3, 0.1 g of KH_2PO_4, and 0.1 g of K_2HPO_4. The constituents are added in the order listed to prevent the formation of precipitates. Medium II, pH 6.8, is medium I with the addition of 3 g/liter of sodium acetate trihydrate and three times the amount of the phosphate buffers. N-free medium is the same as medium I without NH_4NO_3 and KH_2PO_4 and with only enough K_2HPO_4 to bring the pH to 7.6.

Methods

Protein determinations were made by the method of Lowry et al. (12), with a bovine serum albumin standard.

INDUCTION OF GAMETES: To obtain gametes, vegetative cells were grown to a density of 2–4 × 10^6 cells/ml (as determined by use of a Coulter counter or a hemocytometer with iodine-fixed cells), and, after 6 h in the light, the cells were washed into and incubated in N-free medium in continuous light with aeration at 25°C as previously described (3, 19, 22).

ISOLATION OF FLAGELLA: Gametic flagella were isolated from 4-liter cultures grown in medium II by the sucrose-pH shock method of Witman et al. (28) as previously described (19). The isolated flagella were suspended in 7% sucrose in 10 mM tris

buffer, pH 7.8, at 25°C (hereafter called tris buffer) and either used immediately or stored frozen at −20°C. Estimates of the number of flagella were based on protein concentrations of flagellar suspensions (1.7–2.0 × 10^9 flagella/mg protein [22]).

AGGREGATION ASSAY: A Coulter model ZBI electronic particle counter fitted with an electrode with a 100-μm bore aperture was used to determine cell concentration and to assay cell aggregation, as previously described (15, 22).

Cell distribution with respect to size was obtained by determining cell numbers at various window settings of the Coulter counter. The threshold settings were adjusted for each experiment to include at least 75% of the single cells. Typical settings were 1/aperture current = 0.5, 1/amplification = 0.5. Threshold window settings were 15–20 for the lower and 50–60 for the upper window. For counting, 0.125- or 0.250-ml cell samples were diluted into 50 ml of ice-cold medium II.

Two types of cell aggregation were studied: (*a*) aggregation between mt^- and imp mt^+ gametes, where no fusion occurs, and (*b*) aggregation between gametes of a single mating type and flagella isolated from gametes of the opposite mating type. Aggregation was measured by determining the rate of loss of single cells from the suspension.

Cells (number, volume, medium, and method of agitation specified in each experiment) were incubated in 30-ml plastic beakers and swirled occasionally by hand, slowly bubbled with air, or stirred with a magnetic stirrer with a 2.5- × 1.0-cm stirring bar at 25–30 rpm to keep the cells homogenously suspended. At predetermined times, 0.125 ml or 0.250 ml of each suspension was transferred (Gilson 0-1 ml Pipetman Gilson Medical Electronics, Inc., Middleton, Wis.) into 50 ml of cold medium II in a 50-ml plastic beaker. To minimize shear forces, the end of the pipette tip was cut off to leave a bore of 2 mm. To ensure homogeneity, the 50 ml of diluted cells was poured slowly into a 100-ml plastic beaker for counting.

RESULTS AND DISCUSSION

Cycloheximide Inhibition of Cell-Flagella Adhesion and De-adhesion

The kinetics of adhesion and de-adhesion of mt^- gametes mixed with mt^+ flagella are shown in Fig. 1. Aggregation was measured by determining the rate of loss of single cells from a suspension by use of the Coulter counter as described in Materials and Methods. The results (solid line) indicate that the control gametes in N-free medium without inhibitor aggregated when flagella were added, but, as has been shown (22), rapidly de-adhered to become single again. Adding more flagella (arrows) caused the gametes to reaggregate and then to de-adhere again. These control cells showed essentially the same kinetics of adhesion/de-adhesion for seven cycles over a 7-h period.

To determine whether protein synthesis was necessary for adhesion/de-adhesion, mt^- gametes in N-free medium were mixed with isolated mt^+ flagella in the presence of cycloheximide (CH; 1 μg/ml). It has been shown that concentrations ≥1 μg/ml of CH rapidly and effectively (85–100%) inhibit protein synthesis in *Chlamydomonas* (17). The kinetics of adhesion/de-adhesion of the cells in CH were identical to the kinetics of the control cells for the first cycle (broken line). However, in later cycles, the CH-treated cells began to de-adhere more slowly, and at the seventh addition of fresh flagella (345 min), >30% of the cells in CH were still aggregated. Moreover, when the fresh flagella were added, <15% of the remaining single cells aggregated. Similar results were obtained when fresh flagella were added at 390 and 400 min: after seven cycles of adhesion/de-adhesion in CH, nearly 30% of the cells had not de-adhered, and the 70% of the cells that were single were unable to adhere to freshly added flagella. Microscope observation of these cells showed that they were still fully flagellated and motile. Also, control gametes kept in CH for 7 h without flagella being added were still fully capable of interacting with fresh flagella (80% aggregation). These results suggest that, during interactions between gametes and isolated flagella, adhesion sites on both the added flagella and the live cells are lost. However, only if protein synthesis is inhibited, preventing appearance of new adhesion sites, is the loss of sites on the live cells demonstrable.

