Phytochemistry, Vol 23, No 10, pp 2155–2158, 1984
Printed in Great Britain

0031–9422/84 $3 00 + 0 00

REVERSIBLE INACTIVATION OF MAIZE LEAF NITRATE REDUCTASE

CRISTINA ECHEVARRIA, SOFIA G MAURIÑO and JOSE M MALDONADO*

Departamento de Fisiología Vegetal, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain (C E, S G M), *Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Córdoba, Córdoba, Spain (J M M)

(*Revised received* 21 *March* 1984)

Key Word Index—*Zea mays*, Gramineae, maize, nitrate reductase activity, redox regulation

Abstract—Preincubation of maize leaves crude extracts with NADH resulted in a progressive accumulation of nitrite which mimicked a rapid and lineal activation of nitrate reductase Nevertheless, in partially purified preparations it was found that preincubation at pH 8 8 with NADH promoted a gradual inactivation of nitrate reductase At pH 7 5, the enzyme was not inactivated by the presence of NADH alone, but, with the simultaneous presence of a low concentration of cyanide, a fast inactivation took place The NADH-cyanide-inactivated nitrate reductase remained inactive after removing the excess of NADH and cyanide by filtration through Sephadex G-25 However, it could be readily reactivated by incubation with ferricyanide or by a short exposure to light in the presence of FAD Prolonged irradiation caused a progressive inactivation of the photoreactivated enzyme

INTRODUCTION

Nitrate reductase (EC 1 6 6 1) from green algae can exist *in vitro* in two interconvertible forms active and inactive The active form, which corresponds to an oxidized state of the enzyme, is converted into a reduced inactive form when incubated with NADH, the process being markedly enhanced if cyanide or ADP are also present (for review, see [1, 2]) Conversely, the reduced and inactive form is rapidly reactivated when oxidized either by incubation with ferricyanide or by exposure to blue light in the presence of flavins [3, 4]

A similar reversible inactivation of nitrate reductase through a redox mechanism has been demonstrated in higher plants [5–13] Particularly, inactivation either by NADH or NADH and cyanide has been reported for the enzyme of spinach [5–7], rice [8], wheat [9] and pea [10] leaves, and maize scutella [10], this inactivation being reversed by ferricyanide [6–10], blue-light irradiation [3, 4, 9, 11], trivalent manganese ions [12] or peroxidase systems [13] In contrast, other authors have reported that NADH exerts a protective and/or stimulatory effect on nitrate reductase from rice [14, 15] and sorghum [16] leaves, and wheat and maize shoot tips [17] Accordingly, these authors have proposed that, in these plants, the reduced form of the enzyme is active and the oxidized form is inactive

The purpose of this work was to investigate if, *in vitro*, nitrate reductase from maize was inactivated by NADH and reactivated by oxidizing treatments, as found for other higher plant nitrate reductases [3–13]

RESULTS AND DISCUSSION

The effect of NADH on the activity of nitrate reductase from maize was initially studied in crude extracts As shown in Fig 1A, when a crude extract was preincubated at 0° with 3 mM NADH, an apparent activation of nitrate reductase was observed, which was linear from the

