REVERSIBLE INACTIVATION BY NADH AND ADP OF *Chlorella fusca* NITRATE REDUCTASE

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Summary: The active form of *Chlorella fusca* nitrate reductase can be reversibly converted into its inactive form by reduction with NADH in the presence of ADP. Under the experimental conditions used, no inactivation occurs when nitrate is simultaneously present or when the nucleotides act isolately, the inactivating effect being maximal at a concentration of ADP (0.3 mM) equimolecular with that of NADH. The inactive enzyme thus attained can be completely reactivated by reoxidation with ferricyanide. The redox state of the pyridine nucleotide and the phosphorylation degree of the adenine nucleotide are critical for the inactivation process to ensue, since neither NAD^+ nor AMP or ATP do exert any effect. ADP is also a powerful, although rather unspecific, protector against thermal inactivation of the NADH-diaphorase moiety of the NADH-nitrate reductase complex.

Introduction: We have previously reported that the addition of ammonia to a suspension of *Chlorella fusca* (1,2) or *Chlamydomonas reinhardi* (3) cells growing in the light on nitrate determines the rapid inactivation of the second moiety of the nitrate reductase complex, and that the subsequent removal of ammonia brings about an equally rapid reactivation of the inactive enzyme. It is relevant to underline that the ammonia inactivating effect is apparently indirect, in the sense that this inorganic nitrogen derivative seems to act primarily as an uncoupler of noncyclic photophosphorylation, thus leading to a rise in the level of reducing power in the cell (3,4). The conversion of the active form of nitrate reductase into its inactive form depends first of all, both *in vivo* and *in vitro*, on its reduction, and, conversely, the reactivation of the inactive form depends on its oxidation (3-5). Vennesland and her associates have found that the enzyme obtained from *Chlorella vulgaris* appears largely in an inactive

form and that it can be reactivated by oxidation (6,7). They have also shown that the addition of NADH leads to a loss of enzyme activity (6). Inhibition of nitrate reductase from tomato (8) and spinach (9) leaves by ADP has been reported, and possible control by allosteric and kinetic mechanisms has been suggested.

The present work describes further studies of our laboratory on the interconversion mechanism of the active and inactive forms of Chlorella fusca nitrate reductase and clearly shows that the inactivation rate of the enzyme as a result of its in vitro reduction with NADH can be greatly and specifically potentiated by the presence of ADP.

Materials and Methods: Chlorella fusca Shihira et Krauss (=C. pyrenoidosa Chick) was grown in the light in a stream of 5% CO_2 in air with 8 mM KNO_3, as previously described (10), except that the media were buffered with 20 mM sodium phosphate, pH 7.5, and 10 µM molybdate was used. NADH-nitrate reductase in its active form was partially purified by treatment of the cell-free extracts with 7 mM streptomycin sulfate and precipitation with $(NH_4)_2SO_4$ at 50% saturation (cf. 11). The diaphorase and nitrate reductase activities of the enzyme were determined by cytochrome c reduction and nitrite formation, respectively, with NADH as the electron donor, as described elsewhere (12,13).

Results and Discussion: We had previously suggested (14) that the reversible inactivation of nitrate reductase promoted in vivo by ammonia could be a result of the direct action of NADH and ADP on the active form of the enzyme, as a consequence of a change in the redox state and in the energy charge of the cell. More recently evidence was presented that NAD(P)H was an inducer for the in vitro conversion of the active form of the enzyme into its inactive one, a process which was accompanied by the reduction of the molybdoprotein moiety; the transformation was found to be reversible, and the inactive enzyme became again active upon reoxidation (3-5). Finally it was shown that ammonia, the end product of the pathway catalyzed by the assimilatory nitrate-reducing system, promoted the inactivation of nitrate reductase in vivo by acting as an uncoupler of photo-

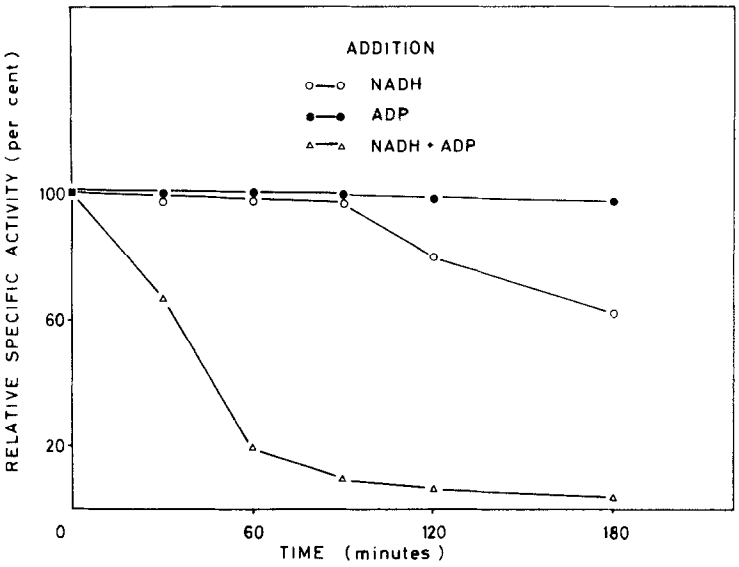

Fig. 1. Reversible inactivation with time of Chlorella nitrate reductase by NADH and ADP. The enzyme preparation (3 mg) was preincubated at 0° in 1 ml of 0.1 M Tris, pH 7.5, with 0.3 mM NADH and (or) 0.3 mM ADP. At the times indicated, NADH-nitrate reductase activity was estimated at 30° on 0.1 ml aliquots of the preincubation mixtures, which were completed, up to a final volume of 1 ml, with the reagents of the standard assay (150 µmoles of Tris-HCl, pH 7.5, 0.3 µmole of NADH and 10 µmoles of KNO_3).

phosphorylation, thus stimulating electron flow and raising the reducing power of the cell (3,4). The results presented below are of significance since they demonstrate that not only NADH but also ADP -the other product of the uncoupling action of ammonia- are required together for achieving inactivation of nitrate reductase at a maximal rate.