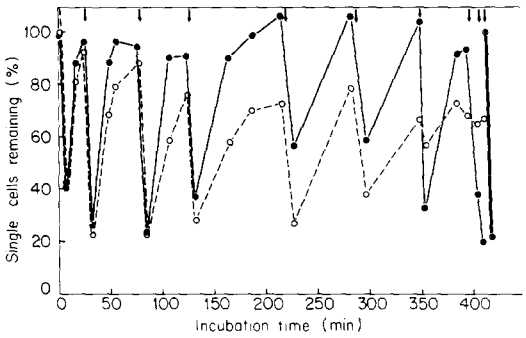

FIGURE 1 Effect of CH on adhesion/de-adhesion. Mt^- gametes (10 ml; 8 × 10^6 cells/ml in N-free medium) were mixed at 22°C with mt^+ flagella in Tris buffer at $t = 0$ and at the times indicated by the arrows to give a final concentration of 6 × 10^6 fresh flagella/ml. The suspensions were stirred at 25 rpm with a magnetic stirrer. At various times after mixing, 0.25-ml portions of the suspension were diluted into 50 ml of ice-cold medium II, and the extent of aggregation was determined with the Coulter counter as described. With CH (1 μg/ml), ○. Without CH, ●. Pretreated with CH, ⊙.

Cycloheximide Effects on De-adhesion

In this and similar experiments, gametes did not lose adhesiveness until each had interacted with 4–8 flagella. (In the experiments shown in Fig. 1,

about 0.75 flagella/cell were added for each of the seven cycles.) This indicates that flagellar de-adhesion involves a stoichiometric rather than a catalytic interaction. Adhesion between flagella of opposite mating types might be the result of the formation of irreversible complexes between complementary molecules. De-adhesion would result from a rapid removal or clearing of the complexes from the flagellar surface, and new protein synthesis would be required to replace the irreversibly complexed adhesion molecules. Our finding that, in the cell-flagella mixtures in CH, the cells appeared to lose their ability not only to adhere but also to de-adhere suggests that the putative clearing activity might also depend upon protein synthesis. In this regard, it has been shown that *Chlamydomonas* cells can actively and rapidly move latex microspheres (1, 2), antibodies (10), and even adherent flagella (8) along their flagellar surface; Bloodgood et al. (2) have reported that this flagellar surface motility is inhibited by CH. Although these may be related phenomena, the relationship, if any, between adhesion/de-adhesion and flagella surface motility remains to be determined.

Adhesion/De-adhesion of Impotent Gametes and Flagella

To determine whether similar protein synthesis is required during the interaction of live gametes of opposite mating types, it was necessary to use a mutant that could aggregate but not fuse. Wild-type cells fuse so rapidly (80–100% fusion within 5–10 min [7, 22]) after mixing at 25°C that it would be difficult to distinguish the biosynthetic requirements for adhesion from those for fusion. To avoid this problem, an impotent mt^+ gamete, imp-1, which can aggregate but not fuse, was used for many of the experiments described below.

It was first necessary to establish that the imp mt^+ cells could aggregate and would show wild-type patterns of adhesion/de-adhesion, both in mixtures of mt^- flagella with imp mt^+ cells and in mixtures of imp mt^+ flagella with mt^- cells. The results shown in Fig. 2 indicate that imp mt^+ cells as well as flagella isolated from these mutant cells show the same pattern of adhesion/de-adhesion as do wild-type cells.

Effect of CH on Adhesion/De-adhesion between Imp Mt^+ and Wild-Type Mt^- Cells

To investigate whether there is a requirement for protein synthesis in prolonged cell-cell interaction, as opposed to the previously described cell-flagella interaction, mt^- and imp mt^+ gametes were mixed in the presence or absence of CH, and aggregation was determined by use of the Coulter counter assay. Without CH the cells stayed aggregated for several hours and did not fuse to form quadriflagellated zygotes, as determined by light microscopy. However, after 200 min, 50% of the cells were singles again (Fig. 3). After 4 h, 25% of the cells were still aggregated and there appeared to be many large clumps of cells, which could still be seen 10–12 h later. Thus, the mutant cells did aggregate for an extended period of time, did not fuse, and, therefore, were suitable for these studies.