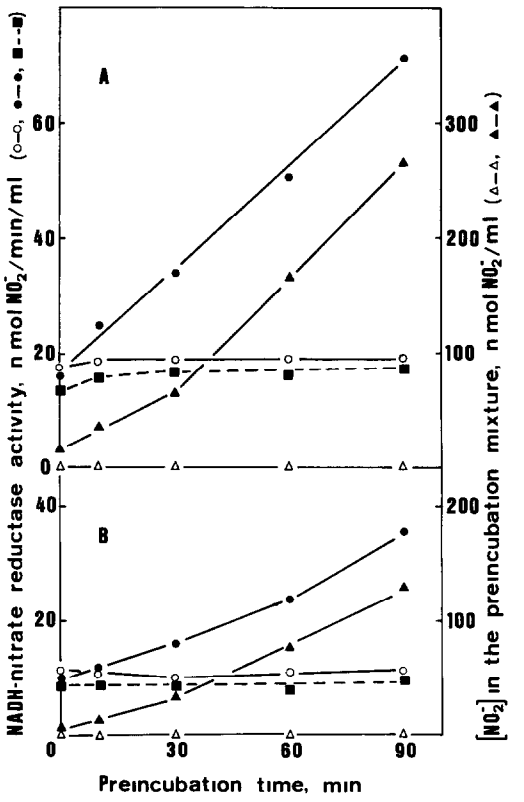

Fig 1 Nitrite appearance and apparent activation of nitrate reductase during preincubation of the crude extract with NADH Undialysed (A) and dialysed (B) crude extracts were preincubated at 0° either in the absence (open symbols) or presence (closed symbols) of 3 mM NADH At the indicated times, two aliquots were taken from each preincubation mixture to measure NADH-nitrate reductase activity (○, ●) and NO_2^- concentration (△, ▲) In those preincubations containing NADH, the true nitrate reductase activity (■) was estimated by subtracting, at each time, the amount of NO_2^- appeared in the preincubation (▲) from the measured nitrate reductase activity (●)

*To whom correspondence should be sent

beginning However, simultaneous determination of nitrite in aliquots taken from the preincubation mixture revealed a progressive accumulation of nitrite in the mixture containing NADH The rate of nitrite appearance was almost equal to the rate of the observed enzyme activation, in such a way that if, at each preincubation time, the amount of nitrite originally present in the aliquot was subtracted from nitrite measured after the activity assay, it became evident that maize nitrate reductase was not appreciably activated by the presence of NADH, in contrast to results reported in rice [14, 15], sorghum [16] and wheat [15, 17]

The fact that nitrite gradually accumulates when the crude extract is preincubated with NADH indicates that maize leaf extracts contain substantial amounts of nitrate which, in the presence of NADH, the physiological electron donor for nitrate reductase [2], would be enzymatically reduced to nitrite In fact, it has been reported [17] that the activity of maize nitrate reductase at 0° was *ca* 33% of that shown at 30° Frequently, the crude extracts have been dialysed in order to remove nitrate [14, 15, 17] Nevertheless, our results demonstrate (Fig 1B) that dialysis in Visking cellulose tubing did not completely remove the nitrate present in the maize leaf extract, as demonstrated by the fact that nitrite was still produced in the preincubation mixture, mimicking, as before, an activation of nitrate reductase by NADH The enzyme activity in the dialysed extract was lower, presumably because inactivation and/or dilution occurred during the prolonged (24 hr) dialysis

In order to examine the *in vitro* redox regulation of nitrate reductase in a nitrate-free preparation, further studies were conducted on partially purified enzyme

As shown in Fig 2, when partially purified nitrate reductase was preincubated at pH 7 5 with 3 mM NADH, inactivation did not take place, at least during the 60 min tested However, nitrate reductase was substantially inactivated (50% in 10 min) when the pH of the preincubation mixture was increased up to 8 8 A dependence of NADH-inactivation on pH has been also observed in *Chlorella* nitrate reductase It has been proposed that ionization of a chemical group of the enzyme, maybe a sulphydryl, is involved in the inactivation process [18]

It has been reported [14–17, 19, 20] that nitrate reductase is very unstable, its activity being protected, and even stimulated, by the presence of NADH In particular, nitrate reductase from maize has been demonstrated to have a half-life of 1 hr at 0° [17] Under our conditions, however, in both crude extracts (Fig 1) and partially purified preparations (Fig 2), nitrate reductase activity retained its original level after 1 hr, presumably because of the protective effect of the cysteine present in the buffer [20] We have also observed [unpublished results] that extraction of maize leaf nitrate reductase in the absence of protectants leads to a low yield of the enzyme activity Nevertheless, inclusion of 3 mM NADH in the extraction medium almost completely prevented the loss of activity These observations suggest that NADH, like cysteine, prevents, during the enzyme extraction, the oxidation of essential sulphydryl groups probably located on the labile NADH-diaphorase moiety [2, 20] It is worth noting that the NADH-diaphorase activity from *Chlorella* nitrate reductase is protected from thermal inactivation by NADH, its activity being even enhanced by the presence of that nucleotide [21, 22] In contrast, the NADH-dependent reversible inactivation of nitrate reductase

