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CHANGES IN ENZYME ACTIVITY DURING DIFFERENTIATION IN *CHLAMYDOMONAS REINHARDTII*

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Summary

The patterns of alanine dehydrogenase, glutamate dehydrogenase and malate dehydrogenase activity were studied during the normal vegetative cell cycle and during the processes of gametic differentiation and dedifferentiation in synchronized cultures of *Chlamydomonas reinhardtii*. During all three phases of growth and differentiation the synthesis of DNA was also measured. During gametic differentiation all three enzyme levels were suppressed compared to vegetative cells although DNA and cell number were comparable. During gametic dedifferentiation no DNA synthesis occurred during the first 24 h cycle and only a doubling during the second. It was not until the third cycle that a normal 4-fold increase in DNA was observed. Cell number followed a similar pattern. Although the levels of alanine dehydrogenase and malate dehydrogenase were uniformly low during the first cycle when glutamate dehydrogenase increased 4-fold, during the second cycle the patterns of these enzymes changed markedly. The enzymes did not attain levels characteristic of vegetative cells until the third cycle.

Introduction

Previous studies have indicated that gametic differentiation in synchronous cultures of *Chlamydomonas reinhardtii* is induced by the removal of ammonium (the nitrogen source) from the culture medium, and that changes in the levels of activity of alanine dehydrogenase and glutamate dehydrogenase occur

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at different times [1–3]. There was, however, no marked difference in the timing of DNA replication in cells undergoing synchronous vegetative growth or gametic differentiation [4]. The source of nitrogen for enzyme and DNA synthesis during gametic differentiation is no doubt supplied from protein turnover [5] and available nitrogenous compounds in the soluble pool of the cells [6]. Although it has been determined that gametes, upon the addition of exogenous nitrogen, lose their ability to mate, and dedifferentiate into vegetative cells after a 24 h period [7], no study was conducted concerning the induction of those enzymes associated with ammonium incorporation.

In the present study, temporal patterns of alanine dehydrogenase (L-alanine : NAD^+ oxidoreductase (deaminating), EC 1.4.1.1), glutamate dehydrogenase (L-glutamate : $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) and malate dehydrogenase (L-malate : NAD^+ oxidoreductase, EC 1.1.1.37) were determined during normal vegetative growth, gametic differentiation and the dedifferentiation of gametes back to vegetative cells. In all cases, bursts of enzyme activity were compared to the timing of DNA synthesis as an index of cell cycle processes.

Materials and Methods

Cell culture

Synchronized vegetative cultures of *C. reinhardtii*, strain 137C-, were grown in 14 l quantities in liquid cultures of high salt minimal media previously described [7], using a New Brunswick Microferm fermentor, aerated with 2.5% CO_2 in air and maintained at a temperature of 25 ± 2° C. Cultures were maintained on a cycle of 12 h light (fluorescent intensity 800 ft-candle) and 12 h dark unless otherwise stated.

Induction of gametic differentiation and dedifferentiation

Gametes were obtained by the following method: At 6 h into the light period, synchronous vegetative cultures ($1 \cdot 10^6$ cells/ml) were harvested under sterile conditions by continuous flow centrifugation at room temperature. The cells were resuspended into an equivalent volume of fresh medium lacking nitrogen; thereafter they were maintained in continuous light (intensity 800 ft-candle). In this strain of *C. reinhardtii*, cells hatched into gametes 18 h from the time of resuspension.

To induce dedifferentiation, gametes, obtained by the above method, were harvested by centrifugation at $5000 \times g$ by continuous flow at room temperature. The cells were resuspended at a concentration of approx. $1 \cdot 10^6$ cells/ml into fresh medium containing 9.3 mM NH_4Cl. The same environmental conditions were employed as in the vegetative cycle.

