STRUCTURAL AND FUNCTIONAL ROLE OF FAD IN THE NADH-NITRATE REDUCING SYSTEM FROM *CHLORELLA*

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1. Introduction

Partially-purified preparations of nitrate reductase from *Chlorella* cells [1] and photosynthetic tissues from higher plants [2, 3] were previously found to utilize reduced FAD or FMN as electron donor for the reduction of nitrate to nitrite. NADPH was only active as reductant when the nitrate reducing system was supplemented with both flavin nucleotide and NADPH diaphorase; NADH, however, could be utilized as electron donor without the addition of other cofactors or enzymes [1, 3, 4]. In the transfer of electrons from NADH to nitrate, the reaction catalyzed by NADH-nitrate reductase (M.W. about 500,000), two enzymatic activities participate sequentially, which, although not physically separable, can be easily and independently assayed: the first, a NADH diaphorase which can use a variety of oxidized compounds (such as cytochrome *c* or dyes) as electron acceptors; and the second, a nitrate reductase proper, or terminal nitrate reductase, which can use reduced flavin nucleotides (or viologens) as electron donors, and has been named therefore FNH_2-nitrate reductase [1–3]. A peculiar characteristic of this second enzyme is that it can exist in two metabolic interconvertible (active or inactive) forms [5].

In agreement with the results from other laboratories [6–8], we found not only that the NADH-dependent reduction of nitrate did not require flavin nucleotide but that the addition of it could result in inhibition [1, 3, 4]. Moreover, the absorption spectrum of highly purified nitrate reductase preparations from *Chlorella* and higher plants did not show any indication of absorption bands for flavin nucleotides but rather a generalized absorption over the entire visible range (except for a shoulder at about 410 nm) quite unlike that of simple flavoproteins [1, 3].

This work presents evidence on the structural and functional role of FAD in the NADH-nitrate reducing system from *Chlorella*. Since we have quite recently found that *Chlorella* nitrate reductase contains also molybdenum [9], it may be concluded that the NADH-dependent assimilatory system from algae as well as that from green plants [10] is, like the NAD(P)H-dependent system from fungi [11] and soybean leaves [12, 13], a molybdoflavoprotein.

2. Materials and methods

Chlorella fusca Shihira et Kraus (= *pyrenoidosa*) 211–15 from Pringsheim's culture collection at Göttingen was grown under the previously described conditions [1]. The cells were collected at the logarithimic phase by centrifugation, washed twice with distilled water, and broken in a vibration homogenator (Bühler) with glass beads of 0.3 mm in diameter. The broken material was resuspended in 5 mM tris-HCl buffer, pH 7.5, freed from glass beads by filtration through cheesecloth, and centrifuged at 20,000 *g* for half an hour.

Nitrate reductase was purified from the resulting supernatant by a procedure which included, as main steps, treatment with streptomycine sulfate, adsorcion

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on calcium phosphate gel, treatment with protamine sulfate, precipitation with $(NH_4)_2SO_4$ at 40% saturation, adsorcion on alumina cγ and, as previously described [1] except for the omission of nitrate from the eluting buffer, gel filtration with agarose.

NADH-nitrate reductase, NADH-diaphorase and FNH_2-nitrate reductase were assayed as previously described [1], using the alumina cγ eluate as enzyme preparation, except if otherwise stated. The heat treatment of the enzyme was performed in a temperature constant bath at 45°. Continuous spectrophotometric measurements were registered with a Beckman DK-2A spectrophotometer.

3. Results and discussion

After gel filtration with agarose, the nitrate reductase which came out of the column was, as previously reported [1], much less active when assayed with NADH than with FNH_2. Apparently, the treatment caused the removal of flavin nucleotide from the enzyme, for, as can be seen in fig. 1, the further addition of flavin coenzyme to the reaction mixture increased activity several times, FAD being more specific than FMN. Using cytochrome *c* as electron acceptor, it was shown that the NADH diaphorase activity of the enzyme was likewise stimulated by the addition of flavin nucleotides, FAD being again more specific (fig. 2). Table 1 shows that FAD, at the concentration

Table 1
Effect of FAD on the enzymatic oxidation of NADH coupled to the reduction of nitrate.

