NITRATE REDUCTASE FROM *Spinacea oleracea*. EFFECTS OF SULFHYDRYL-GROUP REAGENTS ON THE ACTIVITIES OF THE COMPLEX AND THE INACTIVATION BY NADH

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SUMMARY. Sulfhydryl-group reagents inactive the nitrate reductase complex from *Spinacea oleracea*. Most of the reagents used inactivate selectively the NADH-diaphorase moiety. However, at higher concentrations of reagent the FNH_2-nitrate reductase is also affected. Enzyme preparations inactivated by *p*-hydroxymercuribenzoate can be reactivated by dithioerythritol. Nitrate reductase lacking NADH-diaphorase activity, after treatment with *p*-hydroxymercuribenzoate, is inactivated in its FNH_2-nitrate reductase moiety by NADH in the same way as the untreated preparation. This apparent independence of the NADH-inactivation process from NADH-diaphorase activity supports the postulated existence of a binding site for pyridine nucleotides implicated in NADH-inactivation and different from the diaphorase catalytic site.

Nitrate reductase (EC 1.6.6.1) from spinach leaves catalyzes the reduction of nitrate to nitrite using NADH as electron donor (1). This enzyme complex presents two additional activities: an NADH-diaphorase, and an FNH_2-nitrate reductase (1-3). The two activities are affected in different ways by certain treatments and inhibitors: NADH-diaphorase is inactivated by

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Abbreviations: FNH_2, reduced FAD or FMN; pHMB, *p*-hydroxymercuribenzoate; DTNB, 5,5´-dithiobis-(2-nitrobenzoate); NADH-NO_3Rase, NADH-nitrate reductase; FNH_2-NO_3Rase, FNH_2-nitrate reductase.

heating at 45° in the absence of FAD, and by the sulfhydryl-group reagent pHMB, while FNH_2-nitrate reductase is inactivated by cyanide, azide, thiols, and NAD(P)H (1,2,4). The enzyme treated with thiols or NAD(P)H can be reactivated by ferricyanide (4). The inactivation by NAD(P)H seems to be of physiological significance (5,6,7). The susceptibility of the nitrate reductase complex to NAD(P)H-inactivation is abolished by heating at 45° in the absence of FAD, a treatment that also destroys the NADH-diaphorase activity. However, no proportionality was observed between the NADH-diaphorase activity of a preparation and its susceptibility to inactivation by NAD(P)H (4). It was concluded that a portion of the complex structurally related to the active site of the diaphorase moiety is needed for NAD(P)H inactivation.

This paper shows that pHMB-treatment of the nitrate reductase complex inactivates the NADH-diaphorase without affecting the capacity of the complex for being inactivated by NADH, indicating that an active NADH-diaphorase moiety is not required for NADH-inactivation of the FNH_2-nitrate reductase moiety.

MATERIALS AND METHODS

Nitrate reductase was partially purified from spinach leaves (*Spinacea oleracea* L.) by a simplified procedure consisting of the following steps: (I) preparation of the cell-free extract, (II) $(NH_4)_2SO_4$ precipitation, and (III) treatment with calcium phosphate gel. The NADH-nitrate reductase specific activity of the purified preparation was about 400 milliunits/mg, with a ratio of NADH-nitrate reductase to FNH_2-nitrate reductase activities equal to 0.8. Enzyme activity units are expressed as micromoles of substrate (NADH) utilized or product (nitrite) formed per min at 30°. The purified preparation was stored at 0-5°, in 0.2 M potassium phosphate (pH 7.5), 20 μM FAD, 10 mM KNO_3 and 1 mM EDTA -under these conditions the enzymatic activities are maintained for at least a week. The preparation was equilibrated

with the required solution by passage through Sephadex G-25. All the incubations took place in a 0.1 M potassium phosphate buffer solution (pH 7.5), containing 20 μM FAD and 1 mM EDTA.

NADH-nitrate reductase and FNH_2-nitrate reductase activities were determined by measuring nitrite formed, and NADH-diaphorase was estimated spectrophotometrically by following the reduction of cytochrome c at 550 nm (4).

RESULTS AND DISCUSSION

The effects of different sulfhydryl-group reagents on the activities of the nitrate reductase complex are shown in Table I. With all the reagents used, NADH-nitrate reductase and NADH-diaphorase were inactivated to a larger degree than FNH_2-ni-

Table I. Effects of different sulfhydryl-group reagents on the enzymatic activities of the nitrate reductase complex

Addition	Relative activities (%)		
	NADH-NO_3Rase	NADH-Diaphorase	FNH_2-NO_3Rase
pHMB (50 μM)	<1	<1	85
DTNB (0.1 mM)	<1	<1	80
Iodoacetate (100 mM)	7	1	90
Iodoacetamide (100 mM)	49	40	70
N-ethylmaleimide (20 mM)	<1	<1	60

Nitrate reductase (2 mg/ml) was incubated at 30° in the presence of the compounds indicated in the table, for the following times: pHMB, 10 min; DTNB, 1 h; and iodoacetate, iodoacetamide, and *N*-ethylmaleimide, 3 h. After incubation, enzymatic activities were determined on aliquots of the incubation mixtures. The activities are expressed as percentages of the activities of the control without additions after the corresponding incubation time.

trate reductase. pHMB, DTNB, iodoacetate and *N*-ethylmaleimide were very selective on their effect on the diaphorase moiety of the complex. These results agree with those previously reported for pHMB (2). The addition of the thiol dithioerythritol (1 mM) to a pHMB-inactivated enzyme was followed by reactivation.

