Phytochemistry, Vol. 25, No. 4, pp. 799–802, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00

SOME PROPERTIES OF NITRATE REDUCTASE FROM *SCLEROTINIA SCLEROTIORUM*

M. A. RACHIM and D. J. D. NICHOLAS

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, SA 5064, Australia

(*Received* 23 *July* 1985)

Key Word Index—*Sclerotinia sclerotiorum*; Sclerotiniaceae; nitrate reduction; NADPH-nitrate reductase; MVH-nitrate reductase.

Abstract—Nitrate reductase was purified from *Sclerotinia sclerotiorum* and some of its properties studied. The enzyme (M_r 210 000) catalyses the reduction of nitrate to nitrite utilizing either NAD(P)H, MVH, BVH, $FADH_2$ or $FMNH_2$ as an electron donor. In the NAD(P)H-dependent reaction the addition of FAD was required for maximal activity. The K_m values (μM) for nitrate with a range of electron donors were as follows: NADPH, 33; MVH, 1700; $FADH_2$, 150; and $FMNH_2$, 71. Nitrite inhibited the NADPH linked reaction competitively with respect to nitrate but the K_i for nitrite (90 μM) was greater than the K_m for nitrate (33 μM). These results indicate that nitrite is unlikely to inhibit nitrate reduction under physiological conditions. Azide markedly inhibited both the NADPH- and MVH-dependent reactions, but inhibition by *p*-chloromercuribenzoate, *N*-ethylmaleimide and amytal were more marked when NADPH was the reductant.

INTRODUCTION

Nitrate is an important nitrogen source for plants and microorganisms. Nitrate reductase catalyses the reduction of nitrate to nitrite, the first step in the assimilatory reduction of nitrate to ammonia. The enzyme has been characterized in bacteria, fungi, algae and higher plants [1]. The assimilation of nitrate into amino acids appears to be regulated at the level of nitrate reductase [2].

The assimilatory nitrate reductases from fungi, e.g. *Neurospora crassa*, *Aspergillus nidulans* and *Penicillium chrysogenum* have been characterized, but little information is available about the enzyme from plant-pathogenic fungi. The *Neurospora* enzyme is a molybdo-flavohaemprotein utilizing NADPH as an electron donor [3–11]. However, in some yeast strains NADH can also be utilized.

In this paper the purification as well as some properties of the assimilatory nitrate reductase from a pathogenic fungus *Sclerotinia sclerotiorum* are described.

RESULTS AND DISCUSSION

Nitrate reductase was purified 108-fold with a recovery of 39% by ammonium sulphate fractionation and affinity chromatography. The specific activity of the purified enzyme (fraction 3, Table 1) with reduced methyl viologen (MVH) as the electron donor at the optimum pH (7.5) was 1390 units/mg protein. When NADPH was used as an electron donor, in the presence of exogenous FAD, the specific activity at the optimum pH (7.1) was 549 units/mg protein. The purified enzyme has an M_r of 210 000 determined by gel filtration and contains two dissimilar subunits with M_r of 107 000 and 123 000. The M_r for the enzyme from *S. sclerotiorum* is within the range of the M_r of nitrate reductases from other fungi, *N. crassa* [3], *A. nidulans* [8, 9, 12], *P. chrysogenum* [10] and *Rhodotorula glutinis* [13] but is much smaller than those of nitrate reductases from *Torulopsis nitratophila* [14] and *Candida utilis* [15].

Table 1. Purification of nitrate reductase [the activity was measured with either MVH or NADPH (figures in parentheses) as an electron donor]

Fraction	Activity (unit)	Protein (mg)	Specific activity (units/mg protein)	Purification (-fold)	Recovery (%)
1. Crude extract	3140	265	11.8	—	100
	(1850)		(6.99)		
2. $(NH_4)_2SO_4$, 0–40%	3120	154	20.3	2	99
	(1330)		(8.68)	(1)	(72)
3. Blue A column	1240	0.888	1390	118	39
	(488)		(549.32)	(79)	(26)

1 unit of enzyme activity: 1 nmol NO_2^- produced per min.

Electron donors

Several compounds have been tested as electron donors for the purified nitrate reductase (fraction 3, Table 1). The results in Table 2 indicate that both NADPH and NADH function as reductants, but NADPH was more effective, in contrast to the NADH-dependent nitrate reductases from *Ustilago maydis* [16], *Chlorella vulgaris* [17] and from higher plants [18]. The preference for NADPH over NADH as a reductant for the enzyme has been reported for *N. crassa* [3], *A. nidulans* [12, 19], *P. chrysogenum* [10] and *R. glutinis* [13]. In the nitrate reductase assay using NAD(P)H as an electron donor, FAD is required for maximal activity. The requirement for FAD for the NADPH-linked nitrate reductase is similar to that reported for *A. nidulans* [12] and *T. nitratophila* [14]. There was no activity when FMN was substituted for FAD, but this result is different from that for NADPH-nitrate reductases from other fungi [10, 11, 19] where FMN, to varying degrees can substitute for FAD.

