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博 士 論 文

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昭和６３年３月

神戸大学大学院自然科学研究科

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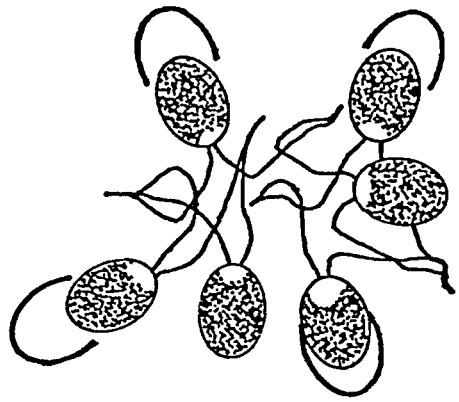
クラミドモナスの性的凝集物質と細胞壁溶解酵素に関する研究



昭和６３年３月

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General introduction

All modern eukaryotic organisms have sexual options in their life cycles. Such sexual options to form diploid organisms have several advantages: (1) Diploidy is usually associated with faster growth rate. (2) Gene complementation results in the correction of the effects of deleterious genes. (3) If two copies of a genome exist in the nucleus, one copy is free to undergo mutational experimentation. (4) Progeny with new gene combinations is generated via meiotic process (cf. Goodenough 1985). Moreover, in the protozoa, zygospore with rigid capsule provides a way of species survival under harsh environmental conditions which would be lethal to vegetative cells.

Among the modern protozoa, the mating-systems of Chlamydomonas and Saccharomyces are probably well understood. Sexual processes in Chlamydomonas also provide good opportunities for the study of various aspects of cell interaction and intercellular communication, such as cell recognition/adhesion, signalling and signal perception, release of cell wall lytic enzyme, and cell and nuclear fusion.

In this thesis I have investigated the differentiation of sexuality in Chlamydomonas reinhardtii where considerable attentions have been given to the studies of synthesis and processing of two key molecules for gametogenesis, sexual agglutinin and cell wall lytic enzyme.

Sexual reproduction

The unicellular biflagellated green alga, C. reinhardtii, occurs as two sexually compatible strains referred to as mating-type plus (mt^+) and minus (mt^-). The two strains grow separately and vegetatively in defined medium and light (Fig. 1A,B). However, gametes are induced when vegetative cells are

placed under a nitrogen-starved condition (Fig. 1C, D) (Sager and Granick 1954; Kates and Jones 1964). When the mt^+ and mt^- gametes are mixed, they instantly recognize each other by the agglutinin molecules located on the flagellar surfaces and agglutinate (Fig. 1E) (Wiese 1969). Their initial agglutinative interaction occurs at all points along the flagellar lengths, but the cells quickly become aligned tip-to-tip by concentrating the agglutinins at their flagellar tips (Goodenough and Jurivich 1978; Mesland et al. 1980). This "tipping" event sends a signal to the cell body, and cells release a cell wall lytic enzyme (gamete wall-autolysin) for shedding of cell walls (Fig. 1E) (Claes 1971; Schlösser 1976; Matsuda et al. 1984, 1985) and activate the mating structures for cell fusion (Goodenough and Weiss 1975). Subsequently, gamete pairs establish a thin protoplasmic connection at their anterior ends (Fig. 1F) (Weiss et al. 1977), then quickly merged, and become quadriflagellated young zygotes (Fig. 1G). At this time the flagella lose adhesiveness, and zygotes swim about for 2-3 h (Goodenough 1977). The flagella are then resorbed, and zygotes are surrounded by zygospore walls (Fig. 1H) (Cavalier-Smith 1976). Two nucleus and two chloroplasts in each zygote fuse after several hours (Cavalier-Smith 1970, 1976). After maturation and germination of zygotes, four to eight zoospores (Fig. 1I) are released by breaking down the zygospore wall.

Purification and characterization of sexual agglutinin

The sexual cell recognition/adhesion in Chlamydomonas is mediated by "agglutinins" located on the flagellar surface. Wiese (1965) and other workers (Bergman et al. 1975; Snell 1976) have shown that gametes, but not vegetative cells, carry mating-type specific agglutinins on their flagellar surfaces and slough off them into the culture medium as membrane vesicles. The isolated vesicles are glycoproteins and have the capacity to isoagglutinate gametes of the opposite mating-type (Wiese 1965). Many authors have then attempted

to purify agglutinins from the membrane vesicles or isolated flagella, but these attempts met with limited success (Bergman et al. 1975; Snell 1976; Musgrave et al. 1979a,b). In 1982 Adair et al. have found that agglutinins can be extracted directly from live gametes of C. reinhardtii with EDTA. They have also developed a simple assay method to quantify the agglutinin activity. Using these techniques, Adair et al. (1983) have purified the plus agglutinin, and Saito and Matsuda (1984b) and Collin-Osdoby and Adair (1985) have purified the minus agglutinin. Both plus and minus agglutinins are glycoproteins with extremely high molecular mass ($\sim 10^6$). In related species, C. eugametos, the plus and minus agglutinins are also purified and identified as high molecular weight glycoproteins (Musgrave et al. 1981; Klis et al. 1985). The purified plus and minus agglutinins show a high degree of homology in the ultrastructure and chemical compositions: they are fibrous molecules and rich in hydroxyproline (Collin-Osdoby and Adair 1985; Samson et al. 1987). However, the plus agglutinin differs from the minus agglutinin in several points: (1) Plus agglutinin is eluted later than minus agglutinin on both hydroxyapatite chromatography and hydrophobic interaction chromatography (Saito and Matsuda 1984b; Collin-Osdoby and Adair 1985). (2) An anion-exchange (Mono Q) column retains only plus agglutinin (Collin-Osdoby and Adair 1985). (3) Chymotrypsin and proteinase K destroy only the activity of minus agglutinin (Collin-Osdoby and Adair 1985; Chapter 1). (4) Minus agglutinin displays a "shepherd's crook" conformation near the head region (Goodenough et al. 1985). These results indicate that two mating-type agglutinins are different in net charge, relative hydrophobicity, susceptibility to proteases and the structure of head. Moreover, tunicamycin, a potent inhibitor of protein glycosylation, blocks specifically the synthesis of plus agglutinin (Matsuda et al. 1981, 1982; Wiese and Mayer 1982; Chapter 1). To investigate the molecular mechanisms for the Chlamydomonas sexual recognition/adhesion, however, it seems to be important to accumulate more data for the heterologous characters which are essential for the activity.

In Chapter 2 I describe the fact that the two agglutinins differ dramatically in their sensitivity to dithiothreitol (DTT) and suggest that sulfhydryls may be important in the active site of plus agglutinin molecule.

Turnover of agglutinin molecules

Snell and Roseman (1979) have found that when gametes adhere to isolated flagella of the opposite mating-type, the isolated flagella, but not live cells, rapidly lose their adhesive activity, and suggested that the flagellar adhesion causes loss and inactivation of agglutinin molecules on the flagellar surface although the live cells can replenish the inactivated agglutinins with new ones. Furthermore, Snell and Moore (1980) have used the imp-1 (mt^+) gametes which continue to agglutinate with mt^- gametes without successive fusion and demonstrated that the addition of cycloheximide to the gamete mixture causes the gradual disadhesion without affecting the cell motility. Similarly, tunicamycin, α, α'-dipyridyl and 3,4-dehydroproline block the continuation of agglutination in the mixture of imp-1 mt^+ and mt^- (Snell 1981; Cooper et al. 1983). It has been assumed, therefore, that protein synthesis, protein glycosylation and proline hydroxylation are required for the maintenance of flagellar adhesiveness, and that gametes must have a pool of agglutinin molecules for the replenishment of agglutinin lost from the flagella (Snell and Moore 1980).

In Chapter 1 the agglutinin pool is directly demonstrated in the cell body of C. reinhardtii gamete. Furthermore, a simple assay method is established to quantify the amount of agglutinin pool in the cell body. Using the newly developed method, synthesis and turnover of agglutinin are examined during mating and after various protease treatments.

Purification and characterization of cell wall lytic enzyme

In C. reinhardtii, the release of cell wall lytic enzyme is observed in at least three different stages of the life cycle. First, a lytic enzyme, referred to as "sporangial wall-autolysin" (Schlösser 1976), is excreted when zoospores (autospores), the products of mitosis in the vegetative cell cycle, hatch by breaking off their enclosing sporangial walls (Fig. 1A,B) (Schlösser 1966; Mihara and Hase 1975; Schlösser 1976; Matsuda 1980). This enzyme has been purified and characterized as a serine protease with a molecular mass of 115 kDa (Koseki, Saito and Matsuda: unpublished results). The second-type lytic enzyme, referred to as "gamete wall-autolysin" (Schlösser 1976) is released during mating as a necessary prelude to cell fusion (Claes 1971; Matsuda et al. 1978). This enzyme is induced by the signal of flagellar agglutination and excreted into the medium by both mating-type gametes concurrently with cell wall release (Fig. 1E) (Claes 1971; Schlösser 1976; Snell 1982). The purified enzyme is distinct from the sporangial wall-autolysin (Matsuda et al. 1984, 1985). Third, when zoospores, the products of meiosis in the sexual cell cycle, hatch by breaking off their enclosing zygospore cell walls, a lytic enzyme is excreted (Fig. 1I). This enzyme has not yet been identified. However, it has been reported that the zygospore cell wall is distinctly different from the vegetative cell wall in chemical composition and arrangment of the components (Catt 1979; Grief et al. 1987) and that the gamete wall-autolysin is unable to dissolve the zygospore cell wall, but does dissolve the vegetative and gametic cell walls and the sporangial wall (Schlösser 1976).

The enzymatic nature of gamete wall-autolysin has been first indicated by Schlösser (1976); the lytic factor is precipitable with $(NH_4)_2SO_4$, non-dializable, heat-labile, and inactivated by papain. Tamaki et al. (1981) have partially purified the cell wall lytic enzyme, and then Matsuda et al. (1984) have succeeded to purify and identify the enzyme as a single glycopolypeptide with a molecular mass of 62 kDa by SDS-polyacrylamide gel electrophoresis. Matsuda et al. (1985) have then characterized the enzyme to be a zinc-containing metalloprotease. Furthermore, it has been shown (Goodenough

and Heuser 1985; Imam et al. 1985; Matsuda et al. 1985) that the sodium perchlorate-insoluble (framework) portion (Catt et al. 1976) of the multilayered structure of the Chlamydomonas cell wall (Roberts et al. 1972) is the target of the lytic enzyme.

Claes (1971) has reported that lytic activity can also be detected in the homogenates of vegetative cells and gametes. To determine whether the cellular enzymes are identical with the gamete wall-autolysin, lytic enzymes are actually isolated and purified from the homogenates of vegetative cells and gametes in Chapter 3. Close examinations reveal that vegetative cells as well as gametes contain the same enzyme molecules as the gamete wall-autolysin. Of quite interesting is the finding that the storage form of lytic enzyme is distinctly different between vegetative cell and gamete: the vegetative enzyme is stored in an insoluble and inactive form and activity is released only when the homogenates are sonicated or the cells are freeze-thawed prior to homogenization, whereas the gametic enzyme is always detected in a soluble and active form. Some indirect evidences suggest that the cellular enzyme is stored outside the plasmalemma, i.e. either in the periplasmic space or attached to the cell wall.

Differentiation and dedifferentiation of gamete

In this alga, withholding a nitrogen source induces gametogenesis, and adding it back converts gamete to vegetative cell (Sager and Granick 1954). Schmeisser et al. (1973) have examined the kinetics of gametogenesis after vegetative cells which have been grown on agar plates or in liquid are transferred to nitrogen-free (-N) medium. Aged cells grown on agar plates for one to several weeks give rise to gametes within 2 h in -N medium. On the other hand, non-synchronized and synchronized vegetative cells grown in liquid medium take 7-12 h until they have fully differentiated into gametes.

Gametogenesis involves the synthesis of agglutinin molecules for

agglutination, the preparation of lytic enzyme for cell wall lysis and the construction of mating structures for cell fusion (Friedmann et al. 1968; Cavalier-Smith 1975; Goodenough and Weiss 1975; Martin and Goodenough 1975). The agglutinin molecules are synthesized in the cell body and then transported to the flagellar surface during gametogenesis. They are visualized as about 220 nm fibers with heads, which protrude from the flagellar surface (Adair 1985). The mating structures look like the desmosome: the mt^+ mating structure consists of curved, electron-dense membrane zone overlaying a doublet zone, while the mt^- mating structure is smaller and lacks a doublet zone (Goodenough et al. 1982). They are located at the apical end of the cell body and activated for protoplasmic connection when two mating-type gametes begin to agglutinate.

In Chapter 1 I have found that gametes prepare agglutinin molecules not only on flagellar surfaces but also in the cell bodies. The activity of cell body-agglutinin can be quantified by a simple bioassay which uses the soluble fraction of cell homogenates. In Chapter 3 I have revealed that cell wall lytic enzyme is detected in an insoluble and inactive form (V-form) in vegetative cells, whereas it is found in a soluble and active form (G-form) in gametes. The lytic activity can also be quantified by a simple assay using the cell homogenates or the soluble fraction. Therefore, in Chapter 4 the kinetics of gametic differentiation and dedifferentiation are studied in relation to the synthesis or processing of agglutinin and cell wall lytic enzyme.

Non-mating mutants

The mating-type (mt) locus which determines plus and minus sexes is located on the left arm of linkage group VI in C. reinhardtii (Ebersold et al. 1962). Galloway and Goodenough (1985) have proposed that the mt locus is a complex region which contains several regulatory genes involved in both gametic

differentiation and chloroplast gene inheritance.

Several classes of gametogenic mutants affecting different steps in mating process have been isolated and analyzed for their biochemistry, ultrastructure, and genetics (Goodenough and Thorner 1983; Snell 1985). The sag-1 (which includes five closely linked mutants, imp-2, imp-5, imp-6, imp-7 and imp-9) and sag-2 (which includes imp-8 mutant) are deficient in flagellar agglutination (Goodenough et al. 1976; Adair et al. 1983). These two mutations are unlinked to the mt locus and to each other, and are expressed only in mt^+ cells; the mt^- cells carrying sag-1 or sag-2 gene can normally agglutinate with wild-type mt^+ cells (Goodenough et al. 1978). On the other hand, the sad-1 (which includes imp-10 and imp-12 mutants) is the non-agglutinating mutant of mt^- and tightly linked to mt^- locus (Hwang et al. 1981). Both imp-1 and fus are the fusion-defective mutants of mt^+: these gametes can normally agglutinate with wild-type mt^- gametes but are unable to fuse so that they continue to agglutinate even for 1 day (Goodenough et al. 1976; Matsuda et al. 1978). The imp-1 locus is tightly linked to the mt^+ allele (Goodenough et al. 1976), and the mutant gamete lacks fringe material on the surface of the fertilization tubule (Goodenough et al. 1982). The gam-1 is a conditional non-fusing mutant: the gametes which have been differentiated at the restrictive temperature (35 ℃) can agglutinate but fail to fuse (Forest and Togasaki 1975). This mutation is unlinked to the mt locus and expressed only in mt^- cells (Forest and Togasaki 1975). The imp-11 mutant, which has been derived from wild-type mt^-, behaves like non-fusing mt^+ in that they agglutinate with mt^- gametes but do not form zygotes (Goodenough et al. 1982). This gene is tightly linked to mt^- allele (Galloway and Goodenough 1985).

Chapter 5 is concerned with the development of a new assay system for classification of non-mating mutants in C. reinhardtii. This system takes advantage of assays of cell body-agglutinin and cell wall lytic enzyme using the soluble fraction of homogenates from nitrogen-starved cells. Many non-mating mutants isolated previously (see above) are analyzed and divided into

four main phenotypic classes.

In Chapter 6 two new conditional sexual differentiation (dif) mutants are isolated. These mutants turn "on" a gametogenic program at the permissive temperature (25 ℃) but turn "off" the program at the restrictive temperature (35 ℃). Genetic analyses are also performed by standard techniques.

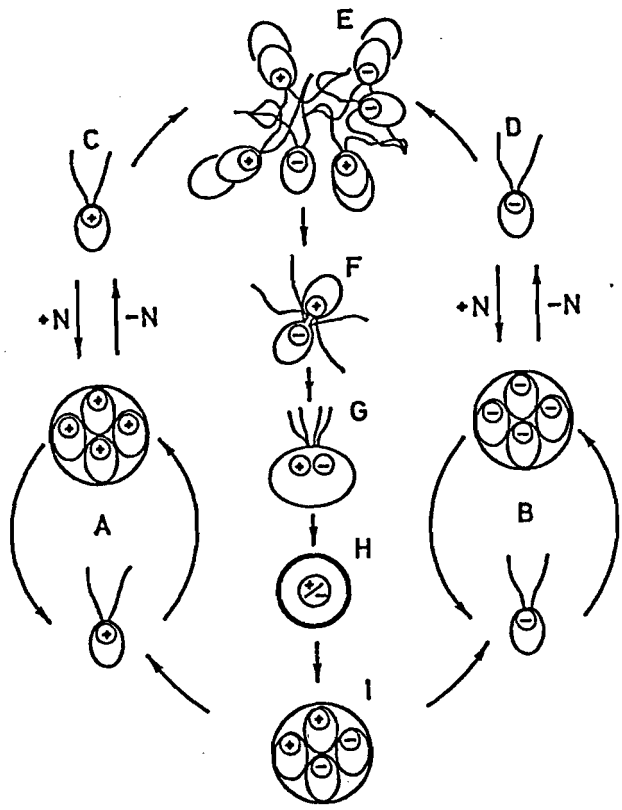

Fig. 1
The life cycle of Chlamydomonas reinhardtii

Chapter 1

Synthesis and turnover of cell body-agglutinin in gamete as a pool of flagellar surface-agglutinin

Introduction

The agglutinin molecules are synthesized in the cell body and transported to the flagellar surface during gametogenesis. Fully competent gametes replace the flagellar surface-agglutinin with new one and the old one is sloughed off into the medium as membrane vesicles (Wiese 1965; Bergman et al. 1975; Snell 1976). Furthermore, when gametes of opposite mating-types agglutinate, the agglutinin molecules on the flagellar surface are continuously lost and replaced (Snell and Moore 1980). It has been assumed, therefore, that gametes must have a pool of agglutinin molecules for the replenishment of agglutinin lost from the flagella (Solter and Gibor 1978; Snell and Moore 1980). In a related species, C. eugametos, Pijst et al. (1983) have reported that the plasma membranes of cells possess 25 times more agglutinin molecules than the flagella. However, it remains obscure on the functional role of agglutinin present in the cell body as a pool of flagellar agglutinin.

In this Chapter, I report that gametes of C. reinhardtii have about 100 times the amount of agglutinin in the cell bodies (cell body-agglutinin) as compared to that in the flagellar surface (flagellar surface-agglutinin) and that the pooled molecules are used to replace the flagellar surface-agglutinin when the latter molecules are inactivated by flagellar agglutination reaction with the opposite mating-type gamete. It is also demonstrated that when live gametes are treated with EDTA or proteases such as trypsin, all of the cell body-agglutinin molecules are lost completely through flagella.

