REGULATION BY AMMONIA OF NITRATE REDUCTASE SYNTHESIS AND ACTIVITY IN Chlamydomonas reinhardi

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Summary: In the green alga Chlamydomonas reinhardi, all the enzymes of the nitrate-reducing system are nutritionally repressed by ammonia. Besides, ammonia promotes in vivo the reversible inactivation of nitrate reductase in an indirect way by primarily raising the cellular level of reducing power, which in turn determines the reversible reduction of the enzyme. Subsequent removal of ammonia from the medium causes reactivation of the inactive enzyme. Interconversion of the active and inactive forms into one another can also be directly achieved in vitro by reducing and oxidizing nitrate reductase with its own physiological substrates.

The assimilatory nitrate-reducing system from algae and higher plants has been thoroughly characterized during the last years and shown to consist of two separate enzymes which catalyze in series the two reductive steps leading from nitrate through nitrite to ammonia (1,2). The reduction of nitrate to nitrite is catalyzed by the flavomolybdoprotein NADH-nitrate reductase, an enzyme complex of high molecular weight (3-5). The reduction of nitrite to ammonia is catalyzed by the ironprotein ferredoxin-nitrite reductase (6,7). In the transfer of electrons from NADH to nitrate, two different enzymatic activities participate sequentially: The first is a FAD-dependent NADH-diaphorase, and the second is the molybdoprotein nitrate reductase proper or terminal nitrate reductase, which has also been named FNH_2-nitrate reductase because it can use exogenous reduced flavin nucleotides as electron donors (3,4).

We have recently shown (8,9) that, when ammonia was added to a suspension of Chlorella cells growing on nitrate, the enzymes of the nitrate-reducing system were fully repressed, and that the second activity of the nitrate reductase complex, i.e. FNH_2-ni-

trate reductase, became rapidly inactivated. The inactive enzyme could be reactivated both *in vivo* (by removal of ammonia from the medium) and *in vitro* (by keeping the crude extract in the cold).

The present work shows that, in *Chlamydomonas reinhardi* cells repressed by ammonia, the enzymes of the nitrate-reducing system start being synthesized when ammonia is absent, provided an adequate source of nitrogen -which does not necessarily have to be nitrate- is added to the medium. It shows besides that ammonia promotes *in vivo* the reversible inactivation of nitrate reductase by indirectly causing the reduction of the enzyme. Evidence for the *in vitro* interconversion of the active and inactive forms of nitrate reductase by reversible reduction and oxidation of the enzyme is also presented.

Materials and Methods: *Ch. reinhardi* (from Laboratorio de Microorganismos Fotosintéticos, CSIC, Madrid) was grown in the light on 8 mM potassium nitrate as described for *Chlorella* (3), except that media were buffered with 20 mM sodium phosphate, pH 7,5, and 10 μM molybdate was used. Preparation of cell-free extracts and estimation of enzyme activities were as previously described (3,6).

Results and Discussion: Table I shows the levels of the enzymes of the nitrate-reducing system in *Chlamydomonas* cells growing on either nitrate, ammonia, or nitrate plus ammonia. It can be seen that all the enzymes of the system were repressed by ammonia even in the presence of nitrate. As shown in Figure 1, when ammonia was removed from the medium, the formation of the nitrate reductase complex did depend on the addition of an adequate source of nitrogen, either nitrate, or aminoacids at low concentration (0.5 mM); at higher concentration (5 mM), aminoacids behaved, however, as nutritional repressors. Ammonia itself acted as repressor even at low concentration (0.5 mM).
Figure 2 shows the *in vivo* inactivation of nitrate reductase (assayed as either FNH_2-NO_3Rase itself or as NADH-NO_3Rase) after addition of ammonia to *Chlamydomonas* cells growing on nitrate, as well as the reactivation of the inactive enzyme that followed ammonia removal from the medium. It is worth emphasizing that

Table I. REPRESSION BY AMMONIA OF THE NITRATE REDUCING SYSTEM IN Chlamydomonas

Nitrogen source	Growth	Specific activities			
		NADH - diaphorase	NADH - NO_3Rase	FNH_2-NO_3RASE	MVH-NO_2Rase
(16 mM)	(O.D. at 660 nm)	(mU per mg protein)			
NO_3^-	1.800	200	40	31	53
NH_4^+	1.850	10	1	1	10
NO_3^- + NH_4^+	1.750	20	2	1	8

The cells were grown for 24 hours in the different media at the concentration indicated. Growth was then measured and the cells were collected by slow speed centrifugation. The activity levels of the pertinent enzymes were determined in the corresponding cell-free extracts. MVH = methyl viologen reduced by dithionite.

only nitrate reductase proper -the second moiety of the two which in series integrate the NADH-NO_3Rase complex- is subjected to this singular control exerted in vivo by ammonia. As can also be seen in the figure, the activity level of the diaphorase moiety of the complex was not at all affected.

