SPECIFIC PROTECTION AGAINST INHIBITORS OF THE NADH-NITRATE REDUCTASE COMPLEX FROM SPINACH

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1. Introduction

In spinach [1, 2], as in algae [2–5] and in most higher plants [2, 6], the reduction of nitrate to nitrite is catalyzed by a NADH-dependent reductase complex. The two enzymatic activities which sequentially participate in the transfer of electrons from NADH to nitrate have been separated by the use of selective inhibitors: the first is a NADH-diaphorase which is completely inhibited by 0.1 mM *p*-chloromercuribenzoate (pCMB) and by heating for 5 min at 45°, and the second is the nitrate reductase proper or terminal nitrate reductase which is totally inhibited by 1 mM cyanide or azide [1, 2, 6]. A peculiar characteristic of this second enzyme is that it can exist, at least in some algae, in two metabolic interconvertible (active or inactive) forms [4, 5, 7, 8].

Until recently, the role of flavin nucleotides in the reduction of nitrate by preparations from green cells was not clear; in fact, it has been reported not only that the reduction of nitrate by NADH was not stimulated by FAD or FMN, but that the addition of flavins could result in inhibition [1–4]. We have lately found, however, that, after gel filtration on agarose of the nitrate reductase from *Chlorella*, FAD is required for activity, and that this nucleotide specifically protects the enzyme against denaturation by heating [3].

This work describes the specific protection by several substrates and cofactors of the NADH-nitrate reductase complex from spinach against several types of inhibition and inactivation, and presents evidence that FAD is the prosthetic group of the enzyme, and that its site of action is on the diaphorase moiety.

2. Materials and methods

NADH-nitrate reductase was purified from a crude homogenate of spinach leaves by a procedure essentially analogous to those previously described for spinach [1, 2] and *Chlorella* [3, 9] which included, as main steps, passage through a DEAE-cellulose bed, adsorption on calcium phosphate gel, treatment with protamine sulfate, precipitation with $(NH_4)_2SO_4$ at 50% saturation, adsorption on alumina Cγ, and gel filtration with agarose.

NADH-nitrate reductase, NADH-diaphorase and FNH_2-nitrate reductase were assayed as previously described [3] using the alumina Cγ eluate as enzyme preparation, except where otherwise stated.

3. Results and discussion

As was previously shown for the *Chlorella* enzyme [3], when FAD was added to a preparation of spinach nitrate reductase which had been purified by gel filtration on agarose, a clear stimulation of the reduction of nitrate by NADH could be observed; here again FAD was only required for the NADH-diaphorase activity of the enzyme complex, whose molecular weight, estimated by calibrating an agarose column with ovalbumin, serum albumin, γ-globulin and apoferritin, was found also to be about 500,000.

Table 1 shows that, among a variety of substrates and cofactors tested, only NADH and FAD were effective as protectors against the inhibition of spinach NADH-nitrate reductase activity by pCMB, if added with pCMB. If, however, the enzyme had been

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Table 1
Protective effect of NADH and FAD against inactivation of spinach NADH-nitrate reductase by pCMB.

Addition	Concentration (μM)	NADH oxidized (nmoles per 5 min)
None	0	100
pCMB	12	12
Plus NADH	35	80
Plus NAD^+	35	16
Plus NADPH	35	32
Plus $NADP^+$	35	10
Plus FAD	35	75
Plus FMN	35	12
Plus NO_3^-	12,000	10

Nitrate reductase was preincubated in a volume of 1.7 ml with 200 μmole Tris-HCl buffer, pH 7.5, and each of the above substrates and cofactors at the concentrations indicated. After 7 min at room temperature, the reaction mixture was completed with the reagents of the enzyme assay (0.3 μmole of NADH and 20 μmole of KNO_3 in a final volume of 2 ml), and NADH oxidation was estimated spectrophotometrically.

previously inhibited by preincubation with pCMB alone, the later addition of either NADH or FAD did not reverse the inactivation. NADPH was much less effective than NADH, and the oxidized forms of the pyridine nucleotides, as well as FMN and nitrate, did not protect. Figs. 1 and 2 show, respectively, the protective effect of different concentrations of NADH and FAD against the inactivation by pCMB. It can be seen that maximal protection was achieved when the concentration of NADH and FAD, reached values of 50 μM and 10 μM, respectively. It was also ascertained that NADH as well as FAD performed their effects by protecting the NADH-diaphorase activity of the nitrate reductase complex.

We have tested different substrates and cofactors as protectors against the thermal inactivation of NADH-nitrate reductase from spinach, and have found, as shown in table 2, that only FAD was effective, and that its protective effect against heat denaturation was on the NADH-diaphorase of the complex.

Table 3 shows that cyanide exerted its very strong inhibitory effect on spinach NADH-nitrate reductase activity only when the enzyme was kept in its reduced state by preincubation with NADH (or alternatively with an electron donor of its second moiety, for example FNH_2). If nitrate was simultaneously present with cyanide and the reductant in the preincubation mixture, the inhibition could be partially or totally abolished, depending on the concentration of nitrate added. However, once the reduced enzyme had been inhibited by cyanide, nitrate was not able to overcome its inhibition. The inhibition was then of

Table 2
Protective effect of FAD against thermal inactivation of spinach NADH-nitrate reductase.