The gametes aggregating in the presence of CH behaved much differently from the control cells: 35–40 min after mixing, they began to de-adhere, and, after 80 min, 90% of the cells were single again. Microscope examination of the suspension showed that the cells were fully flagellated and motile. Gametes incubated with anisomycin, another inhibitor of protein synthesis (4), gave identical results (data not shown). In addition, mixing isolated flagella of either mating type with de-adhered cell suspensions did not cause any aggregation that could be detected with the microscope or with the Coulter counter assay (data not shown).

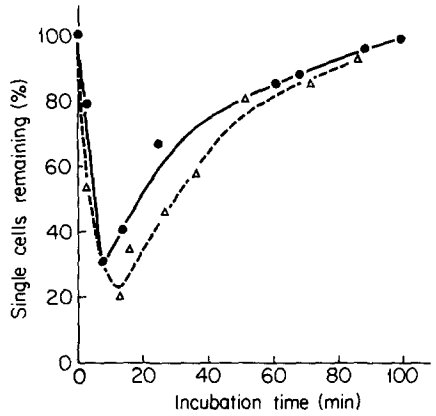

FIGURE 2 Kinetics of aggregation. Gametes (8×10^6 cells/ml in N-free medium) were mixed at 22°C with flagella of the opposite mating type in tris buffer and stirred at 25 rpm with a magnetic stirrer. At various times after mixing, 0.25-ml portions were diluted into 50 ml of ice-cold medium II, and the extent of aggregation was measured as described. Mt^- gametes mixed with imp mt^+ flagella, final concentration 7×10^7 flagella/ml, ●. Imp mt^+ gametes mixed with mt^- flagella, final concentration 3×10^7 flagella/ml, △.

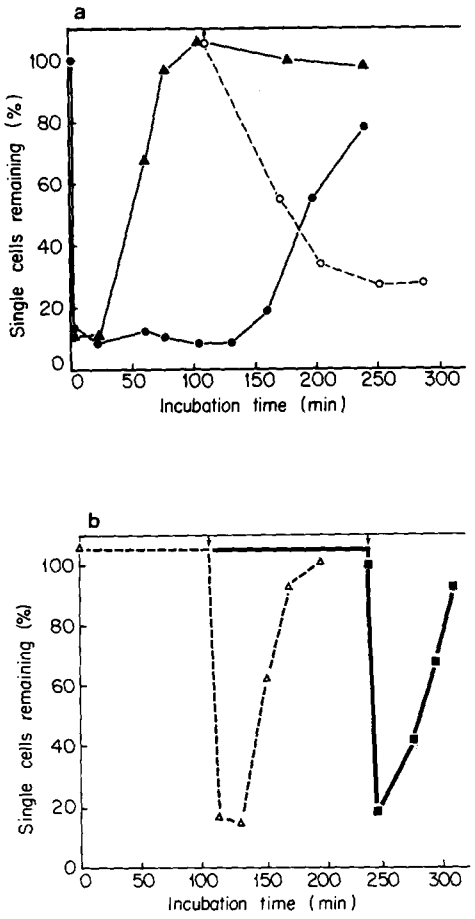

FIGURE 3 (*a*) Effect of CH on aggregation of mt^- and imp mt^+ gametes. Mt^- and imp mt^+ gametes (5 ml of each, 8×10^6 cells/ml in N-free medium) were mixed together at 22°C without (●) and with CH (10 μg/ml) (△). The cells were swirled gently every 5 or 10 min to keep them in suspension. Aggregation was measured with the Coulter counter. At 111 min after mixing, when 100% of the cells in CH were singles again, a portion of the suspension was washed out of the CH by adding 5 ml of the suspension to 9 ml of N-free medium and centrifuging at 2,000 *g* (3,000 rpm, IEC CRU 5000 centrifuge, rotor no. 264, Damon Corp., I.E.C. Div., Needham Heights, Mass.) for 2 min at 25°C in 50-ml conical polycarbonate centrifuge tubes. The sedimented cells were washed two times with N-free medium, resuspended in N-free medium to a concentration of 8×10^6 cells/ml, and bubbled slowly with air to keep them in suspension. (*b*) Pretreatment of mt^- and imp mt^+ gametes with CH. Mt^- and imp mt^+ gametes (10 ml of each, 8×10^6 cells/ml in N-free medium with 10 μg/ml CH) were incubated with aeration at 24°C for 104 min (△) or 239 min (■) before mixing (arrows).

