NITRATE REDUCTASE FROM *Spinacea oleracea*. FAD AND THE INACTIVATION BY NAD(P)H

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SUMMARY. Treatment by *p*-hydroxymercuribenzoate of nitrate reductase from spinach leaves causes the disappearance of NADH-diaphorase activity and the appearance of an FAD-requirement for the inactivation by NAD(P)H of FNH_2-nitrate reductase. The diaphorase activity of the treated preparation is not affected by incubation with FAD or the addition of this nucleotide to the assay mixture. Conversely, filtration of the native preparation through a column of Sepharose 6B produces the appearance of an FAD-stimulation of the diaphorase activity, but no effect of FAD on the NADH-inactivation was observed. These differences between the FAD-requirement of NADH-diaphorase activity and NADH-inactivation agree with the postulated independence of the two processes.

The nitrate reductase complex from spinach leaves catalyzes the reduction of nitrate to nitrite using NADH as electron donor (EC 1.6.6.1) (1). The enzyme complex presents two additional activities: NADH-diaphorase and FNH_2-nitrate reductase (1-3). Each of these two activities is affected independently by certain treatments and inhibitors (1,2,4). When the complex lacks either of them, no NADH-nitrate reductase activity is detected.

Abbreviations: FNH_2, reduced FAD or FMN; FNH_2-NO_3Rase, FNH_2-nitrate reductase; pHMB, *p*-hydroxymercuribenzoate.

Spinach nitrate reductase, like other nitrate reductases of the assimilatory type, is subjected to interconversion between an oxidized active form and a reduced inactive one (1,5). This interconversion affects the FNH_2-nitrate reductase activity in a specific way. Inactivation is achieved by NADH, NADPH and thiols, and reactivation by ferricyanide (4). It is interesting to note that, while NADH but not NADPH acts as electron donor in the catalytic reduction of nitrate or artificial acceptors, both pyridine nucleotides are effective in the inactivation of FNH_2-nitrate reductase (4).

The NAD(P)H-inactivation does not take place if the complex has been previously heated at 45° in the absence of FAD, with loss of the NADH-diaphorase activity (4). However, a pHMB-treated complex, that lacks diaphorase activity, can be inactivated by NADH as well as the untreated one (6). This result seems to indicate that the capacity for NADH-inactivation is independent from the NADH-diaphorase activity.

The present paper reports the various requirements of added FAD for NADH-diaphorase activity and NAD(P)H-inactivation in preparations of nitrate reductase treated in different ways.

MATERIALS AND METHODS

Nitrate reductase was partially purified from spinach leaves (*Spinacea oleracea* L.) by a previously summarized procedure (6). The purified preparation was stored at 0-5° in 0.2 potassium phosphate (pH 7.5), 20 µM FAD, 10 mM KNO_3 and 1 mM EDTA. Under these conditions the enzymatic activities of the nitrate reductase complex are maintained for at least a week. The preparation was equilibrated with the required solution by passage through a Sephadex G-25 column. All the incubations were conducted at 30°. Enzyme activity units are expressed as micromoles of substrate (NADH) utilized or product (nitrite) formed per min at 30°. The NADH-nitrate reductase specific activity of the purified preparation was about 400 milliunits/mg, with ratios of NADH-nitrate reductase to NADH-diaphorase and FNH_2-nitrate reductase activities equal to 0.2 and 0.8, respectively.

The enzymatic activities were determined as described previously (4): NADH-nitrate reductase and FNH_2-nitrate reductase

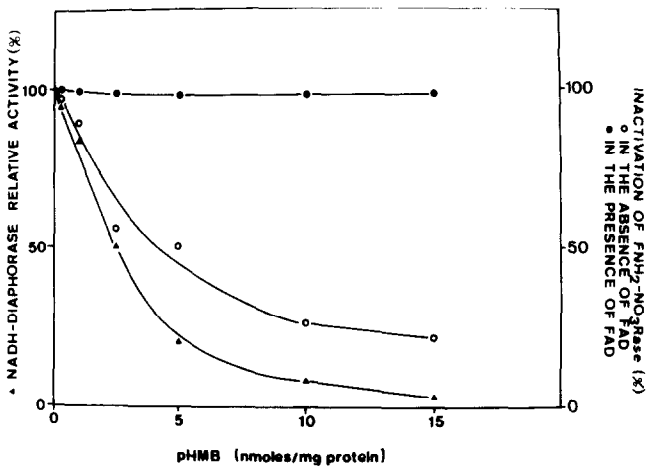

Figure 1. *Effect of FAD on the inactivation by NADH of pHMB-treated nitrate reductase.* The enzyme preparation (5 mg/ml) was incubated in the presence of 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA, 20 µM FAD and pHMB at the concentrations indicated in the figure. After incubation for 10 min, the preparations were passed through Sephadex G-25 columns to get rid of the free pHMB and FAD. At this point, NADH-diaphorase activity and NADH-inactivation were determined.

