INACTIVATION AND REPRESSION BY AMMONIUM OF THE NITRATE REDUCING SYSTEM IN CHLORELLA

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Summary: Addition of ammonium to a suspension of Chlorella cells growing autotrophically in the light with nitrate causes a striking inactivation of nitrate reductase in less than 1 hour. Neither the $NADH_2$-specific diaphorase which catalyzes the first step of the reduction of nitrate to nitrite by $NADH_2$ nor nitrite reductase are affected by the ammonium treatment. However, all the enzymes of the nitrate reducing system, including nitrite reductase, are fully repressed by ammonium.

The *in vivo* and *in vitro* reactivation of nitrate reductase and the derepression of all the enzymes of the nitrate reducing system are also described.

Recently, it was shown (1) that the nitrate reducing system of the alga Chlorella is quite similar to that previously found in the photosynthetic tissues of higher plants (2-4). The reduction of nitrate to ammonium proceeds in two independent enzymatic steps: 1) reduction of nitrate to nitrite catalyzed by $NADH_2$-nitrate reductase, and 2) reduction of nitrite to ammonium catalyzed by ferredoxin-nitrite reductase. In the transfer of electrons from $NADH_2$ to nitrate, two enzymatic activities participate sequentially. Both can be easily and independently assayed: the first, a $NADH_2$-diaphorase which can use oxidized cytochrome c or benzyl viologen as the electron acceptor, and the second, a nitrate reductase proper which can use reduced flavin nucleotides or methyl viologen as the electron donor and which has, the-

refore, been named FNH_2-nitrate reductase.

Syrett and Morris (5) have shown that nitrate assimilation by Chlorella vulgaris is completely inhibited by the addition of a small quantity of ammonium, and that the inhibition is relieved as soon as the ammonium is assimilated. No specific conclusions could be drawn as to the mechanism of this process, but it was inferred that ammonium inhibits the first step of nitrate assimilation, namely the reduction of nitrate to nitrite. In a second paper, Morris and Syrett (6) established further that the formation of nitrate reductase in Chlorella vulgaris is repressed by ammonium. In higher plants, by contrast, ammonium ions were found by Schrader and Hageman (7) not to be effective as inhibitors of nitrate reductase activity or synthesis.

The present work shows that ammonium ions causes in Chlorella fusca a drastic blockade of the nitrate reducing system, by affecting exclusively the activity of nitrate reductase proper. After removal of ammonium ions, an equally striking reversal of this process, i.e. the reactivation of nitrate reductase, occurs in cell-free extracts as well as in intact cells. The regulation by ammonium of the synthesis of all the enzymes of the nitrate reducing system is also considered.

Materials and Methods: Chlorella fusca Shihira et Krauss 211-15 from Pringsheim's culture collection at Göttingen was grown as described previously (1) but either KNO_3 or equivalent amounts of nitrogen in the form of NH_4NO_3 or $(NH_4)_2SO_4$ were used. Preparation of cell-free extracts and measurement of enzyme activities were also carried out as in the previous paper.

Results and Discussion: Incidentally, we found that the addition

of ammonium sulfate to Chlorella fusca cells growing autotrophically in the light with potassium nitrate as the only nitrogen source caused a rapid and pronounced decrease of the level of nitrate reductase while the other two activities of the nitrate reducing system ($NADH_2$-diaphorase and ferredoxin-nitrite reductase) were not significantly affected. In these experiments, specific activity of each enzyme was determined inmediately after preparation of the cell-free extracts.

In order to define this striking effect of ammonium ions, Chlorella cells growing on nitrate in the light were harvested, washed and transferred to illuminated culture media containing either nitrate, ammonium, or nitrate plus ammonium (Table I). By preparing cell-free extracts at the times indicated, it was found that in less than one hour the level of nitrate reductase assayed with either $NADH_2$ or $FMNH_2$ as electron donor fell in the cultures where ammonium was present (either alone or with nitrate) but remained high in its absence. In all cases, however, the content of $NADH_2$-diaphorase and nitrite reductase remained high, essentially unaffected by the presence of ammonium.

The rapid decline in the level of nitrate reductase promoted by ammonium could not be caused by repression or degradation of the enzyme but rather by its inactivation, which was found to be reversible. Cell free extracts prepared from cells in which the nitrate reductase had been inactivated by 1-2 hours prior exposure to ammonium could be completely reactivated simply by keeping the extracts for some hours at about 0ºC. Reactivation did not occur, however, if the extracts were first pass over Sephadex G-25.

