EFFECTS OF REDUCED DEHYDROGENASE ELECTRON ACCEPTORS ON THE VARIOUS NITRATE DEPENDANT ACTIVITIES OF SPINACH (*SPINACEA OLERACEA* L.) NITRATE REDUCTASE

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1. Introduction

NADH-Nitrate reductase (EC 1.6.6.1) reduces nitrate to nitrite using NADH as electron donor. The enzyme also displays two mutually independent partial activities:

(i) The NADH-dehydrogenase function, demonstrated by reduction of ferricyanide, ferricytochrome *c* or dichlorophenolindophenol (DCPIP);

(ii) The reduction of nitrate using $FMNH_2$ or reduced methyl viologen (MV°) as electron donor (see scheme, fig.1).

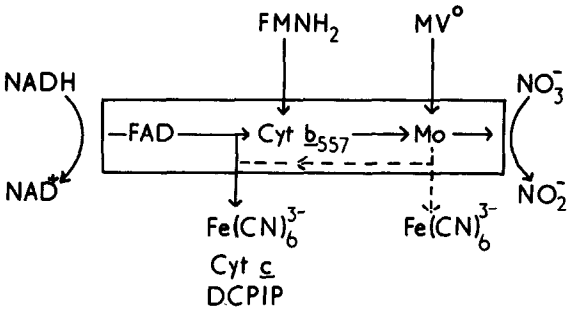

Fig.1. Schematic representation of nitrate reductase showing electron flow pathways for reduction of nitrate and various dehydrogenase electron acceptors (———) and alternative pathways for reoxidation by ferricyanide (– – –) of over-reduced enzyme.

Nitrate reductase is slowly inactivated during a pseudo first-order reaction by incubation with NADH in the absence of nitrate [1–3] and especially in the presence of cyanide [3,4] and over-reduction of molybdenum to a valency state less than in normal turnover, resulting in enhanced cyanide liganding, is the most generally favoured explanation [5]; NADH-dehydrogenase activity is not affected by this treatment. The inactivated enzyme is rapidly reactivated by ferricyanide [4] but the reoxidation site has not been identified. As ferricyanide is a dehydrogenase electron acceptor [5] reactivation might occur therefore either by electron flow from molybdenum to the dehydrogenase site or by direct oxidation of the metal [5] (see scheme, fig.1). Evidence provided by reactivation of the NADH-treated enzyme by $Mn^{(III)}$ pyrophosphate [6] supports the second possible mechanism.

On the assumption that reduced forms of dehydrogenase electron acceptors may combine competitively at the sites at which electron donation to the oxidised forms occurs, we tested the effects of ferrocyanide, reduced DCPIP and ferrocytochrome *c* on the various nitrate reductase activities fulfilled by NADH, $FMNH_2$ or reduced methyl viologen (MV°) and on the course of inactivation by NADH. We find that the reduced dehydrogenase electron acceptors have different types of effects on these reductive activities and on the course of inactivation, and the results indicate that each reacts with the enzyme in different respective ways.

2. Materials and methods

Spinach (*Spinacea oleracea* L. cv Noorman) were grown using a nutrient film technique with Long Ashton nitrate type solutions [7] and leaves sampled after 5–6 weeks growth. Nitrate reductase was extracted and purified using streptomycin sulphate, $(NH_4)_2SO_4$ and hydroxylapatite as in [8]. The enzyme was precipitated with $(NH_4)_2SO_4$ at 50% saturation, re-dissolved in 0.1 M phosphate buffer, pH 7.5, 1 mM EDTA and stored at −40°C. NADH-nitrate reductase activity was determined either by nitrite formation or by NADH oxidation [9]. Nitrite formation was measured after 5 min incubation at 27°C in 1.0 ml containing 50 mM phosphate buffer, pH 7.5, 1 mM EDTA, 10 mM nitrate, 0.3 mM NADH and the enzyme preparation. Colour was developed with the Greiss-Ilosvay reagent. The rate of NADH oxidation was followed spectrophotometrically at 340 nm using the same reaction mixture as above except that NADH was 150 μM in 2.0 ml 6.22 × 10^3 l. mol^{-1} . cm^{-1} was used). $FMNH_2$-nitrate reductase was determined by nitrite formation in 1.0 ml containing 0.2 mM FMN reduced by 0.1 ml $S_2O_4^{2-}$ (8 mg/ml in 50 mM $NaHCO_3$) [10], and MV°-nitrate reductase in 2.0 ml containing 50 μM MV reduced with 0.2 ml $S_2O_4^{2-}$ solution, as electron donor systems, respectively. Colour was developed after autoxidation of dithionite and alkaline iodine treatment [11]. Protein concentration was calculated from $A_{280\,nm}$ and $A_{260\,nm}$ values [12]. All spectrophotometric measurements were made at room temperature in a Pye-Unicam SP1700 spectrophotometer with an AR 25 chart recorded or in a SP800 spectrophotometer adapted to accommodate Thunberg cuvettes.