Materials and methods

Strains and cell culture

The wild-type Chlamydomonas reinhardtii strain 137c, mt^+ and mt^-, and the non-fusing mutant strain, fus mt^+ (Matsuda et al. 1978), were used. Vegetative cells were grown in continuous light (3,000 lux) at 25 ℃ in a minimal (M) liquid medium (Matsuda et al. 1971). Gametes were obtained after 6 days of growth on 1.5 % agar plates containing M medium, suspended in nitrogen-free induction medium to a density of 5×10^7 cells/ml and shaked for 2-3 h (Matsuda et al. 1978; Saito and Matsuda 1984b). Cell number was determined by using a hemocytometer. Mating efficiency was determined by mixing gametes of two mating-types in equal numbers as described previously (Matsuda et al. 1978, 1981) and usually exceeded 90 %.

Cell wall digestion

The cell wall lytic enzyme was prepared by the method of Tamaki et al. (1981) and was used in a crude form in this study. For digestion of cell walls, cells were harvested by centrifugation (1,500 xg, 2 min), resuspended in the nitrogen-free medium containing the crude enzyme and incubated at 35℃ for 30 min. Protoplast formation was monitored by the heating method (Matsuda et al. 1978) and was regularly > 95 %.

Deflagellation

Flagella were detached from cells by the pH shock method (Witman et al. 1972). The detached flagella were separated from the cell bodies by loading the mixture (30 ml) onto 15 ml of 30 % sucrose, 10 mM Tris-HCl pH 7.0 and by centrifuging at 1,500 xg for 30 min in a swinging bucket-type rotor. The white

band at the interface was collected and centrifuged at 100,000 xg for 30 min to sediment the flagella.

Preparation of soluble fraction

Whole cells, cell bodies or flagella were resuspended in 10 mM Tris-HCl to a final density of 1 x 10^8 cells/ml or 2 x 10^8 flagella/ml, and broken by passing through a French pressure cell at 400 kg/cm^2 (this preparation is termed "pressate"). The pressate was centrifuged at 100,000 xg for 60 min, and the supernatant was saved as a soluble fraction.

Agglutination assay

The ability of live cells to agglutinate was determined by mixing them in equal numbers with tester gametes of the opposite mating-type. The activity was scored in a hemocytometer within 5 min after mixing as: - (no agglutination); + (less than ⅓ cells were agglutinated); ++ (⅓- ⅔ cells were agglutinated) and +++ (more than ⅔ cells were agglutinated). Isolated flagella were examined for their ability to isoagglutinate gametes of the opposite mating-type when the flagella were given at about the twice the number of gametes (Matsuda et al. 1978). The bioassay of agglutinin was carried out by the method described previously (Saito and Matsuda 1984a). A binary dilution series of the sample to be tested was prepared, 1μl aliquot from each dilution was dried on a cover slip, and contacted with 5 μl of tester gametes. Agglutination activity (titer) is expressed as 2 to the power of the number of the highest dilution still showing activity.

Treatment of cells with chemicals

EDTA and cycloheximide (CHI) were obtained from Nakarai Chemicals Ltd.

Tunicamycin (TM), trypsin, chymotrypsin (TLCK-treated), and soybean trypsin inhibitor (TI) were from Sigma Chemical Co, thermolysin from Protein Research Foundation, and proteinase K and phenylmethylsulphonyl fluoride (PMSF) from Boehringer Mannheim. EDTA was dissolved in 10 mM Tris-HCl pH 7.0 at 100 mM. Thermolysin, proteinase K, and TI were dissolved in 10 mM Tris-acetate, pH 7.5, at 10 mg/ml. Trypsin and chymotrypsin were dissolved in 1 mM HCl at 10 mg/ml, CHI in distilled water at 1 mg/ml, TM in 25 mM NaOH at 1 mg/ml, PMSF in isopropanol at 40 mM. In all cases, the specified amounts of these stock solutions were added to the cell suspensions at 1 x 10^8 cells/ml. The protease action was stopped by adding inhibitors: TI (0.25 mg/ml) for trypsin, PMSF (1 mM) for chymotrypsin and proteinase K, and EDTA (4 mM) for thermolysin.

Results

Pool size of cell body-agglutinin

Whole cells, wall-digested protoplasts, flagella-amputated cells and isolated flagella were passed through a French pressure cell, and the soluble fractions of pressates were tested for agglutinin activity (Table 1). Walled-gametes and the protoplasts with or without flagella yielded practically the same level of activity in their soluble fractions, whereas no activity was found in the soluble fractions of vegetative cells and the isolated flagella from gametes. The results indicate that the agglutinin found in the soluble fraction of pressates from C. reinhardtii gametes is derived from cell body, but not from flagella.

Previous studies (Saito and Matsuda 1984a) have shown that when mt$^-$ gametes are treated with EDTA at a low concentration (2 mM), they lose their flagellar agglutinability within 15 min after the addition and, at the same time, release agglutinin into the medium. The quantitative bioassay of agglutinin activity (Table 2) showed that the EDTA extract contained the same amount of agglutinin as that found in the cell bodies before the EDTA treatment, and that nothing was left in the cell bodies after the EDTA treatment. Furthermore, when the isolated mt$^-$ flagella were treated with 2 mM EDTA, their agglutinability was lost completely, but the EDTA extract contained only less than 1 % of the activity of cell body-agglutinin (Table 2). These results indicate that gametes, when they are treated with EDTA, lose both flagellar surface- and cell body-agglutinins into the medium, and that the quantity of cell body-agglutinin is about 100 times more than that of the flagellar surface-agglutinin.

The cell body-agglutinin of mt$^+$ gametes was also extracted with EDTA although the concentration (10 mM) was much higher than that required for the extraction of mt$^-$ agglutinin (Table 2; cf. Saito and Matsuda 1984a).

Cell body-agglutinin as a pool of flagellar surface-agglutinin

To determine whether the cell body-agglutinin in the soluble fraction actually functions as a pool of flagellar surface-agglutinin of gametes, the turnover of the cell body-agglutinin was examined in the following experiments. When the wild-type gametes of opposite mating-types are mixed, flagellar agglutination occurs very intensively for the initial few minutes and then disappears after cells undergo fusion (Goodenough 1977). In contrast, cells continue to agglutinate for at least several hours when gametes of the non-fusing mt^+ mutant, fus, are mixed with the wild-type mt^- (Matsuda et al. 1978). In the mixture of the wild-type gametes, the cell body-agglutinin was reduced rapidly after mixing and lost almost completely within 30 min (Fig. 2A), whereas in the mixture of the mutant gametes, the molecules were first reduced to 10 % level, then increased gradually and reached the premixing level after 4 h (Fig. 2B). When cycloheximide (CHI), a potent inhibitor of protein synthesis, was given to the mutant mixture, the flagellar agglutination and also the cell body-agglutinin disappeared completely by 30 min after mixing (Fig. 2B). These results indicate that the cell body-agglutinin is actually used as a reservoir of flagellar surface-agglutinin, the half life of which is less than 15 min, and that if gametes fail to fuse such as seen in fus mt^+ x mt^-, the agglutinin molecules are resynthesized for continuation of agglutination.

To see the turnover of cell body-agglutinin in the gametic cells, CHI was added to the medium of fully competent gametes of mt^+, and the activity of flagellar surface- and cell body-agglutinins were examined at given times of incubation (Fig. 3). Both activities decreased with essentially the same kinetics and were lost after 24 h. This suggests that the half life of cell body-agglutinin in gametes, when unmixed, is about 12 h, which is almost 50 times longer than that in the mixed gametes (< 15 min).

Loss of cell body-agglutinin by protease treatment

Wiese (1974) has reported that addition of various proteases to gamete cultures causes a complete loss of flagellar agglutinability without affecting cell motility. To determine whether the gametes after protease treatment lose also the cell body-agglutinin and release it into the medium, the treated cells were separated from the culture medium by centrifugation, and the agglutinin activities were assayed for the soluble fractions of cell homogenates and the culture medium (Table 3). When mt^+ gametes were treated with trypsin or thermolysin for 5-15 min and lost completely their flagellar agglutinability, neither the soluble fractions nor culture medium contained agglutinin activity. However, when the mt^+ gametes lost their agglutinability by chymotrypsin or proteinase K treatment, an active agglutinin derived from cell bodies was released into the medium (Table 3). In contrast, no agglutinin activity was detected in both soluble fractions and culture medium when mt^- gametes were treated with the above 4 proteases (Table 3).

Synthesis of cell body-agglutinin after trypsinization

As described above, gametes, when treated with trypsin, lose not only their flagellar surface-agglutinin but also their cell body-agglutinin. When the trypsinized gametes were given with trypsin inhibitor (TI) at 0.25 mg/ml, the flagellar agglutinability recovered rapidly and reached the level of untreated cells within 1 h incubation (Fig. 4). However, the amount of the cell body-agglutinin was still low at that time and reached the untreated cell level after 2 h incubation (Fig. 4). CHI (4μg/ml) completely blocked the recovery of activity of the cell body-agglutinin in both mating-type gametes, whereas TM (2μg/ml), a potent inhibitor of glycosylation of proteins, only inhibited the restoration of plus-type activity. Above results indicate that the synthesized agglutinin is initially transported to the flagellar surface, and

then a large pool of the cell body-agglutinin is gradually built up.

Discussion

In this Chapter I show that gametes of C. reinhardtii contain about 100 times the amount of agglutinin in their cell bodies as compared to the flagellar surface. The cell body-agglutinin appears to function as a reservoir of flagellar surface-agglutinin. This is indicated by a rapid turnover of the cell body-agglutinin during mating; (1) when the wild-type gametes of opposite mating-types began to agglutinate, the amount of cell body-agglutinin was decreased rapidly and almost used up within 30 min (Fig. 2A) and (2) in the mixture of the fus mt$^+$ and mt$^-$ gametes, the amount of cell body-agglutinin was decreased quickly to about 10 % of the unmixed level, then increased and reached the unmixed level (Fig. 2B). Since flagellar agglutination causes a rapid inactivation of the flagellar surface-agglutinin (Snell and Moore 1980), the large pool of cell body-agglutinin molecules may be used to replace the inactivated flagellar surface-agglutinin molecules with new ones. It is estimated that the half life of the cell body-agglutinin is only 15 min when gametes agglutinate (Fig. 2), whereas it is about 12 h when gametes do not (Fig. 3).

The definite location of cell body-agglutinin is yet unknown. Since almost all of the agglutinin activity is found in the soluble fraction of cell homogenate, the cell body-agglutinin might be contained either freely in the cytoplasm or within an organelle such as the contractile vacuole. Another possibility is that they are loosely attached to membranes, for example, the outer surface of plasma membrane as mentioned in C. eugametos by Pijst et al. (1983). However, since the deflagellated protoplasts prepared from gametes were unable to agglutinate tester gametes of the opposite mating-type, the cell body-agglutinin in C. reinhardtii may not be localized on the outer surface of the plasma membrane.

The present study also shows that when gametes are treated with EDTA, both the cell body- and flagellar surface-agglutinins are released into the medium

(Table 2). Since EDTA did not affect cell motility, it might not penetrate into the cell body. Therefore, the EDTA-treated cells may continuously replace the stripped flagellar surface-agglutinin by cell body-agglutinin until all of the reserved molecules are exhaused. The results suggest that the mt^+ and mt^- agglutinins, which have been purified from the EDTA extracts of gametes previously (Saito and Matsuda 1984b; Adair 1985), are mostly originated from the agglutinin pool in the cell bodies.

The loss of cell body-agglutinin also occurred when mt^+ and mt^- gametes were treated with proteases such as trypsin, chymotrypsin, proteinase K and thermolysin (Table 3). Since these protease treatments did not affect cell motility at all, the cell body-agglutinin may be lost into the medium via flagellar surface as in the case of EDTA treatment (see above). The transport mechanism of cell body-agglutinin to the flagellar surface, however, is not known at present. Among 4 proteases examined, chymotrypsin and proteinase K acted on the mt^+ and mt^- gametes differently; the former cells released active agglutinin into the medium by the treatment, whereas the latter cells yielded inactive agglutinin (Table 3). This suggests that the plus and minus agglutinins are different in the sensitivity to chymotrypsin and proteinase K. It is possible that the functional domain of plus agglutinin is resistant to these proteases while that of minus agglutinin is sensitive. Trypsin and thermolysin appears to destroy both plus and minus agglutinins released into medium (Table 3). Similarly, Adair (1985) has reported that purified plus and minus agglutinins are inactivated by trypsin and thermolysin, whereas chymotrypsin destroys only the activity of minus agglutinin.

When the trypsinized gametes were added with trypsin inhibitor, the cell body-agglutinin, which had been lost by trypsin treatment, was again accumulated (Fig. 4). This recovery process was blocked by CHI for both mating-type gametes and by TM for only mt^+ cells, suggesting the involvement of protein synthesis and protein glycosylation. The results also suggest that the plus agglutinin molecules contain TM-sensitive sugar moieties which are

responsible for cell-cell recognition in C. reinhardtii (cf. Matsuda et al. 1981, 1982).

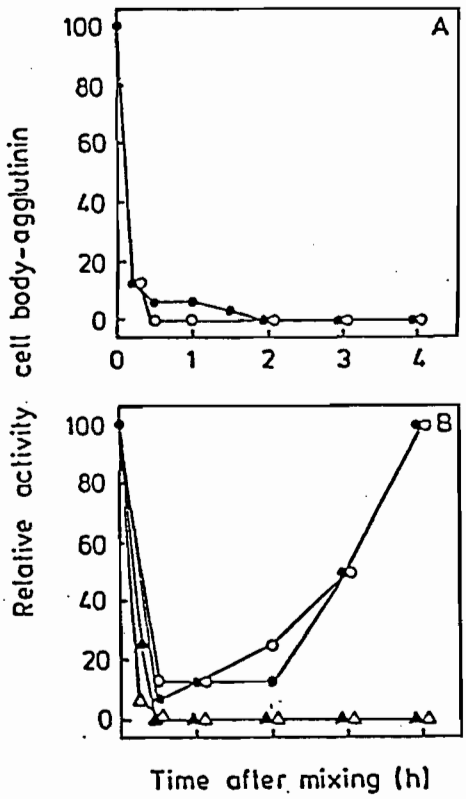

Fig. 2

Kinetics of turnover of cell body-agglutinin during mating

A, gametes of the wild-type mt$^+$ and mt$^-$ or B, fus mt$^+$ and mt$^-$ were mixed together. At the times indicated, portions of cells were harvested, the soluble fractions were prepared, and activities of the plus-type (●) and minus-type (○) cell body-agglutinins were bioassayed. In B, cycloheximide (4 μg/ml) was given to the cell culture 15 min before mixing, and the plus-type (▲) and minus-type (△) activities were also examined.

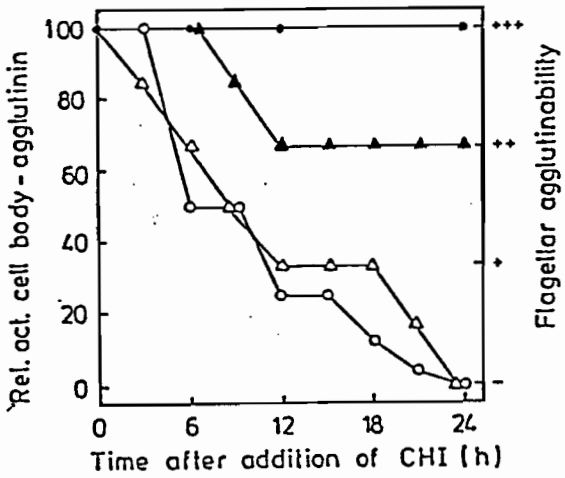

Fig. 3

Kinetics of turnover of cell body-agglutinin in mt$^+$ gametes

The single culture of mt$^+$ gametes was incubated in the absence (▲, ●) or presence (△, ○) of 4 μg/ml cycloheximide. Symbols: △ and ▲, flagellar agglutinability of live cells; ○ and ●, activity of cell body-agglutinin.

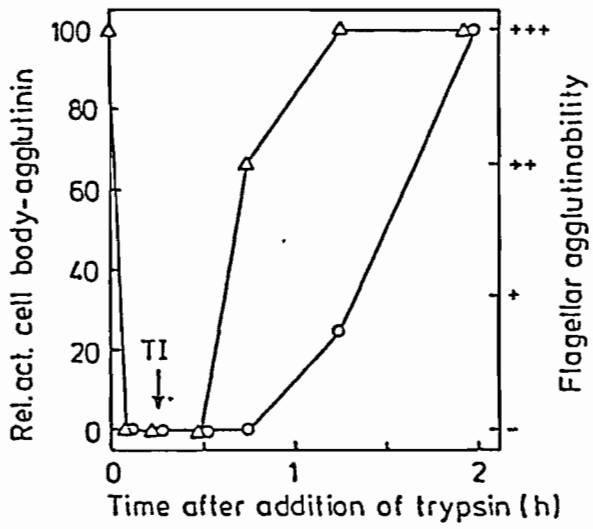

Fig. 4

Kinetics of recovery of flagellar agglutinability and cell body-agglutinin in trypsin-treated mt$^+$ gametes after addition of trypsin inhibitor

Cells were treated with 0.25 mg/ml trypsin for 15 min, and then 0.25 mg/ml trypsin inhibitor was added to neutralize the trypsin. Symbols: △ , flagellar agglutinability of live cells; ○ , activity of cell body-agglutinin.

Table 1
Agglutinin activity in the soluble fractions of pressates
of whole cells, cell bodies and flagella

Source	Agglutinin activity (titer)	
	Plus (*mt*$^+$)	Minus (*mt*$^-$)
Vegetative cells		
Whole cells	<2^0	<2^0
Gametes		
Whole cells	2^9	2^8
Cell bodies	2^7	2^9
Protoplasts	2^9	2^7
Flagella[a]	<2^0	<2^0

Agglutinin activity was expressed as a titer per 10^8 cells or per 2×10^8 flagella. <2^0, no activity without dilution.

[a] Flagella agglutinability before disruption was scored as +++.

Table 2
Treatment of cells and flagella with EDTA

Source	EDTA (mM)	Agglutinin activity (titer)	
		Soluble fraction	Culture medium
Gametes (mt$^+$)	0	2^{10}	$<2^0$
	10	$<2^0$	2^9
Gametes (mt$^-$)	0	2^8	$<2^0$
	2	$<2^0$	2^8
Flagella (mt$^-$)	0	-	$<2^0$
	2	-	2^1

Gametes (1 x 10^8 cells/ml) or flagella (2 x 10^8 flagella/ml) were incubated in the presence or absence of EDTA for 30 min at 25℃. The cells were separated from the culture medium by centrifugation at 1,000 xg for 5 min. The soluble fractions of cell homogenates were prepared as described under Materials and methods.

Table 3

Agglutinin activity in the soluble fractions of cell homogenate and culture medium after various protease treatment

Treatment	Agglutinin activity (titer)			
	mt^+ gamete		mt^- gamete	
	Soluble fraction	Culture medium	Soluble fraction	Culture medium
Trypsin	<2°	<2°	<2°	<2°
Chymotrypsin	<2°	2^8	<2°	<2°
Proteinase K	<2°	2^5	<2°	<2°
Thermolysin	<2°	<2°	<2°	<2°

Gametes (1×10^8 cells/ml) were treated with 0.25 mg/ml various proteases at 25℃.