Assimilatory nitrate reductases from other fungi [3, 10, 12, 13] have also been shown to utilize reduced viologen dyes as reductants. In the present work, the most effective electron donor was MV, chemically reduced with $Na_2S_2O_4$. In contrast to NAD(P)H-nitrate reductase, the MVH-linked enzyme showed no dependence on exogenous FAD, in agreement with the results for the enzyme from other fungi [12, 13]. Chemically reduced flavin nucleotides functioned as electron donors; $FMNH_2$ was more effective than $FADH_2$. Similar results were observed for *N. crassa* [3], *P. chrysogenum* [11], *R. glutinis* [13] and *Acinetobacter calcoaceticus* [20].

Table 2. Electron donors for nitrate reductase activity (fraction 3, Table 1)

Electron donors	Activity (%)
1. NADPH	0
2. NADPH, FAD	100
3. NADPH, FMN	0
4. NADH	0
5. NADH, FAD	24
6. NADH, FMN	0
7. $Na_2S_2O_4$	0
8. $Na_2S_2O_4$, MV	242
9. $Na_2S_2O_4$, BV	149
10. $Na_2S_2O_4$, FAD	21
11. $Na_2S_2O_4$, FMN	43

Enzyme activity with NADPH as an electron donor, in the presence of FAD was 551 units/mg protein. When NAD(P)H was used as an electron donor the reaction mixture (in 1 ml final volume) was: 50 mM phosphate buffer (pH 7.1); 1 mM KNO_3; 10 μM FAD or FMN; 0.2 mM NAD(P)H and 25 μl enzyme (11 μg protein). The reaction mixture with MVH, BVH, $FADH_2$ or $FMNH_2$ as an electron donor was 50 mM phosphate buffer (pH 7.5); 10 mM KNO_3; 0.2 mM MV, BV, FAD or FMN, 1 mM $Na_2S_2O_4$ (in 1% w/v $NaHCO_3$) and 25 μl enzyme (11 μg protein).

Kinetic constants for nitrate reduction

The effects of various concentrations of nitrate on enzyme activity with various electron donors were examined. Based on Lineweaver–Burk plots, the K_m values for the substrate with the following reductants were (μM): NADPH, 33; MVH, 1700; $FMNH_2$, 150 and $FADH_2$, 71. The small K_m value for nitrate with NADPH as an electron donor indicates that this reductant functions under physiological condition. The K_m values for NADPH and FAD (in the NADPH-dependent reaction) were 40 and 0.2 μM, respectively, whereas the K_m value for MVH was 1.8 μM.

From double reciprocal plots of the effects of various concentrations of either chlorate or bromate at different levels of nitrate on the MVH-dependent enzyme activity (Fig. 1A and B), it can be seen that both compounds inhibited the reaction competitively with respect to nitrate. The apparent K_i values estimated from Dixon plots [21] were 18.1 mM for chlorate and 15.6 mM for bromate. These K_i values were larger than the K_m value for nitrate, thus these compounds are unlikely to inhibit nitrate reduction *in vivo*.

Nitrite, the product of nitrate reduction, also inhibited NADPH-dependent nitrate reductase competitively, with respect to nitrate (Fig. 2) with a K_i value for nitrite of 90 μM. In *S. sclerotiorum* the K_i value for nitrite was 3-fold greater than the K_m value for nitrate in agreement with the data for the *Chlorella* enzyme [17]. The K_i values for nitrite determined in *R. glutinis* [13] and *A. nidulans* [9] were, however, similar to the K_m values for nitrate indicating that nitrite might well inhibit nitrate reduction *in vivo*.

Effect of inhibitors

Several compounds were tested for their inhibitory effects on nitrate reductase either at pH 7.5 for MVH-dependent activity or at pH 7.1 for the NADPH-linked reaction. The results in Table 3 show that azide strongly inhibited both MVH- and NADPH-dependent enzyme activities; at 0.5 mM NaN_3, the inhibition was similar at about 90%. Similarly, cyanide inhibited these activities, but to a lesser extent. Sulphydryl group inhibitors such as *p*-chloromercuribenzoate (*p*-CMB) and *N*-ethylmaleimide (*N*EM) also restricted enzyme activities. The extent of inhibition by *p*-CMB of the NADPH-dependent activity was similar to that of azide but less for the MVH-linked reaction. Amytal and rotenone restricted the NADPH-dependent reaction.

