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Light-mediated Activation of Nitrate Reductase in Synchronous *Chlorella*

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ABSTRACT

The mechanism underlying the sharp increase in activity of nitrate reductase (EC 1.6.6.1) in *Chlorella vulgaris forma tertia* (strain 211 8k) during the first hour of the 7 hours/5 hours light/dark cycle was investigated. Using the method of density labeling and isopycnic centrifugation, it could be demonstrated that this rapid increase in activity is based on light-mediated activation rather than *de novo* synthesis of the enzyme. The problematic nature of cycloheximide specificity and models of nitrate reductase activation are discussed.

The reduction of nitrate to ammonia, requiring a total of eight electrons, is catalyzed during the first two steps by the enzymes nitrate reductase (EC 1.6.6.1) and nitrite reductase (EC 1.6.6.4); both enzymes have been frequently investigated in a variety of organisms. During the cell cycle of *Chlorella vulgaris forma tertia* (20), it was found that the activities of both enzymes exhibited a 28-fold increase in specific activity during the 1st hr of illumination. The cells grew completely synchronously in a light/dark cycle of 7 hr/5 hr. Such an increase in enzymic activity can be due either to *de novo* synthesis of the enzyme protein or to activation by light of a protein already present during the dark. For a differentiation between the two possibilities, we used the method of density labeling of the enzyme with subsequent isopycnic sedimentation (8, *cf.* 10).

MATERIALS AND METHODS

Chlorella vulgaris forma tertia (strain 211 8k of the algal collection of the Institute of Plant Physiology, University of Göttingen) was cultivated as described by Pirson *et al.* (16) and Lorenzen (14). The alga grows completely synchronously in a LD[1] 7/5 and produces eight autospores/cell each cycle. Measurement of enzyme activities and preparation of cell-free extract were previously described (20). Protein was estimated by the Lowry method (15). Density labeling was achieved by culturing the algae in the presence of deuterated amino acids (4 mM, 98% deuterated, obtained from Merck, Sharp & Dohme of Canada). Separation was done in both metrizamide (for nitrate reductase) and rubidium chloride (for glutamate dehydrogenase, acid phosphatase) according to Hüttermann and Wendlberger (13). Density calculation followed the measurement of refractive indices using the formula:

$$\rho^{25} = 3.0534\ \eta_D^{25} - 2.9541$$

for metrizamide-^2H_2O solutions.

[1] Abbreviation: LD: light/dark changes.

RESULTS

Conditions of Density Labeling. The usual way of performing density labeling studies is to apply the label of heavy amino acids together with the change in physiological conditions which are to be studied (12). This strategy could not be used during the course of this study because introductory experiments showed that even a small amount of external amino acids completely suppressed the increase in nitrate reductase activity after onset of the light. In addition, it was found that supplementing the growth medium with amino acids (4 mM) did not result in an increase in the amino acid pool of the organism (data not shown). Therefore the labeling scheme had to be reversed. The cells used in our experiments had to be pregrown in the presence of deuterated amino acids, then transferred to the standard medium and illuminated without heavy label. Inasmuch as the activity increase of nitrate reductase was repressed by any external amino acids, the heavy amino acids should be depleted from the pool before the expected synthesis of nitrate reductase. Any protein being synthesized before illumination thus should contain deuterated amino acids, later produced proteins should be composed mainly by light amino acids.

Internal Markers. As internal density markers and indicators for over-all protein synthesis, NAD-glutamate dehydrogenase (EC 1.4.1.2) and acid phosphatase (EC 3.1.3.2) were chosen which both could be found as single bands in the density gradients. Furthermore, these specific activities behave in a way considerably different from those of nitrate reductase (Table I). Assuming the 1st hr of the light cycle, where nitrate reductase exhibits an increase in activity of 2,850%, both glutamate dehydrogenase and acid phosphatase decrease slightly in their specific activities. The same behavior was observed in the presence of actidion, whereas the nitrate reductase was inhibited in its activity of 98%. The residual enzyme activity was much higher in the case of the two other enzymes. These differences in the behavior of the specific activities, 28.5-fold increase in the case of nitrate reductase, and a slight decrease in the two other enzymes, should be measurable by density labeling experiments, if actual *de novo* synthesis of the respective enzymes is involved.

Density Labeling Experiments. In all density labeling experiments the cells were grown in the presence of deuterated amino acids (4 mM) for three generations in order to reach equilibrium labeling. For the induction experiments the cells were harvested at the end of the last dark period, washed twice with the standard nutrient solution, resuspended in the same solution, and illuminated for 1 hr. A parallel culture grown in the absence of deuterated amino acids and handled in the same way was used as reference. Aliquots of the extracts were centrifuged both in metrizamide-^2H_2O (for nitrate reductase) and RbCl-H_2O (for glutamate dehydrogenase and acid phosphatase) density gradients. The fractions were then analyzed for appropriate enzyme activity. Initial

studies had shown that nitrate reductase is unstable in the presence of CsCl or RbCl. Therefore, metrizamide-^2H_2O gradients had to be used for its isopycnic banding. Unfortunately, the capacity of that gradient was too small to permit assay of the activities of all three enzymes. So parallel samples of the same extract were centrifuged in RbCl gradients.