NADH-diaphorase activity was determined on aliquots of the preparations. The activities (▲) were the same whether determined in the presence or in the absence of 20 µM FAD. They are expressed as percentages of the activity of a control not treated with pHMB.

To evaluate the degree of inactivation by NADH, the preparations were incubated for 1 h in the presence of 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA and 0.5 mM NADH, with (•) and without (o) 20 µM FAD. At the end of this incubation FNH_2-nitrate reductase activity was determined on aliquots of the incubation mixtures.The percentages of inactivation were calculated with respect to the corresponding controls incubated without NADH. All the controls had nearly the same FNH_2-nitrate reductase specific activity.

by measuring nitrite formed, and NADH-diaphorase by following spectrophotometrically the reduction of cytochrome *c*. Protein concentration was evaluated by the method of Lowry *et al.* (7).

RESULTS

Incubation with pHMB of the nitrate reductase complex produces the inactivation of NADH-diaphorase and the appearance of an FAD-requirement for NADH-inactivation. Figure 1 shows the inactivation of NADH-diaphorase after incubation with different

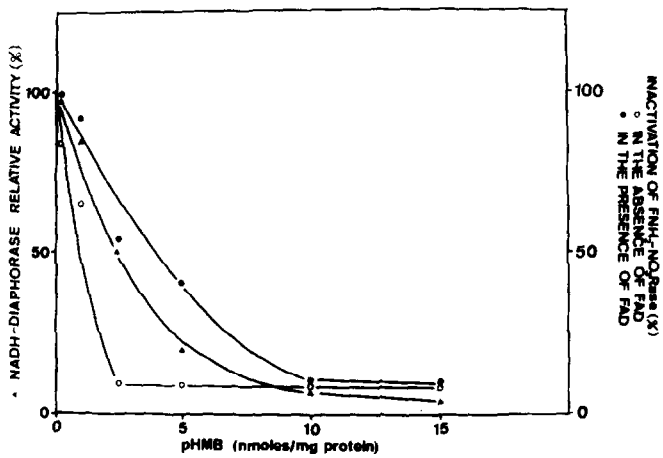

Figure 2. *Effect of FAD on the inactivation by NADPH of pHMB-treated nitrate reductase.* The experiment was conducted as described in the legend of Figure 1, but using NADPH instead of NADH.

concentrations of pHMB. The NADH-diaphorase activity decreased with increasing concentrations of pHMB. The enzymatic activity was the same whether determined in the presence or in the absence of FAD. Even incubation with FAD for 1 h prior to enzymatic assay did not affect the diaphorase activity. This figure also shows that the inactivation of FNH_2-nitrate reductase by NADH was complete (100%) in the presence of 20 μM FAD, independently of the concentration of pHMB in the previous treatment. However, in the absence of FAD, the degree of inactivation decreased as the concentration of pHMB in the previous treatment increased. These results indicate that, in a pHMB-treated preparation, the FAD-requirement for NADH-inactivation is different from that for NADH-diaphorase activity.

An effect of FAD on the inactivation by NADPH of the pHMB-treated preparation was also observed (Fig. 2). However, in this case the extent of inactivation in the presence of FAD decreased as the pHMB-concentration in the previous treatment increased.

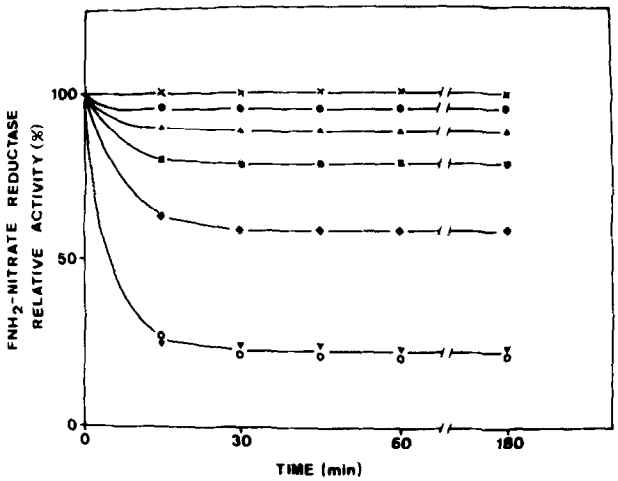

Figure 3. *NADPH-inactivation of a pHMB-treated nitrate reductase.* The enzyme preparation (5 mg/ml) was incubated in the presence of 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA, 20 µM FAD and pHMB (15 nmoles/mg protein). After incubation for 10 min the enzyme preparation was passed through a Sephadex G-25 column to get rid of free pHMB. The preparation obtained in this way was incubated with NADPH at the following concentrations: zero (X), 0.3 mM (●), 1 mM (▲), 1.5 mM (■), 2 mM (◆), and 5 mM (▼). As a control, an aliquot of the preparation was incubated with 0.3 mM NADH (o). FNH_2-nitrate reductase activity was determined on aliquots of the incubation mixtures at the times of incubation indicated in the figure.