3. Results

Ferrocyanide inhibited both NADH- and MV°-nitrate reductase activities in a concentration-dependant manner (table 1), which was not affected by the time of incubation of enzyme and inhibitor. The inhibition was of the competitive type with respect to nitrate for the NADH-, $FMNH_2$ and MV°-dependant systems (fig.2) with K_i values of 35 μM, 40 μM and 10 μM, respectively.

Reduced DCPIP (59 μM) had no effect on NADH-nitrate reductase activity even when preincubated with the enzyme for 10 min before assay and had no effect on the inactivation which was produced when the enzyme was preincubated with NADH (176 μM) over a similar period before adding nitrate (table 2). The effect of reduced DCPIP on the $FMNH_2$- and MV°-dependant systems was not tested because of the interference of the reduced dye on nitrite estimation.

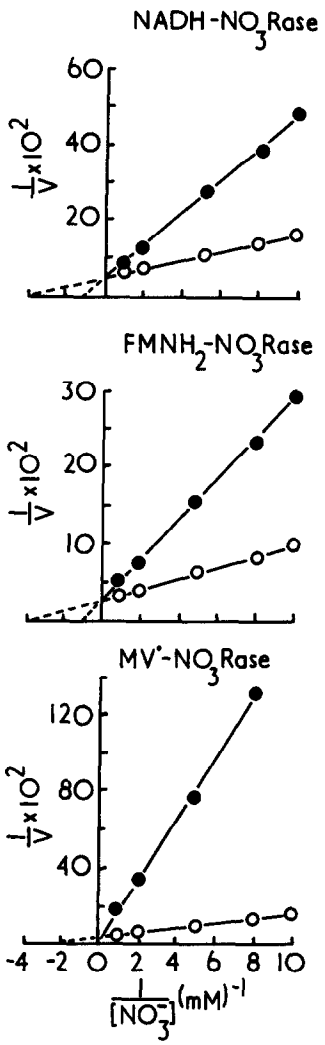

Fig.2. Competitive inhibition by ferrocyanide with respect to nitrate of spinach nitrate reductase activity. Experimental conditions as in the standard assays in section 2, except that nitrate was added as indicated. Reaction velocities are expressed as nmol NO_2^- formed/min. (○) Control, no ferrocyanide; (●) + ferrocyanide (0.1 mM).

Table 1
Effect of ferrocyanide on spinach nitrate reductase activity

Ferro-cyanide (mM)	Inhibition (%)	
	NADH-NO_3 reductase	MV°-NO_3 reductase
1×10^{-3}	6.3	9.0
5×10^{-3}	6.6	19.8
1×10^{-2}	9.2	26.4
5×10^{-2}	19.2	32.1
1×10^{-1}	28.5	36.6
1×10^{0}	44.7	53.8

Experimental conditions as in the standard assays in section 2, except that potassium ferrocyanide was added at the indicated final concentration

Ferrocytochrome *c* had no effect on MV°-dependant nitrate reductase activity. This activity was partially inactivated by preincubation of the enzyme with MV° for 10 min in the absence of nitrate, but this effect was also independent of ferrocytochrome *c* (table 3).