Addition	NADH oxidized (nmoles)	Nitrite formed (nmoles)
FAD, NO_3^-	43.6	42.2
NO_3^-	8.6	8.4
FAD	0	0
None	0	0

The reaction was carried out under air for 5 min using an enzyme preparation just eluted from an agarose column. The reaction mixture contained, in a final volume of 2 ml, 200 μmoles of tris-HCl buffer, pH 7.5, 0.6 μmole of NADH and 70 μg of protein. Where indicated, 0.04 μmole of FAD and 20 μmoles of KNO_3 were added.

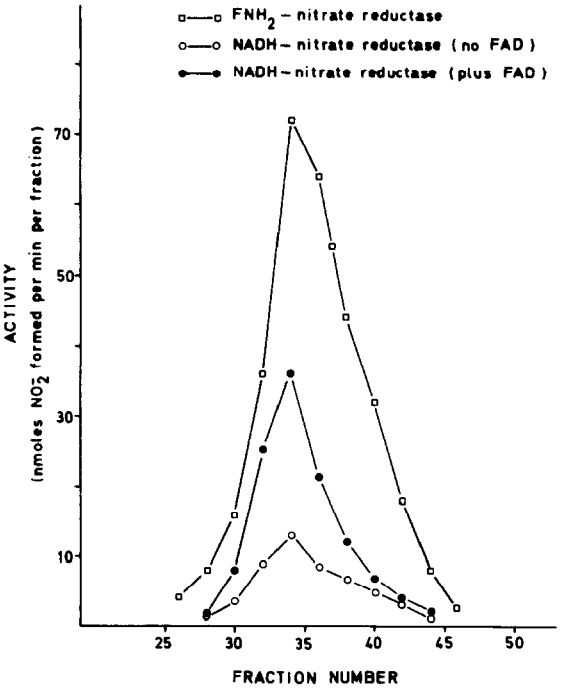

Fig. 1. Stimulation by FAD of NADH-nitrate reductase activity after gel filtration with agarose. The fractions were assayed for FNH_2-nitrate reductase and for NADH-nitrate reductase (plus and minus 0.02 mM FAD) immediately after elution from the column (2.5 × 30 cm).

used, stimulated the enzymatic oxidation of NADH only when it was coupled with the reduction of nitrate. In fact, NADH oxidation was strictly dependent on the presence of nitrate and stoichiometric with its reduction to nitrite.

It has been recently reported by Kaplan et al. [14] that a NAD(P)H diaphorase from *Clostridium kluyveri* which contains FMN as prosthetic group can be protected against denaturation at elevated temperatures by NADH, NADPH or FMN, but not by NAD^+, $NADP^+$ or FAD. We knew from previous work [1, 3, 15] that, when NADH-nitrate reductase is heated at 45° for 5 min, the NADH diaphorase activity of the enzyme (not the nitrate reductase proper) is completely destroyed, thus causing the total inhibition of nitrate reduction with NADH. We have now tested as protectors against the thermal inactivation of NADH-nitrate reductase from *Chlorella* a variety of its natural

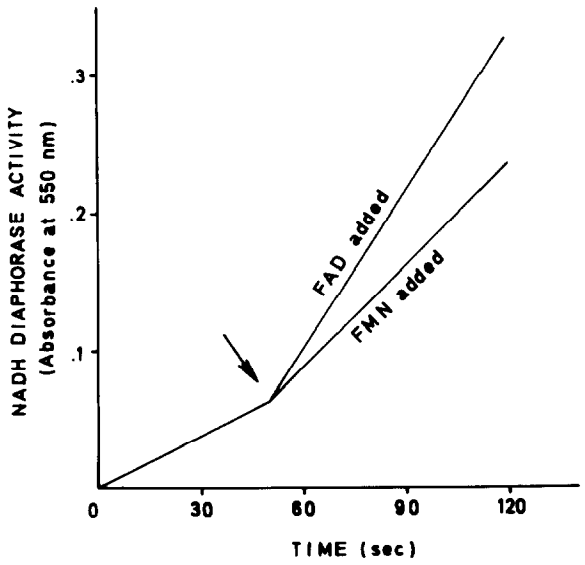

Fig. 2. Stimulation by FAD or FMN of NADH diaphorase activity after gel filtration with agarose. The reaction mixture contained, in a final volume of 2 ml, 200 μmoles of tris-HCl buffer, pH 7.5, 0.3 μmoles, of NADH, 2 mg of cytochrome *c* and 30 μg of an enzyme preparation just emerged from the column. At the time indicated by the arrow, 0.04 μmole of either FAD or FMN were added.

artificial substrates and cofactors, and have found, as shown in table 2, that only FAD (not even FMN) was effective. Maximal protection was achieved at about 10^{-5} M FAD; if the FAD concentration was further increased, the protection against heat denaturation continued being maximal, as measured by NADH oxidation, but the electron flow to nitrate diminished, resulting in the formation of less nitrite (fig. 3). Fig. 4 demonstrates that FAD performs in fact its effect by protecting NADH diaphorase against inactivation; FMN did not protect at all. The addition of FAD to the reaction mixture after the heat treatment of the enzyme was completely ineffective.