Chapter 2

Difference in sensitivity of plus and minus agglutinin to dithiothreitol

Introduction

The plus and minus agglutinins have recently been purified and identified as high molecular weight glycoproteins in C. reinhardtii (Adair et al. 1983; Saito and Matsuda 1984b; Collin-Osdoby and Adair 1985) and in a related species, C. eugametos (Musgrave et al. 1981; Klis et al. 1985). The purified plus and minus agglutinins show a high degree of homology although there are some differences in net charge, relative hydrophobicity and susceptibility to proteases (Saito and Matsuda 1984b; Collin-Osdoby and Adair 1985; Samson et al. 1987).

To investigate the molecular mechanism for the Chlamydomonas sexual cell recognition/adhesion, the homologous and heterologous sites between the plus and minus agglutinins need further characterization. Especially, it is important to accumulate data for the heterologous characters which are responsible for the recognition/adhesion activity. This study presents the results that the plus agglutinin differs from the minus agglutinin in relative sensitivity to disulfide-reducing agents, dithiothreitol (DTT) and β-mercaptoethanol. Plus agglutinin is shown to be inactivated by DTT in vivo and in vitro, whereas minus agglutinin is stable.

Materials and methods

Strains and cell culture

The wild-type C. reinhardtii strain 137c, mt^+ and mt^-, was used in most studies. In some experiments, CC-620 (mt^+) and CC-621 (mt^-) (Adair et al. 1982) obtained from the Chlamydomonas Genetics Center, S11-32b (mt^+) and S11-32c (mt^-) obtained from the Sammlung von Algenkulturen at Göttingen, and 6301B (mt^+) and 6301D (mt^-) (Matsuda et al. 1978; Matsuda 1980) were used. As Claes (1977) has already pointed out, S11-32b, which corresponds to Texas Collection no. 90 (Schlösser 1982), mated with 137c mt^- and, therefore, was labeled as mt^+ in the present study. Cells were grown in continuous light (3,000 lux) at 25 ℃ for 6 days on 1.5 % agar plates containing minimum medium (Matsuda et al. 1971). Gametes were prepared by suspending the cells in nitrogen-free induction medium (Matsuda et al. 1978) to 1 x 10^7 cells/ml and shaked for 2-3 h. Cell number was determined in a hemocytometer.

Assay for agglutinability, cell wall loss and fusing ability

The ability of live cells to agglutinate was determined by mixing them in equal numbers with tester gametes of the opposite mating-type. Agglutinability was scored in a hemocytometer within 1 min after mixing as - (no agglutination), + (less than ⅓ cells were agglutinated), ++ (⅓ − ⅔ cells were agglutinated) and +++ (more than ⅔ cells were agglutinated) (Matsuda et al. 1981). All visual scores were done in duplicate samples and expressed as the average. Wall loss and cell fusion (mating efficiency) were determined after allowing the mixed cells to mate for 30 min. The number of cells before and after heat treatment (60 ℃, 2 min) was counted to determine the number of cells that have lost their cell walls (Matsuda et al. 1978). The number of biflagellated and quadriflagellated cells was counted by phase-contrast microscopy to

determine the mating efficiency (Matsuda et al. 1978).

Isolation of flagella

Gametes were washed with 10 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.5 and resuspended in the same solution containing 7 % sucrose to make a final density of 2 x 10^8 cells/ml. Flagella were amputated from cells by pH shock (Witman et al. 1972) and isolated by centrifugation in discontinuous sucrose gradient as described previously (Saito and Matsuda 1984b).

Preparation of crude agglutinin

Plus and minus agglutinins were extracted from live gametes with EDTA by the method of Adair et al. (1982) with modification (Saito and Matsuda 1984a, b). Extraction of plus agglutinin was carried out at 25 ℃ with 20 mM EDTA for 45-60 min and minus agglutinin with 4 mM EDTA for 15-30 min until the cells at a density of 5 x 10^7 cells/ml had lost completely the agglutinability with the tester gametes of the opposite mating-type. The soluble fraction of cell homogenate was prepared in the following ways and used as a source of cell body-agglutinin (Chapter 1). Cells resuspended in 10 mM Tris-HCl, pH 7.0, at 1 x 10^8 cells/ml were broken completely by passing through a French pressure cell at 400 kg/cm^2. The cell homogenate was centrifuged at 100,000 xg for 60 min, and the supernatant was saved as the soluble fraction. Bioassays of agglutinin in the EDTA and dithiothreitol (DTT) extracts and in the soluble fraction of cell homogenate were carried out by the dried spot method of Adair et al. (1982) with slight modification (Saito and Matsuda 1984a). Agglutinin activity (titer) is expressed as 2 to the power of the number of the highest dilution still showing activity.

Treatment of cells with chemicals

All chemicals were reagent grades and purchased from Nakarai Chemicals Ltd. DTT and diamide were dissolved in 10 mM Tris-HCl, pH 7.0, at 100 mM. β-Mercaptoethanol was adjusted to pH 7.0 by addition of 0.5 M KOH and diluted to 10 M. In all cases, the specified amounts of these solutions were added to the cell suspensions at 1 x 10^7 cells/ml, unless otherwise described.

Results

Effects of disulfide-reducing agents on flagellar agglutination

Fig. 5A shows that dithiothreitol (DTT) causes a selective loss of agglutinability of 137c mt^+ gametes. The mt^+ gametes lost their agglutinability during 30 min incubation with DTT partially at 0.75-1.5 mM and completely at 2 mM and above, whereas 137c mt^- gametes showed no significant loss of agglutinability at a DTT concentration as high as 50 mM (Figs. 5A and 6; Table 4). Cell motility was not affected at all. This interesting difference in sensitivity between the two mating-type gametes was also seen with β-mercaptoethanol: the agglutinability of mt^+ gametes demonstrated a partial loss of activity at 100 mM and complete loss of activity at 200 mM, whereas the agglutinability as well as cell motility of mt^- gametes was unaffected by even 300 mM (Table 4). Furthermore, all of other mt^+ strains (CC-620, S11-32b and 6301B) examined were sensitive to DTT treatment, but their partners, mt^- strains (CC-621, S11-32c and 6301D) were insensitive.

Effects of disulfide-reducing agents on zygote formation

Fig. 5B shows that pretreatment with DTT also inhibits the cell wall loss and fusion of mt^+ gametes at low concentrations where there is no inhibition of pretreated mt^- gametes. Wall loss and fusion appeared to be a little more sensitive to disulfide-reducing agents than flagellar agglutination. For example, pretreatment with 1 mM DTT or 100 mM β-mercaptoethanol resulted in a partial loss of agglutinability but a complete loss of activity of wall release and fusion (Fig. 5 and Table 4).

Above results are related to the recent report of Forest (1985) who showed that DTT (0.5-2 mM) inhibits preferentially the ability of mt^+ gamete to fuse to form zygote and suggested that DTT is an inhibitor of sexual signalling in

the mt$^+$ gamete. However, that author did not find a clear loss of flagellar agglutinability of mt$^+$ gamete by DTT treatment as reported in the present study. The differences of results may be due to the differences in experimental conditions. Forest (1985) preincubated mt$^+$ gametes with DTT for 30 min, spun them down, resuspended them in DTT-free medium, mixed them with untreated gametes of mt$^-$, and then examined their agglutination and fusion. When I followed this procedure (Fig. 6), it was noted that the flagellar agglutinability of DTT-pretreated gametes began to recover soon after they were resuspended in DTT-free medium and reached the untreated cell level within 60 to 80 min. However, the ability of gametes to fuse was still low (mating efficiency, 11 %) at the time of full recovery of agglutinability.

Release of agglutinin from DTT-treated gametes

It has been shown in Chapter 1 that fully competent gametes have a pool of large amount of agglutinin molecules in their cell bodies for the replenishment of agglutinin lost from the flagella. The activity of cell body-agglutinin can be quantified by a simple bioassay of the soluble fraction of cell homogenate (Chapter 1). To determine whether live mt$^+$ gametes actually lose their agglutinin by DTT treatment, the DTT-treated cells were separated from the culture medium by centrifugation, and the amounts of agglutinin activity in the soluble fraction of cell homogenate and culture medium were examined (Table 5). DTT-untreated control cells yielded 2^8 agglutinin activity in the soluble fraction and no activity was found in the culture medium. In contrast, neither the soluble fraction nor culture medium obtained from DTT-treated gametes (4 mM, 30 min) exhibited any detectable agglutinin activity. It was noticed, however, that when the culture medium from DTT-treated cells was dialyzed against distilled water, an activity titer of 2^5 was generated after a 1-day incubation.

It was further found that when the culture medium from DTT-treated cells was

added with a sulfhydryl-oxidizing agent, diamide (4 mM), 2^{e} agglutinin activity occurred within 30 min after addition, whereas none of the agglutinin activity was generated if the diamide was added to the culture medium from DTT-untreated cells or to the soluble fraction from DTT-treated cells (Table 5). These results indicate that the loss of cell body-agglutinin from DTT-treated mt^{+} gamete is not due to its inactivation by the reducing agent that penetrates into the cell, but is due to its release into the medium by the cell in an inactive form.

The mt^{-} gametes treated with 50 mM DTT did not lose their cell body-agglutinin at all (Table 5).

In vitro inactivation of agglutinin with DTT

Table 6 shows that plus agglutinin both in the EDTA extract and in the soluble fraction of cell homogenate was inactivated completely by addition of DTT at 1-5 mM. Therefore, the plus agglutinin in vitro is just as sensitive as that in vivo. By contrast, minus agglutinin in solution was insensitive to DTT (Table 6). Addition of diamide to the DTT-treated plus agglutinin solution at equal concentrations with DTT resulted in a full restoration of activity within 15-30 min.

Isolated flagella of plus origin were also inactivated by incubating them with 2 mM DTT for 30 min. However, if the inactivated flagella obtained by a 1-day incubation with DTT were collected by centrifugation, washed, and then resuspended in fresh medium containing diamide, activity was fully recovered within 15 min incubation. The results indicate that the DTT-treated flagella still retain their agglutinin components in an inactive and reversible form so that the activity can be fully recovered by the reformation of disulfide bridges with diamide.

Discussion

The present study demonstrates that there is a distinct difference between the C. reinhardtii plus and minus agglutinins with respect to the sensitivity to a disulfide-reducing agent, DTT. By treatment with DTT in vivo, the mt^+ gametes lost their agglutinability without losing cell motility and, at the same time, released into the medium cell body-agglutinin in an inactive form. Plus agglutinin in solution was also inactivated by DTT. In contrast, minus agglutinin was totally insensitive to DTT both in vivo and in vitro. Since isolated plus flagella treated with DTT retained inactivated agglutinin molecules on the surface, they may not be anchored extrinsically to the flagellar membrane by disulfide bridges. It is therefore assumed that live mt^+ gametes may shed actively the flagellar surface-agglutinin inactivated by DTT and replace it with cell body-agglutinin until the cells use up the reserve. Recently, Goodenough (1986) has also reported that the agglutinability of isolated plus flagella is inactivated by 1 mM DTT whereas minus flagella are insensitive to DTT as high as 100 mM.

Similar unilateral sensitivity to disulfide-reducing agents has been reported in two mating-type sexual agglutination factors of ascosporogenous yeasts (Burke et al. 1980; Yamaguchi et al. 1984). In yeasts one factor, which is a large, heat-stable, mostly carbohydrate component, is inactivated by reducing agents, whereas the other, which is a smaller, heat-labile, glycoprotein component, is stable to reducing agents (Burke et al. 1980; Pierce and Ballou 1983). Interestingly, the reducing agent-sensitive factor of Hansenula wingei consists of a large core to which several small recognition sites are attached by disulfide linkage. Reduction with DTT causes the release of the recognition sites in a monovalent form with concomitant abolition of activity (Taylor and Orton 1971; Yen and Ballou 1974). Unlike Hansenula, the Chlamydomonas plus agglutinin may not release any recognition sites by DTT treatment since the activity was restored immediately and completely even

when plus flagella were treated with DTT, washed and then added with diamide. Similarly, Collin-Osdoby et al. (1984) have shown that the plus agglutinin conjugated to agarose beads, which has been inactivated by exposure to reducing agent (100 mM DTT or 120 mM β-mercaptoethanol) and then washed, is fully reactivated after a 3-day incubation in reagent-free medium.

Plus agglutinin, but not minus agglutinin, might abolish the activity by altering the conformation of the recognition sites caused by the cleavage of disulfide bonds.

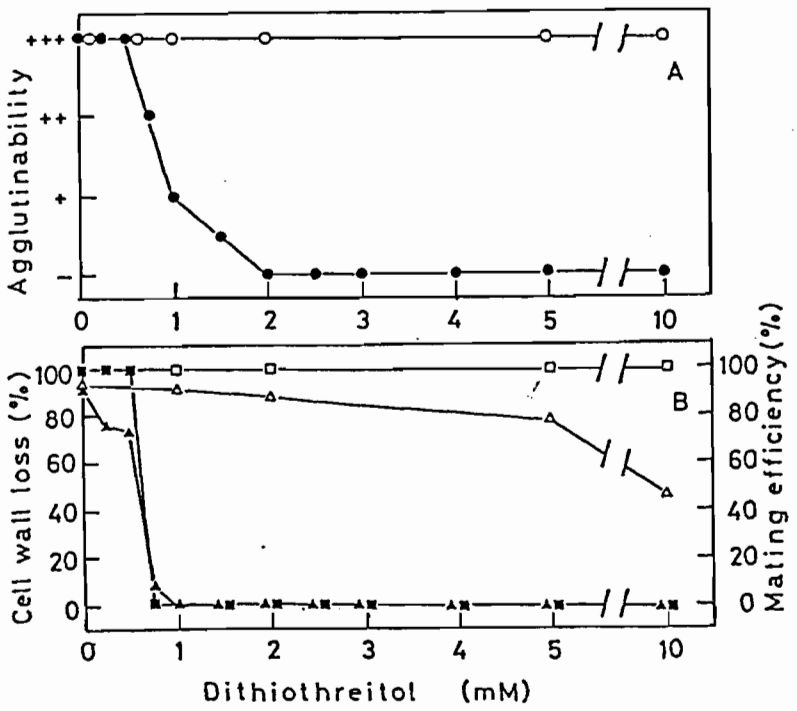

Fig. 5
Effects of dithiothreitol (DTT) on the mating activity of mt$^+$ and mt$^-$ gametes

Various concentrations of DTT (0.25-10 mM) were given to mt$^+$ and mt$^-$ gametes, and they were incubated for 30 min. A, 5μl aliquots of DTT-treated mt$^+$ (●) and mt$^-$ (○) cells in DTT-containing medium were mixed with 5 μl of untreated gametes of opposite mating-type, and their agglutinability was scored quickly. B, DTT-treated cells of mt$^+$ (■, ▲) and mt$^-$ (□, △) were mixed with tester gametes of opposite mating-type, left to stand for 30 min, and cell wall loss (■, □) and mating efficiency (▲, △) were examined.

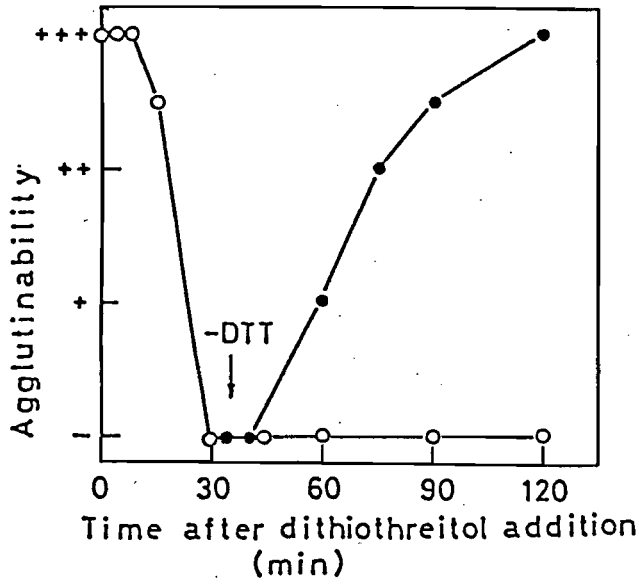

Fig. 6
Kinetics of recovery of flagellar agglutinability
in DTT-treated mt$^+$ gametes

Cells were treated with 2 mM DTT for 30 min, spun down by centrifugation, and resuspended in DTT-free (●) or DTT-containing (○) induction medium, and incubation was continued from the time indicated by an arrow.

Table 4

Effects of disulfide-reducing agents on the mating activity of gametes

Reagent (mM)	Activity					
	mt⁺ gamete			mt⁻ gamete		
	Agglutination	Wall loss (%)	Fusion (%)	Agglutination	Wall loss (%)	Fusion (%)
None	##	100	90	##	100	90
Dithiothreitol						
10	—	0	0	##	100	39
20	—	0	0	##	100	17
50	—	0	0	##	100	5
β-Mercaptoethanol						
50	##	100	88	##	100	87
100	#	52	0	##	100	54
200	—	31	0	##	100	17
300	—	0	0	##	79	0

Experimental conditions were the same as those described in the caption to Fig. 5.

Table 5

Agglutinin activity in the soluble fractions of cell homogenates and culture medium obtained from dithiothreitol (DTT)-treated gametes

Mating-type	DTT (mM)	Flagellar agglutinability	Agglutinin activity (titer)			
			Soluble fraction		Culture medium	
			None	+Diamide	None	+Diamide
mt^+	0	##	2^8	2^8	$<2^0$	$<2^0$
mt^+	4	—	$<2^0$	$<2^0$	$<2^0$	2^8
mt^-	0	##	2^8	ND	$<2^0$	$<2^0$
mt^-	50	##	2^8	ND	$<2^0$	$<2^0$

Gametes (1 x 10^8 cells/ml) were treated with DTT for 30 min at 25 ℃. The culture medium was separated from cells by centrifugation at 1,000 xg for 5 min. Half portions of the soluble fraction of cell homogenates and culture medium were added with 4 mM diamide and incubated for 30 min before the examination of agglutinin activity.

$<2^0$, no activity without dilution; ND, not determined.

Table 6

Treatment of plus and minus agglutinins in solution with DTT

DTT (mM)	Activity (titer)			
	Source of mt⁺ agglutinin		Source of mt⁻ agglutinin	
	EDTA extract	Soluble fraction	EDTA extract	Soluble fraction
0	2^6	2^6	2^5	2^6
0.5	2^7	2^5	2^5	2^6
1.0	2^7	$<2^0$	2^5	2^6
2.0	2^5	$<2^0$	2^5	2^6
5.0	$<2^0$	$<2^0$	2^5	2^6
10.0	$<2^0$	$<2^0$	2^5	2^6

The mt⁺ and mt⁻ agglutinins were incubated with DTT for 30 min at 25℃, and activities were bioassayed.

Chapter 3

Topography of cell wall lytic enzyme

Introduction

The pioneering work on the Chlamydomonas lytic factor by Claes (1971) has shown that lytic activity found in the mating medium can also be detected in the homogenates from vegetative and gametic cells. However, the identity of the gamete wall-autolysin with the cellular enzymes remains to be clarified. In addition, the form and location of the stored enzyme in these two cell types are still unknown. This study presents some topographic aspects of cell wall lytic enzyme. It is demonstrated by purification and characterization studies that the same enzyme molecules as gamete wall-autolysin exist both in vegetative and gametic cells. However, the storage form of lytic enzyme is quite different between the two cell types; the vegetative enzyme is stored in an insoluble and inactive form, whereas the gametic enzyme is, at least partly, in a soluble and active form. It is also suggested that the lytic enzyme is localized outside the plasmalemma and excreted into the medium when gametes undergo mating.

