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# INACTIVATION OF NITRATE REDUCTASE FROM WHEAT AND RICE LEAVES

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**Key Word Index**—*Oryza sativa*; *Triticum aestivum*; Gramineae; rice; wheat; nitrate reductase; inactivating factor; NADH.

**Abstract**—In fresh leaves, the inactivation of nitrate reductase was rapid at high temperatures as compared to low temperatures. In leaves subjected to freeze–thaw treatment, the loss of enzyme activity was extremely rapid particularly at high temperatures. Pre-incubation with NADH not only protected the enzyme against inactivation, but also stimulated its activity. In dialysed extracts of rice leaves, NADH alone offered some protection while nitrate alone did not protect the enzyme from inactivation. Addition of both NADH and nitrate during pre-incubation enhanced the enzyme activity considerably. It is suggested that stimulation of nitrate reduction by NADH and nitrate may be of physiological significance to the plant, in the sense that in the presence of sufficient supplies of reductant and nitrate, the process of nitrate assimilation would be accelerated.

## INTRODUCTION

Nitrate reductase from higher plants is extremely unstable and is subject to rapid turnover. The activity determined at any given time is the result of the reversible processes of synthesis/activation and degradation/inactivation. Rapid loss of enzyme activity when light-grown plants are transferred to dark has been observed by a number of workers [1]. Jolly and Tolbert [2] attributed this effect to the presence of an inhibitor (MW 31 000) of nitrate reductase in soybean leaves, which was activated in the dark and inactivated in light. However, the identity of the inhibitor as well as its possible physiological significance in different plants is controversial. Wallace [3] suggested that the inactivator from maize root (MW 44 000) is a protease. On the other hand, Yamaya and Ohira [4] found that the inhibitor from rice cells (MW 100 000) did not have any proteolytic activity. Kadam *et al.* [5] reported that purified horse radish peroxidase (MW 40 000) inactivated rice leaf nitrate reductase and isolated a similar factor from the roots of rice seedlings. They also found that pre-incubation with NADH protected the enzyme from inactivation by both horse radish peroxidase and the inhibitor. Yamaya and Ohira [6] showed that pre-incubation of nitrate reductase from rice cells with NADH reversed the inhibitory effect of the inactivating factor. They suggested that the enzyme–inhibitor complex is dissociated by NADH and the inhibitor combines with nitrate reductase only in the oxidized form. Sherrard *et al.* [7] purified one inhibitory and two stabilizing factors from extracts of wheat leaves. Sherrard *et al.* [8] further found that the inhibitory factor was active towards the NADH-nitrate reductase. Yamaya *et al.* [9] observed that the nitrate reductase inactivating protein from corn roots (MW 75 000) was a protease while the one from rice cells (MW 150 000) was not a protease. Both of them inhibited NADH-nitrate reductase but had no effect on methyl viologen-nitrate reductase. While NADH had no effect on the maize inactivator, it reversed the effect of rice cell inhibitor.

In view of these conflicting reports regarding the identity of inhibitors and activating factors of nitrate reductase in leaves, no conclusive evidence is available about the physiological role of these factors. Under *in vivo* conditions, leaf discs are impermeable to large MW metabolites such as NADH and hence, it is difficult to study the interactions between NADH, nitrate reductase and its inhibitors. On the other hand, in leaf extracts, the physiological *in vivo* regulatory mechanism is disrupted and hence the observed interactions could be non-specific without any physiological significance. We have therefore used the freeze–thaw technique [10] for this study, because it is known that after freezing in liquid nitrogen, leaf discs become easily permeable to externally added metabolites [10, 11]. Freeze–thaw assay has been regarded as an intermediate between normal *in vivo* assays and a crude homegenate [11]. Thus, using leaf discs frozen in liquid nitrogen, we report that rapid loss of nitrate reductase activity can be prevented by addition of NADH and nitrate together. We also report that pre-incubation with NADH enhances enzyme activity. These results have further been confirmed by *in vitro* experiments.