Cell number and DNA determination

Cell number was estimated with a Levy hemocytometer. For DNA determinations, 1 ml of cells ($1 \cdot 10^6$ ml) was harvested in quadruplicate every 2 h across the life cycle by centrifugation in 6 × 50 mm test tubes in an Hb4 swinging bucket rotor at $3000 \times g$ for 10 min. All but 50 µl of the supernatant was decanted from each tube using a Pasteur pipet and the cells were resus-

pended using a Vortex mixer and 0.5 ml of 80% acetone (v/v) added. The samples were then centrifuged at 6000 × *g* for 10 min and washed in 80% acetone four times, or until colorless, after which they were stored at 5°C in 80% acetone until assayed. The amounts of DNA were estimated using a rapid filter microfluorometric assay shown to be reliable with *Chlamydomonas* [8]. Metricel Alpha-6 cellulose filters, 0.45 μm pore size, were purchased from Gelman Instrument, Ann Arbor, MI. Polyethylene BEEM capsules, size 00, were obtained from Ted Pella, Tustin, CA. Calf thymus DNA (used as a standard) and 3,5-diamino benzoic acid were purchased from Sigma Chemical Co., St. Louis, MO.

Preparation of cell-free extracts

Frozen samples were thawed at room temperature and the pellets were resuspended in a volume of 50 mM potassium phosphate buffer (pH 7.5) equivalent to 2 vols. of packed cells. The homogeneous, green suspension was centrifuged for 30 min at 27 000 × *g* at 4°C and the light yellow supernatant was decanted and immediately placed on ice. Enzymes in the crude extract were found to be stable for at least 5 days at 4°C.

When harvested cells were sonicated using an M.S.E. Ultrasonic Disintegrator at approx. 12–20 kcycles for 1 min on ice cell rupture was found to be complete by light microscopy. This method was carried out across an entire vegetative cell cycle and the activity patterns of the enzymes were found to be identical to those for the freeze-thaw method described above. Because the freezing technique was more convenient for both harvesting and extract preparation it was routinely used for all experiments.

Enzyme assays

All enzymatic reactions were measured spectrophotometrically by following the oxidation of NADH at 340 nm with a Gilford 2400-2 spectrophotometer. A unit of activity was defined as the amount of enzyme required to produce a change of 0.001 *A* unit/min under the conditions employed. All reactions were measured during the first 5 min where the rate remained linear. Enzymes were assayed at their pH optima. The following reaction mixtures were employed:

(a) alanine dehydrogenase: 160 mM NH_4Cl, 15 mM sodium pyruvate, 0.28 mM NADH, 30 mM Tris-HCl buffer (pH 8.0), 0.05 ml crude extract.

(b) glutamate dehydrogenase: 160 mM NH_4Cl, 15 mM alpha-keto glutarate (NaOH neutralized), 0.28 mM NADH, 40 mM Tris-HCl buffer (pH 9.0), 0.1 ml crude extract.

(c) malate dehydrogenase: 0.72 mM oxalacetate (NaOH neutralized), 0.28 mM NADH, 40 mM potassium phosphate buffer (pH 7.5), 0.025 ml crude extract (40 × dilution). Total volumes were 2.5 ml in each case. Reaction mixtures were zeroed before the addition of NADH directly into the cuvette. All assays were found to be linear with respect to the amount of extract added. The following were purchased from Sigma Chemical, St. Louis, MO: NADH, sodium pyruvate, α-keto glutarate and oxalacetate (*cis-enol* form).

Results

Fig. 1 illustrates the pattern of activity of the enzymes alanine dehydrogenase, glutamate dehydrogenase and malate dehydrogenase, and the timing of DNA

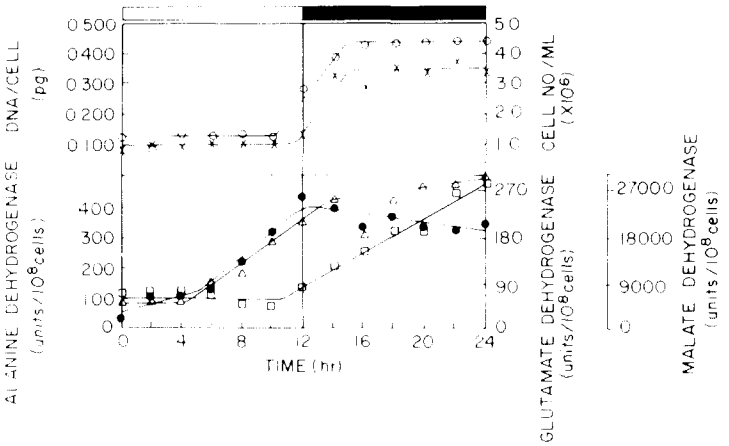

Fig. 1. Variation in cell number (×), DNA (○), alanine dehydrogenase activity (●), glutamate dehydrogenase activity (□) and malate dehydrogenase activity (○) during synchronous vegetative growth of *C. reinhardtii*. Open and closed bars represent light and dark periods, respectively.