The primary site of action of azide and cyanide is probably molybdenum [9], when either NADPH or MVH was the electron donor, in agreement with the data for *A. nidulans* [12]. Sulphydryl or flavin inhibitors showed less inhibition with MVH than with NADPH as an electron donor. This is in agreement with the result for the purified enzyme from *N. crassa* [3, 4] where electrons are transferred from MVH to Mo, whereas NADPH donates reducing equivalents first to FAD. The involvement of sulphydryl group(s) in the transfer of electrons from NADPH to the flavin component is in agreement with the data for *N. crassa*.

EXPERIMENTAL

Growth conditions. Sclerotinia sclerotiorum was maintained on Czapek-Dox agar plates. Mycelial discs (1 cm^2) from the outer-

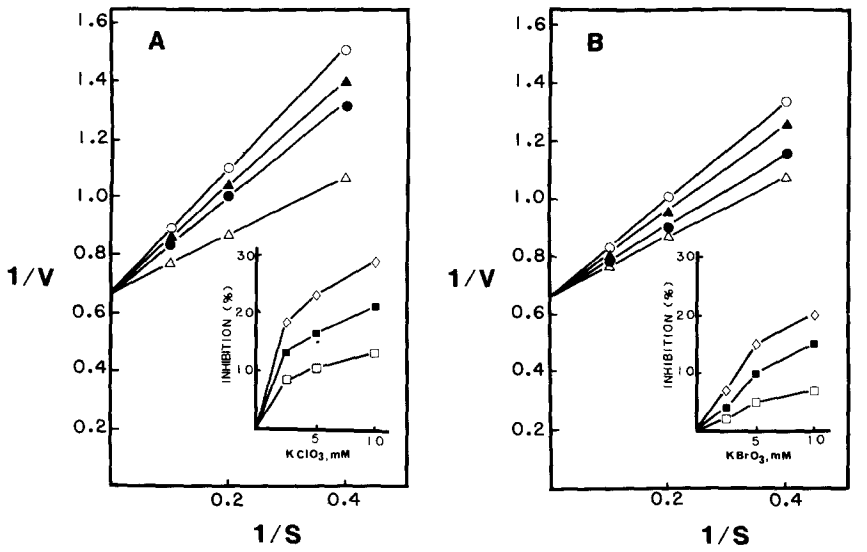

Fig. 1. Double reciprocal plots of the effects of chlorate (A) and bromate (B) on nitrate reductase activity with reduced methyl viologen as an electron donor (fraction 3, Table 1). Concentration of inhibitors: 0 mM, △; 2.5 mM, ●; 5 mM, ▲; and 10 mM, ○. Insets: effect of inhibitors with nitrate at 2.5 mM, ◇; 5 mM, ■; and 10 mM, □. V = activity (μmol NO_2^- produced/min/mg protein); S = substrate (nitrate, mM).

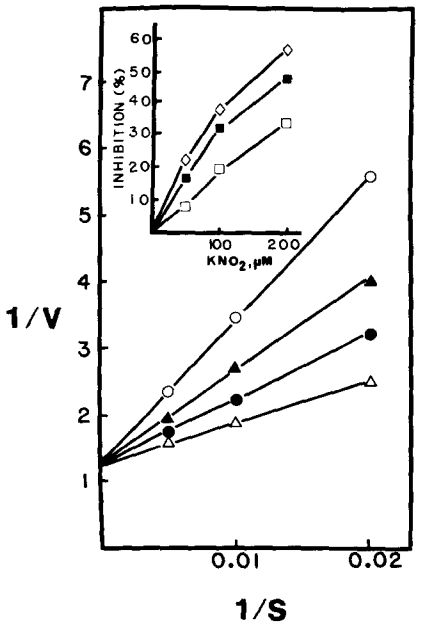

Fig. 2. Double reciprocal plots of the effects of nitrite on nitrate reductase activity with NADPH as an electron donor (fraction 3, Table 1). Concentration of nitrite: 0 μM, △; 50 μM, ●; 100 μM, ▲; and 200 μM, ○. Inset: effect of nitrite with nitrate at 50 μM, ◇; 100 μM, ■; and 200 μM, □. V = activity (μmol NADPH oxidized/min/mg protein). The activity was determined by following the rate of nitrate-dependent oxidation of NADPH spectrophotometrically at 340 nm. S = substrate (nitrate, μM).