The result of such an experiment is given in Figure 1. Nitrate reductase of cells pregrown with heavy amino acids bands at a density of ρ^{25} equals 1.2422, whereas the enzyme from algae grown in the absence of amino acids is significantly lighter (ρ^{25} = 1.2300). Mixed gradients made with both extracts show a double peak.

Identical results were found for the two internal markers. Figure 2 shows the results only for the mixed gradients. The data of this experiment show that in general a very low degree of labeling with heavy amino acids can be achieved with this organism. This is probably due to a rather high rate of basal amino acid synthesis during the labeling time. A similar situation has been reported for the slime mold *Physarum polycephalum* (7, 21), for the filamentous *Fomes annosus* (11), and for cultured chick myotubes (4). In all of these cases, labeling with ^2H- or ^{14}C-amino-acids shows, even after very prolonged labeling times, no equilibration between the external supplied amino acids with the pool of it.

Nevertheless, the degree of labeling reached in our experiments was enough to produce a significant shift in density being detectable not only by a band widening but even by double peaks in mixed gradients.

Although the specific activities of nitrate reductase and the other enzymes are very different (Table I), their labeling as shown in Figures 1 and 2 was identical. From this it is obvious that the 28.5-fold increase in activity of nitrate reductase was not due to synthesis but rather to light-mediated activation of an inactive protein already present in the dark.

A longer illumination up to 4 hr of the algae pregrown with deuterated amino acids results into a double peak of nitrate reductase in the gradient (Fig. 3), with one band in the position of the labeled and the other in that of the light enzyme. The same result was obtained for the other two enzymes (data not shown). This also points to the interpretation that light-mediated activation rather than *de novo* synthesis is due to the observed rapid increase of enzyme activity. The doubling time of nitrate reductase can be estimated from the data to be about 4 hr, which is in good agreement with the cell cycle of the organism under the experimental conditions.

Table I. Changes of the specific activity of three enzymes in Chlorella strain 211 8k after different growth condition

	dark	1 hr light	increase %	1 hr light+ actidion	inhibition %
Nitrate reductase	0.9	26.8	+ 2850	0.54	98
NAD-glutamate-dehydrogenase	0.3	0.32	- 16	0.24	30
Acid phosphatase	0.5	0.40	- 2	0.40	18

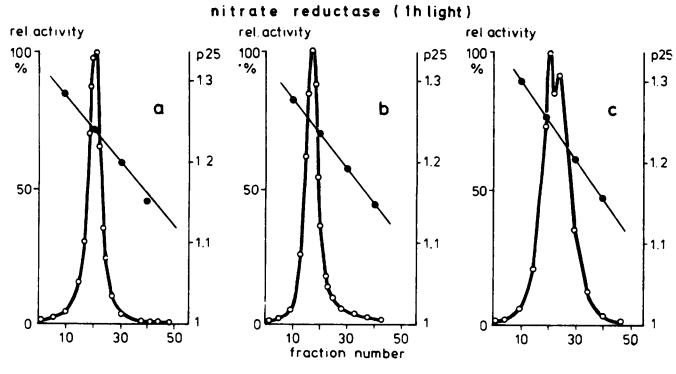

FIG. 1. Equilibrium density gradient sedimentation of nitrate reductase in metrizamide-D_2O density gradients. Line indicates slope of gradients. Conditions: a, normal cultivation; b, precultivation in presence of heavy amino acids; c, hybrid band a + b. Induction phase: 1 hr of light.

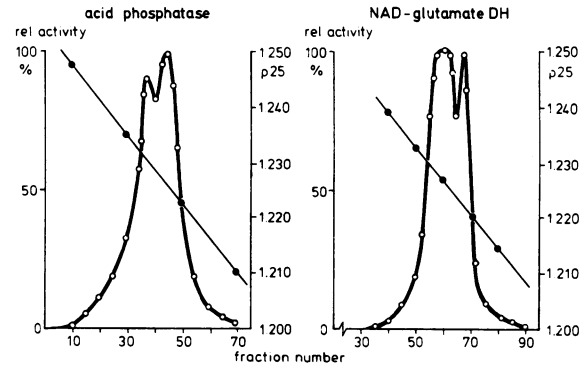

FIG. 2. Equilibrium density gradient sedimentation of a: acid phosphatase, b: NAD-glutamate dehydrogenase, both in RbCl. Figure shows hybrid bands taken mixing extracts of algae pregrown ± deuterated amino acids. Cells were harvested after 1 hr of light.