Materials and methods

Strains and culture conditions

The wild-type strain 137c mt$^+$ and mt$^-$ of C. reinhardtii and the cell wall-less mutant strains, cw-2 mt$^+$, cw-3 mt$^+$, cw-8 mt$^+$, cw-10 mt$^-$, cw-15 mt$^+$ and cw-15 mt$^-$ (Hyams and Davies 1972) were used. All the mutant strains were obtained from the Chlamydomonas Genetics Center, Department of Botany at Duke University (Durham NC), with the exception of cw-15, obtained from the Cambridge Algae Collection. Vegetative cells were grown in constant light in liquid minimal (M) medium for about 40 h (Matsuda et al. 1978). Gametes were obtained by flooding 6 day-old cultures on 1.5 % agar plates containing M medium with nitrogen-free induction medium at 5 x 10^7 cells/ml and by incubating for 2-3 h (Saito and Matsuda 1984a). Agglutinability and fusing ability (mating efficiency) of cells were determined by mixing them with tester gametes of the opposite mating-type in equal numbers as described previously (Matsuda et al. 1978, 1981) and usually exceeded 90 %.

Preparation of cell homogenates and their fractionation

Vegetative or gametic cells were harvested by centrifugation, resuspended in ice cold 10 mM Tris-acetate, pH 7.5, at 1 x 10^8 cells/ml, and homogenized by passing through a French pressure cell at 400 kg/cm^2 at 4℃. Complete breakage of cells by French press was regularly monitored by phase-contrast microscopy. In some experiments, the pelleted cells were frozen at -80℃ overnight and thawed before homogenization. Fractionation of homogenates was carried out either by differential centrifugation ranging from 1,000 to 100,000 xg or by density gradient centrifugation using Percoll (Pharmacia Fine Chemicals) at 4 ℃. Homogenates were centrifuged at 8,000 xg for 15 min to remove large cell debris, and the supernatant was added with Percoll and

sucrose to give a final concentration of 15 % and 0.25 M, respectively. After centrifugation at 50,000 xg for 30 min with a Hitachi RP50T2 rotor, fractions of 1 ml were collected. The density was determined from refractive index. Sonication of homogenates before and after fractionation was performed using a Tomy Seiko Ultrasonic Vibrator (model UR-200 P) at 4℃ for 1 min at designated powers.

Methods of lytic enzyme purification

Gamete wall-autolysin was purified from the mating medium by the procedure modified from that described previously (Matsuda et al. 1984, 1985). The mt^+ and mt^- gametes were mixed in equal numbers and allowed to mate for 10 min at 25℃. The mating mixture (1 liter) was centrifuged at 8,000 xg for 10 min. The supernatant was applied to DEAE-cellulose gel (Whatman Inc; DE52; 2.2 x 30 cm column) at a flow rate of 60 ml/h, which had been equilibrated with 10 mM Tris-acetate (pH 7.5). The flow-through fraction, which contained almost all the lytic activity, was saved and added with one-fourth volume of 50 mM Tris-acetate, pH 7.5, and 1 M NaCl. The solution was applied to concanavalin A (Con A)-Sepharose 4B gel (Pharmacia Fine Chemicals; 2.2 x 12 cm column) at a flow rate of 25 ml/h. After extensive washing of the column with 10 mM Tris-acetate, pH 7.5, and 200 mM NaCl (solution A), enzyme was eluted with 0.1 M methyl- α-D-mannoside in solution A. Fractions containing lytic activity were pooled, concentrated by collodion bag (Sartorius) to ～1 ml, and centrifuged at 100,000 xg for 30 min. The supernatant was fractionated by gel filtration over Sephacryl S-200 Superfine (Pharmacia Fine Chemicals) packed in a 2.2 x 30 cm column at a flow rate of 30 ml/h. The most active fractions were pooled, bound to a 2-ml bed of hydroxyapatite (Bio-Rad), and eluted with 50 mM sodium phosphate in solution A. The fractions containing lytic activity were pooled, concentrated, rechromatographed through a second gel filtration column (2.2 x 60 cm) at a flow rate of 10 ml/h. The purified enzyme thus obtained was

concentrated by collodion bag and stored at -80 ℃.

Vegetative and gametic enzymes were purified in the following ways. Harvested mt$^+$ cells were frozen, thawed, resuspended in 10 mM Tris-acetate at 2-5 x 10^8 cells/ml, and broken by French press as described above. After 60-min centrifugation at 100,000 xg, the supernatant (~30 ml) was applied to DEAE-cellulose column (2.2 x 60 cm column). The flow-through fraction, in which most of lytic enzyme was recovered, was added with one-fourth volume of 5 x solution A. Subsequently, Con A affinity chromatography, gel filtration, hydroxyapatite chromatography and the second gel filtration were carried out by the same procedures as those described for the purification of gamete wall-autolysin.

Enzyme assay

The activity of cell wall lytic enzyme was estimated with glutaraldehyde-fixed zoosporangia as substrates by the method described previously (Tamaki et al. 1981; Matsuda et al. 1984). The reaction mixtures contained 10 mM Tris-acetate, pH 7.5, 0.5 mg/ml bovine serum albumin (BSA), 2.5 x 10^5 glutaraldehyde-fixed zoosporangia and 0-240 μl enzyme solution in a total volume of 250 μl. The reaction was run at 35℃ for 30 min and stopped by addition of EDTA to give a final concentration of 20 mM. After stirring vigorously in a Vortex mixer for 15 sec, the extent of zoospore liberation was determined by phase-contrast microscopy. One unit of activity is defined as the amount of enzyme which liberates daughter cells at the 50 % level (Tamaki et al. 1981).

Acid phosphatase activity was determined by the method of Loppes and Matagne (1973) modified from MacIntyre (1971) using 1-naphthylphosphate as a substrate. Cell homogenates were centrifuged at 100,000 xg for 60 min and the supernatants (soluble fractions) were used for enzyme assay.

Gel electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was carried out in the discontinuous buffer system of Neville (1971) using 7.5-15 % linear acrylamide gradient with 3.5 % acrylamide stacker. Samples were lyophilized, dissolved in 2 % SDS containing 10 % glycerol, 0.004 % bromphenol blue, and 70 mM Tris-HCl, pH 6.8, and heated for 5 min in a boiling water bath. The gels were run at a constant current of 12 mA for 5-6 h at 15℃. Protein standards (Pharmacia) were ferritin half-unit (220 kDa), BSA (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa), and ferritin (19 kDa). Following gel electrophoresis, gels were stained with silver stain (Matsuda et al. 1984; Saito and Matsuda 1984b).

Protoplast formation

Walled vegetative cells or gametes (~5 x 10^6 cells/ml) were incubated with 20-50 units/ml crude lytic enzyme and 0.5 mg/ml BSA at 25 ℃ for 30 min (Matsuda et al. 1984). Protoplast formation was monitored by the heating method as described previously (Matsuda et al. 1978) and was usually above 95 %. For the reformation of cell walls after enzyme treatment of vegetative cells, the lytic enzyme was washed away by centrifugation, and the cells were incubated in M medium supplemented with 1 g/liter sodium acetate at 5 x 10^6 cells/ml.

Inhibitors

Phenylmethylsulfonyl fluoride (PMSF) was dissolved in isopropanol at 40 mM, p-chloromercuribenzoic acid in 40 mM NaOH at 50 mM, and 1,10-phenanthroline in 95 % ethanol at 100 mM (Gray 1982). $HgCl_2$ and iodoacetate were dissolved in 10 mM Tris-HCl, pH 7.0, at 200 mM. The specified amounts of these stock solutions were added to the enzyme solutions.

Results

Lytic enzyme in vegetative cell

Vegetative cells of C. reinhardtii were homogenized in a French pressure cell, the homogenate was spun at 100,000 xg for 1 h, and the supernatant termed soluble fraction was saved. In this experiment, half the amount of cells was frozen and thawed before homogenization. It was found that neither the homogenate (Table 7) nor the soluble fraction prepared from unfrozen cells (Table 8) had any detectable activity of lytic enzyme, whereas both preparations from frozen cells yielded practically the same level of activity (~ 40 units/ 10^8 cells). Since unfrozen cells as well as frozen cells were observed by phase-contrast microscopy to be disrupted completely after passing through the French press, the lack of activity in the former preparation was not due to non-breakage of cells. Furthermore, the activity of the soluble acid phosphatase, which is located in the vacuoles in Chlamydomonas (Matagne et al. 1976), was equally found in the soluble fractions of homogenates from unfrozen and frozen cells (Table 8). These results indicate that the lytic enzyme in vegetative cells is released into the soluble fraction only by freeze-thawing the cells before homogenization.

Fig. 7 shows that sonication is also effective to release the lytic activity from the homogenate of unfrozen vegetative cells. At an appropriate power setting (~50 W), the sonicated homogenates yielded the same amount of lytic activity as the homogenates from frozen cells. Almost all the activity was observed in the soluble fraction after sonication.

The homogenate from unfrozen cells was first fractionated by differential centrifugations and then each fraction was sonicated to localize the inactive enzyme (Table 9). The results showed that the 15,000 xg and 30,000 xg pellets contain a considerable amount of inactive enzyme. Fig. 8 shows the distribution pattern of the inactive enzyme after Percoll density gradient

centrifugation. Cell homogenate was spun at 8,000 xg to remove the large cell fragments, and the supernatant was mixed with Percoll, centrifuged and fractionated. By sonicating each fraction, lytic activity was detectable as two peaks at a density of 1.035 and 1.048. These two activity peaks were reproducible in repeated experiments although their relative activity ratio was changed.

Lytic enzyme in gamete

Different from vegetative cells, both unfrozen and frozen cells of gametic origin yielded the same level of lytic activity in the homogenates (Table 7) and in the soluble fractions (Table 8). It was noticed, however, that homogenization by the French press is required to release the gametic enzyme into the soluble fraction since no activity was found in the soluble fraction of frozen cells without homogenization (Table 8). This was a sharp contrast to the soluble acid phosphatase in which the activity could be released into the soluble fraction only by freeze-thawing the cells (Table 8).

Purification and characterization of intracellular lytic enzyme

To see whether the lytic enzymes found in vegetative cells and gametes are identical to gamete wall-autolysin, the intracellular enzymes were purified from the soluble fractions of frozen cells, and their characters were compared with each other or with gamete wall-autolysin purified from the mating medium.

Both vegetative and gametic enzymes were not adsorbed to DEAE-cellulose at low salt condition, but a large amount of protein impurities and some inhibitor(s) of lytic activity were bound to the resin. Thus, after the DEAE-cellulose chromatography, about 90 % of the protein impurities in the soluble fractions could be removed, and the total lytic activity was increased three- to fivefold. No such an increase in total activity was observed when mating medium containing

gamete wall-autolysin was passed through the DEAE-cellulose column.

Similar to the gamete wall-autolysin (Matsuda et al. 1984), both cellular enzymes were then bound specifically to a Con A-Sepharose column (the flow-through did not contain any lytic activities) and eluted with 0.1 M methyl-α-D-mannoside, indicating that these enzymes have mannose and/or glucose residues.

Further purification of the enzyme was achieved by gel filtration using Sephacryl S-200 (Fig. 9). Vegetative (Fig. 9A) and gametic (Fig. 9B) enzymes eluted in one peak of activity at the same position with an apparent molecular mass of 67±2 kDa (mean value with SD in three separate analyses). Furthermore, this value was quite similar to that of gamete wall-autolysin (65±4 kDa in three separate analyses) (Fig. 9C). After rechromatography of the vegetative and gametic enzymes on a second gel filtration column, the enzyme peak fractions were visible after SDS-PAGE and silver staining as a single 62 kDa polypeptide accompanied by two to three faint bands around this main band (Fig. 10, lanes 1 and 2). The 62 kDa polypeptide corresponded exactly to the gamete wall-autolysin on SDS-PAGE (lane 3). This molecule is therefore considered to be the vegetative and gametic enzymes.

Table 10 lists the effects of potent inhibitors of serine, thiol, and metalloproteases on the purified vegetative and gametic enzymes. Chelators of metal ions (EDTA, 1,10-phenanthroline) and SH-blocking agents (p-chloro-mercuribenzoic acid, iodoacetate, $HgCl_2$) have previously been shown to inactivate the gamete wall-autolysin (Matsuda et al. 1984) and did also inactivate the two cellular enzymes. In contrast, PMSF, which is a potent inhibitor of serine protease and ineffective against gamete wall-autolysin (Matsuda et al. 1984), did not abolish the vegetative and gametic enzymes.

From all the above results it is concluded that both vegetative and gametes contain the same cell wall lytic enzyme as gamete wall-autolysin.

Localization of lytic enzyme

To determine whether the lytic enzyme is located within the cytoplasmic membrane or outside, the cell walls of vegetative cells and gametes were removed by gamete wall-autolysin. The protoplasts thus obtained were harvested, washed to remove the digested cell walls and added enzyme, frozen, homogenized, and then the lytic activity was determined. The homogenates from vegetative cells and gametes after enzyme treatment had only 10 % or less of lytic activity as compared to those from untreated cells (Table 11). These results indicate that the lytic enzyme is localized largely outside the cytoplasmic membrane. This was also indicated by use of the cell wall-deficient mutants. Vegetative cells and gametes of the cell wall-deficient strains, cw-2, cw-3, cw-8, cw-10, and cw-15 contained less than 5 % level of the lytic activity as compared to the wild-type cells (Table 11).

To see whether the lytic enzyme localized outside the plasmalemma is actually released into the medium during mating, kinetics of enzyme release into the medium was examined (Fig. 11). As Snell (1982) has first reported, when the wild-type mt^+ and mt^- gametes were mixed, the lytic enzyme was released into the medium as a pulse, and the excretion was almost complete after 5 min (Fig. 11). When the mating reaction began, the amount of lytic activity in cell homogenates was decreased to less than 4 % level within 10 min.

When the two mating-type gametes were de-walled with exogenously added lytic enzyme and then mixed together, agglutination and cell fusion occurred normally (mating efficiency, 87 %), but the amount of lytic activity released was only 10 % that of the walled cells (Fig. 11). This proportion was practically in agreement with that of lytic activity observed in the wall-digested cells before mixing (Table 11). Similarly, gametes of cw-15 cells showed good agglutination and fusion (mating efficiency 76 %), whereas the level of lytic enzyme released was < 10 % that of the wild-type cells (Fig. 11).

It has been reported that when the wall-digested protoplasts are incubated in lytic enzyme-free medium, they reform new walls over a period of several hours (Robinson and Schlösser 1978; Goodenough and Heuser 1985). Here I followed

this process to see the correlation of lytic enzyme restoration with wall regeneration. The development of lytic activity occurred after wall construction was almost complete (Fig. 12).

Discussion

The storage form of cell wall lytic enzyme in vegetative cell and gamete

The present study demonstrates that cell wall lytic activity is present both in vegetative cells and gametes of C. reinhardtii. Quantitative assays show that these two cell types and also both mt^+ and mt^- cells have the same level of lytic activity (Table 7). Purification studies show that both vegetative and gametic enzymes are a glycoprotein with an apparent molecular mass of 67 kDa on gel filtration (Fig. 9) and 62 kDa on SDS-PAGE (Fig. 10). Moreover, these values are in agreement with those of gamete wall-autolysin which is excreted into the medium by mating gametes (Figs. 9 and 10; Matsuda et al. 1984). Further characterization of the purified enzymes by use of various protease inhibitors (Table 10) shows that the cellular enzymes are, like gamete wall-autolysin (Matsuda et al. 1984), sensitive to SH-blocking agents and metal ion chelators, but are insensitive to the inhibitor of serine protease. It is, therefore, concluded that vegetative cells and gametes possess the same enzyme molecules as gamete wall-autolysin.

Although the vegetative and gametic enzyme molecules are identical, the storage form appears to be quite different between the vegetative cell and gamete. In vegetative cell, both freezing and homogenization are required to activate and solubilize the lytic enzyme (Table 8). Sonication of the homogenate from unfrozen vegetative cell is also effective to activate the enzyme (Fig. 7). The inactive enzyme is localized in the particulate fractions (15,000 to 30,000 xg pellets) of cell homogenate (Table 9). It is assumed, therefore, that the vegetative enzyme may be stored in the vesicles or particles in an inactive state and that freeze-thawing or sonication of cells or homogenates may result in the breakage of these vesicles or granules and release of the active enzyme into the soluble fraction. Another possibility is that the enzyme is inactivated since it might be tightly attached to the cell structure

such as the cell wall. An analogous situation is found in yeast (Hien and Fleet 1983a,b) and Chlorella (Loos and Meindl 1985), where the lytic enzymes show two forms during the cell cycle, the soluble form and the cell wall-associated form which is presumably the inactive one.

In contrast, the lytic enzyme of gametes may be either contained freely or associated loosely with the cell structures in that it can be solubilized and activated by French press disruption alone (Table 7). In this study, I also analyzed the soluble acid phosphatase activity and found that this vacuolar enzyme (Matagne et al. 1976) is released into the soluble fraction with similar ease by French press in vegetative cells and gametes (Table 8). In addition, this enzyme comes out if only cells are frozen. On the other hand, homogenization by French press is required to solubilize the lytic enzyme even in gametes (Table 8). Since active enzyme is known to form a large aggregate of 62 kDa subunits in the culture medium or low salt buffer (Tamaki et al. 1981; Matsuda et al. 1984), it is possible that the gametic enzyme remains in the freeze-thawed cells due to large size of the enzyme aggregate although the acid phosphatase can be released from the partially broken cells. Another possibility is that the lytic enzyme is associated with cellular components still in frozen gametes until the cells are homogenized drastically.

The present study also shows that homogenates or their soluble fractions of vegetative cells and gametes have only 20 % of the lytic activity present in the medium of mating gametes (Tables 7 and 8). This difference is due to the presence of some, if not all, inhibitor(s) of lytic activity in the soluble fraction. The inhibitor(s) is bound to DEAE-cellulose so that the total activity in soluble fraction increases and reaches the level of the activity present in the mating medium. A recent preliminary experiment showed that the inhibitor can be eluted from the DEAE-cellulose column with 0.2 M NaCl and causes a reversible inhibition of the activity of purified lytic enzyme. It is possible that the endogenous inhibitors act on the lytic enzyme in gametic cell to keep the activity below the critical level until its release by mixing

with the opposite mating-type gamete.