Table 3. Effects of inhibitors on nitrate reductase activity (fraction 3, Table 1)

Inhibitors	Final concentration (mM)	Inhibition (%) MVH-NR	Inhibition (%) NADPH-NR
NaN_3	0.50	91	90
KCN	2.00	41	52
p-CMB	0.50	64	90
N-Ethylmaleimide	2.50	30	60
Amytal	0.50	9	45
	1.00	16	—
	2.00	31	—
Rotenone	0.10	—	62

Inhibitors, added to the assay mixtures (see Table 2) at the concentration indicated, were incubated for 10–15 min prior to start reaction.

most growth zone of 4–5 day-old cultures on agar plate grown at 25° were used as inocula. Into 100 ml sterile cultures in 25 ml conical flasks stoppered with cotton wool was added three discs of inocula. After incubating at 30° on a gyratory shaker for 4 days the contents of each flask were transferred into 1 l. conical flasks containing 300 ml of sterile culture medium, and incubation was continued for a further 2 days. The felts, harvested by centrifugation at 9000 *g* for 5 min at 4° in a Sorvall RC-2B refrigerated centrifuge, were washed three times with cold 0.1 M NaPi buffer (pH 7.5) and then blotted between towel paper to remove H_2O. The blotted mycelial pads could be stored at −15° for at least 1 month without loss of enzyme activity.

Preparation of crude extracts. Cell-free extracts were prepared at 4° and subsequent purification of the enzyme was carried out at this temp. Frozen mycelial pads were thawed and homogenized in cold 0.1 M NaPi buffer (pH 7.5) containing 5 mM EDTA, 5 mM cysteine, 10 μM FAD and 10% (v/v) glycerol. The homogenate was passed twice through a French pressure cell at 20 000 psi and then centrifuged at 27 000 *g* for 20 min. The supernatant fraction (S_{27}) was collected and used as a crude extract.

Nitrate reductase assay. Enzyme activity was determined by measuring the production of nitrite from nitrate at 30°. Nitrite was determined by the procedure of ref. [22]. The red azo dye in 1 ml of aliquot was developed by adding 1 ml of 1% (w/v) sulphanilamide in 1.5 M HCl followed by 1 ml of 0.02% (w/v) *N*-1-naphthylethylenediamine dihydrochloride. After standing for 15 min the absorbance at 540 nm was read in a 1 cm cuvette.

When NAD(P)H was used as an electron donor the reaction mixture contained (in 1 ml final vol.): 50 mM NaPi buffer (pH 7.1); 1 mM KNO_3; 10 μM FAD; 0.2 mM NAD(P)H and the enzyme. The reaction was started by the addition of NAD(P)H and stopped after 15 min by adding 0.1 ml M $Zn(OAc)_2$ followed by 1.9 ml 90% (v/v) EtOH. The mixture was centrifuged and nitrite determined in a 1 ml aliquots.

The reaction mixture for nitrate reductase assay with either MVH, BVH, $FADH_2$ or $FMNH_2$ as an electron donor was 50 mM NaPi buffer (pH 7.5); 10 mM KNO_3; 0.2 mM MV, BV, FAD or FMN; 1 mM $Na_2S_2O_4$ and the enzyme. The assay was carried out in test-tubes (10 × 1 cm) fitted with subaseals. The tubes were evacuated via needles inserted in the caps and then flushed with argon for 2 min. The reaction was started by adding $Na_2S_2O_4$ [freshly prepared in 1% (w/v) $NaHCO_3$ under argon] and stopped after 15 min by rapid oxidation of the electron donor system by stirring the reaction mixture in a Vortex mixer.

Protein content. Protein was determined by the method of ref. [23] using bovine serum albumin as a protein standard.

Enzyme purification. Nitrate reductase was purified by the procedure described below. All operations were carried out at 4°. To the crude extract (S_{27}), in 0.1 M NaPi buffer (pH 7.5), solid $(NH_4)_2SO_4$ was slowly added with constant stirring under argon to make 40% $(NH_4)_2SO_4$ satn. During the addition of $(NH_4)_2SO_4$, the pH of the soln was maintained at 7.5 by adding 0.1 M NaOH. After standing in ice for 30 min the mixture was centrifuged at 20 000 *g* for 15 min. The pellet redissolved in the same buffer was dialysed for 4 hr against 3 l. of the buffer and then loaded onto a Matrex Gel Blue A column (100 × 15 mm). Prior to loading the enzyme 50 ml of BSA (2 mg/ml in the buffer containing 0.2 mM EDTA and 0.1 mM DTT) was passed through the column and washed with 150 ml of the same buffer. After loading the enzyme, the column was washed with 20 ml of the buffer containing 0.5 mM ATP and 0.5 mM NADP and then with 0.1 M NaPi buffer (pH 7.5). The enzyme was eluted with the buffer containing 1 mM NADPH. Details of the purification of the enzyme are presented in Table 1.