synthesis during a typical 24 h vegetative cell cycle. Whereas malate dehydrogenase continues to increase in activity throughout the cycle, alanine dehydrogenase increases over the light period and prior to completion of DNA synthesis, while glutamate dehydrogenase remains relatively constant during the 12 h light period but increases during the time of DNA synthesis and continues throughout the dark period of the cycle. Each enzyme increases in activity approx. 4-fold, as does the DNA and cell number as a result of cell division. Hatching of the cells from the mother cell occurs at hour 24.

Following suspension of vegetative cells, from hour 6 of the light period, into nitrogen-free medium and exposing them to continuous light rather than a light/dark cycle, the cells undergo DNA synthesis and a 4-fold increase in cell number to produce gametes, which mate with gametes similarly induced but of the opposite mating strain (137C+). As can be seen from Fig. 2a, the timing of

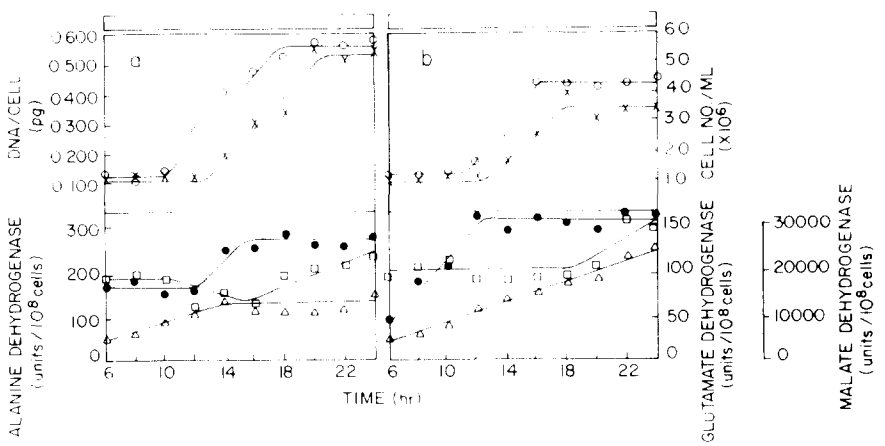

Fig. 2. Variation in cell number (×), DNA (○), alanine dehydrogenase activity (●), glutamate dehydrogenase activity (□) and malate dehydrogenase activity (○) during (a) gametic differentiation and (b) vegetative cells maintained in continuous light of *C. reinhardtii*. Open bars represent light periods.

DNA synthesis and increase in cell number follows a pattern similar to typical vegetative cells (Fig. 1). Even in the control situation (Fig. 2b) where cells were resuspended in full medium but maintained in continuous light, DNA synthesis and increase in cell number were similar to that found for a normal light/dark vegetative cycle.

During the process of gametic differentiation (Fig. 2a) alanine dehydrogenase does not show any increase in activity until hour 12, which is contrary to the normal vegetative cycle or for the control cells in continuous light (Figs. 1, 2b). Glutamate dehydrogenase increases only slightly during the latter 6—8 h of the light period in both differentiating gametes and control cells. Malate dehydrogenase, however, shows increase in activity for some 6—8 h and then remains somewhat constant but at a level lower than that attained in the control cells (Fig. 2b) or normal vegetative cells. Control cells in continuous light (Fig. 2b) exhibit a pattern of malate dehydrogenase activity similar to that of vegetative cells on a light/dark cycle (Fig. 1).