Storage of lytic enzyme outside the plasmalemma

The present results suggest that lytic activity is stored either in the periplasmic space or attached to the cell wall. Removal of the cell walls of vegetative cells or gametes by the exogenously added enzyme causes almost complete loss of the enzyme from the cells (Table 11). When these de-walled gametes are mixed together, they normally agglutinate and fuse, but release into the medium only small amount of the enzyme (Fig. 11). Similarly, in the cell wall-deficient mutants (cw), the amount of lytic activity in the homogenates of cells and in the medium after mating is practically negligible (Table 11 and Fig. 11). Therefore, the lytic enzyme, which has been stored within the periplasmic space or in the cell walls, may be actually released into the medium as the gamete wall-autolysin when mating reactions begin. When vegetative cells are de-walled with exogenously added lytic enzyme, and then incubated after washing out of the enzyme, cell walls are regenerated and lytic enzyme is again accumulated in the cells after the wall reformation is almost complete (Fig. 12). Goodenough and Heuser (1985) have analyzed the wall regeneration using the quick-freeze, deep-etch technique and shown that after lytic enzyme is washed away, cells quickly regrow radial fibers to constitute the "warp", and the elements of the central triplet are then assembled to form the "weft" within a period of few hours. It is therefore predicted that the newly synthesized lytic enzyme would be organized outside the plasmalemma after the "weft" is formed.

In this organism, insoluble phosphatases and carbonic anhydrase are also located outside the plasmalemma (Kimpel et al. 1983; Lien and Knutsen 1973; Loppes 1976; Matagne et al. 1976). As for the carbonic anhydrase, treatment of walled cells with trypsin causes a quantitative release of this enzyme into the medium, indicating that its locality is on the outer surface of the cell

wall (Yagawa et al. 1986). I found recently that treatment of gametes with trypsin does not cause any release of lytic activity from the cells, although this treatment has been shown to cause a complete release of agglutinins (Chapter 1). Therefore, the lytic enzyme may not be located on the cell surface. Since the innermost layer of the central triplet (W2 layer) has been proposed to be the target of lytic enzyme (Goodenough and Heuser 1985; Imam et al. 1985; Matsuda et al. 1985), it is plausible to assume that the enzyme is stored in the vicinity of the W2 layer. In this context, Millikin and Weiss (1984) have analyzed the binding of Con A to the Chlamydomonas gametic cells and proposed that periplasmic Con A-binding sites represent lytic enzyme or a precursor of enzyme.

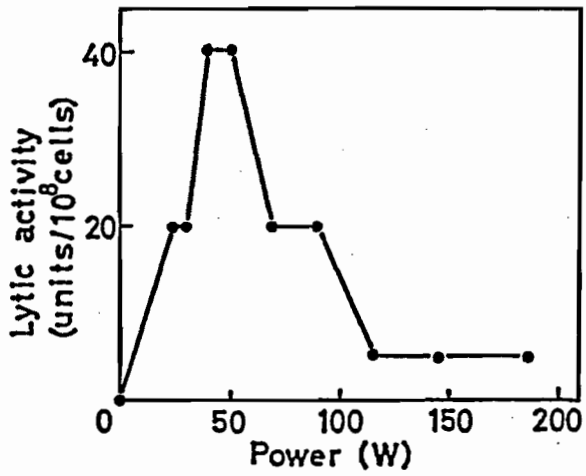

Fig. 7
Sonication of homogenates from unfrozen vegetative cells

Unfrozen vegetative cells were homogenized completely by French press at a density of 1 x 10^8 cells/ml. Aliquots (2 ml) were sonicated at different power indicated on the abscissa for 1 min at 4℃, and lytic activities were determined.

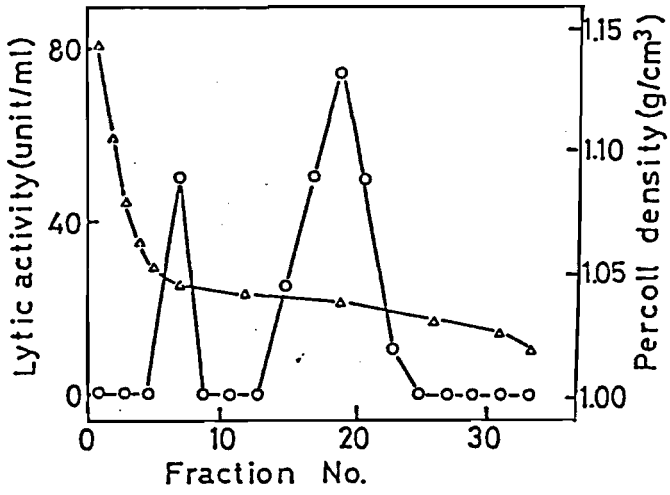

Fig. 8
Percoll density gradient fractionation of homogenate
from unfrozen vegetative cells

Cells were broken, centrifuged at 8,000 xg for 15 min to remove the large cell fragments, and the supernatant (20 ml) was mixed with Percoll and sucrose to give final concentrations of 15 % and 0.25 M. After centrifugation at 50,000 xg for 30 min, fractions of 1 ml were collected, and analyzed for the Percoll density (△) and lytic activity (○) after sonicating at 50 W for 1 min.

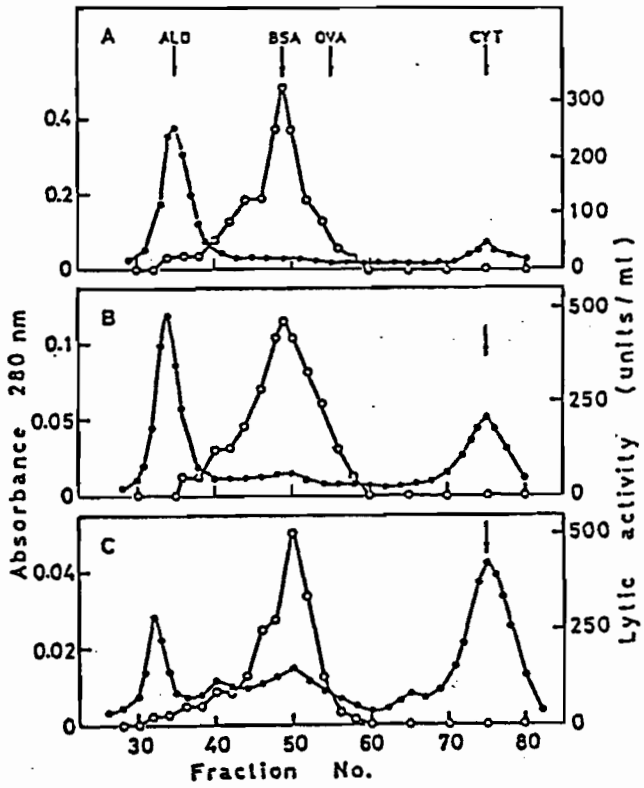

Fig. 9

Purification of lytic enzymes from vegetative cells, gametes, and mating medium by gel filtration

After Con A affinity chromatography and concentration, 1 ml of vegetative enzyme (A), gametic enzyme (B), and gamete wall-autolysin (C) were fractionated by Sephacryl S-200 chromatography. Before fractionation, samples were mixed with 0.5 mg cytochrome C used as a marker. Fractions of 1 ml were collected and analyzed for the absorbance at 280 nm (●) and lytic activity (○). Standard proteins used for the determination of the molecular mass of lytic enzymes were aldolase (ALD, 155 kDa), bovine serum albumin (BSA, 66 kDa), ovalbumin (OVA, 45 kDa), and cytochrome C (CYT, 12.5 kDa). Arrows indicate the peak positions of standard proteins.

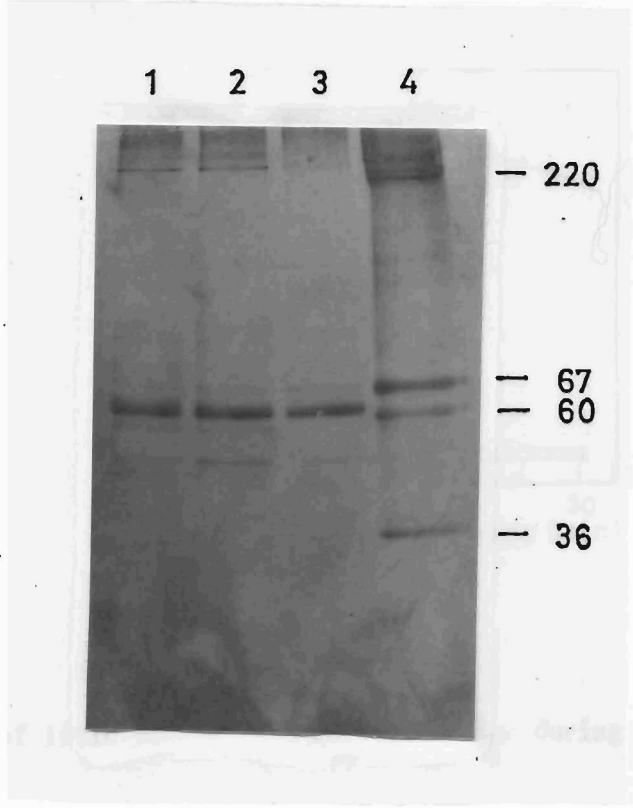

Fig. 10

SDS-polyacrylamide gel electrophoresis of purified lytic enzymes

After the second gel filtration, the activity peak fractions were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining. Lane 1, vegetative enzyme; lane 2, gamete enzyme; lane 3, gamete wall-autolysin in mating medium; lane 4, marker proteins.

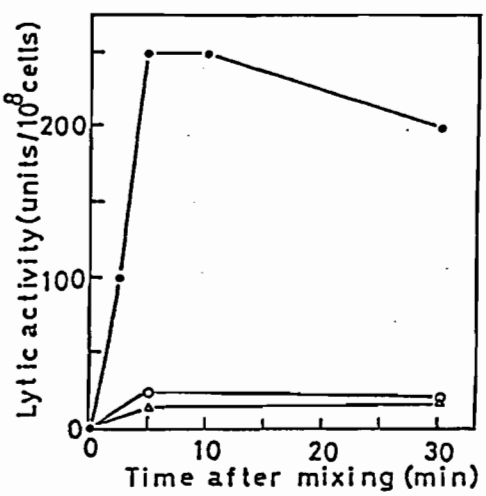

Fig. 11

Release of lytic enzyme into culture medium during mating of gametes

The wild-type cells (●), their wall-digested cells (○), and the cw-15 mutant cells (△) of mt^+ and mt^- were mixed together at 5 x 10^7 cells/ml. At the times indicated, 1 ml portions were taken, centrifuged quickly, and the supernatants were assayed for lytic activity.

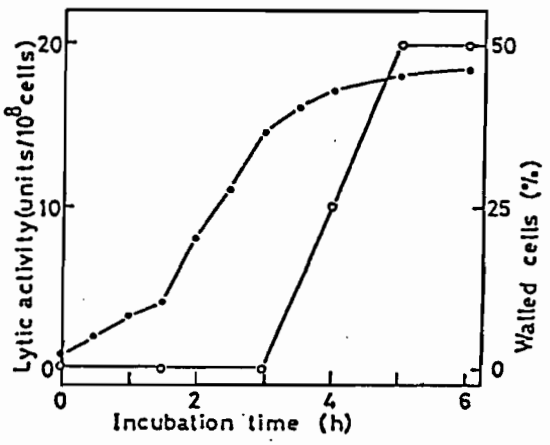

Fig. 12

Re-accumulation of lytic enzyme after wall regeneration

Vegetative cells were treated with gamete wall-autolysin to digest their walls, centrifuged, resuspended in enzyme-free medium. At the times indicated, aliquots were taken to determine the proportion of walled cells (●) and lytic activity (○). The proportion of walled cells was quantified by the heating method. Lytic activity was assayed using homogenates from frozen cells.

Table 7

Activity of cell wall lytic enzyme in cell homogenates and mating medium

Source	Lytic activity (units/10^8 cells)		
	Homogenate		
	Unfrozen cell	Frozen cell	Medium
Vegetative cell			
mt$^+$	<1	46±7	−
mt$^-$	<1	40±8	−
Gamete			
mt$^+$	50±6	40±8	−
mt$^-$	37±8	43±6	−
Gamete mixture	−	−	256±18

Unfrozen and frozen cells were resuspended in 10 mM Tris-acetate buffer, homogenized, and the lytic activities were determined.

Table 8

Activities of lytic enzyme and acid phosphatase in the soluble fraction obtained before and after homogenization of mt$^+$ cells

Cell	Lytic enzyme (units/10^8 cells)		Acid phosphatase (μ mol naphthol/10^8 cells·h)	
	Before FP[a]	After FP	Before FP	After FP
Vegetative				
unfrozen	<1	<1	0	1.2
frozen	<1	40	1.2	1.3
Gametic				
unfrozen	<1	50	0	0.6
frozen	<1	50	0.4	0.5

[a] FP, French press

Table 9

Distribution of lytic activity after fractionation of homogenate from unfrozen vegetative cells by differential centrifugation

Fraction	Relative activity of lytic enzyme (%)
1,000 xg pellet	6.8
8,000 xg pellet	6.8
15,000 xg pellet	56.4
30,000 xg pellet	17.1
100,000 xg pellet	8.5
100,000 xg supernatant (soluble fraction)	4.3

Homogenate was prepared from $\underline{mt}^+$ vegetative cells without freeze-thawing. Centrifugations were carried out for 15 min with the exception of 100,000 xg, done for 60 min. Pellets were resuspended in 10 mM Tris-acetate buffer and sonicated at 50 W for 1 min to solubilize the enzyme.

Table 10

Effects of inhibitors on lytic activity of enzyme purified from vegetative cells and gametes

Inhibitor	Concentration (mM)	Percent inhibition	
		Vegetative enzyme	Gametic enzyme
PMSF[a]	1.0	0	0
EDTA	0.02	12	8
	0.05	100	100
1,10-Phenanthroline	0.05	18	100
	0.1	59	100
$HgCl_2$	0.2	6	100
	0.5	100	100
Iodoacetate	4.0	67	68
	5.0	100	100
PCMB[a]	2.5	10	36
	5.0	100	100

Purified enzymes after the second gel filtration were used. Inhibitors were added to reaction mixtures containing 0.25-0.5 units of enzyme activity, and reaction was started by adding zoosporangia as substrate.

[a] PMSF, phenylmethylsulfonyl fluoride; PCMB, p-chloromercuribenzoic acid.

Table 11

Lytic activity in wild-type cells treated with gamete wall-autolysin and in wall-less mutant cells

Strain		Culture	Lytic activity (units/10^8cells)	
			Gamete wall-autolysin	
			Untreated	Treated
Wild-type	mt$^+$	V	40	2
		G	45	4
	mt$^-$	G	44	2
cw-2	mt$^+$	V	<1	ND
cw-3	mt$^+$	V	<1	ND
cw-8	mt$^+$	V	<1	ND
cw-10	mt$^-$	V	<1	ND
cw-15	mt$^+$	V	<1	ND
		G	3	ND
cw-15	mt$^-$	V	<1	ND
		G	2	ND

Abbreviations: V, vegetative culture; G, gametic culture; ND, not determined.
Activities were measured by use of homogenates from frozen cells.

Chapter 4

Differentiation and dedifferentiation of gamete : Kinetic analysis of activity of agglutinin and lytic enzyme

Introduction

Vegetative cell and gamete of C. reinhardtii are indistinguishable in the level of light microscope. In the ultrastructural and biochemical levels, however, these two cell types can be distinguished. Gamete of both mating-types, but not vegetative cell, possesses the mating structure for cell fusion at the apical portion of the cell body and fibrous agglutinin molecules at the surface of flagella (Friedmann et al. 1968; Goodenough et al. 1982; Adair 1985). Biochemically, vegetative cell does not have any agglutinin activity, but gamete has the activity in the cell body (cell body-agglutinin) and on the flagella (flagellar surface-agglutinin) (Chapter 1). Moreover, vegetative cell contains cell wall lytic enzyme in an inactive and insoluble form (V-form), whereas gamete has the enzyme in an active and soluble form (G-form) (Chapter 3).

In this alga, differentiation of vegetative cell to gamete is a reversible process which can be controlled experimentally. Withholding a nitrogen source induces gametogenesis, and adding it back converts gamete to vegetative cell (Sager and Granick 1954; Kates and Jones 1964). In this study I therefore used the differentiation and dedifferentiation processes to examine the kinetics of agglutinin and lytic enzyme activities using the soluble fraction of cell homogenate (see Chapters 1 and 3).

Materials and methods

Strain and culture condition

The wild-type strain 137c, *mt*$^{+}$ and *mt*$^{-}$, was used. Vegetative cultures were grown at 25 ℃ under continuous illumination in liquid minimal (M) medium (Matsuda *et al.* 1971). To obtain gametes, vegetative cells were harvested by centrifugation at 700 xg for 2 min, washed twice in nitrogen-free induction medium (-N ; Matsuda *et al.* 1978), resuspended in -N medium at about 5 x 10^6 cells/ml and incubated at 25℃. In certain experiments, gametes were obtained from plate cultures. Cells were grown on 1.5 % agar plates containing M medium for 6 days, flooded in -N medium at 5 x 10^7 cells/ml and incubated at 25℃. Agglutinability and mating efficiency of cells were determined by mixing them with tester gametes of the opposite mating-type in equal numbers as described in Chapter 2 (Matsuda *et al.* 1981).

Agglutinin and lytic enzyme assay

Cells were harvested by centrifugation, resuspended in ice cold 10 mM Tris-acetate, pH 7.5, at 1 x 10^8 cells/ml, and broken completely by passing through a French pressure cell at 400 kg/cm^2. The cell homogenate was centrifuged at 100,000 xg for 60 min and the supernatant was saved as the soluble fraction. Bioassay of cell body-agglutinin in the soluble fraction was carried out as described in Chapter 1. Agglutinin activity determined by a dried spot method (Adair *et al.* 1982; Saito and Matsuda 1984a) is expressed as 2 to the power of the number of the highest dilution still showing the activity. Activity of cell wall lytic enzyme in the soluble fraction was estimated as described in Chapter 3. One unit of activity is defined as the amount of enzyme which breaks the mother cell walls of glutaraldehyde-fixed zoosporangia at the 50 % level (Tamaki *et al.* 1981).

Results

Gametogenesis from liquid culture

Fig. 13A shows the kinetics of development of flagellar agglutinability and fusing ability during gametogenesis that occurs when vegetative cells in non-synchronized liquid culture are transferred to -N medium. Both activities were not observed during first 3-4 h of gametic induction, then developed rapidly and reached the maximum level after about 7 h (Fig. 13A). The kinetics is in agreement with that reported previously by many authors (Kates and Jones 1964; Martin and Goodenough 1975; Matsuda 1980). During the gamete induction, the increase in cell number was negligible.

Fig. 13B shows the activities of cell body-agglutinin and cell wall lytic enzyme, which were assayed by use of the soluble fractions from unfrozen cells. The activity of cell body-agglutinin developed after 5 h and reached the gametic levels after 8 h. Therefore, the accumulation of agglutinin molecules in the cell body appeared to occur slightly later than the acquisition of flagellar agglutinability. The activity of lytic enzyme was not detectable during the first 4 h of gametic induction, then developed rapidly and reached the level of gametes after 8 h (Fig. 13B). This indicates that cell wall lytic enzyme stays in V-form (i.e. inactive form) during the first 4 h and then shifts rapidly to G-form (i.e. active form).

Gametogenesis from plate culture

When vegetative cells which have been growing on agar plates for one or several weeks are flooded in -N medium, they give rise to gametes within 2 h (Schmeisser et al. 1973; Martin and Goodenough 1975). Since the 2-h period is much shorter than the period of time which is required for gametogenesis from liquid culture (see above), many authors have assumed, with little evidence,

that the agar-cultured, "aged" cells may differentiate into gametes on the plate medium and the 2-h period is required for the acquisition of motility since the plate cells have been losing completely their flagella (cf. Martin and Goodenough 1975). The following experiments were therefore conducted to determine whether differentiation actually occurs on plates or whether it occurs after suspension in -N medium, by the assay of cell body-agglutinin and cell wall lytic enzyme.