Addition	Concentration (mM)	NADH oxidized (nmoles per min)
None	0	3.8
FAD	0.066	57.2
FMN	0.066	3.8
Methyl viologen	3.3	3.2
NADH	1.0	8.0
NO_3^-	33.3	3.2
None, not heated	0	66.7

Nitrate reductase was heated at 45° for 5 min in 0.5 ml of 0.2 mM Tris-HCl buffer, pH 7.5, with each of the above substrates and cofactors at the concentrations indicated. After the heat treatment, the reaction mixture was completed with the reagents of the enzyme assay (200 μmole Tris-HCl buffer, pH 7.5, 0.3 μmole NADH, 10 μmole KNO_3 and 20 nmole FAD, in a final volume of 2 ml), and NADH oxidation was estimated spectrophotometrically.

Table 3
Inhibition by cyanide and protection by nitrate of spinach nitrate reductase reduced with NADH.

Addition	NADH oxidized (nmoles per min)
None	47
CN^-	46
NADH	32
$CN^- + NAD^+$	47
CN^- + NADH	3
CN^- + NADH + NO_3^-	29

The preincubation mixture included, in a final volume of 2 ml, nitrate reductase, 200 μmole Tris-HCl buffer, pH 7.5, 20 nmole FAD, and, where indicated, 3 nmole KCN, 300 nmole NAD^+ or NADH, and 2.5 μmole KNO_3. After 7 min at room temperature, the reaction mixture was completed with the reagents of the enzyme assay, as described in table 2, and enzyme activity was determined spectrophotometrically by NADH oxidation.

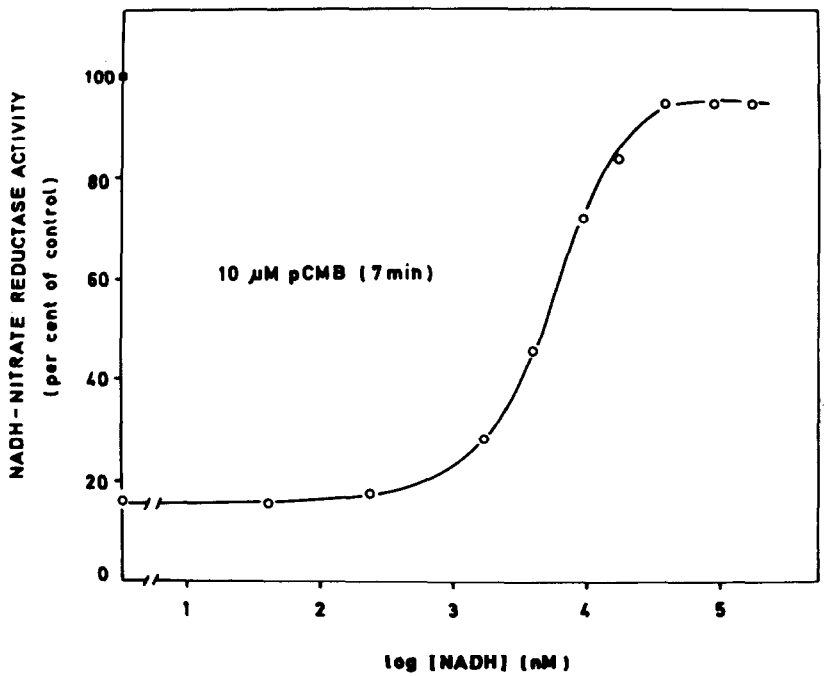

Fig. 1. Effect of NADH concentration on the inhibition of spinach NADH-nitrate reductase by pCMB. Experimental conditions as described for the NADH system of table 1, except that the concentration of pCMB was 10 μM and that of pyridine nucleotide was varied as indicated. Activity at 100% corresponds to the control value obtained in the absence of inhibitor.

the noncompetitive type, with a K_i value of 0.2 μM (fig. 3). We have recently found that, in *Chlorella*, the second activity of the nitrate reductase complex, i.e. nitrate reductase proper, depends absolutely on molybdenum [9, 10]. It seems, thus, likely that when the metal is in its reduced state, cyanide can bind irreversibly to it in the absence of nitrate, leading to inactivation of the enzyme. In regard to these properties of the spinach enzyme, it is of interest to recall the observation by Vennesland and Jestschmann [4] that, in *Chlorella*, the addition of NADH alone in the absence of nitrate (cf. table 3) led to a loss of nitrate reductase activity.

In contrast with cyanide, the inhibition by azide did not depend on the presence of NADH and was of the competitive type with respect to nitrate (K_i = 0.3 μM). Carbamyl phosphate, which may well prove to be an inhibitor of physiological significance [11], was also found to be a competitive inhibitor with nitrate (K_i = 13 μM). Finally, it is significant that the second activity of the nitrate reductase complex was also found to be sensitive to pCMB, but, in this case, in competition with nitrate (K_i = 66 μM). However, this activity was not protected by FAD against pCMB inhibition. The spinach enzyme could use not only nitrate but also chlorate as substrate (K_m = 3 mM), and in this respect is similar to the bacterial nitrate-reductases studied by Pichinoty and his group [12]; however, it could not use cyanide or azide as electron acceptors, as reported for nitrogenase [13].