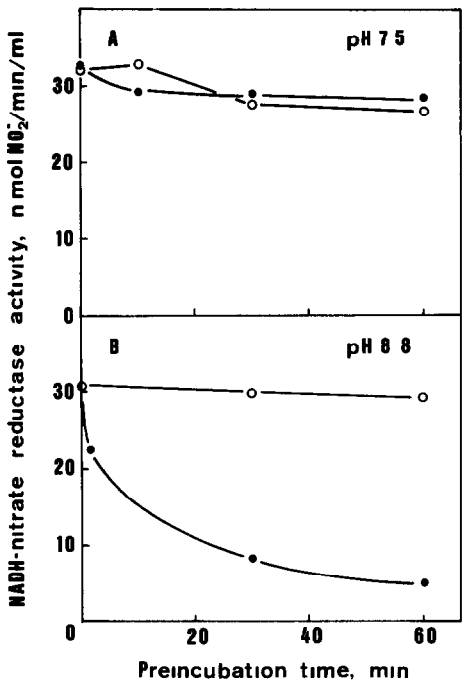

Fig 2 pH-dependent inactivation of nitrate reductase by NADH A nitrate reductase preparation, partially purified as described in the Experimental, was preincubated at 0° either at pH 7 5 or 8 8 both in the absence (○) or presence (●) of 3 mM NADH At the indicated times, aliquots were taken out from the different mixtures for NADH-nitrate reductase assay

affects exclusively its terminal molybdenum-containing component [1, 2, 6–10] Accordingly, we believe that the reported stabilization and activation of nitrate reductase by NADH [14–17] might be rather the result of the aforementioned effect of NADH on the diaphorase activity, moreover, when the experiments were conducted on crude extracts, the appearance of nitrite during preincubation might have also contributed to the observation of an untrue activation, as demonstrated in this work

In green algae, the reductant-dependent inactivation of nitrate reductase is enhanced by the presence of low levels of cyanide [1] Our results show (Fig 3) that inactivation of maize leaf nitrate reductase was also considerably stimulated when NADH and cyanide were simultaneously present in the preincubation system A 50% inactivation occurred in only 1 min In the absence of reductant, cyanide did not affect the enzyme activity Similar inactivation by NADH *plus* cyanide has been observed on nitrate reductase from other higher plants [4, 5, 8–10] It is worth noting that inactivation could readily proceed at pH 7 5 In fact, we have found with nitrate reductase from spinach leaf [23] that, in contrast to NADH-inactivation, NADH–CN^--inactivation proceeds faster at pH 7 5, indicating that the mechanisms of both processes were different In this context, it has been proposed [1, 24, 25] that, under reducing conditions, cyanide can complex with an 'over-reduced' form of the molybdenum component of nitrate reductase, giving rise to the inactivation of the terminal activity of the enzyme

We have observed that inactivation of maize leaf nitrate reductase by NADH *plus* cyanide also affected its terminal