Cells from 6 day-old cultures on M plates were scraped using a razor blade, suspended in ice cold Tris-buffer, homogenized, and the soluble fraction was obtained. Neither cell body-agglutinin activity nor cell wall lytic activity was found in the soluble fraction. When the remaining cells on plates were suspended in -N medium and incubated at 25℃, more than 90 % of the cells became normally flagellated after 30 min, a full agglutinability was acquired after 1 h, and both cell body-agglutinin and lytic enzyme activities reached the gametic levels after 2 h (Fig. 14A). Cycloheximide (CHI) at 4μg/ml, a potent inhibitor of protein synthesis, blocked completely the development of cell body-agglutinin and lytic enzyme activities although the treated cells became flagellated (slightly shorter flagella than normal gametes) and motile. These results clearly indicate that the cells grown on plates stay vegetative, and they differentiate into gametes without apparent lag after suspension in -N medium.

Martin and Goodenough (1975) have shown that the aged cells grown on plates can become gametes even when they are suspended in nitrogen-containing (+N) medium. As shown in Fig. 14B, when vegetative cells grown on agar plates were suspended in +N medium, both cell body-agglutinin and lytic enzyme activities developed in parallel with the development of mating ability although at a slower rate than the cells suspended in -N medium (Fig. 14A), and reached the normal gametic levels after 2-3 h. The gametes, however, began to dedifferentiate after 4 h in +N medium (Fig. 14B).

Dedifferentiation of gamete on plate

Although the aged cells grown on M plates can differentiate into gametes within 2 h after suspension in liquid -N medium or even in +N medium (see above), a trace amount of nitrogen might be still left in cells and/or agar medium, which causes the cells to stay vegetative on plates. To deprive of nitrogen completely, I used the agar plates with nitrogen-free M medium (M-N) during the cell growth. The agar plates contained no detectable amount of inorganic nitrogen, as determined by Nessler's Reagents (Burris and Wilson 1957).

Vegetative cells were plated on M-N medium, incubated for 3 days, and then suspended in liquid -N medium. The cells on plates yielded neither agglutinin activity nor lytic activity, and these activities as well as motility and agglutinability developed rapidly after suspending them in -N medium (Table 12). The kinetics resembled closely that in cells grown on M plates (Fig. 14A). Quite surprisingly, when gametes with normal agglutinin and lytic enzyme activities were plated on M-N medium, these two activities were lost completely in the cells after 3 days. Following resuspension in liquid -N medium the plate cells again differentiated into gametes, as defined by the development of agglutinin and lytic enzyme activities (Table 12). During the incubation of vegetative cells and gametes on N-free plates, a lawn of cells became yellow-green, but most of cells appeared to survive without apparent growth.

Dedifferentiation in liquid condition

Fig. 15 shows the kinetics of dedifferentiation of gametes after they are transferred to liquid +N medium. Both cell body-agglutinin and lytic enzyme activities, which had been contained in gametes (Fig. 15B), were decreased in parallel with the loss of mating ability (Fig. 15A). After a 14-h incubation, cells lost completely all of these activities. When the dedifferentiated cells

were harvested and frozen before homogenization, the lytic activity was detected normally in the soluble fraction of cell homogenate (Table 13). This indicates that the lytic enzyme returns to V-form during gametic dedifferentiation. During dedifferentiation the increase in cell number was at most 20 %. Table 13 also shows that CHI blocks gamete dedifferentiation, that is, loss of mating ability, disappearance of agglutinin, and the transition of lytic enzyme from G-form to V-form.

Discussion

In this study the kinetics of gametic differentiation and dedifferentiation was examined in relation to the changes in agglutinin and lytic enzyme activities. When vegetative cells grown non-synchronously in liquid medium are transferred to -N medium, flagellar agglutinability develops after about 3 h and reaches the maximal level at 7 h (Fig. 13A), while the accumulation of agglutinin in the cell body occurs between 4 and 8 h after nitrogen starvation (Fig. 13B). This slight time lag has been also observed in trypsin-treated gametes; the trypsinized gametes recover the flagellar agglutinability within 1 h after addition of trypsin inhibitor, whereas the cell body-agglutinin accumulates after 1 h (Chapter 1). It is considered, therefore, that agglutinin molecules which synthesized during gametogenesis or after trypsinization are initially transported to the flagellar surface, and only then can large pool of the cell body-agglutinin be built up (see also Chapter 1).

The processing of lytic enzyme from its V-form to G-form occurs in parallel with the development of mating ability during gametogenesis (Fig. 13), and the processing in the opposite direction (from G-form to V-form) occurs again in parallel with the loss of mating ability (Fig. 15). Therefore, the transition in the storage form of lytic enzyme may be one of the crucial events which are involved in the gametic differentiation and dedifferentiation in C. reinhardtii.

The present study also demonstrates that the "aged" cells grown on agar plates stay vegetative, as defined by the lack of activities of cell body-agglutinin and lytic enzyme in the soluble fraction of cell homogenates, but can differentiate into fully competent gametes within 2-3 h after suspension in -N or even in +N medium (Fig. 14). The difference in the induction time between the liquid culture (7 h) and plate culture (2-3 h) may be due to a difference in cellular pool sizes of nitrogen: the liquid-cultured cells, when transferred to -N medium, still have a pool of nitrogen so that they do not respond to nitrogen starvation and stay vegetative until it is exhausted by

the cells during the initial few hours in -N medium, whereas the aged cells on plates already use up their nitrogen reserves so that they are ready to run a gametogenic program just as suspended in -N medium. Since gamete differentiation occurs even after suspending the plate-cultured cells into +N medium (Fig. 14B), it is predicted that the "aged" cells may already get a signal for differentiation, but stay vegetative on plates, and differentiate into gametes if only they are placed in liquid medium (even if it is +N medium). The gametes which have differentiated in +N medium begin to dedifferentiate after 4 h incubation (Fig. 14B). This indicates that the cells may switch off the signal for differentiation soon after suspension in +N medium.

Even more surprising are the present observations that when gametes in liquid culture are plated on -N medium, they not only lose their flagella, but also dedifferentiate into vegetative cells and that the plate "gametes" (actually vegetative cells) again differentiate into gametes after suspension in liquid medium (Table 12). It is therefore concluded that:

(1) C. reinhardtii cells stay vegetative on solid medium even when they are starved for nitrogen.

(2) Gametes dedifferentiate into vegetative cells when placed on solid medium even in the absence of nitrogen; when motility is lost on solid medium, the cells lose not only flagella but also agglutinin and lytic enzyme activities which are essential for mating.

(3) Gametic differentiation occurs only in liquid medium.

The bioassays of agglutinin and lytic enzyme constitute useful analytical tools to investigate the sexual differentiation and dedifferentiation in C. reinhardtii.

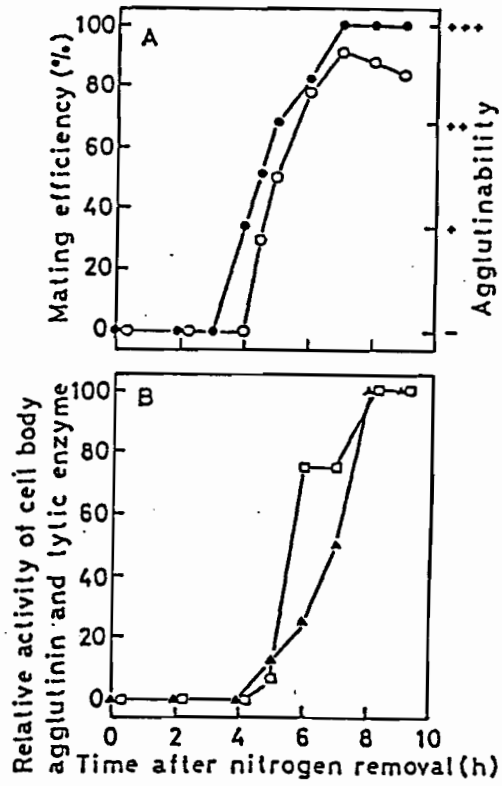

Fig. 13

Time course of gametogenesis from non-synchronous liquid culture

Wild-type mt$^+$ cells grown in liquid medium (5 x 10^6 cells/ml) were transferred to -N medium. At the times indicated, agglutinability (● in A), mating efficiency (○ in A), cell body-agglutinin activity (▲ in B) and lytic enzyme activity (□ in B) were determined.

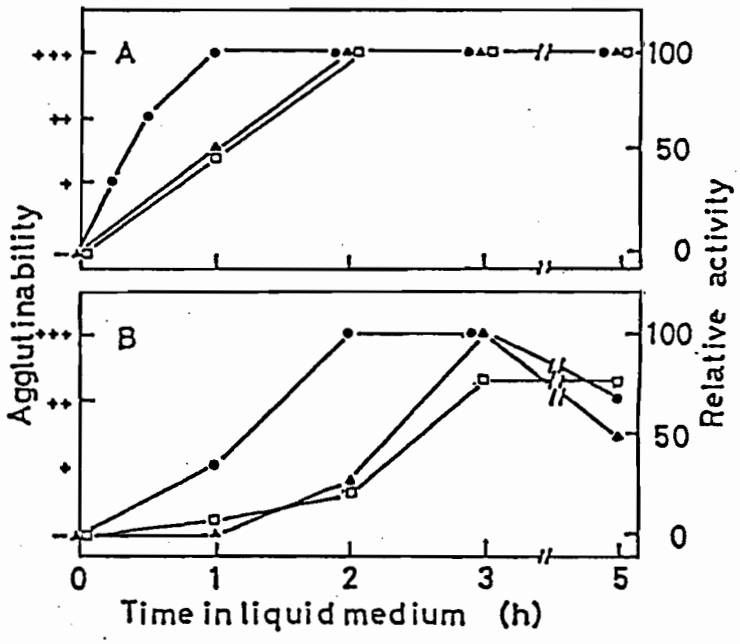

Fig. 14

Time course of gametogenesis from plate culture

Wild-type mt$^+$ cells were cultured on M agar plates for 6 days and suspended in -N medium (A) or +N medium (B) at a density of 5 x 10^7 cells/ml. At the times indicated, agglutinability (●), cell body-agglutinin activity (▲) and lytic enzyme activity (□) were determined.

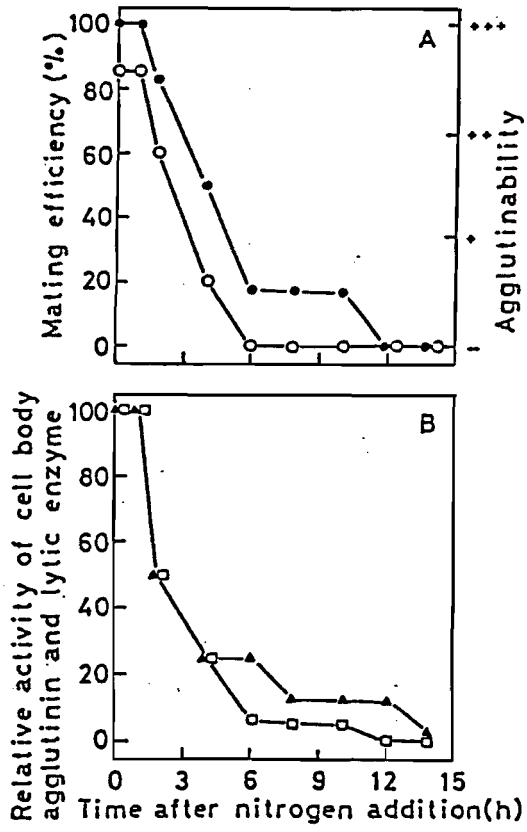

Fig. 15

Time course of dedifferentiation in liquid culture

Fully differentiated gametes of mt$^+$ (5×10^7 cells/ml) were transferred to 9 volumes of nitrogen-containing medium. At the times indicated, agglutinability (● in A), mating efficiency (○ in A), cell body-agglutinin activity (▲ in B) and lytic enzyme activity (□ in B) were determined.

Table 12

Activities of cell body-agglutinin and lytic enzyme in the soluble fraction of cell homogenates from cells grown on nitrogen-free plates and then suspended in liquid

Plated cell	Time in liquid (min)	Flagellated cell (%)	Agglutinability	Activity (/10^8 cells)	
				Agglutinin	Lytic enzyme
Vegetative	0	0	−	$<2^0$	0
cell	30	92	++	2^6	15
	60	100	+++	2^8	46
	120	100	+++	2^8	100
Gamete	0	0	−	$<2^0$	0
	30	88	+	ND	ND
	60	90	+++	2^6	25
	120	96	+++	2^7	63

Vegetative cells and gametes of mt$^+$ were plated on M-N agar medium. After 3 days on plates, aliquots of cells were sampled and suspended in ice cold 10 mM Tris-acetate, pH 7.5, to determine percent flagellated cells and activities of cell body-agglutinin and lytic enzyme in the soluble fraction of cell homogenates. The remaining plate cells were suspended in -N medium, incubated at 25 ℃ and determined for their activities at the times indicated. ND, not determined.

Table 13

The effect of cycloheximide on dedifferentiation of gametes

Condition	Agglutination	Fusion (%)	Activity (/10^8cells)		
			Agglutinin	Lytic enzyme	
				unfrozen	frozen
-N ,-CHI	##	61	2^8	25	ND
-N ,+CHI	##	26	2^6	50	ND
+N ,-CHI	—	0	$<2^0$	0	40
+N ,+CHI	##	18	2^6	40	40

Gametes (mt$^+$) were incubated in nitrogen-free (-N) or nitrogen-containing (+N) medium for 14 h. Cycloheximide (CHI, 4μg/ml) was added at the beginning of incubation. ND, not determined.

Chapter 5

Classification of non-mating mutants by the assay of agglutinin and lytic enzyme

Introduction

Sexual reproduction in C. reinhardtii involves gametic differentiation from vegetative cells under a nitrogen deprived condition and the mating reaction between gametes of two mating-types, mt^+ and mt^- (see General Introduction and Fig. 1). Since the Chlamydomonas mating is a complex process, mutants are useful tools for studying the individual steps involved in mating. Several classes of gametic mutants have been isolated and analyzed for their biochemistry, ultrastructure and genetics (Goodenough and Thorner 1983; Snell 1985). In view of mating process in C. reinhardtii, the non-mating mutants can be divided into at least four distinct classes (cf. Goodenough et al. 1976): class I mutations which prevent cells from responding to nitrogen starvation and/or block all events of gametogenesis; class II mutations which lack agglutinin molecules; class III mutations which lack cell wall lytic enzyme; and class IV mutations which cannot fuse (Table 14). Among these four classes the phenotypes of class I and class II mutations might be similar: neither type of mutant cells agglutinates, excretes lytic enzyme, fuses to form zygotes even when they are starved for nitrogen and mixed with wild-type gametes of opposite mating-type (Table 14).

In this Chapter I report a promising system for classification of the non-mating mutants of C. reinhardtii into four classes, especially for distinguishing between the mutations belonging to class I and class II. This system is the combination of simple bioassays for cell body-agglutinin (Chapter 1) and cell wall lytic enzyme (Chapter 3) by use of the soluble

fractions of homogenates from nitrogen-starved cells. Using this system many non-mating mutants isolated previously (Matsuda et al. 1978; Goodenough and Thorner 1983) are classified. I also report the isolation and characterization of new non-agglutinating mutants belonging to class II.

Materials and methods

Strains and culture conditions

The wild-type strain is 137c, *mt*$^{+}$ and *mt*$^{-}$. The non-mating mutant strains, *imp-1* *mt*$^{+}$ (CC-462), *imp-2* *mt*$^{+}$ (CC-463), *imp-8a* *mt*$^{+}$ (CC-475) (Goodenough *et al.* 1976), *imp-11* *mt*$^{-}$ (CC-1147) (Goodenough *et al.* 1982) and *imp-12* *arg-7* *mt*$^{-}$ (CC-1310) (Hwang *et al.* 1981) were obtained from the *Chlamydomonas* Genetics Center, Department of Botany, Duke University. Cells of *fus* *mt*$^{+}$, 7430B (*arg-7* *actr* *sr-u-2-60* *mt*$^{-}$) and 7457B (*arg-2* *msr* *spr-u-1-27* *mt*$^{-}$) were those isolated by Matsuda *et al.* (1978, 1983) and Tsubo and Matsuda (1984).

Vegetative cultures were grown at 25℃ with continuous illumination in liquid TAP medium (Gorman and Levine 1965) or arginine (100μg/ml)-supplemented medium (TAPA). To obtain gametes, vegetative cells were harvested by centrifugation at 700 xg for 2 min, washed twice in nitrogen-free (-N) induction medium (Matsuda *et al.* 1978), resuspended in -N medium at about 5 x 10^{6} cells/ml and incubated overnight at 25℃. Agglutinability of cells was determined by mixing them with tester gametes of the opposite mating-type in equal numbers as described in Chapter 1 (Matsuda *et al.* 1981).

Isolation of non-mating mutants

The method for selection of the non-mating mutants of *mt*$^{-}$, which took advantage of a uniparentally inherited chloroplast marker *spr*, was essentially that described by Forest and Togasaki (1975). Vegetative cells of 7457B grown in TAPA medium were exposed to UV light for 90 sec (3,800 erg/cm^{2} x sec) at a density of 1 x 10^{6} cells/ml. The UV-irradiated cells were diluted with 3 volumes of medium and incubated in the dark for 24 h. They were then centrifuged, washed in -N medium, resuspended in the medium to induce gametogenesis and mixed with an excess of 137c *mt*$^{+}$ gametes. After overnight incubation, the pellicle of

zygotes was carefully removed and the remaining single cells were spread on plates containing TAPA and 200 μg/ml spectinomycin dihydrochloride. Since the wild-type (mt$^+$) cells and most of the remaining zygotes are phenotypically spectinomycin sensitive, they should be killed with spectinomycin, whereas non-mated 7457B (spr mt$^-$) cells would survive. About 160 colonies developed on plates were picked, and their ability to mate with either mating-type gametes were retested. Each clone was also examined for requirement of arginine and resistance against DL-methionine-dl-sulfoximine (500 μg/ml) and spectinomycin (100 μg/ml).

Assay for agglutinin activity and lytic enzyme activity

Cells were grown in +N medium or -N medium, and the soluble fractions of cell homogenates were prepared (Chapter 1). The activities of cell body-agglutinin and cell wall lytic enzyme in the soluble fractions were assayed quantitatively by the methods described in Chapter 4. Agglutinin activity is expressed as 2 to the power of the number of the highest dilution still showing activity (Adair et al. 1982; Saito and Matsuda 1984a). One unit of cell wall lytic enzyme is defined as the amount of enzyme which liberates daughter cells from 1 x 10^6 zoosporangia at the 50 % level (Tamaki et al 1981).