Vega et al. (10) had previously found that purified Chlorella nitrate reductase inactivated by preincubation with NADH in the presence of cyanide could be immediately reactivated by the addition of tiny amounts of ferricyanide, which apparently was able to reoxidize the reduced enzyme. It was therefore relevant to test if Chlamydomonas nitrate reductase inactivated in vivo by ammonia could also be reactivated in vitro by ferricyanide in the same way. As shown in Fig. 3, this was found to be the case, and interesting enough not only ferricyanide but also nitrate, the physiological oxidant, could convert the inactive enzyme in the crude extract into its active form. At 0º, reactivation by ferricyanide was an instantaneous process, whereas reactivation by nitrate was progressive with time.

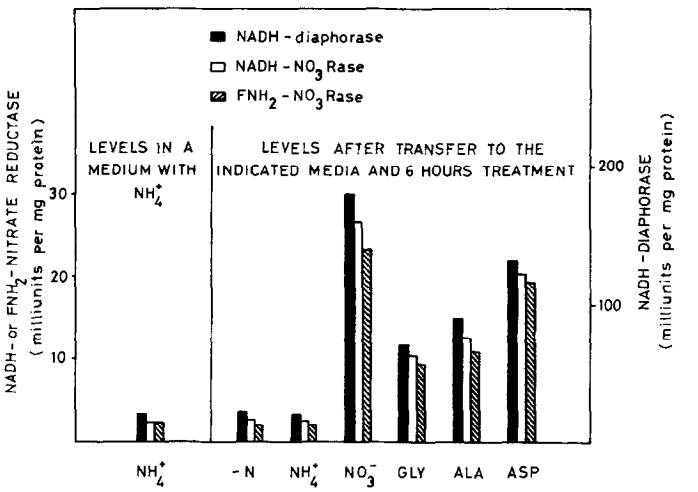

Fig. 1. Derepression of the nitrate reductase complex in Chlamydomonas. Left: Activity levels in an extract from cells grown on 16 mM ammonia for 18 hours. Right: Activity levels in extracts from the same cells after collection by centrifugation and transfer to media with either no nitrogen, ammonia (0.5 mM), nitrate (16 mM), or the indicated aminoacids (0.5 mM).

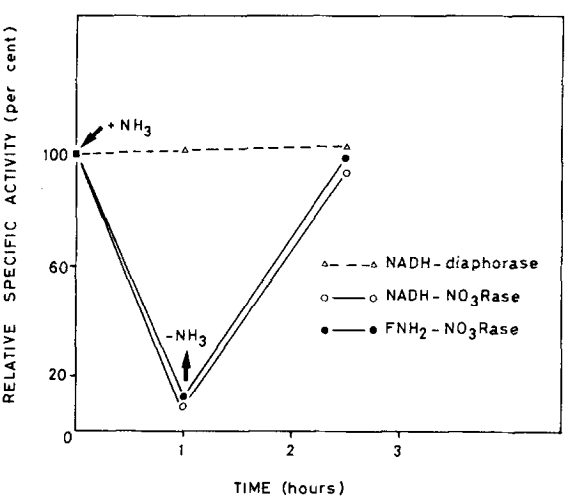

Fig. 2. In vivo inactivation and reactivation of nitrate reductase in Chlamydomonas. The first arrow indicates the time when ammonia (16 mM) was added to cells logarithmically growing on nitrate. The second arrow indicates the time when the cells were harvested and resuspended in a medium with nitrate (4 mM). Specific activities were estimated in the corresponding cell-free extracts at the times indicated.

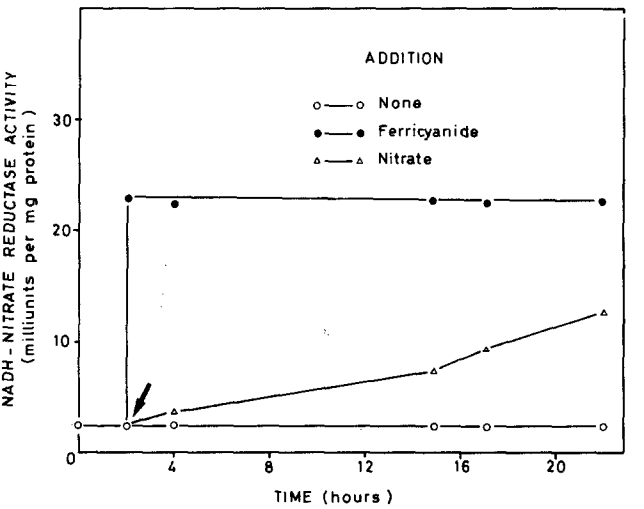

Fig. 3. In vitro reactivation of nitrate reductase. An inactive crude extract was prepared from cells treated with 16 mM ammonia for one hour. At the time indicated, ferricyanide (0.3 mM) or nitrate (10 mM) were added to the ice-cold extract. NADH-NO_3Rase activity was determined with time in aliquots of the corresponding incubation mixtures.