Figure 1 shows that, under the experimental conditions chosen, inactivation of nitrate reductase by NADH depended on the simultaneous presence of ADP. ADP alone (or even NADH) was not effective. Conversion of the resulting inactive form of the enzyme into its active one after reoxidation with ferricyanide (cf. 3,5,15) was subsequently corroborated. Using the following range of ADP concentrations: 0.01, 0.03, 0.1, 0.3, 1, and 3 mM, it was established that the ADP inactivating effect occurred rather distinctly at 0.3 mM, a concentration which was equimolecular with that of NADH used. In agreement with previous

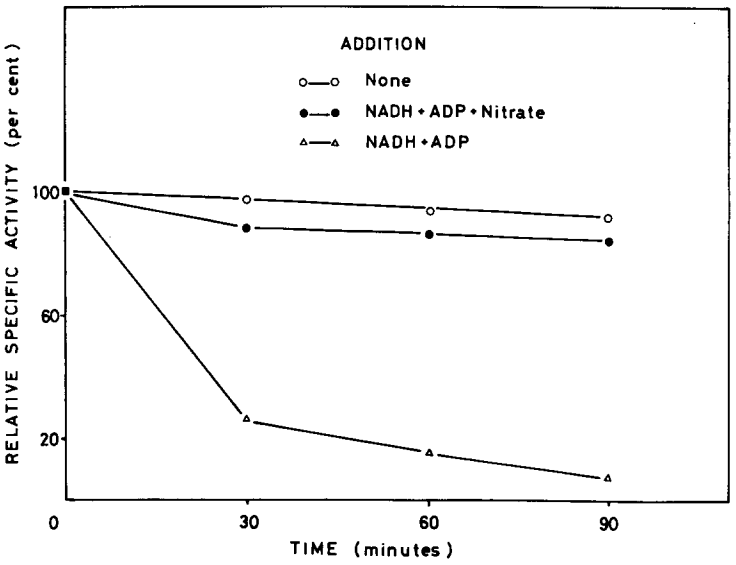

Fig. 2. Protection by nitrate of Chlorella nitrate reductase against inactivation by NADH and ADP. The experimental conditions were the same as in Figure 1. When the preincubation mixture contained nitrate (10 mM), NADH was progressively oxidized by the enzyme, but it was checked that at the end of the preincubation period about half of the nucleotide remained yet reduced.

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results (3,5,13,15), nitrate completely prevented inactivation by NADH and ADP (Figure 2).

As can be seen in Table I, the effect of ADP was quite specific. Under identical conditions, AMP, ATP and GDP were much less effective. With respect to nicotinamide adenine dinucleotide specificity, little cooperation seemed to exist between NADPH and ADP, whereas NAD^+ was completely ineffective. EDTA (5 mM) had no effect.

In previous work, it was reported the specific protection by NADH and FAD of the diaphorase moiety of nitrate reductase from spinach (13) and Chlorella (12,15) against several kinds of inactivation. We can now add (Table II) that ADP -which, as mentioned above, specifically contributes to the inactivation by NADH of the second moiety of the enzyme complex- is by itself (either in the presence or in the absence of Mg^{++}) a powerful protector of the diaphorase moiety against thermal inactivation. In this respect, however, its effect is rather

Table I

EFFECT OF DIFFERENT REDOX- AND ENERGY-NUCLEOTIDES ON THE REVERSIBLE INACTIVATION OF NITRATE REDUCTASE FROM *Chlorella*

Addition	Relative specific activity (per cent)
None	100
NADH	75
NADH + ATP	88
NADH + ADP	10
NADH + AMP	77
NADH + GDP	90
NADPH	79
NADPH + ADP	86
NAD^+ + ADP	115

The enzyme was preincubated for 1.5 hours with the specified nucleotides at a concentration of 0.3 mM each. Other experimental conditions as in Figure 1.

unspecific as compared to others purine, pyrimidine and pyridine nucleotides tested, except NAD(P)H. Inorganic phosphate or EDTA (5 mM) did not exhibit any protection.

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Table II

PROTECTIVE EFFECT OF DIFFERENT NUCLEOTIDES AGAINST THERMAL INACTIVATION OF THE NADH-DIAPHORASE MOIETY OF THE NADH-NITRATE REDUCTASE COMPLEX FROM *Chlorella*

Addition	Relative specific activity (per cent)
None	3
NADPH	5
$NADP^+$	4
NADH	145
NAD^+	112
ATP	130
ADP	123
AMP	118
GDP	110
CDP	102
UDP	129
NADH + ADP	145

The enzyme was preincubated for 4 hours at room temperature with the specified nucleotides at a concentration of 0.3 mM each. Other experimental conditions as in Figure 1. NADH-diaphorase activity was estimated at room temperature on 0.1 aliquots of the preincubation mixtures, which were completed up to 2 ml with the reagents of the standard assay (200 µmoles of Tris-HCl, pH 7.5, 0.3 µmole of NADH and 1.5 mg of cytochrome *c*). Activities are expressed as percentages of the control at zero time.

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