Diploid construction

Diploid strains were constructed using polyethylene glycol-induced cell fusions (Matagne et al. 1979; Matsuda et al. 1983). Two complementing haploid arginine auxotrophs (arg-2 and arg-7) were de-walled by cell wall lytic enzyme, and the protoplasts were mixed together in a solution containing 30 % polyethylene glycol, 50 mM $CaCl_2$ and 10 mM HEPES, pH 7.8 (Matsuda et al. 1983). After plating on 1 % agar-TAP medium, the plates were illuminated continuously at about 3,000 lux for 7-10 days, and 20 diploid colonies that

derived from fusion products were picked. Each colony was subcultured for 4-5 days in TAP liquid medium, and then the mating ability was tested. Verification of diploidy was made by measurement of cell size (Matsuda et al. 1983).

Results

Method for classification of non-mating mutants

Nitrogen-starvation triggers in the wild-type cells of C. reinhardtii a gametogenic program (Martin and Goodenough 1975) leading to flagellar agglutinability and fusing ability. According to this program, cells synthesize agglutinin in their cell bodies, which is then transported to flagellar surfaces for agglutination (Chapter 1), and construct the mt^+ and mt^- mating structures for cell fusion (Goodenough et al. 1982). The agglutinin molecules are also accumulated in the cell bodies during gametogenesis, as a pool of flagellar surface-agglutinin and the activity of cell body-agglutinin can be quantified by a simple assay using the soluble fraction of cell homogenates (Chapters 1 and 4). Moreover, vegetative cells and gametes have the same cell wall lytic enzyme, but the storage form is quite different between the two cell types (Chapters 3 and 4). Of particular importance is the previous finding (Chapter 4) that the storage form correlates with the state of differentiation; nitrogen starvation of vegetative cells causes a shift in enzyme localization to G-form in parallel with the acquisition of mating ability, and dedifferentiation of gametes by addition of nitrogen is accompanied by a shift to V-form. In other words, we can now clearly distinguish between vegetative cells and gametes by a simple determination of lytic activity in the soluble fraction of cell homogenates. If this enzyme assay is combined with the cell body-agglutinin assay, the system will become a valuable tool to characterize the Chlamydomonas non-mating mutants and classify them into the four classes; the assay will be especially useful for distinguishing between the class I and class II mutations (see Introduction). The idea is as follows:

Since class I mutations block all events of gametic differentiation under a nitrogen (N)-starved condition, that is, the mutant cells in this class stay vegetative, the soluble fraction of cell homogenates will contain neither

agglutinin activity nor lytic activity (Table 14). In contrast, the soluble fraction from the N-starved cells of class II mutants, although lacking agglutinin, will retain the activity of lytic enzyme since the cells become gametic for the enzyme (Table 14). The mutant cells of class III (lytic enzyme-less) will have only agglutinin activity in the soluble fraction, while the non-fusing mutants (class IV) will exhibit both agglutinin and lytic enzyme activities (Table 14). In addition to these four main classes, other types of mutations, for example, those affecting sexual signalling or agglutinin transportation could also be identified by the examination of phenotypes and assay of the two activities.

Table 15 summarizes the agglutinin and lytic enzyme activities found in the soluble fractions prepared from wild-type cells and many non-mating mutants isolated previously (Goodenough et al. 1976; Matsuda et al. 1978; Hwang et al. 1981). N-starved cells of the non-agglutinating mutants, imp-2 (mt^+), imp-8 (mt^+) and imp-12 (mt^-), all lacked detectable activities of cell body-agglutinin in their soluble fractions, but yielded nearly normal levels of lytic enzyme activity. The results indicate that these mutants can become non-agglutinable "gametes" by nitrogen starvation, so they belong to class II, but not to class I (Table 15).

Phenotypically non-fusing mutants, fus (mt^+), imp-1 (mt^+), and imp-11 (mt^-) were found to possess normal agglutinin and lytic enzyme activities in their soluble fractions, indicating that these mutant cells belong to class IV (Table 15).

Isolation of new non-agglutinating mutants

Since the non-mating mutants isolated previously all belong to class II and class IV (see above), I tried to isolate new non-mating mutants of mt^- belonging to class I (sexual differentiation mutant; see above). In the present study 7457B (arg-2 msr spr-u-1-27) was used as the parent for

mutagenesis to facilitate the selection (see Materials and methods). About 160 clones were isolated by the initial screening, and finally three clones were proved to be non-agglutinating mutants. These three mutants, named agl-1, agl-2 and agl-3, were capable of normal growth and motility, and carried the nuclear arg-2 and msr markers and the chloroplastic spr marker. When starved for nitrogen, however, they failed to agglutinate and fuse with tester gametes of mt^+ or mt^-. To determine whether the agl mutants belong to class I or class II, the activities of cell body-agglutinin and lytic enzyme in the soluble fractions of N-starved cells were examined. All the N-starved cells yielded no cell body-agglutinin activity, but had normal lytic enzyme activity (Table 16), indicating that agl-1, agl-2 and agl-3 all belong to class II, but not to class I.

Genetic analysis of agl-1 mutant

One of the three agl mutants, agl-1 (mt^-) was further analyzed for the genetic characters. Hwang et al. (1981) have shown that the imp-12 mutation, which belongs to class II (Table 15), is closely linked to the mt^- mating-type locus in linkage group VI. To see whether agl-1 mutation is also linked to the mt^- locus, an artificial fusion was tried between the wild-type (mt^+) and agl-1 (mt^-) gametes by the centrifuging method of Goodenough et al. (1976). Gametes of two mating-types were mixed, de-walled by exogenously added cell wall lytic enzyme, and centrifuged at 700 xg for 15 min to let them fuse. The cell mixture was plated, incubated in the dark for a week, exposed to chloroform vapor to kill non-zygotic cells and then transferred to light. However, no zygospore colonies were developed on the plates.

Artificial fusions were then carried out between two complementing arginine auxotrophs (arg-2 and arg-7) using polyethylene glycol (PEG) solution (Matsuda et al. 1983). PEG-induced fusions were made between 7430B (mt^-) and agl-1 (mt^-), and 20 diploid clones (agl-1 mt^-/ + mt^-) were obtained. All clones behaved

like wild-type mt⁻; the N-starved cells yielded both agglutinin and lytic enzyme activities (Table 16), and normally mated with haploid mt⁺ gametes, indicating that the agl-1 is a recessive allele. Moreover, when diploids were constructed by PEG-induced fusions between CC-1310 (imp-12 mt⁻) and agl-1 (mt⁻), none of 20 diploid clones obtained were able to acquire agglutinability and fusing ability in a N-starved condition. These cells lacked cell body-agglutinin activity, but yielded a nearly normal level of lytic enzyme activity (Table 16). The result indicates that agl-1 and imp-12 are allelic which are closely linked to the mt⁻ locus.

Discussion

In this study I developed a simple assay system to classify non-mating mutants of C. reinhardtii into four classes. This system is especially effective to distinguish between class I (differentiation mutation) and class II (agglutinin-less mutation) mutants both of which are phenotypically the same, i.e. non-agglutinable (Table 14). Using this system, three non-agglutinable mutants isolated in Goodenough's laboratory, imp-2 (mt^+), imp-8 (mt^+) and imp-12 (mt^-) are confirmatively classified in class II (Table 15). Goodenough and Jurivich (1978) have reported that these mutants may simply lack a functional agglutinin since the treatment with a polyclonal antiserum raised against isolated flagella enables the N-starved mutant cells to undergo agglutination and fusion with normal gametes of the opposite mating-type. Furthermore, Adair (1985) has shown that the mutant cells starved for nitrogen lack agglutinin bands on SDS-PAGE of EDTA extracts. As compared to these previous methods, my newly developed assay system that uses the soluble fraction of cell homogenate appears to be very simple, rapid and reliable for mutant classification.

In this study I also tried to isolate a new mutant which belongs to class I but failed. Three new non-agglutinable mutants, named agl, belong to class II but not to class I, as defined by the assay system (Table 16). Genetic analyses by employing the PEG-induced cell fusion method suggest that one of the mutants, agl-1, is a recessive gene and closely linked to the mt^- locus, as in the case of imp-12 (Hwang et al. 1981).

Although it could not be obtained any non-conditional class I mutant strains after several trials, I recently succeeded to isolate conditional sexual differentiation (i.e. class I) mutants. This has been described in Chapter 6.

Table 14

Four classes of mating mutations in Chlamydomonas reinhardtii

Class	Mutation	Nitrogen (N)-starved cell				
		Phenotype[a]			Activity in the soluble fraction	
		Agglutination	Wall loss	Fusion	Agglutinin	Lytic enzyme
I	no response to N-starvation	no	no	no	no	no
II	lack of agglutinin molecule	no	no	no	no	yes
III	lack of cell wall lytic enzyme	yes	yes[b]	yes	yes	no
IV	inability of cell fusion	yes	yes	no	yes	yes

[a] Events which occur when mutant cells are mixed with wild-type gametes of opposite mating-type.

[b] Although the mutant cells lack lytic enzyme, their walls are digested from the outside with the enzyme excreted into the medium by wild-type gametes of opposite mating-type.

Table 15

Classification of various mating mutants by determining agglutinin and lytic enzyme activity in the soluble fraction of cell homogenates

Strain	Phenotype	Activity (/10^6 cells)				Class
		+N medium		-N medium		
		Agglutinin	Lytic enzyme	Agglutinin	Lytic enzyme	
137c (mt$^+$)	wild-type	<2[0]	0	2^9	50	
137c (mt$^-$)	wild-type	<2[0]	0	2^8	50	
imp-2 (mt$^+$)	non-agglutinating	<2[0]	0	<2[0]	20	II
imp-8 (mt$^+$)	non-agglutinating	<2[0]	0	<2[0]	38	II
imp-12 (mt$^-$)	non-agglutinating	<2[0]	0	<2[0]	50	II
fus (mt$^+$)	non-fusing	<2[0]	0	2^9	52	IV
imp-1 (mt$^+$)	non-fusing	<2[0]	0	2^{10}	50	IV
imp-11 (mt$^-$)	non-fusing	<2[0]	0	2^{10a}	50	IV

Cells were cultured in nitrogen-containing (+N) or nitrogen-free (-N) medium at a density of 5 x 10^6 cells/ml for 14 h at 25 ℃. The activities of agglutinin and lytic enzyme were examined using the soluble fraction.

[a] The mutant gametes contain plus-type cell body-agglutinin activity.

Table 16

Properties of newly isolated non-agglutinating mutants, agl and diploid strains containing agl-1 marker

Strain	Flagellar agglutinability	Mating efficiency (%)	Activity (/10^6cells)	
			Agglutinin	Lytic enzyme
agl-1 mt^-	−	0	<2^0	50
agl-2 mt^-	−	0	<2^0	50
agl-3 mt^-	−	0	<2^0	50
agl-1$^+$ mt^-/agl-1 mt^-	##	80	2^7	25
agl-1 mt^-/imp-12 mt^-	−	0	<2^0	50

Vegetative cells were transferred to nitrogen-free medium at a density of 5 x 10^6 cells/ml and incubated overnight. Agglutinability and mating efficiency of cells were determined by mixing with tester gametes of mt^+ in equal numbers.

Chapter 6

Isolation and characterization of conditional mutants affecting gamete differentiation

Introduction

Unconditional gametogenic mutants have previously been isolated in other and our laboratories (see Chapter 5). In Chapter 5 these mutants have been successfully classified by the assay system which uses the soluble fraction of cell homogenates from nitrogen (N)-starved cells: all the phenotypically non-agglutinable mutants belong to class II, and all the phenotypically non-fusable mutants are classified as class IV. I have also tried but failed to isolate new unconditional mutant strains which belong to class I, that is, mutations that block all events of gametic differentiation under a N-starved condition. However, I felt that it was necessary to isolate conditional class I mutants to facilitate analyses of gamete differentiation and dedifferentiation in C. reinhardtii genetically, morphologically and biochemically. Such conditional mutants may turn "on" the differentiation program at the permissive temperature, and turn "off" its program and/or turn "on" the dedifferentiation program at the restrictive temperature. In addition, genetic analysis will be manipulated easily since the mutant cells can mate normally at the permissive temperature.

The present report is concerned with the first isolation and characterization of two conditional class I mutants, dif-1 and dif-2.

Materials and methods

Strains and culture conditions

The wild-type strain is 137c, mt$^+$ and mt$^-$. The mutant strains, 7457B (arg-2 msr spr-u-27 mt$^-$) and 7430A (arg-7 actr sr-u-2-60 mt$^+$) were those described in previous papers (Matsuda et al. 1983; Tsubo and Matsuda 1984).

Vegetative cultures were grown at 25℃ under continuous illumination in liquid TAP (Gorman and Levine 1965) or arginine (100 μg/ml)-supplemented medium (TAPA). To obtain gametes, vegetative cells were harvested by centrifugation at 700 xg for 2 min, washed twice in nitrogen-free induction medium (-N ; Matsuda et al. 1978), resuspended in -N medium at about 5 x 10^6 cells/ml, and incubated for 14-16 h at either 25℃ or 35℃. Agglutinability and mating efficiency of cells were determined by mixing them with tester gametes of opposite mating-type in equal numbers as described in Chapter 2. Flagellar length was determined by using a micrometer.

Mutagenesis and selection of conditional non-mating mutants

Vegetative cells of 7457B (arg-2 msr spr-u-27 mt$^-$) were subjected to UV irradiation (3,800 erg/cm^2 x sec, 90 sec) at a density of 1 x 10^6 cells/ml, then diluted with 3 volumes of TAPA medium, and incubated in the dark for 24 h. The UV-irradiated cells were centrifuged, washed in -N medium, resuspended in the medium, shaked for 7 h at 35 ℃ to induce gametes, and then mixed with an excess of 137c mt$^+$ gametes. After overnight incubation at 35 ℃, the zygote pellicle was carefully removed and the remaining single cells were spread on plates containing TAPA plus 200μg/ml spectinomycin dihydrochloride. Since all wild-type mt$^+$ cells and most of the remaining zygotes are spectinomycin sensitive, they should be killed on the spectinomycin-containing plates, while unmated 7457B (spr mt$^-$) cells should be survived (cf. Forest and

Togasaki 1975). Therefore, 160 colonies developed were picked and retested for their mating abilities at 25℃ (the permissive temperature) and 35℃ (the restrictive temperature). Twelve clones were found to be the conditional mutants, and their characters were further analyzed (see Results).

Assay for classification of non-mating mutants

To classify the conditional non-mating mutants into four classes, especially to distinguish between the mutations belonging to class I and class II, the simple assay system which has been developed previously (Chapter 5) was used. Cells were cultured in -N medium at 35℃ overnight, harvested by centrifugation, homogenized by passing through a French pressure cell, and the soluble fractions of cell homogenates were prepared (Chapter 1). In certain experiments the harvested cells were frozen at -80℃ overnight and thawed prior to homogenization. The activities of cell body-agglutinin and lytic enzyme in the soluble fraction were assayed quantitatively by the methods described in Chapter 4. By this assay, for example, the conditional mutant cells which lack the two activities in their soluble fractions are defined as class I (see Chapter 5, for details).

Genetic analysis

The mt^+ and mt^- gametes were mixed together, incubated for 0.5-1 h, and spread on 3 % agar plates containing arginine and 1/5 the concentration of minimal medium (Matsuda et al. 1971). Zygotes were matured in darkness for a week, then exposed to chloroform vapor for 40 sec to kill non-zygotic cells, and transferred to defined positions of 1.5 % agar plate containing TAPA. After 24 h illumination at 20 ℃ to allow zygote germination, four or eight meiotic zoospores were separated and manipulated into a line using a glass needle under a dessecting microscope (Sager and Tsubo 1961). After growth on the

plate, colonies derived individual zoospores were picked, suspended in TAPA medium, cultured for 4-5 days, and examined for the motility, flagellar length, mating-type, nuclear genotypes (arginine requirement and actidione resistance) and mating ability at 25℃ and 35 ℃.

Diploid construction

Diploid strains were obtained by sexual crosses according to the method of Matsuda et al (1983). Two complementing haploid arginine auxotrophs (arg-2 and arg-7) of opposite mating-type were crossed, plated on TAP medium, and illuminated continuously at 3,000 lux for a week. Twenty colonies that had developed from vegetative zygotes were picked, suspended in TAP medium, and cultured for 4-5 days. Verification of diploidy was made by measurement of cell size (Matsuda et al. 1983).

Results

Phenotypes of conditional non-mating mutants

Twelve mt⁻ clones obtained by UV mutagenesis were phenotypically conditional non-mating mutants: they normally agglutinated and formed zygotes with tester gametes of mt⁺ at 25℃, but failed to mate at 35℃ (see Materials and methods). Further examinations showed that 11 clones lose flagella at 35℃ in both liquid +N and -N medium, and only one clone has normal flagella and motility at the restrictive temperature. When activities of cell body-agglutinin and cell wall lytic enzyme were assayed for cells cultured in -N medium at 35 ℃, 10 of the 11 flagella-less clones yielded normal levels of two activities, whereas one flagella-less and one flagellated clones possessed neither agglutinin activity nor lytic activity in the soluble fractions of cell homogenates. When the cells from the latter two clones were freeze-thawed prior to homogenization, the soluble fractions yielded a normal level of lytic enzyme activity. It is concluded from the above results that: (1) 10 clones are the conditional flagella-less mutants; they lose flagella at 35 ℃ but are able to differentiate into flagella-less gametes in -N medium. (2) two clones, named dif-1 and dif-2, are the conditional sexual differentiation (class I) mutants; they differentiate into gametes in -N medium at 25 ℃, whereas they stay vegetative at 35 ℃. The dif-1 mutant also loses the flagella at 35℃, while the dif-2 does not. The phenotypes of dif-1 and dif-2 mutants are termed DIF-1 and DIF-2, respectively.

Table 17 summarizes the properties (flagellar length, agglutinability, mating efficiency, cell body-agglutinin activity and lytic enzyme activity) of wild-type (mt⁻), dif-1 (mt⁻) and dif-2 (mt⁻) cells when they are starved for nitrogen at 25℃ and 35 ℃. It was noted that although the wild-type cells can differentiate into gametes at 35℃, their flagella are slightly shorter than those in gametes which differentiated at 25℃.

Kinetics of differentiation and dedifferentiation in dif mutants

When vegetative cells of dif-1 (mt^-) mutant in non-synchronized liquid culture were transferred to -N medium and incubated at 25 ℃, the activities of cell body-agglutinin and lytic enzyme as well as the ability to mate developed rapidly after 3-4 h, and reached the maximal levels after 6-8 h (Fig. 16), the kinetics being very similar to that in wild-type cells (Chapter 4, Fig. 13). After then the temperature was shifted up to 35℃. Quite dramatically, the flagella of dif-1 gametes were quickly resorbed (Fig. 16A), and both agglutinin and lytic enzyme activities in their cell bodies were decreased and lost completely after 3-5 h (Fig. 16B), indicating that they return to flagella-less vegetative cells. When the cells were placed again at 25℃, the flagella were regenerated, and the mating ability was recovered by activating agglutinin and lytic enzyme synthesis/processing (Fig. 16).

This dramatic shift from vegetative cells to gametes and vice versa was also observed when the dif-2 mutant cells were subjected to the temperature shift between 25℃ and 35 ℃ in -N medium although their flagella were not resorbed at 35 ℃ (data not shown).