Figure 3 shows the inactivation by different concentrations of NADPH of a pHMB-treated nitrate reductase. At the concentration of NADPH that inactivates completely the FNH_2-nitrate reductase of the native complex, the pHMB-treated preparation was only slightly inactivated (*cf.* Fig. 2). Higher concentrations of NADPH were required to produce nearly complete inactivation, indicating that the NADPH-binding site has probably been affected by the pHMB-modification.

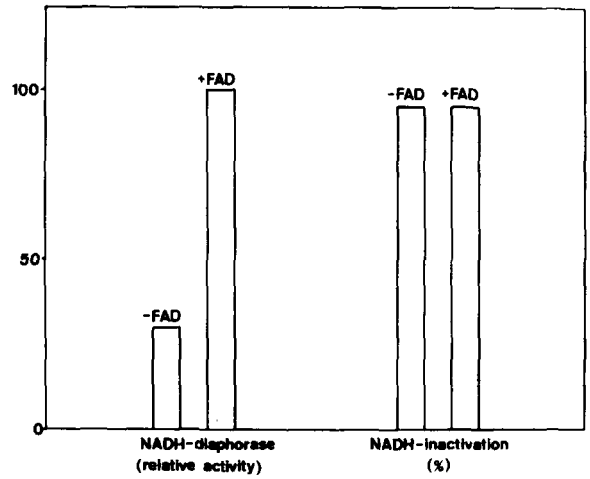

Figure 4. *Effect of FAD on the NADH-diaphorase activity and NADH-inactivation of a nitrate reductase preparation passed through a Sepharose column.* Nitrate reductase (4 ml with 10 mg/ml) was passed through a column of Sepharose 6B (2 cm diameter and 65 cm high) equilibrated with 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl and 2 mM KNO_3. The fractions with higher FNH_2-nitrate reductase activity were pooled, and the NADH-diaphorase activity of the mixture determined (-FAD). An aliquot of the pooled Sepharose fractions was incubated with 0.1 mM FAD for 30 min. At the end of the incubation NADH-diaphorase activity was determined on an aliquot of the incubation mixture (+FAD), the concentration of FAD in the assay mixture being 5 µM.

To determine the inactivation by NADH, KNO_3 was eliminated from the pooled Sepharose fractions by passage through a Sephadex G-25 column, and the nitrate-free preparation incubated with 0.5 mM NADH for 1 h, in the presence (+FAD) and absence (-FAD) of 0.1 mM FAD. At the end of the incubation, FNH_2-nitrate reductase activity was determined on aliquots of the incubation mixtures. NADH-inactivation is expressed as the percentage of inactivation relative to a control incubated without NADH and FAD.

After passage through a Sepharose column, the NADH-diaphorase activity was stimulated by incubation with FAD (Fig. 4) (*cf.* 2). However, this same preparation did not show any requirement of added FAD for NADH-inactivation. Therefore, in this preparation, the phenomenon that presents a requirement of added FAD is the NADH-diaphorase activity, instead of the NADH-inactivation.

DISCUSSION

All nitrate reductases from fungi, algae and higher plants seem to contain FAD, which is required for catalytic activity of the diaphorase moiety of the complex (1). However, the requirement of added FAD for catalytic activity varies with the preparation being studied, indicating that at least in some cases FAD is firmly bound to the enzyme.

In native nitrate reductase from *Neurospora crassa*, an FAD-requirement has been described for the inactivation of FNH_2-nitrate reductase by NADPH plus cyanide (8). In this case, the NADPH-diaphorase activity also requires the addition of FAD.

The present paper reports different requirements of added FAD for NADH-diaphorase activity and NADH-inactivation in differently treated preparations of spinach nitrate reductase. In the purified preparation used in this work, neither NADH-diaphorase activity nor NADH-inactivation was affected by added FAD. On the contrary, in preparations treated with pHMB or passed through Sepharose, added FAD affected one of the two processes. However, the process affected by FAD was different in each case. The pHMB-treated preparations showed an FAD-requirement for NADH-inactivation, but no effect of added FAD on NADH-diaphorase activity. Conversely, in the Sepharose-filtered preparation, NADH-diaphorase activity was stimulated by added FAD, and no effect of FAD on NADH-inactivation was observed. These differences in FAD-requirement of the two phenomena seem to indicate that they do not correspond to the same basic process, and are in agreement with the postulated existence of an independent regulatory site (4,6).

Since NADH causes inactivation by reduction of the complex, it is possible that FAD acts as a cofactor in the inactivation,

apparently not being required in the spinach native enzyme because of its presence in a tightly bound way.

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