M_r *determination.* The M_r of the purified enzyme was determined by gel filtration on a Sepharose 6B column, according to the method of ref. [24]. The following proteins were used as markers: ferritin, M_r 440 000; catalase, 232 000; aldolase, 158 000 (from Pharmacia) and albumin, 68 000 (from Sigma).

Electrophoresis. The M_r of the enzyme subunit was determined by polyacrylamide (12.5%, w/v) slab gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulphate. The enzyme soln was boiled for 3 min in the presence of 1% (w/v) SDS and 5% (v/v) β-mercaptoethanol. To facilitate layering, glycerol (5%, v/v) was added to each sample and bromophenol blue was used as a tracking dye. The running buffer used was 0.025 M Tris, 0.192 M glycine and 0.1% w/v SDS. Electrophoresis was performed at a constant current of 20 mA through the stacking gel and then increased to 30 mA in the resolving gel. The tracking dye was allowed to travel *ca* 0.5 cm from the bottom of the gel before termination of electrophoresis. The proteins used as markers were phosphorylase *b* (subunit M_r 94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 000) and L-lactalbumin (14 400).

REFERENCES

1. Hewitt, E. J. (1975) *Ann. Rev. Plant Physiol.* **26**, 73.
2. Beevers, L. and Hageman, R. H. (1969) *Ann. Rev. Plant Physiol.* **20**, 495.
3. Garrett, R. H. and Nason, A. (1969) *J. Biol. Chem.* **244**, 2870.
4. Pan, S. and Nason, A. (1978) *Biochim. Biophys. Acta* **523**, 297.
5. Nason, A. and Evans, H. J. (1955) *Methods Enzymol.* **II**, 411.
6. Nicholas, D. J. D. and Nason, A. (1954) *J. Biol. Chem.* **211**, 183.
7. Kinsky, S. C. and McElroy, W. D. (1958) *Arch. Biochem. Biophys.* **73**, 466.
8. Downey, R. J. (1971) *J. Bacteriol.* **105**, 759.
9. McDonald, D. W. and Coddington, A. (1974) *Eur. J. Biochem.* **46**, 169.
10. Renosto, F., Ornitz, D. M., Peterson, D. and Segel, I. H. (1981) *J. Biol. Chem.* **256**, 8616.
11. Renosto, F., Schmidt, N. D. and Segel, I. H. (1982) *Arch. Biochem. Biophys.* **219**, 12.
12. Minagawa, A. and Yoshimoto, A. (1982) *J. Biochem.* **91**, 761.
13. Guerrero, M. G. and Gutierrez, M. (1977) *Biochim. Biophys. Acta* **482**, 272.
14. Rivas, J., Guerrero, M. G., Paneque, A. and Losada, M. (1973) *Plant Sci. Letters* **1**, 105.
15. Sims, A. P., Folkes, B. F. and Bussey, A. H. (1968) in *Recent Aspects of Nitrogen Metabolism in Plants* (Hewitt, E. J. and Cutting, C. V., eds) p. 91. Academic Press, New York.
16. Lewis, C. M. and Fincham, J. R. S. (1970) *J. Bacteriol.* **103**, 55.
17. Solomonson, L. P. and Vennesland, B. (1972) *Biochim. Biophys. Acta* **267**, 544.
18. Dunn-Coleman, N. S., Smarrelli, J., Jr. and Garrett, R. H. (1985) *Int. Rev. Cytol.* **92**, 1.
19. Cove, D. J. and Coddington, A. (1965) *Biochim. Biophys. Acta* **110**, 312.
20. Villalobo, A., Roldán, J. M., Rivas, J. and Cárdenas, J. (1977) *Arch. Microbiol.* **112**, 127.
21. Dixon, M. and Webb, E. C. (1964) *Enzymes*, 2nd edn. Longmans Green, London.
22. Hewitt, E. J. and Nicholas, D. J. D. (1964) in *Modern Methods of Plant Analysis* (Liskens, Y. F., Sanwal, B. D. and Tracey, M. V., eds) Vol. 7, p. 67. Springer, Berlin.
23. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.
24. Andrews, P. (1970) *Methods Biochem. Analysis* **18**, 1.