In the presence of NADH as reductant the effects of ferrocytochrome *c* were entirely different (table 4). Ferrocytochrome *c* alone inactivated the enzyme incompletely in a progressive manner during 5 min

Table 2
Effect of reduced DCPIP on spinach NADH-nitrate reductase activity

Addition (before preincubation)	NADH-NO_3-reductase act. (nmol NADH oxidised/min)
None	16.1
NADH	9.3
DCPIP (reduced)	15.9
NADH + DCPIP (reduced)	9.6

Nitrate reductase (0.13 mg) was preincubated in 1.7 ml containing 100 μmol potassium phosphate buffer, pH 7.5, 1 μmol EDTA, either alone or with the addition of the compounds indicated. After 10 min at room temperature the reaction mixture was supplemented with reagents of the standard assay (nitrate alone or nitrate followed immediately by NADH) up to 2 ml. Enzyme activity was determined spectrophotometrically by NADH oxidation. NADH, 0.3 μmol; DCPIP (reduced), 0.1 μmol. DCPIP was reduced chemically by addition of dithionite (8 mg/ml in 50 mM $NaHCO_3$) and excess dithionite removed by auto-oxidation during gentle agitation

Table 3
Effect of reduced methyl viologen (MV°) and ferrocytochrome *c* on MV°-nitrate reductase activity

Addition (before preincubation)	MV°-NO_3-reductase act. (nmol MV° oxidised/min)
None	149
MV°	64
Ferrocytochrome *c*	153
MV° + Ferrocytochrome *c*	68

Nitrate reductase (0.17 mg) was preincubated for 10 min at room temperature in the main compartment or sidearm (as appropriate) of a Thunberg (1 cm light path) cuvette with 200 μmol potassium phosphate buffer, pH 7.5, 2 μmol EDTA, either alone or with 1.6 μmol reduced methyl viologen, or with 0.1 μmol reduced cytochrome *c* or with both in final vol. 2 ml. After repeated evacuation and flushing with N_2 gas, methyl viologen was chemically reduced by addition, into the main cuvette compartment through a rubber GLC septum, of small volumes of sodium dithionite (2 mg/ml in 50 mM $NaHCO_3$) until the methyl viologen was ~90% reduced as shown by increase in $A_{730\,nm}$ as predetermined experimentally. After preincubation the remaining components of the reaction mixture were added by tipping the sidearm. The rate of MV oxidation was followed spectrophotometrically at 730 nm using an extinction coefficient of $\epsilon = 2.3 \times 10^3$ l. mol^{-1} . cm^{-1} (D. P. Hucklesby, personal communication).

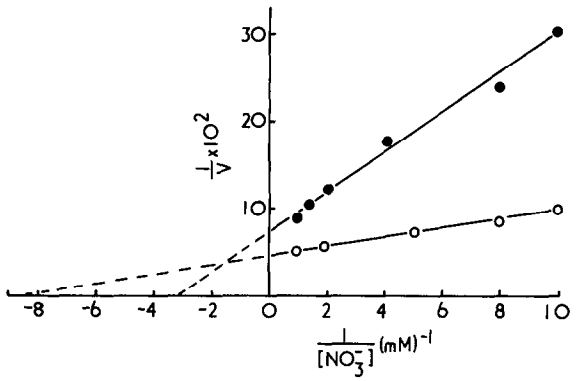

Fig.3. Inhibition of nitrate reductase by ferrocytochrome *c* in the presence of NADH. The assays were carried out after preincubation at room temperature for 5 min of nitrate reductase with NADH and inhibitor. The reaction was started by addition of nitrate at the concentration indicated. Other experimental conditions as in table 2. Reaction velocities are expressed as nmol NADH oxidised/min. (○) Control, no ferrocytochrome *c*; (●) + ferrocytochrome *c* (50 μM).