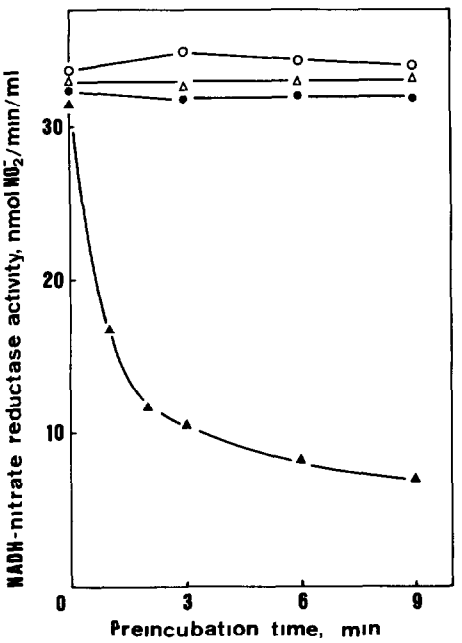

Fig 3 Inactivation of nitrate reductase by NADH *plus* cyanide A sample of partially purified nitrate reductase was preincubated at 0° and pH 7 5 either alone (○) or in the presence of 0 3 mM NADH (●), 5 μM KCN (△) or 0 3 mM NADH *plus* 5 μM KCN (▲) Other conditions as in Fig 2

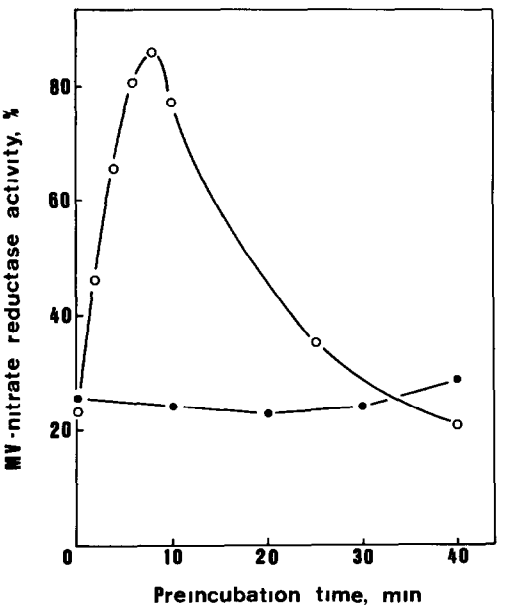

Fig 4 Effects of light on *in vitro* inactivated nitrate reductase A sample of nitrate reductase, treated with NADH and KCN, and subsequently passed through Sephadex G-25, was preincubated under air, and in the presence of 10 μM FAD, either in the dark (●) or under 100 W/m² white light (○) At the indicated times, aliquots were taken to measure MV -nitrate reductase activity Irradiation conditions were similar to those described in ref [29], except that pH was 7 5 Activities are expressed as percentages of the activity of the enzyme before inactivating treatment

activity, assayed with reduced methyl viologen (MV) as electron donor Furthermore, after removing the excess of NADH and cyanide by filtration through Sephadex G-25, nitrate reductase remained largely (*ca* 90%) inactive, which suggests that cyanide was firmly bound to the enzyme molecule Nevertheless, a short (5 min) incubation with ferricyanide (0 3 mM) reactivated the enzyme up to 90% of its original activity In rice [8], however, nitrate reductase was spontaneously reactivated when NADH and cyanide were removed by gel filtration, indicating that NADH–CN^--inactivated nitrate reductase was less stable in rice than in green algae [4, 26], spinach [7] and wheat [9] Reactivation by ferricyanide presumably involves oxidation of the very stable Mo(IV)–CN complex to the unstable Mo(VI)–CN complex, from which cyanide would be spontaneously dissociated [1, 12, 25]

It has been demonstrated that inactive nitrate reductase from *Chlorella* [3, 4], *Chlamydomonas* [27], *Neurospora* [28], spinach [3, 4, 11, 29] and wheat [9] is also rapidly reactivated when exposed to light in the presence of flavins, blue light being more effective than red light Our results show (Fig 4) that irradiation with white light of NADH–CN^--inactivated nitrate reductase from maize leaves in the presence of 10 μM FAD, initially promoted a fast reactivation of its terminal activity Nevertheless, upon longer light exposure, the reactivated enzyme suffered a progressive loss of activity In the dark, FAD did not appreciably modify the original activity We have postulated [4, 11, 29] that the flavin-mediated photoreactivation of nitrate reductase involves the reoxidation of the Mo(IV)–CN complex by light-excited FAD, which seems to have a more positive redox potential than in the ground state Recently we have observed that reactivation of NADH–CN^--inactivated nitrate reductase from spinach can also be achieved by irradiation of the enzyme with red light in the presence of methylene blue, a sensitizer whose redox potential becomes likewise more positive when photoexcited [30] On the other hand, the inactivation (irreversible) which takes place with prolonged irradiation has been demonstrated, in the spinach enzyme, to be caused by singlet oxygen generated through energy transfer from excited triplet-state flavin to molecular oxygen [31]