When a portion of the de-adhered cell suspension was washed out of the CH into fresh medium, gametes began to reaggregate. Within 70 min, 50% of the cells had reaggregated.

This loss of adhesiveness during aggregation in the presence of CH could simply be the result of a natural turnover of adhesive molecules on the flagella, inasmuch as Bloodgood has reported that some flagella surface proteins turn over quite rapidly (2). On the other hand, the results of cell-flagella interaction shown in Fig. 1 suggest that it is adhesion per se that stimulates the much more rapid turnover of adhesive sites. To test this in cell-cell mixtures, gametes were pretreated separately with CH for 100 or 250 min and then mixed in the continuous presence of CH. The results shown in Fig. 3*b* indicate that the pretreated cells could still aggregate just as well as the nonpretreated cells (cf. Fig. 3*a*). Furthermore, the pretreated cells stayed aggregated for the same length of time as the nonpretreated cells. In all three cases, 45 ± 5 min elapsed from the time the cells were mixed until 50% were single again. These results, in combination with those from the cell-flagella experiments (Fig. 1), indicate that flagellar adhesion between live gametes of opposite mating types is a dynamic interaction in which adhesive sites are continuously being lost and replaced. Furthermore, although there may be a low rate of turnover of these molecules in the absence of adhesion, it is only during adhesion that the molecules are lost at a rapid rate.

Temperature Effects on De-adhesion in CH

Previous studies (22) on de-adhesion of cell-flagella mixtures have shown that de-adhesion is a temperature-sensitive process. To determine whether there were similar effects of temperature on the rapid loss of adhesiveness of live gametes in CH, mt^- and imp mt^+ gametes were incubated with CH at 10°, 16°, and 21°C, and aggregation was measured as described above. Fig. 4 shows that the time required for inhibition to be expressed was lengthened and that the rate of de-adhesion was reduced when the incubation temperature was lowered, indicating that these are temperature-dependent processes.

Pool Size of Proteins Involved in Adhesion

The results shown in Fig. 3, which indicate that mt^- and imp mt^+ gametes mixed with CH began to de-adhere after 30–40 min of aggregation, suggest that the cells have a reservoir of the molecules

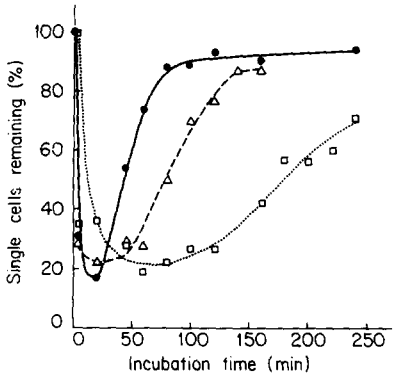

FIGURE 4 Effect of temperature on adhesion/de-adhesion of mt⁻ and imp mt⁺ gametes in CH. Mt⁻ and imp mt⁺ gametes (5 ml of each, 8×10^6 cells/ml with 1 μg/ml CH) were incubated at 21° (●), 16° (△), and 10°C (□). The cells were slowly bubbled with air, and aggregation was measured with the Coulter counter.

involved in adhesion. In the presence of CH, this pool of adhesive sites is completely depleted, whereas, in the absence of inhibitor, the cells apparently regenerate enough sites to maintain flagellar adhesiveness. To further investigate pool size, mt⁻ and imp mt⁺ gametes were mixed, and, at 0, 10, 20, 30, 40, 55, and 80 min after mixing, CH was added and aggregation was measured as described above. The results shown in Fig. 5 *a* indicate that, independent of the time of addition of CH, the cells eventually began to de-adhere and that the rate of de-adhesion was the same for all samples. Of more interest, though, was the finding that the length of time until inhibition was expressed varied with the length of time the cells had been aggregating. This is shown in Fig. 5 *b*, which presents results averaged from three experiments in which protein synthesis inhibitors were added at various times after the cells were mixed. In two of the experiments, the inhibitor used was CH, and, in the third, anisomycin was used. The time until inhibition was expressed (defined as the time at which the cells first began to de-adhere minus the time of addition of inhibitor) was plotted vs. incubation time. If the assumption is made that the rate of utilization of adhesion sites is constant, then the time until inhibition is expressed will be related to the pool size of adhesive molecules. Fig. 5 *b* shows that, when inhibitor was added when the cells were mixed ($t = 0$), aggregation continued for 35 min whereas when inhibitor was added at $t = 20$ min, the cells began to de-adhere within 15 min. By $t = 55$ min, the cells were again able to stay aggregated for ~35 min after addition of the inhibitor. These results suggest that, immediately after adhesion starts, the pool of adhesive proteins begins to be reduced until ~25 min have passed, when the pool apparently reaches a minimum. After 25–30 min of aggregation, the pool rapidly increases and, at $t = \sim 55$ min, returns to its premixing level. Therefore, although aggregation causes rapid utilization of adhesion molecules, the gametes are able to regulate the pool of these molecules, both before and during the adhesion process. Future experiments using radioactive precursors may reveal which, if any, of the flagellar surface proteins show an increased rate of synthesis during adhesion.