Table 18 shows that cycloheximide (CHI), an inhibitor of protein synthesis, blocks both gametic differentiation and dedifferentiation in dif-1 mutant. When CHI (4μg/ml) was given to the gametes before transferring to 35℃, their flagella were resorbed but the activities of cell body-agglutinin and lytic enzyme were retained even after 7 h (Table 18). When the antibiotic was added to the cells dedifferentiated at 35 ℃ and then they were transferred to 25 ℃, the development of agglutinin and lytic enzyme activities as well as flagellar regeneration was blocked completely (Table 18).

Genetic analysis of dif-1 and dif-2

The dif-1 (mt^-) or dif-2 (mt^-) cells were crossed with wild-type (mt^+) or

7430A (*arg-7 actr mt*$^{+}$). The tetrad analyses (Table 19) showed that: (1) *dif-1*$^{+}$ and *dif-1* segregate strictly in the ratio of 2:2, indicating a Mendelian mode of inheritance. On the other hand, *dif-2*$^{+}$ and *dif-2* segregate in the ratio of 3:1 in 10 of 48 tetrads. I had an experience that the *dif-2* marker is, unlike *dif-1*, unstable when the mutant stocks are maintained on agar plates although it was stable in liquid culture. It is therefore possible that some of meiotic zoospores of *dif-2* might revert to wild-type during growth to form colonies on agar plates. (2) All of *dif-1* progenies are conditional mutants for both flagella and gametic differentiation, indicating that these two mutant characters are derived from a single mutation or two closely-linked mutations. (3) Both *dif-1* and *dif-2* mutations segregate independently of other nuclear genetic markers examined (Table 19). It appears, therefore, that the two genes are unlinked to *arg-2/7* marker in linkage group I, *actr* marker in linkage group II, and mating-type loci in linkage group VI. (4) The *dif-1* and *dif-2* markers are not expressed sex-limitedly. The *dif-1 mt*$^{+}$ and *dif-2 mt*$^{+}$ strains obtained by above crosses are also conditional class I mutants.

Crosses between *dif-2 mt*$^{+}$ and *dif-1 mt*$^{-}$ yielded three phenotypic classes of tetrads (Table 20, cross #1); class A which is composed of two DIF-1 and two DIF-2 phenotypes, class B which is composed of two DIF-1 and two wild-type phenotypes, and class C which is composed of two DIF-1, one DIF-2 and one wild-type phenotypes. Assuming that *dif-1* and *dif-2* are unlinked and that the *dif-1 dif-2* double mutant produces DIF-1 phenotype, class A can be recognized as parental ditype (PD), class B as non-parental ditype (NPD) and class C as tetratype (T). If the above assumption is correct, one of the two DIF-1 clones in class C tetrad should have a *dif-1 dif-2* genotype. To confirm this, the two DIF-1 clones were back-crossed with wild-type. The resulting tetrads indicate that the genotype of the first clone is *dif-1 dif-2*$^{+}$ (Table 20, cross #2), whereas that of the second clone is *dif-1 dif-2* (Table 20, cross #3).

Complementation test between *dif-1* and *dif-2* mutants

Diploid strains were constructed by crosses between the two dif mutants. The dif-1 mt^+/dif-1 mt^- and dif-2 mt^+/dif-2 mt^- diploid cells behaved like mt^- gametes at 25 ℃, but failed to differentiate into gametes at 35℃ (Table 21). In contrast, the dif-2 mt^+/dif-1 mt^- diploid strain was able to differentiate into mt^- gametes even at the restrictive temperature (Table 21). This indicates that the dif-1 and dif-2 alleles are recessive and complementing to each other.

Discussion

In this study, I succeeded the first isolation of two conditional class I mutants. The temperature-sensitive gametic differentiation mutants, dif-1 and dif-2, have some very interesting properties. These strains can differentiate from vegetative cells to gametes upon nitrogen starvation at the permissive temperature (25 ℃), accompanied with the synthesis of agglutinin and processing of lytic enzyme from V-form to G-form (Table 17). However, when the mutant cells are placed in -N medium at the restrictive temperature (35℃), they stay vegetative without activating agglutinin and lytic enzyme synthesis/processing (Table 17). In the case of dif-1 strain, the flagella are also resorbed at the restrictive temperature. Temperature shift experiments show that gametes, which differentiate from vegetative cells at 25 ℃, dedifferentiate into vegetative cells after transfer to 35 ℃ and again differentiate at 25℃ (Fig. 16). It should be noticed that these temperature-sensitive events occur thoroughly under a nitrogen-starved condition and that CHI block the events (Table 18). A possible explanation for these results is that the dif-1 and dif-2 are temperature-sensitive regulatory genes which control the gamete differentiation and dedifferentiation. At the permissive temperature nitrogen starvation may trigger the synthesis of regulatory proteins from dif genes which turn "on" the differentiation program and turn "off" the vegetative or dedifferentiation program. The differentiation program, which contains the synthesis of agglutinin, construction of mating structure and shift in the storage form of lytic enzyme (Chapter 4), might be turned "off" if the regulatory proteins are not produced at the restrictive temperature.

Both dif-1 and dif-2 genes are unlinked to arg-2/7, actr and mt loci, and expressed in both mating-types (Table 19). Moreover, they themselves are unlinked to each other and recessive genes; the dif-1 dif-2 double mutant has DIF-1 phenotype (Table 20) and the dif-2 mt^+/dif-1 mt^- diploid cells behave like wild-type mt^- gametes at 35℃ (Table 21).

The dif-1 mutation affects not only the gametic differentiation but also flagellation at the restrictive temperature. These two characters are strictly cotransferred to progenies through sexual reproduction, therefore, if they are controlled by two regulatory genes, the two may be closely linked in dif-1 locus. Although dif-1 cells lose flagella at 35 ℃, this event may not be directly related to or required for the sexual dedifferentiation. The reason for this is: (1) Several other temperature-sensitive isolates, although they are not of further interest in this study, become flagella-less gametes at 35 ℃. (2) Flagellar resorption of dif-1 occurs also in +N medium at 35℃. (3) CHI blocks the dedifferentiation of gametes at 35 ℃, but does not inhibit their flagellar resorption (Table 18).

Further studies on the molecular and genetical basis of sexual differentiation and dedifferentiation in C. reinhardtii are necessary by using the temperature-sensitive class I mutants which are now available.

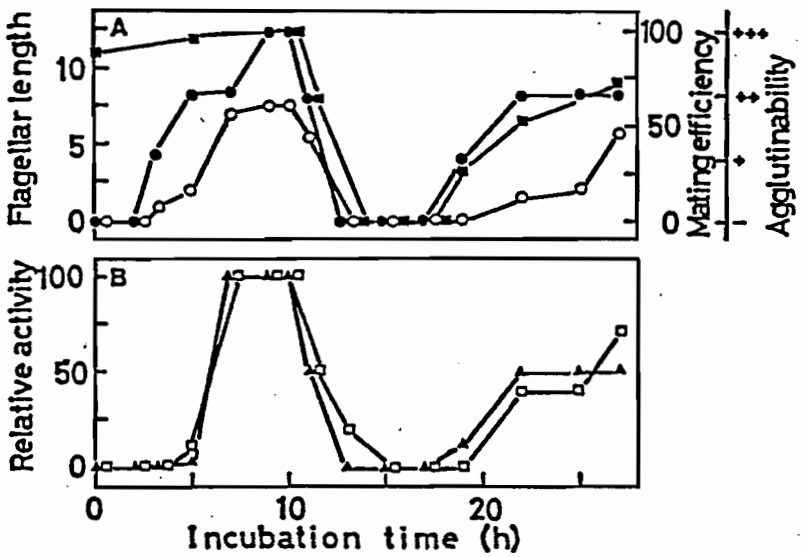

Fig. 16
Kinetics of gametic differentiation and dedifferentiation in dif-1 mutant cells

Vegetative cells of dif-1 (mt^-) grown in TAPA medium were transferred to nitrogen-free medium at 5 x 10^6 cells/ml. After 10 h incubation at 25 ℃, the temperature was shifted up to 35℃. After 5 h incubation, the temperature was again shifted down to 25℃, and incubation continued for 12 h. At the times indicated, flagellar agglutinability (● in A), mating efficiency (○ in A), flagellar length (■ in A), cell body-agglutinin activity (▲ in B), and lytic enzyme activity (□ in B) were determined.

Table 17

Properties of the conditional non-mating mutants, dif-1 and dif-2 at 25 ℃ and 35 ℃

Strain	Condition	Flagellar length (μm)	Agglutinability	Fusion (%)	Activity (/10^8cells)		
					Agglutinin	Lytic enzyme	
						unfrozen	frozen
137c mt⁻	25℃	12.1	##	84	2^8	37	43
	35℃	10.1	##	60	2^8	37	50
dif-1 mt⁻	25℃	12.0	##	74	2^8	50	50
	35℃	0	—	0	<2^0	0	37
dif-2 mt⁻	25℃	11.9	##	64	2^9	50	50
	35℃	10.0	—	0	<2^0	0	50

Cells (5 x 10^6 cells/ml) were cultured in -N medium for 14 h at either 25 ℃ or 35 ℃. Half portions of cells after harvest were freeze-thawed before the determination of lytic enzyme activity.

Table 18

The effect of cycloheximide on gametic differentiation and dedifferentiation in dif-1 mutant

Condition		Incubation time (h)	Flagellar length (μm)	Agglutinability	Activity (/10^8 cells)	
					Agglutinin	Lytic enzyme
25℃→35℃	-CHI	0	12.3	##	2^8	50
	-CHI	7	0	—	$<2^0$	0
	+CHI	7	0	—	2^6	25
35℃→25℃	-CHI	0	0	—	$<2^0$	0
	-CHI	7	10.6	#	2^6	20
	+CHI	7	0	—	$<2^0$	0

Cycloheximide (CHI, 4μg/ml) was added at the time of temperature shift-up (25 ℃→35℃) or shift-down (35 ℃→25℃).

Table 19
Linkage analysis

Combination	Number of tetrad			Linkage* (%)
	PD	NPD	T	
+ mt^+ x dif-1 mt^-	6	9	23	53.9
+ + x dif-1 arg-2	8	16	14	60.5
+ actr x dif-1 +	5	3	13	45.2
+ mt^+ x dif-2 mt^-	3	6	23	54.7
+ + x dif-2 arg-2	4	10	18	59.3
+ actr x dif-2 +	3	3	13	50.0

Crosses: 137c (mt^+) x dif-1 arg-2 msr (mt^-)
7430A (arg-7 actr mt^+) x dif-1 (mt^-)
137c (mt^+) x dif-2 arg-2 msr (mt^-)
7430A (arg-7 actr mt^+) x dif-2 (mt^-).

Abbreviations: PD, parental ditype; NPD, non-parental ditype; T, tetratype; $mt^{+/-}$, mating-type plus or minus; arg-2, arginine requirement actr, actidione resistance; dif-1/2, conditional differentiation mutation.

*Linkage was calculated by the equation; (NPD + ½ T) x 100/(PD + NPD + T) (Levine and Ebersold 1960).

Table 20

Tetrad analysis

Cross	Phenotypic tetrad class		
	A	B	C
1. *dif-2* mt^+ x *dif-1 arg-7 actr* mt^-	3	3	2
2. wild-type mt^+ x DIF-1 #1 from class C mt^-	0	8	0
3. wild-type mt^+ x DIF-1 #2 from class C mt^-	1	2	6

Phenotypic class: A, two DIF-1 and two DIF-2
B, two DIF-1 and two wild-type
C, two DIF-1, one DIF-2 and one wild-type.

Table 21

Agglutinin and lytic enzyme activity in diploids

Diploid	Condition	Flagellar length (μm)	Agglutinability	Activity (/10^8cells)	
				Agglutinin	Lytic enzyme
dif-1 arg-7 mt$^+$/dif-1 arg-2 mt$^-$	25℃	10.4	++	2^7	25
	35℃	0	−	<2^0	0
dif-2 arg-7 mt$^+$/dif-1 arg-2 mt$^-$	25℃	11.1	+++	2^7	38
	35℃	10.4	+++	2^8	50
dif-2 arg-7 mt$^+$/dif-2 arg-2 mt$^-$	25℃	11.6	+++	2^7	50
	35℃	9.8	−	<2^0	0

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Summary

The unicellular biflagellated green alga, Chlamydomonas reinhardtii, occurs as two sexually compatible strains, mating-type plus (mt^+) and minus (mt^-). Vegetative cells of mt^+ and mt^- differentiate separately into gametes in nitrogen-free (-N) medium. When gametes are mixed, they instantly recognize and adhere to each other by mating-type specific agglutinins located on the flagellar surface. This agglutination event triggers release of a lytic enzyme for shedding of cell walls. The resulting naked gametes soon establish a thin protoplasmic connection at their anterior ends and quickly merged to form a quadriflagellated zygote.

The purposes of the present study are: First, to get more information on the molecular structure and location of agglutinin and lytic enzyme, which are key molecules for the gametogenesis and mating in C. reinhardtii. Second, to elucidate the temporal and spatial controls of the synthesis/processing of agglutinin and lytic enzyme during gametic differentiation and dedifferentiation. Third, to develop a simple assay system for classification of non-mating mutants and to isolate and characterize the first mutants affecting gametic differentiation.

In Chapter 1 gamete was found to contain large amount of agglutinin molecules in the cell body as a pool of flagellar surface-agglutinin. The cell body-agglutinin was quantified by a simple assay which used the soluble fraction of cell homogenate, and the amount was estimated to be about 100 times more than that of flagellar surface-agglutinin. In unmixed gametes the cell body-agglutinin molecules were turned over slowly with a half life of about 12 h. However, when the two mating-type gametes of wild-type were mixed, both plus and minus cell body-agglutinins were turned over quite rapidly during agglutination and fusion, and used up within 30 min. When fus mt^+ gametes

agglutinated with mt^- gametes without successive fusion, the amount of cell body-agglutinin was first reduced to about 10 % of the unmixed level, then increased and reached the unmixed level: the recovery was blocked by cycloheximide. The cell body-agglutinin as well as flagellar surface-agglutinin was also lost when gametes were treated with EDTA or proteases, such as trypsin. The medium after EDTA treatment contained the same amount of agglutinin as observed in the cell body before the treatment. The mt^+ gametes released active cell body-agglutinin molecules into the medium by chymotrypsin and proteinase K treatments. Gametes, which had lost their cell body-agglutinins by trypsin treatment, reaccumulated the molecules after addition of trypsin inhibitor: the recovery was inhibited by cycloheximide for both mating-types and by tunicamycin for only mt^+.

In Chapter 2 the plus and minus agglutinins were found to be different with respect to the sensitivity to a disulfide-reducing agent, dithiothreitol (DTT). The mt^+ gametes lost completely their flagellar surface- and cell body-agglutinins within 30 min after addition of DTT at 2 mM : the medium contained agglutinin molecules in an inactive form. Isolated plus agglutinin was also inactivated with DTT, and reactivated rapidly after addition of a sulfhydryl-oxidizing agent, diamide. When isolated flagella from mt^+ gametes were treated with DTT, agglutinin molecules were inactivated but retained on the flagella. In contrast, the minus agglutinin was totally insensitive both *in vivo* and *in vitro* to DTT even at higher concentrations. It was suggested, therefore, that disulfide bonds are important for the active site of plus agglutinin.

In Chapter 3 it was found that vegetative cells as well as gametes contain cell wall lytic enzyme, however, the former enzyme is inactive (V-form) while the latter is active (G-form). V-form enzyme was activated when vegetative cells were freeze-thawed beforehand or the cell homogenates were subjected to sonication. The purified enzyme after activation was a glycoprotein with an

apparent molecular mass of 67 kDa on gel filtration and 62 kDa on SDS-PAGE, and sensitive to metal ion chelators and SH-blocking agents. These properties were the same as those of G-form enzyme purified from gametic cell homogenates and gamete wall-autolysin purified from mating medium. The V-form enzyme before activation appeared to be larger than the activated enzyme: the inactive enzyme was precipitated by centrifugation at 15,000-30,000 xg, while the activated enzyme was found in the soluble fraction.

It was also suggested that both V-form and G-form enzymes are stored outside the plasmalemma, i.e. in the periplasmic space or attached to the cell wall. When vegetative cells and gametes were de-walled by exogenously added enzyme, the resulting protoplasts contained neither V-form enzyme nor G-form enzyme. Many cell wall-deficient mutants yielded very little activity of lytic enzyme in the cell homogenates. When the protoplasts regenerated their walls, V-form enzyme was reaccumulated after the wall reformation was almost complete. When the de-walled or wall-deficient mutant gametes of opposite mating-types were mixed together, they normally agglutinated and fused, but the release of lytic enzyme into the medium was practically negligible.

In Chapter 4 the temporal and spatial changes of agglutinin and cell wall lytic enzyme during gametic differentiation and dedifferentiation were examined. When vegetative cells of liquid culture were transferred to -N medium, flagellar agglutinability and fusing ability were developed between 4 and 7 h. Cell body-agglutinin was accumulated about 1 h later than the acquisition of flagellar agglutinability. Cell wall lytic enzyme stayed in V-form during the initial 4 h, and then a rapid processing to G-form occurred in parallel with the development of mating ability. When gametes were transferred to nitrogen-containing (+N) medium, the mating ability disappeared in parallel with the loss of cell body-agglutinin and the processing of lytic enzyme from G-form to V-form. Aged cells grown on +N agar plates for 6 days differentiated into gametes within 2 h after suspension in -N or even +N medium. The flagella-less

cells on the plates were classified as vegetative since the soluble fraction of cell homogenates yielded no activities of agglutinin and lytic enzyme. It was also found that gametes dedifferentiate into vegetative cells after plating on -N agar medium. It was therefore concluded that the Chlamydomonas cells stay vegetative under a "solid" environment, and gametic differentiation occurs only in "liquid".

In Chapter 5 a new assay system was proposed to classify non-mating mutants of C. reinhardtii into four main classes (I, non-differentiation mutation; II, agglutinin-deficient mutation; III, lytic enzyme-deficient mutation; IV, fusion-defective mutation). This system was the combination of simple bioassays for cell body-agglutinin and lytic enzyme by use of the soluble fraction of homogenates from nitrogen-starved cells. The assay system is especially effective to classify phenotypically the same non-agglutinable mutants into class I and class II, and all of the non-agglutinable mutants isolated previously (imp-2, imp-8, imp-12) and newly (agl-1, agl-2, agl-3) were found to be class II mutants, but not class I mutants. Phenotypically non-fusable mutants (fus, imp-1, imp-11) belonged to class IV. Genetic analyses which used polyethylene glycol-induced artificial fusions indicated that agl-1 is a recessive gene and closely linked to the mt^- allele.

In Chapter 6 the first isolation of two conditional mutants (dif-1, dif-2) affecting gametic differentiation (i.e. class I) was successful, and their physiological, biochemical and genetic characters were examined. The two mutant cells were found to differentiate normally into mt^- gametes at 25℃, but stay vegetative, as defined by the new assay system, at 35℃ in -N medium. The dif-1 cells also lost their flagella at 35℃. Temperature shift experiments showed that when gametes at 25℃ were transferred to 35 ℃, they dedifferentiated into vegetative cells within 3-5 h, and again differentiated into gametes at 25℃. The differentiation and dedifferentiation processes were

blocked by cycloheximide. Tetrad analyses and complementation tests indicated that both dif-1 and dif-2 were recessive genes, and unlinked to each other and to the mt allele.

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