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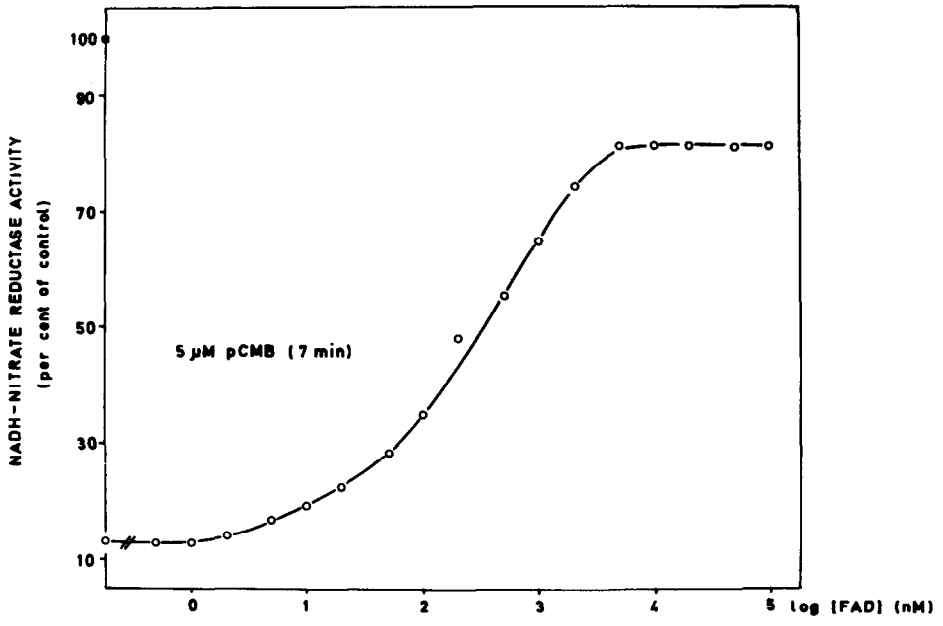

Fig. 2. Effect of FAD concentration on the inhibition of spinach NADH-nitrate reductase by pCMB. Experimental conditions as described for the FAD system of table 1, except that the concentration of pCMB was 5 μM and that of flavin nucleotide was varied as indicated. Activity at 100% corresponds to the control value obtained in the absence of inhibitor.

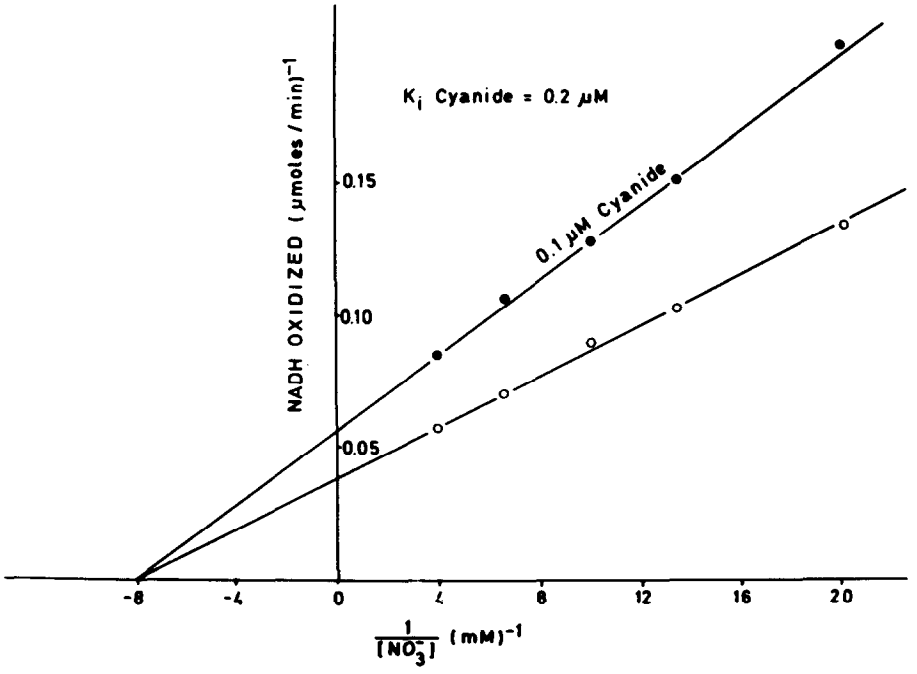

Fig. 3. Noncompetitive inhibition with respect to nitrate of reduced spinach nitrate reductase previously treated with cyanide. Experimental conditions as described for the cyanide-NADH system of table 3, except that cyanide concentration was 1 μM. After preincubation, nitrate was added at the concentrations indicated.

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