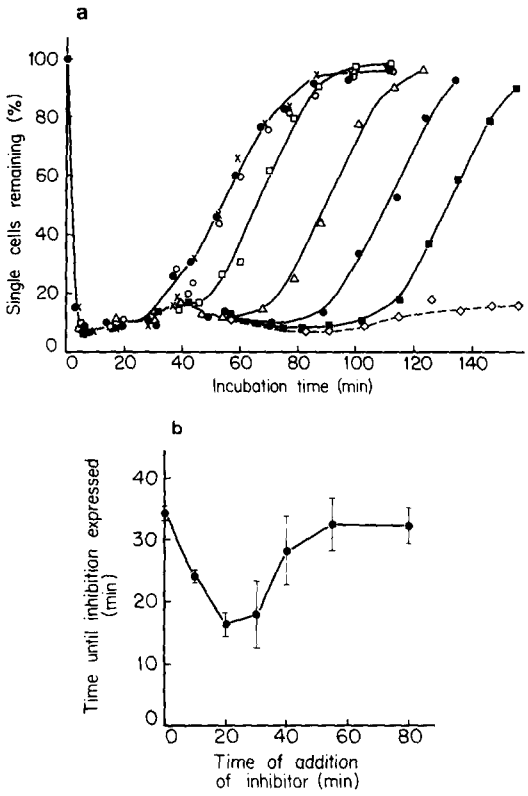

FIGURE 5 (*a*) Effect of time of addition of CH on de-adhesion. Mt⁻ and mt⁺ gametes (5 ml of each, 8×10^6 cells/ml in N-free medium) were mixed at 22°C, and, at 0 (●), 10 (×), 20 (○), 30 (□), 40 (△), 55 (⊙), and 80 min (■) after mixing, CH was added to give 1 μg/ml. A control suspension had no CH added (◇). Suspensions were stirred at 25 rpm with a magnetic stirrer and aggregation was measured as described. (*b*) Replot of the data shown in *a* averaged with data from two similar experiments done on a different day, one performed with CH (1 μg/ml) and one with anisomycin (12.5 μM). Bars represent average deviation from the mean.

The experiments described above raise the question of the mechanism of regulation of the adhesion proteins. Rosenbaum and co-workers (11, 17) and Weeks and Collis (25) have shown that deflagellation or methods used to induce flagellar resorption stimulate flagellar protein synthesis and that the rate of new synthesis is carefully regulated. In fact, gametes may be able to detect the absence of the adhesion molecules by a mechanism similar to the one that permits them to recognize the absence of a flagellum. In this regard, several workers have shown that trypsinization of gametes destroys their flagellar adhesiveness (7, 14, 20, 27). Within 1–4 h after the trypsin has been washed out, the cells regain their aggregating ability (14, 20). One explanation for the reappearance of new adhesion sites is that the reappearance simply reflects the normal turnover of adhesion sites and that these sites would have appeared whether or not the cells had been trypsinized. However, the data presented in this report show that, in the absence of adhesion, the adhesion molecules turnover very slowly, if at all (Figs. 1 and 3). Therefore, the reappearance of adhesiveness after trypsinization and maintenance of adhesiveness during aggregation may be caused by new synthesis initiated in response to depletion of the adhesion molecules. The signal to generate new adhesion sites may be the same as the one controlling cell wall release and mating structure activation (7). This idea could be tested with mutants appropriately defective in signal generation.

In conclusion, the results presented in this report indicate that flagellar adhesion in *Chlamydomonas* involves the continuous loss and replacement of flagellar surface molecules. These observations were possible because adhesion could be effectively separated from fusion by use of a mutant and by use of isolated flagella. Similar adhesion/de-adhesion phenomena may also be discovered in other systems in which cell-cell interactions are studied, but, to determine this, it will be necessary to attempt to separate initial recognition and adhesion from later junction formation.

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