Acknowledgements

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Table 2
Protective effect of different substrates on the thermal inactivation of NADH-nitrate reductase.

Addition	Concentration (mM)	Nitrite formed (nmoles)
None	–	0
NADH	0.6	0
MV	0.8	0
BV	0.4	0
NO_3^-	10.0	0
FMN	0.02	2.5
FAD	0.02	44
None, not heated	–	48

30 μg of nitrate reductase in 0.5 ml of 0.2 M tris-HCl buffer, pH 7.5, were heated at 45° for 5 min 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 standard assay up to 1 ml and the NADH-nitrate reductase activity was estimated.

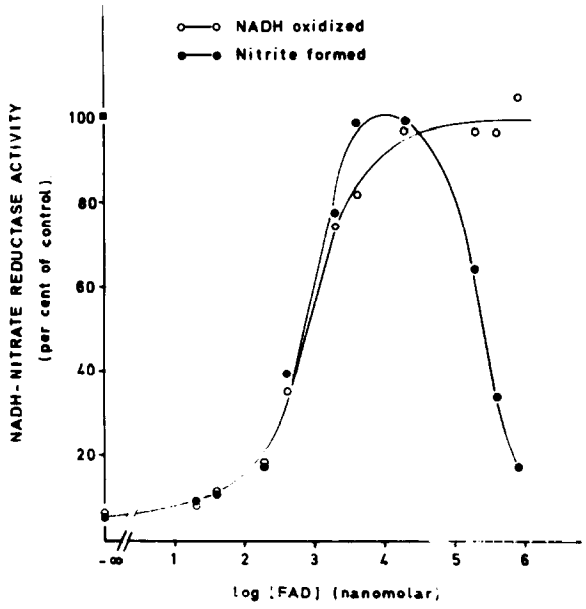

Fig. 3. Effect of FAD concentration on the thermal inactivation of NADH-nitrate reductase. Experimental conditions as described for the FAD system of table 2, except that the concentration of the nucleotide was varied as indicated. Activity at 100% corresponds to the control value obtained with non-heated enzyme.

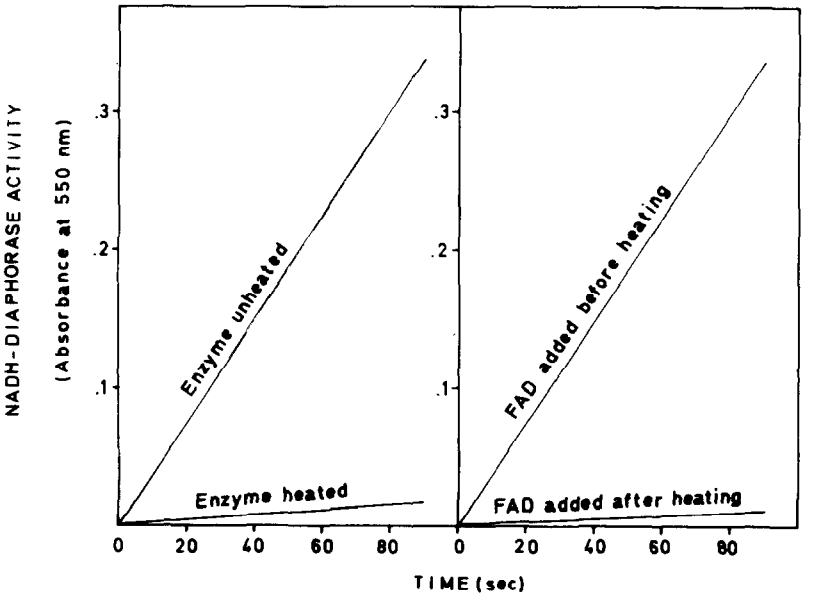

Fig. 4. Protective effect of FAD on the thermal inactivation of NADH diaphorase. 15 μg of enzyme was heated in the absence and in the presence of FAD as described in table 2. After the heat treatment, NADH diaphorase was assayed with cytochrome *c* as described in fig. 2.

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