## RESULTS

Freezing in liquid nitrogen completely abolished the *in vivo* nitrate reductase activity without affecting the *in vitro* activity in wheat leaves (Table 1). The basic difference between these two methods lies in the fact that whereas *in vivo* activity is dependent upon endogenous generation of NADH, *in vitro* activity requires addition of exogenous NADH. Thus, freezing and thawing did not affect the enzyme as such, but the NADH generating system was disrupted. Subbalakshmi *et al.* [12] also reported that when rice leaves were frozen in liquid nitrogen *in vivo*

Table 1. Effect of freeze–thaw treatment on nitrate reductase activity in wheat leaves

| Treatment | Nitrate reductase activity | |
|---|---|---|
| | *in vivo* | *in vitro* |
| Fresh leaves | 2.1 | 5.2 |
| Frozen leaves | nil | 5.2 |
| Frozen leaves + NADH (1.25 mM) | 3.1 | — |

Enzyme activity in fresh and frozen leaves was determined as described in methods. The activity was expressed as $\mu$mol $NO_2^-$ formed/g fr. wt in 30 min.

nitrate reductase activity was completely destroyed and that it could be restored by supplying NADH. After freeze–thaw treatment, the leaf discs become permeable to exogenous NADH [10, 11].

In fresh wheat leaves, *in vivo* nitrate reductase activity was quite stable when they were incubated at temperatures between 0° and 10° (Table 2). However when the temperature was increased to 20° or above, rapid loss of *in vivo* enzyme activity was observed. This could be due to the inactivation of nitrate reductase at higher temperatures. As mentioned above, leaves frozen in liquid nitrogen lost the capacity to generate the NADH required for *in vivo* nitrate reduction. The activity was therefore assayed by adding NADH exogenously. Thus, NADH supply was not a limiting factor in frozen leaves and in this respect, it resembled *in vitro* assay in leaf extracts. Thus, in effect, a rapid loss of *in vitro* enzyme activity was observed particularly at higher temperatures, and at 30° the enzyme activity was completely destroyed (Table 2).

The time course of inactivation of nitrate reductase in wheat and rice leaf discs subjected to freeze thaw treatment is given in Table 3. When wheat leaf discs were incubated at 0°, the rate of loss of activity was rather slow and after 1 hr, only 44% of the activity was lost. The destruction of the enzyme activity was extremely rapid at 30° and in 20 min, almost all the activity disappeared. In the case of rice leaf discs, although considerable loss of activity was observed, the enzyme appears to be more stable than in wheat leaves. In a 2 hr incubation less than 50% of the activity was lost. The higher initial activity (zero time) in frozen leaves as compared to the activity in fresh leaves is obviously due to the fact that in the former, NADH is supplied in adequate quantities exogenously, while in fresh leaves, the reduction of nitrate is dependent upon endogenous generation of NADH, which appears to be a limiting factor.

Table 2. Effect of pre-incubation temperature on the stability of nitrate reductase in wheat leaves

| Pre-incubation temperature | Nitrate reductase activity (*in vivo* assay) + NADH | |
|---|---|---|
| | fresh leaves | frozen leaves |
| Control | 3.20 | 3.40 |
| 0° | 3.28 | 1.92 |
| 10° | 2.96 | 1.92 |
| 20° | 0.72 | 1.00 |
| 25° | 0.59 | 0.44 |
| 30° | 0.37 | nil |
| 40° | 0.37 | nil |

Intact leaves isolated from 20-day-old wheat seedlings grown in 15 mM $KNO_3$ were incubated at different temperatures for 20 min. *In vivo* nitrate reductase activity was assayed as described in the Experimental. Leaf discs prepared from another lot of leaves were placed in thin walled dry test tubes and immersed in liquid nitrogen for 2 min. The frozen leaf discs were allowed to thaw. These leaf discs were incubated at different temperatures for 20 min and then nitrate reductase activity was assayed after supplying 1.25 mM NADH. The enzyme activity was expressed as $\mu$mol $NO_2^-$ formed/g fr. wt in 30 min.