EXPERIMENTAL

Plant culture Maize (*Zea mays* L) was grown under a 24 hr regime of 12 hr light, 12 hr dark at 25° and 18°, respectively Light (8000 lux) was supplied by intermixed Sylvania 'daylight' and 'cool white' fluorescent lamps supplemented with incandescent bulbs Plants were grown on vermiculite in plastic containers perforated on the bottom to allow free drainage, and were watered on alternative days with a nitrate-type Long Ashton nutrient soln [32]

Enzyme extraction and partial purification Healthy leaves from 3–4 week-old plants were excised, cut in 1 cm-length segments, and ground with sand and grinding medium (5 ml/g leaf fr wt) in a chilled pestle and mortar The grinding medium consisted of 25 mM Pi buffer, pH 7 5, 1 mM EDTA, 10 mM cysteine and 1% (w/v) PVP The homogenate was centrifuged at 37 000 *g* for 15 min and the resulting supernatant (crude extract) was used as enzyme preparation in some expts Where indicated in the Results section, the crude extract was dialysed for 24 hr against the extraction buffer using 1 cm-diameter Visking cellulose dialysis tubings

Partial purification of nitrate reductase was performed by fractional pption with 40% satd $(NH_4)_2SO_4$ After standing for 20 min with gentle shaking, the mixture was centrifuged at 37000 *g* for 10 min The supernatant was discarded and the pellet resuspended in one fifth of the original vol with 50 mM Pi buffer, pH 7 5, containing 0 1 mM EDTA Finally, the resuspended fraction was passed through a Sephadex G-25 column equilibrated with the same buffer All operations were carried out at 4°

Enzyme assays and analytical methods NADH-nitrate reductase activity was estimated by measuring nitrite formed in an assay system containing 0 1 M Pi buffer, pH 7 5, 1 mM EDTA, 10 mM KNO_3, the enzyme preparation and 0 3 mM NADH, in a final vol of 1 ml After 10 min incubation at 30°, the reaction was terminated by addition of 0 1 ml of 1 M barium acetate and 1 9 ml of 96% (v/v) EtOH After vigorous shaking in a Supermixer, the mixture was left to stand for 5 min at 4°, centrifuged at 2300 *g* for 5 min and then nitrite measured on an aliquot from the clear supernatant soln by addition of 1 ml of sulphanilamide reagent followed by 1 ml of *N*-(1-naphthyl)ethylenediamine reagent [33]

MV-nitrate reductase activity was also determined by nitrite formation but using, as electron donor system, 0 1 mM MV reduced by 0 1 ml $Na_2S_2O_4$ (8 mg/ml in 0 1 M $NaHCO_3$) Reaction was stopped by vigorous shaking until the reduced MV was reoxidized

Protein was determined according to the method of ref [34]

Acknowledgements—We wish to thank Professor Losada and collaborators for valuable help and discussions, and Miss C Santos for her secretarial assistance We are also grateful to the Ministerio de Educación y Ciencia, Spain, for a Research Studentship to S G M