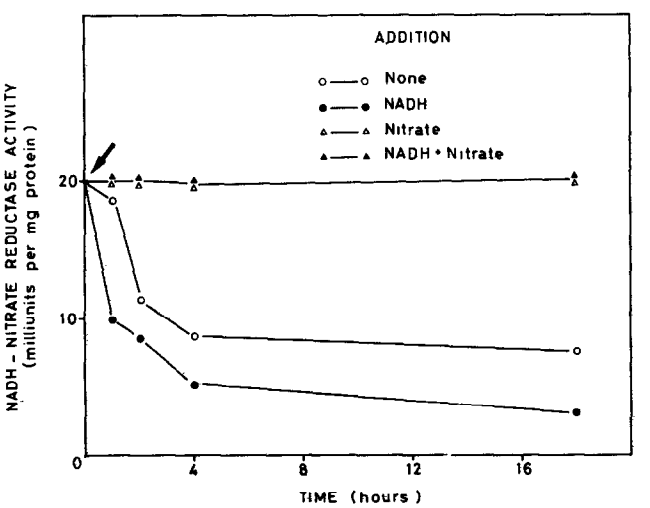

Fig. 4. In vitro inactivation and protection of nitrate reductase. An active fresh crude extract from cells logarithmically growing on nitrate was incubated at 0º with either 0.3 mM NADH, 10 mM nitrate, or 0.3 mM NADH plus 10 mM nitrate. NADH-NO_3Rase activity was determined with time in aliquots of the corresponding incubation mixtures.

Fig. 4 shows the time course of nitrate reductase spontaneous inactivation in Chlamydomonas crude extracts kept in the cold. Addition of NADH greatly stimulated the conversion of active nitrate reductase into its inactive form, but inactivation was prevented in the presence of nitrate. In all cases, the inactive enzyme could be reactivated by ferricyanide. These results are in agreement with the recent findings of Moreno et al. (11) using a purified preparation from Chlorella that NADH causes by itself a pH-dependent inactivation of nitrate reductase, which can be both protected and reversed by nitrate.

When Chlamydomonas cells at the stationary phase of growth, which exhibited negligible content of NADH-nitrate reductase, were transferred for 4 hours to media containing increasing amounts of nitrate, the activity level of the de novo synthesized nitrate reductase revealed a striking dependence on nitrate concentration, being maximal, under the selected conditions, at 4 mM. Upon addition of ferricyanide, nitrate reductase became fully active and followed the pattern of NADH-diaphorase (Fig.5). From these data, it can be concluded: a) the synthesis of NADH-nitrate reductase requires a certain minimal concentration of nitrate to attain its constant, high level; b) the terminal moiety of the NADH-nitrate reductase formed is initially in its inactive state (probably because lack of protection by insufficient nitrate), and becomes progressively active with nitrate concentration up to an optimum value at 4 mM; c) above this concentration, terminal nitrate reductase appears again gradually in its inactive form (probably because accumulation of increasing amounts of ammonia).

With respect to the mechanism of ammonia inactivation, our present evidence (12) indicates that ammonia exerts in vivo its effect by acting as an uncoupler of photosynthetic phosphorylation (13), increasing as a consequence the redox level of the cell. Reversible inactivation of nitrate reductase can thus be equally achieved, in the light, by arsenate, another chemically unrelated uncoupler (13), and, in the dark, by stopping aeration of the culture. On the other hand, in vivo inactivation by arsenate or ammonia does not occur when accumulation of reducing power is hindered, either by speeding up its reoxidation or by

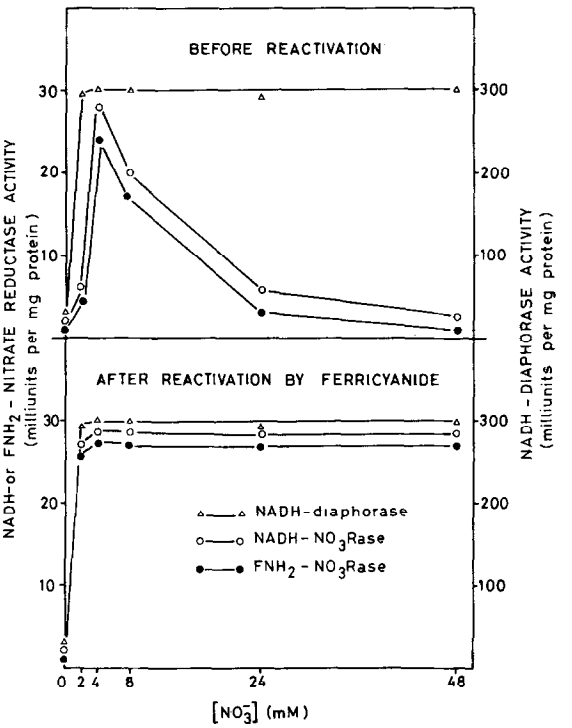

Fig. 5. Effect of nitrate concentration on the activity levels of the NADH-nitrate reductase complex in Chlamydomonas. Cells grown for 48 hours on nitrate were transferred for 4 hours to media with nitrate at the concentration indicated. Enzyme activities were determined in the corresponding cell-free extracts before and after reactivation for 2 min with 0.3 mM ferricyanide.

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blocking the non-cyclic electron flow of photosynthesis (13).

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