Table I. EFFECT OF AMMONIA ON THE ENZYMATIC LEVELS OF THE NITRATE REDUCING SYSTEM IN CHLORELLA

Nitrogen source	Time (min)	FNH_2-NO_3^- reductase	$NADH_2$-NO_3^- reductase	$NADH_2$-diaphorase	NO_2^- reductase
		Relative specific activity (%)			
NO_3^- (control)	zero	100	100	100	100
NO_3^-	45	225	190	113	115
	90	83	72	72	140
NH_4^+	45	18	14	100	75
	90	7	3	117	140
NO_3^-, NH_4^+	45	20	23	105	154
	90	6	8	130	135

Cells grown on 8 mM KNO_3 were harvested in the logarithmic phase, washed and transferred at zero time into media containing equivalent amounts of nitrogen in the form of KNO_3, $(NH_4)_2SO_4$ or NH_4NO_3. The specific activity of the enzymes was determined in the extracts of the corresponding cultures at the times indicated. Specific activities at 100% for $FMNH_2$-NO_3^- reductase, $NADH_2$-NO_3^- reductase, $NADH_2$-diaphorase (using cytochrome c) and nitrite reductase were in nanomoles of NO_2^- formed, cytochrome c reduced, or NO_2^- disappeared, per minute per mg protein, 24, 64, 539 and 114, respectively

Figure 1 shows the time course of the *in vivo* inactivation of nitrate reductase (assayed with both $NADH_2$ and $FMNH_2$) caused by the addition of ammonium to cells growing on nitrate, as well as the *in vivo* reactivation of the inactive enzyme, which follows removal of ammonium. The duration of the complete process is rather brief as compared with the generation time (about 8 hours) of Chlorella cells. It is worth emphasizing that only the second of the two activities which in series integrate $NADH_2$-nitrate reductase, *i.e.* nitrate reductase proper, is subjected to this singular metabolic control promoted by ammonium.

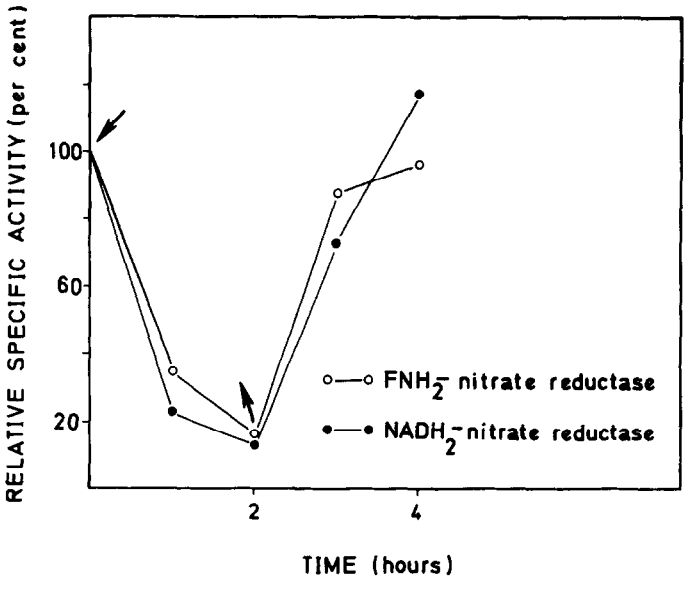

Figure 1. Time course of inactivation and reactivation of nitrate reductase in living Chlorella cells. Experimental conditions as described for the NH_4NO_3 system in Table I. The first arrow indicates the time of NH_4NO_3 addition. As indicated by the second arrow, at 2 hours the cells were harvested and washed, and KNO_3 was again added. Specific activities at 100% for $FMNH_2$-NO_3 reductase and $NADH_2$-NO_3 reductase, 12 and 35 nanomoles per minute per mg protein, respectively.

Besides the rapid inactivation of nitrate reductase by ammonium, an independent process, i.e. the repression by ammonium of all the enzymes of the nitrate reducing system, occurs (Figure 2).