Fig. 3a illustrates the results obtained when gametes were resuspended in minimal medium plus NH_4^+ as the source of nitrogen and maintained in periods of 12 h light and 12 h dark for two complete cycles. Fig. 3b, on the other hand, shows the results obtained when gametes are maintained in medium lacking nitrogen for the two cycles. During the process of dedifferentiation of gametes into vegetative-like cells (Fig. 3a) only a 2-fold increase in DNA occurs, and not until hours 32—36, while number, likewise, only doubles. Alanine dehydrogenase remains relatively constant over the first 28 h but then increases until hour 40, after which a distinct decrease in activity is noted. During the period of increasing activity a 2-fold rise occurred. Glutamate dehydrogenase, however, increases immediately and continuously over the first 24 h period to a level approx. 4 times that of its initial value, but then decreases to a constant

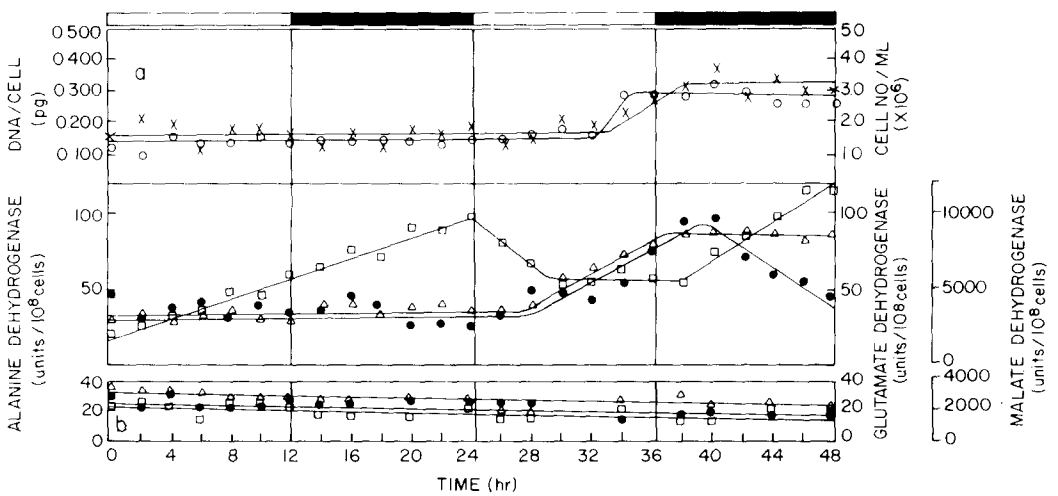

Fig. 3. Variation in cell number (X), DNA (○), alanine dehydrogenase activity (●), glutamate dehydrogenase activity (□) and malate dehydrogenase activity (△) during (a) gametic dedifferentiation and (b) gametes maintained in nitrogen-free medium of *C. reinhardtii*. Open and closed bars represent light and dark periods, respectively.

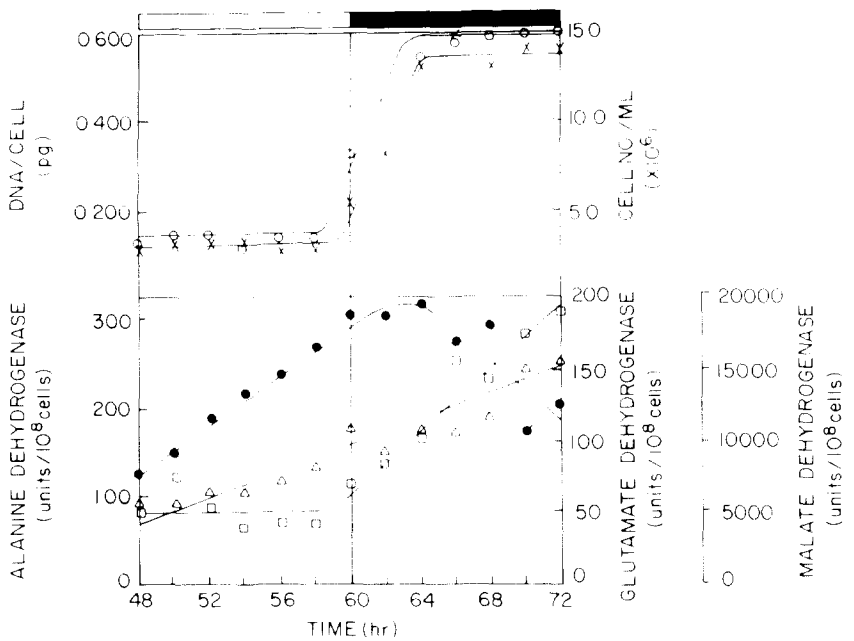

Fig. 4. Variation in cell number (△), DNA (□), alanine dehydrogenase activity (●), glutamate dehydrogenase activity (○) and malate dehydrogenase activity (▽) during the third cycle of gametic dedifferentiation of *C. reinhardtii*. Open and closed bars represent light and dark periods, respectively.