Figure 1 shows the effects of pHMB on the three enzymatic activities at different concentrations of reagent, after incubation for 10 min. From 20 to 50 nmoles of pHMB per mg of protein the inactivation of NADH-nitrate reductase and NADH-diaphorase was practically complete, whereas FNH_2-nitrate reductase was not affected at all. Al higher concentrations of pHMB FNH_2-nitrate reductase was also inactivated.

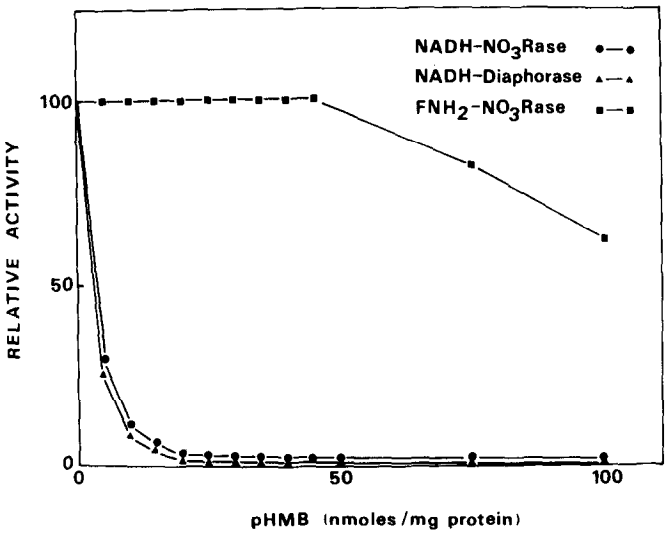

Figure 1. *Effects of different concentrations of pHMB on the enzymatic activities of the nitrate reductase complex.* Nitrate reductase (2 mg/ml) was incubated at 30°, for 10 min, in the presence of pHMB at the concentrations indicated. After incubation, enzymatic activities were determined on aliquots of the incubation mixtures.

A previous pHMB-treatment that inactivates the NADH-diaphorase has no effect on the inactivation by NADH of FNH_2-nitrate reductase. After treatment with 25 nmoles of pHMB per mg of protein for 10 min, the FNH_2-nitrate reductase of this preparation was inactivated by NADH in the same way as that from the untreated enzyme (Fig. 2).

In previous papers (4,8), we postulated the existence in the

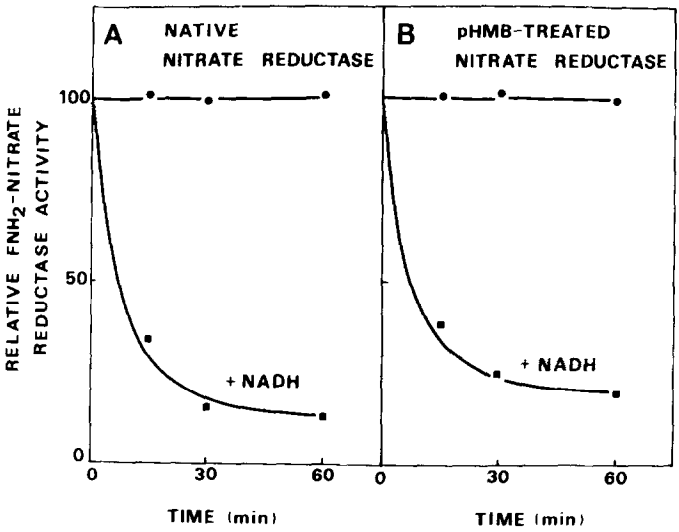

Figure 2. *Effect of pHMB-treatment on the inactivation by NADH of FNH_2-nitrate reductase.* Native nitrate reductase (A) and pHMB-treated nitrate reductase (B) (1 mg/ml) were incubated at 30° with (■) and without (●) 0.5 mM NADH. Enzymatic activity was determined on aliquots of the incubation mixtures at the indicated times of incubation.

To obtain the pHMB-treated nitrate reductase, the native preparation (2 mg/ml) was incubated at 30° for 10 min, in the presence of 50 µM pHMB (25 nmoles of pHMB/mg protein). After this treatment, the preparation was passed through a Sephadex G-25 column to eliminate free pHMB. The control preparation was subjected to the same operations as the treated one but in the absence of pHMB. The NADH-diaphorase activity of the pHMB-treated preparation was less than 1% of that of the untreated control. During incubation with NADH no reactivation of the NADH-diaphorase was observed in the pHMB-treated preparation.

nitrate reductase complex of two different binding sites for pyridin nucleotides: one at the catalytic site of NADH-diaphorase, and the other at a different site of possible regulatory significance. The binding of NADH or NADPH at the second site would be followed by reduction, probably of disulfide bridges, and inactivation of the FNH_2-nitrate reductase moiety.

The apparent independence of the NADH-inactivation process from NADH-diaphorase activity favors the postulated existence of a regulatory site. The portion of the complex that includes this site might act as a converting enzyme, responsible for the conversion by reduction of an active form of the complex into an inactive one. Very little is known of the reverse process, the oxidation of the inactive form to produce the active complex.

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