INTERCONVERSION OF THE ACTIVE AND INACTIVE FORMS OF *CHLORELLA* NITRATE REDUCTASE

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1. Introduction

In the green alga *Chlorella*, the reduction of nitrate to nitrite is catalyzed by the flavomolybdoprotein NADH-nitrate reductase, an enzyme complex of high molecular weight with two different enzymatic activities participating sequentially in the transfer of electrons from NADH to nitrate: the first is a FAD-dependent NADH-diaphorase, and the second is the molybdoprotein nitrate reductase or terminal nitrate reductase, also named FNH_2-nitrate reductase for it can use exogenous reduced flavin nucleotides as electron donors [1,2].

Both activities are affected in different ways by appropriate treatments or by selective inhibitors. Thus, NADH-diaphorase is heat-labile and sensitive to -SH binding reagents, such as *p*CMB; FAD and NADH specifically protect against inactivation by heating and *p*CMB, respectively [3,4]. On the other hand, FNH_2-nitrate reductase is inactivated by incubation with NAD(P)H, particularly when minimal amounts of cyanide are simultaneously present; nitrate prevents against this inactivation that, once it has occurred, can be reversed by ferricyanide [4].

This paper presents evidence that *Chlorella* nitrate reductase can exist in two interconvertible forms, active and inactive, according to the redox state of the enzyme, the conversion being affected by temperature and pH. A preliminary report of this work has appeared previously [5].

2. Materials and methods

Chlorella fusca Shihira et Kraus (= C. *pyrenoidosa* Chick) grown autotrophically [1] was used throughout this work. The enzyme was partially purified from crude extracts by a modification of the procedure used by Aparicio et al. [6] consisting of the following steps: i) treatment with 7 mM estreptomycine sulfate; ii) adsorption on calcium phosphate gel (1 mg/mg protein); iii) treatment with 0.5% protamine sulfate; iv) precipitation with $(NH_4)_2SO_4$ (45% saturation); v) filtration through a Sephadex G-25 column; vi) chromatography on a DEAE-Sephadex A-50 column using a linear gradient of NaCl for the elution. The fractions with highest specific activities were precipitated with $(NH_4)_2SO_4$, dissolved in 70 mM potassium phosphate (pH 7.5), 150 mM KCl and 10 μM FAD, and filtered through a Sephadex G-25 column to obtain 3–5 mg of nitrate reductase II (specific activity 600 milliunits/mg). In some experiments, nitrate reductase I, obtained after step 5 of purification, was used (specific activity 140 milliunits/mg).

The enzymatic activities of the nitrate reductase complex (i.e., NADH-nitrate reductase, NADH-diaphorase, and FNH_2-nitrate reductase) were assayed as described previously [1]. Enzyme activity units are expressed as micromoles of substrate utilized (NADH) or product formed (nitrite) per min at 25°.

3. Results and discussion

Nitrate reductase from *Chlorella* is reversibly inactivated by incubation with NADH. Table 1 shows that

Table 1

Reversible inactivation of nitrate reductase by NADH and its protection by nitrate.

Addition	Relative activities (%)		
	NADH-Diaphorase	FNH_2-NO_3Rase	NADH-NO_3Rase
None	100	100	100
NADH	93	25	3
NADH, Nitrate	95	100	100
NADH, Ferricyanide*	–	100	65
NAD^+	106	125	87
DTE	106	120	100

Nitrate reductase I (3 mg/ml) was incubated at 45° for 7 min in the presence of 5 mM Tris-HCl (pH 7.5), 10 μM FAD, and the compounds indicated in the table, except ferricianide*, at the following concentrations: NADH, NAD^+, and DTE, 0.6 mM; and KNO_3, 10 mM. After incubation, enzymatic activities were determined on aliquots of the incubation mixtures. Activities are expressed as percentages of the activities of the control without addition.

* Ferricyanide (0.3 mM) was added after incubation at 45° and before determination of activities.

NADH- and FNH_2-nitrate reductase activities, but not NADH-diaphorase, are affected by this treatment, indicating that the second or terminal moiety of the complex, but not the first, is altered by NADH. This inactivation is prevented by the presence of nitrate and reversed by addition of ferricyanide, in agreement with results obtained in crude extracts of *Chlamydomonas* [7]. Neither the oxidized form of the pyridine nucleotide nor the reducing compound dithioerythritol (DTE) produced any significant inactivation under the conditions of the experiment. Table 2 shows that when the NADH-diaphorase moiety of the complex is previously inactivated by pCMB, the NADH treatment produces a much lower decrease in FNH_2-nitrate reductase activity, indicating that the activity of the first moiety is required to obtain maximal inactivation of the second.

The inactivation by NADH of nitrate reductase was found to be dependent on pH. Fig. 1 shows the effect of pH on the inactivation of NADH-nitrate reductase by NADH. When incubation took place at pH 6.0, the enzyme retained its total original activity, whereas at pH 9.0, 50% of the activity was lost. This inactivation was also completely reversed by addition of ferricya-

Table 2

Requirement of NADH-diaphorase activity for FNH_2-nitrate reductase inactivation by NADH.