level until hour 38 when it doubles in activity. There appears to be little increase in malate dehydrogenase activity during the first 24 h cycle but thereafter the activity increases and follows a pattern similar to that found for vegetative cells (Fig. 1), however exhibiting only a 2-fold increase by the end of the second cycle. Gametes that were resuspended in nitrogen-free medium and maintained for two light/dark cycles (Fig. 3b) showed no changes in enzyme activities. During this period no DNA synthesis or cell division occurred either (data not shown).

When the gametes undergoing dedifferentiation in nitrogen-containing medium were allowed to continue for a third 12 h light/12 h dark cycle (Fig. 4), the enzymes exhibited patterns of activity that were typical of vegetative cells as were the 4-fold increases in DNA and cell number.

All results presented are averages of two sets of experiments which were in close agreement with each other.

Discussion

The present results indicate that both temporal sequences and levels of activity of alanine dehydrogenase, glutamate dehydrogenase and malate dehydrogenase vary with the particular state of cell differentiation in *C. reinhardtii*.

Gametic differentiation resulting from the removal of exogenous sources of nitrogen results in the suppression of increases in levels of enzyme activities characteristic of the normal vegetative cell cycle (compare Figs. 1 and 2). During this particular process, both protein turnover and RNA degradation

take place [5,9]. As shown here (Fig. 2), increases in the enzymes alanine dehydrogenase and glutamate dehydrogenase, normally active in amino acid metabolism because of their pivotal roles in ammonium incorporation, are suppressed even though the genome is replicated twice [4]. This suggests that regulation in the synthesis of these two enzymes occurs.

When ammonium is added back to the newly hatched gametes, dedifferentiation into vegetative cells is not immediate but requires a period of time equivalent to at least two normal light/dark cycles before the levels and sequence of these enzymes begin to return to normal (Fig. 3). It is interesting to note that during the first 24 h cycle, although DNA synthesis does not take place, the level of glutamate dehydrogenase does increase four times and this increase is initiated immediately upon ammonium readdition (Fig. 3). This rapid response is not characteristic of a normal vegetative cycle nor of cells undergoing gametic differentiation (Figs. 1 and 2). During the period when glutamate dehydrogenase increases, both alanine dehydrogenase and malate dehydrogenase are maintained at their low gametic levels. Only during the second cycle accompanying a 2-fold increase in DNA and cell number is there a concomitant increase in alanine dehydrogenase and malate dehydrogenase activities, with alanine dehydrogenase displaying a distinct decrease in activity near the end of the cycle, suggesting inactivation of the enzyme. On the other hand, after its initial increase, glutamate dehydrogenase exhibits a temporary decrease in activity during the light period and a proportional increase after the time of DNA synthesis (Fig. 3). It is not known to what extent enzyme turnover or regulation is responsible for the pattern of activity exhibited by glutamate dehydrogenase during the early phases of dedifferentiation. During the third cycle, all processes follow a program of synthesis and activity comparable to typical vegetative cells (compare Figs. 1 and 4).

Although the patterns of enzyme activity and timing of DNA synthesis for vegetative cells of this particular strain (137C—) are similar to those found for the Dangeard strain [2—4], during the dedifferentiation of gametes, the Dangeard strain underwent two rounds of DNA replication but only doubled in cell number at the end of the second cycle, while the current strain only replicated once during the second cycle. Other differences, both biochemical and physiological, between these strains have been noticed in the authors' laboratory.

The data reported here support the notion that *C. reinhardtii* lends itself to further studies of the regulation of metabolic and physiological processes during several states of growth and differentiation common to many eukaryotes.

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