Table 4
Effect of NADH and ferrocytochrome *c* on NADH-nitrate reductase activity

Addition (before preincubation)	NADH-NO_3-reductase act. (nmol NADH oxidised/min)
None	12.5
NADH	3.8
Ferrocytochrome *c*[a]	6.4
NADH + Ferrocytochrome *c*[a]	0.9
NADH + NO_3^-[b]	12.5
Ferrocytochrome *c*[a] + NO_3^-	8.8
NADH + Ferrocytochrome *c*[a] + NO_3^-[b]	9.9

[a] Cytochrome *c* was reduced as for DCPIP
[b] Rates were measured after a reaction turnover period of 5 min equivalent to the preincubation in the other treatments

The enzyme (0.10 mg) was preincubated at room temperature for 5 min, alone or with the addition of the following compounds: NADH, 0.3 μmol; cytochrome *c* (reduced), 0.1 μmol; potassium nitrate, 20 μmol. Other experimental conditions as in table 2

preincubation before adding NADH or nitrate. Corresponding inactivation by NADH alone during a similar period was greater and the two together acted synergistically when tested according to Webb [13]. We found that the inactivation by ferrocytochrome *c* alone does not tend to completion with time, by contrast with the effects of NADH [2]. The presence of nitrate added at the same time protected the enzyme against the effects of NADH but had only a small, though quite consistent, protective effect against ferrocytochrome *c*. Incubation of the enzyme with both ferrocytochrome *c* and NADH before adding nitrate increased the K_m for nitrate about 3-fold as well as decreasing V_{max} (fig.3), whereas in similar tests, adding NADH and nitrate together after preincubation with ferrocytochrome *c* alone produced no difference in K_m for nitrate and there was no competition or other interference with nitrate binding.

4. Discussion

The effects of ferrocyanide differed from ferrocytochrome *c* and reduced DCPIP in consistently altering nitrate binding for all three donor systems in an apparently simple competitive manner and we suggest therefore that ferricyanide can react directly with the molybdenum site possibly in addition to its reaction as a dehydrogenase electron acceptor. This view avoids the need to postulate electron flow from molybdenum to the dehydrogenase site during reactivation [5] and is in accordance with conclusions derived from the experiments with $Mn^{(III)}$ pyrophosphate [6].

Ferricytochrome *c* either fails to reactivate the inactive form of the enzyme from *Chorella vulgaris* [14] and, as we have found, from spinach or it reactivates only weakly as for the enzyme from *Neurospora crassa* [15]. Thus we believe that ferricytochrome *c* reacts only at the dehydrogenase site and it evidently does not interfere, in the reduced (ferro) state, with nitrate binding in the presence of MV° as reductant.

Reduced DCPIP had no effects on NADH-nitrate reductase and, as we have now confirmed with spinach during this work, DCPIP reactivates inactivated enzyme only incompletely as well as slowly [14,15]. We believe therefore that DCPIP reacts only at the dehydrogenase site and that electron flow from molybdenum to the dehydrogenase site occurs only to a limited extent to produce limited reactivation.

It is therefore of particular interest that ferrocytochrome *c*, which is the reduced form of a naturally occurring dehydrogenase electron acceptor (unlike ferricyanide and DCPIP), influences nitrate binding when the enzyme is reduced by the physiological reductant NADH (unlike MV°). Additionally it can enhance synergistically the inactivation produced by NADH. We therefore believe that ferrocytochrome *c*

has a regulatory function in nitrate reductase which would operate synergistically and progressively under circumstances of nitrate depletion to inactivate the enzyme reversibly and possibly protect it from in vivo denaturation. As there is no effect of ferrocytochrome *c* on nitrate binding when the enzyme is reduced by MV°, the interaction observed when NADH is the reductant is most probably the result of an allosteric change induced by NADH which allows ferrocytochrome *c* to influence nitrate binding indirectly, which might be described as partial competition [13]. The nature of a naturally occurring oxidant substituting for ferricyanide to reactivate the enzyme is of similar interest. We believe that this function might be fulfilled by manganese, deficiency of which causes substantial nitrate accumulation [16].

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