A culture of cells was prepared by growing the alga in a medium containing nitrate as the only nitrogen source, thus maintaining high the level of all the enzymes involved in nitrate reduction. After harverting and washing, the cells were resuspended in an ammonium medium, and the pertinent enzyme activities were then periodically estimated in cell-free extracts. Nitrate reductase was rapidly inactivated and remained so. The level of $NADH_2$-diaphorase and nitrite reductase decreased more slowly reaching negligible values in about 12 hours. At this time the

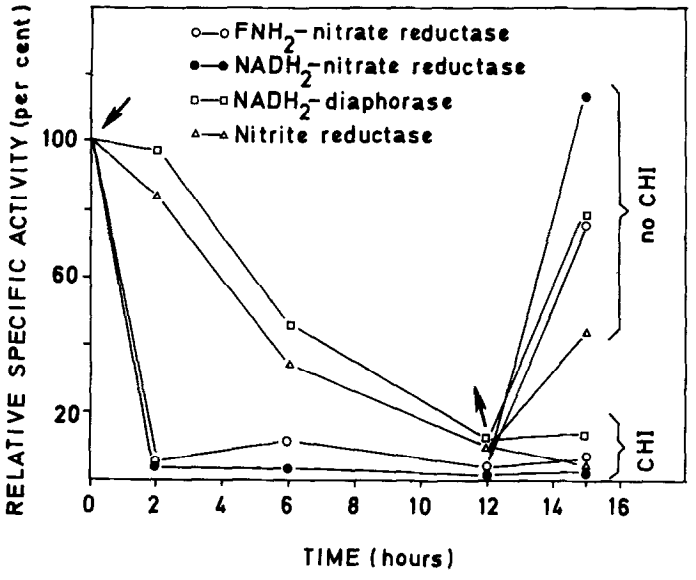

Figure 2. Time course of inactivation, repression and derepression of the enzymes of the nitrate reducing system in Chlorella. Experimental conditions as described for the $(NH_4)_2SO_4$ system in Table I. The first arrow indicates the time of $(NH_4)_2SO_4$ addition. As indicated by the second arrow, at 12 hours ammonium was removed by centrifuging and washing the cells, and nitrate was again added, with and without cycloheximide (10 micrograms/ml). Specific activities at 100% for $FMNH_2$-NO_3 reductase, $NADH_2$-NO_3 reductase, $NADH_2$-diaphorase and NO_2 reductase, 10, 28, 235 and 117 nanomoles per minute per mg protein, respectively.

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cells were again harvested, washed and transferred to a medium containing nitrate. The removal of ammonium clearly caused an increase in the content of all the enzymes of the nitrate system. This rise was inhibited by cycloheximide under the same experimental conditions, thus suggesting a possible involvement of 80 S cytoplasmic ribosomes in the de novo synthesis of these proteins (4,8,9).

Since similar results were obtained when the experiment was repeated with ammonium nitrate instead of ammonium, it may be concluded that, in Chlorella, ammonium is the nutritional repressor of the entire nitrate reducing system rather than nitrate being its inducer.

We are now studying if the inactivation of nitrate reductase by ammonium in Chlorella corresponds to a mechanism similar to that known for the regulation of glutamine synthetase in Escherichia coli (10).

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REFERENCES

1. Zumft, W.G., A. Paneque, P.J. Aparicio, and M. Losada, Biochem. Biophys. Res. Commun., 36, 980 (1969).
2. Paneque, A., P.J. Aparicio, J. Cárdenas, J.Mª Vega, and M. Losada, FEBS Letters, 3, 57 (1969).
3. Losada, M., P.J. Aparicio, and A. Paneque, in Progress in Photosynthesis Research vol. III, edited by H. Metzner, Springer Verlag, 1969, p. 1504.
4. Beevers, L., and R.H. Hageman, Ann. Rev. Plant Physiol., 20, 495 (1969).
5. Syrett, P.J. and I. Morris, Biochim. Biophys. Acta, 67, 566 (1963).
6. Morris, I., and P.J. Syrett, Arch. Mikrobiol., 47, 32 (1963).
7. Schrader, L.E., and R.H. Hageman, Plant Physiol., 42, 1750 (1967).
8. Smillie, R.M., and N.S. Scott, in Progress in Molecular and Subcellular Biology vol. 1, edited by F.E. Hahn, Springer Verlag, 1969, p. 136.
9. Vazquez, D., T. Staehelin, M.L. Celma, E. Battaner, R. Fernandez-Muñoz, and R.E. Monro, in Inhibitors Tools in Cell Research, edited by Th. Bücher and H. Sies, Springer Verlag, 1969, p. 100.
10. Holzer, H., Advances in Enzymology, 32, 297 (1969).