REFERENCES

1 Vennesland, B and Guerrero, M G (1979) in *Encyclopedia of Plant Physiology, New Ser* (Gibbs, M and Latzko, E, eds) Vol 6, p 425 Springer, Berlin
2 Guerrero, M G, Vega, J M and Losada, M (1981) *Annu Rev Plant Physiol* **32**, 169
3 Aparicio, P J, Roldán, J M and Calero, F (1976) *Biochem Biophys Res Commun* **70**, 1071
4 Aparicio, P J and Maldonado, J M (1979) in *Nitrogen Assimilation of Plants* (Hewitt, E J and Cutting, C V, eds) p 207 Academic Press, London
5 Relimpio, A M, Aparicio, P J, Paneque, A and Losada, M (1971) *FEBS Letters* **17**, 226
6 Palacián, E, de la Rosa, F, Castillo, F and Gómez-Moreno, C (1974) *Arch Biochem Biophys* **161**, 441
7 Maldonado, J M, Vargas, M A, Mauriño, S G and Aparicio, P J (1981) *Biochim Biophys Acta* **661**, 112
8 Leong, C C and Shen, T C (1979) *Experientia* **35**, 584
9 Aryan, A P, Batt, R G and Wallace, W (1983) *Plant Physiol* **71**, 582
10 Wallace, W (1975) *Biochim Biophys Acta* **377**, 239
11 Roldán, J M, Calero, F and Aparicio, P J (1978) *Z Pflanzenphysiol* **90**, 467
12 Maldonado, J M, Notton, B A and Hewitt, E J (1980) *Planta* **150**, 242
13 Maldonado, J M, Notton, B A and Hewitt, E J (1982) *Planta* **156**, 289
14 Gandhi, A P, Sawhney, S K and Naik, M S (1973) *Biochem Biophys Res Commun* **55**, 291
15 Ramarao, C S, Srinivasan and Naik, M S (1981) *Phytochemistry* **20**, 1487
16 Kadam, S S, Johari, R P, Ramarao, C S and Srinivasan (1980) *Phytochemistry* **19**, 2095
17 Datta, N, Rao, L V M, Guha-Mukherjee, S and Sopory, S K (1983) *Phytochemistry* **22**, 821
18 Moreno, C G, Aparicio, P J, Palacián, E and Losada, M (1972) *FEBS Letters* **26**, 11
19 Tischler, C R, Purvis, A C and Jordan, W R (1978) *Plant Physiol* **61**, 714
20 Hageman, R H and Reed, A J (1980) *Methods Enzymol* **69**, 270
21 Maldonado, J M, Herrera, J, Paneque, A and Losada, M (1973) *Biochem Biophys Res Commun* **51**, 27
22 Maldonado, J M, Pueyo, M C and Chaparro, A (1974) *Rev Real Acad Cien Madrid* **68**, 633
23 Vargas, M A (1983) Ph D thesis, University of Seville, Spain
24 Losada, M (1976) *J Mol Catal* **1**, 245
25 Hewitt, E J and Notton, B A (1980) in *Molybdenum and Molybdenum-containing Enzymes* (Coughlan, M, ed) p 273 Pergamon Press, Oxford
26 Sólomonson, L P (1974) *Biochim Biophys Acta* **334**, 297
27 Azuara, M P and Aparicio, P J (1983) *Plant Physiol* **71**, 286
28 Roldán, J M and Butler, W L (1980) *Photochem Photobiol* **32**, 375
29 Mauriño, S G, Vargas, M A, Aparicio, P J and Maldonado, J M (1983) *Physiol Plant* **57**, 411
30 Julliard, M and Le Petit, J (1982) *Photochem Photobiol* **36**, 283
31 Vargas, M A, Mauriño, S G, Maldonado, J M and Aparicio, P J (1982) *Photochem Photobiol* **36**, 223
32 Hewitt, E J (1966) *Sand and Water Culture Methods Used in the Study of Plant Nutrition*, 2nd edn, Commonwealth Bureaux of Hort, Tech Commun No 22 Farnham Royal
33 Snell, F D and Snell, C T (1949) *Colorimetric Methods of Analysis*, 3rd edn, Vol 2, p 804 D van Nostrand, Reinhold, Princeton, New Jersey
34 Bailey, J L (1967) in *Techniques in Protein Chemistry*, 2nd edn, p 340 Elsevier/North Holland, Amsterdam