		Relative activities (%)	
Treatment I	Treatment II	NADH-Diaphorase	FNH_2-NO_3Rase
None	None	100	100
None	NADH	126	20
pCMB	NADH	6	60

Nitrate reductase I (1.5 mg/ml) was incubated at 0° for 15 min, in the presence of 5 mM Tris-HCl (pH 7.5), and 10 μM FAD, with and without 20 μM pCMB (treatment I). After treatment I, 1 mM NADH was added as indicated in the table, and the mixtures were incubated at 45° for 7 min (treatment II). Enzymatic activities were determined on aliquots of the incubation mixtures after treatment II.
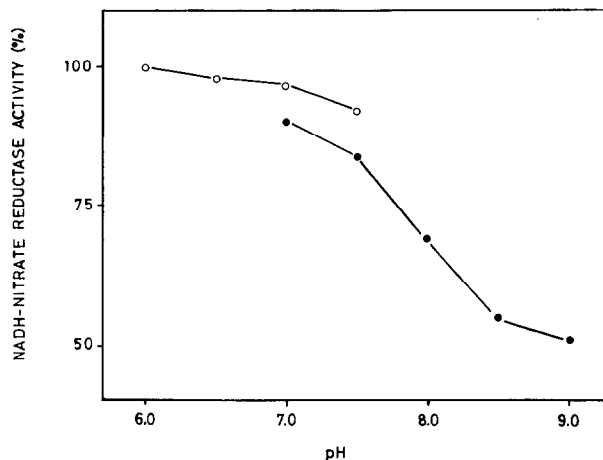

Fig. 1. Effect of pH on the inactivation by NADH of NADH-nitrate reductase. Nitrate reductase II (0.1 mg/ml) was incubated at 25° for 10 min with 150 μM NADH, 10 μM FAD, and 100 mM potassium phosphate (o—o—o) or Tris-HCl (●—●—●) buffer at the pH indicated. After incubation, 10 mM KNO_3 was added, and the oxidation of NADH was measured spectrophotometrically. Enzymatic activities are expressed as percentages of the activity of the control at the corresponding pH without NADH.

nide. The dependence of NADH-inactivation on pH seems to indicate that ionization of a chemical group of the enzyme, maybe a sulphydryl, is involved in the inactivation process. The kinetics of inactivation by NADH with time, at pH 6.5 and 8.5, are shown in fig. 2.
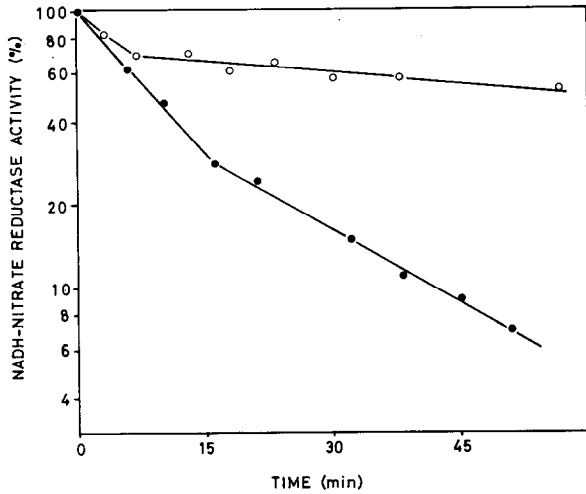
Fig. 2. Inactivation with time of NADH-nitrate reductase by NADH at pH 6.5 (o—o—o) and 8.5 (•—•—•). Nitrate reductase II (0.25 mg/ml) was incubated at 25° with 200 μM NADH, 10 μM FAD, and 250 mM 2(N-morpholino)-ethane sodium sulfonate buffer (pH 6.5) or Tris-HCl (pH 8.5). Enzymatic activities were determined on aliquots of the incubation mixtures at the times indicated. Activities are expressed as percentages of the activity of the control at the corresponding pH without NADH.
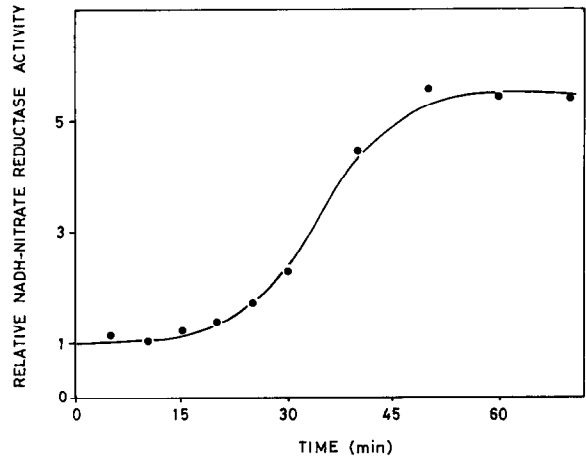

Fig. 3. Reactivation with time of NADH-nitrate reductase in the presence of nitrate. The assay mixture contained inactivated nitrate reductase (0.1 mg/ml), 1 mM NADH, 20 mM KNO_3, and 150 mM Tris-HCl buffer, pH 7.0. The inactivated enzyme was obtained by incubating nitrate reductase II (1.5 mg/ml) at 25° for 1 hr in the presence of 150 mM Tris-HCl (pH 8.5), 0.3 mM NADH, and 10 μM FAD. Enzymatic activity with time was followed spectrophotometrically by measuring oxidation of NADH.

In both cases, two phases of inactivation can be distinguished, the first taking place with a higher rate constant that the second. This phenomenon might be explained either in terms of two different forms of the enzyme having different sensitivity to NADH-inactivation, or assuming the existence of an intermediate form with a V_{max} lower than that of the original complex. Further work is being done to elucidate this point.

The nitrate reductase complex inactivated by NADH can be reactivated in the presence of nitrate during the assay of NADH-nitrate reductase activity, as it is shown in fig. 3 (cf. [8]).

The interconversion of nitrate reductase active and inactive forms in vitro, using a purified preparation from Chlorella, has a special physiological significance, for it can explain the reversible inactivation of the enzyme produced in vivo by ammonia in Chlorella [9] as well as in Chlamydomonas [7] cells. Our present evidence [7, 10] indicates that ammonia exerts its effect indirectly by acting as an uncoupler of photosynthetic phosphorylation, increasing as a consequence the redox level of the cell.

Acknowledgements

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