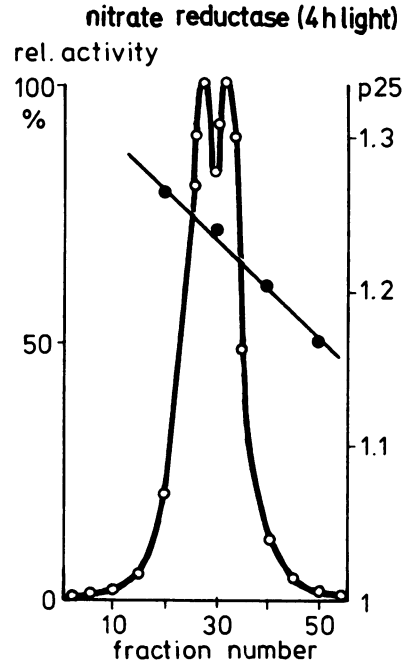

FIG. 3. Equilibrium density gradient sedimentation of nitrate reductase from algae after 4 hr of light, precultivated with deuterated amino acids.

Oscillation of Nitrate Reductase Activity during Short Periods. The interpretation of the density labeling studies is supported by the following experiment. As shown previously (20) a quick decrease of nitrate reductase activity occurred after darkening the cultures. The "half-life time" was about 1.5 hr. Cultivating the algae in a light-dark program, in which the duration of each phase was 1 hr (0.5 hr) we found (Fig. 4) that the increase and decrease of enzyme activity fit the onset and the offset of the light. This was reproducible several times. Naturally the amplitude of the oscillations is higher at 1-hr phases than at 0.5-hr phases, but even there the effect is clearly discernible.

DISCUSSION

The results of our density labeling studies clearly indicate that nitrate reductase in synchronous *C. vulgaris forma tertia* (strain 211 8k) has a doubling time of about 4 hr and is synthesized both during the light and dark phases of the light/dark cycle. The rapid

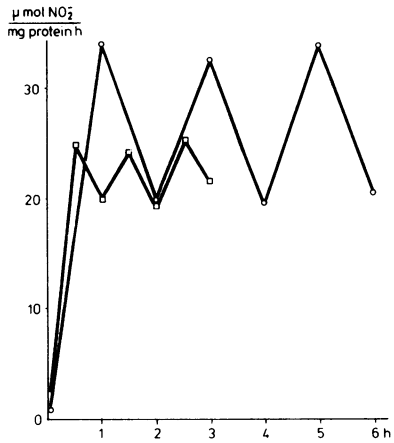

FIG. 4. Specific activity of nitrate reductase during a LD program of 1/1 or 0.5/0.5. Samples are taken at each change of conditions.

increase of activity after onset of illumination is due to light-mediated activation. The data on synthesis of this enzyme are identical to those of two other enzymes which were studied: glutamate dehydrogenase and acid phosphatase. These synthesis data are in rather conspicuous contrast to the 28-fold increase of specific activity of the enzyme due to illumination. Short light/dark changes result in a perfect oscillation of nitrate reductase activity (Fig. 4). It is now quite clear that these activity changes are the result of a light-mediated activation and are not caused by *de novo* synthesis of the enzyme protein in quest.

The mechanisms underlying this light-mediated activation are not understood at present. Several observations must be taken into account. One is the fact that the activity increase is inhibited by cycloheximide, which was used as a strong argument for a *de novo* synthesis under the conditions of illumination (20). The action of cycloheximide can be interpreted in several ways: it may inhibit the synthesis of a rapidly synthesized protein with negligible turnover during illumination, which in turn activates the enzyme. On the other hand the action of cycloheximide probably is not specific in this case, and it could employ sites different from the ribosomes. It may act directly on nitrate reductase as it was shown for glutamate dehydrogenase from *Physarum polycephalum* (21) and from beef liver (Hüttermann, unpublished data); in both cases the mol wt was decreased by the drug. On the other hand, cycloheximide may change the amino acid pool (6), which would, according to our observations, influence the nitrate reductase activity.

The action of light in this activation may be based on a promoted permeation of NO_3^- which enhanced enzyme activity. The model given by Butz and Jackson (2) points at the correlation between NO_3^- uptake and the activity of the nitrate-reducing system. Some results, gained with barley by Prasad and Rains (17), and by Huffaker *et al.* (9), show this interaction. The same group (1) demonstrated a promotion of transport of NO_3^- from an inactive pool to an active pool, which could induce nitrate reductase activity. Solomonson and Sephar (18) show some possibilities of light regulation of nitrate reductase. They summarized that activation/inactivation rather than *de novo* synthesis is the regulating mechanism. That agrees well with our results.

Our data indicating activation rather than *de novo* synthesis as the basic mechanism underlying the activity of nitrate reductase agree well with the findings of Funkhouser *et al.* (5). These authors reported that nitrate reductase is induced in *Chlorella* by replacing NH_4^+ by NO_3^- in the medium. The increase in nitrate reductase activity is sensitive to cycloheximide, and it was shown that [^{14}C]arginine, applied during the induction period, was incorporated in the enzyme. These two results led to the hypothesis that precursors of this enzyme are present in ammonia-grown cells.

Our results, obtained in continual presence of nitrate, show that darkening the cultures leads to an inactivation and illumination results in an activation of nitrate reductase. This process is sensitive to cycloheximide too (20) and is probably based on dissociation and association of precursors of the enzyme. With the data shown here it is quite sure that *de novo* synthesis is not involved in this process.

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