In Table 4, comparison of stability of nitrate reductase in leaf extracts at 0° and 30° has been shown. It is seen that the rates of inactivation of the enzyme in leaf extracts (Table 4) and in leaves subjected to freeze–thaw pretreatment (Table 3) are almost the same. As a result of these treatments, nitrate reductase probably comes into contact with the inactivator due to the disruption of cells Inactivation of the enzyme at 0° suggests that to some extent, non-enzymatic reactions are involved. It is likely that phenolic compounds released from vacuoles may be inactivating nitrate reductase by forming complexes with the enzyme. Phenolics form a hydrogen-bonded complex with the enzyme resulting in decreased activity [13]. Grasmanis and Nicholas [14] found that nitrate reductase in extracts of apple roots was inactivated by phenolic compounds, and addition of polyvinyl pyrrolidone during extraction protected the enzyme. Inactivation at 30° could be due to both chemical and enzymatic reactions. The rate of inactivation at 30° is considerably greater than at 0°.

It was also seen (results not shown) that the presence of NADH during pre-incubation of wheat leaf discs at 0° provided some protection against slow inactivation. Activity after 30 min was 50% greater with an optimal concentration of 0.75 mM than with its omission, and induced a small activation (28%) compared to the control, which was not subjected to pre-incubation. Further, it was observed that the presence of the optimal concentration of NADH (0.75 mM) induced progressive activation in wheat leaf discs for a period up to 30 min of pre-incubation at 0°.

In the experiments described so far with fresh leaves, frozen leaves and leaf extracts, nitrate, NADH and other metabolites which are likely to affect enzyme activity are always present. In order to study the effect of NADH and nitrate, together or separately, it was therefore necessary to remove these substances from the extracts. In Table 5, the stability of nitrate reductase in dialysed extracts of rice leaves in the presence of NADH and nitrate is shown. It is seen that dialysed extract also lost activity during incubation at 30° almost at the same rate as the undialysed extract. Addition of nitrate alone to the dialysed extract did not offer any protection against inactivation, but at 30°, NADH alone offered some protection against inactivation in the dialysed extract. It is interesting to note that when dialysed extracts were pre-incubated at 0° with NADH and nitrate together, the enzyme activity was stimulated by *ca* 100%. The effect of pre-incubation with NADH and nitrate at 30° could not be studied because at this temperature considerable quantities of nitrite were released to the medium as a result of the activity of nitrate reductase.

Table 3. Time course of inactivation of nitrate reductase in wheat and rice leaves

| Pre-incubation time (min) | Nitrate reductase activity: wheat leaves (frozen leaves + NADH) at 0° | wheat leaves (frozen leaves + NADH) at 30° | rice leaves at 30° in fresh leaves | rice leaves at 30° in frozen leaves + NADH |
|---|---|---|---|---|
| 0 | 5.2 | 5.2 | 3.1 | 6.6 |
| 10 | 4.9 | 0.9 | — | — |
| 20 | 4.0 | 0.3 | — | — |
| 30 | 3.8 | 0.1 | 3.6 | 6.6 |
| 40 | 3.2 | nil | — | — |
| 60 | 2.9 | nil | 2.2 | 5.7 |
| 120 | — | — | 1.6 | 3.8 |
| 180 | — | — | 1.6 | 3.8 |

Wheat leaf discs were initially subjected to freeze–thaw treatment and then incubated at 0° and 30° for different time intervals. Rice leaves subjected to freeze–thaw treatment were incubated only at 30° for different time intervals. The activity in frozen leaves was compared with the *in vivo* activity in fresh rice leaves. Nitrate reductase activity was then assayed as in Table 2. Activity was expressed as μmol $NO_2^-$ formed/g fr. wt in 30 min.

## DISCUSSION

Study of inactivation of nitrate reductase under physiological conditions in intact leaves is difficult because of the non-permeability of leaf cells to externally supplied metabolites. On the other hand, investigations in cell-free extracts or with purified enzyme and inactivating factors may not necessarily have any relevance to the situation in intact leaves, as explained in the introduction. We have therefore used the freeze–thaw technique which avoids the drawbacks of both these methods because the leaf discs become permeable to metabolites such as NADH. By using this method, it is now shown that pre-incubation with NADH not only protected the enzyme against inactivation, but also stimulated the activity. Conflicting reports are available about the effect of NADH on nitrate reductase activity. It has been postulated that nitrate reductase from bacteria [15], yeast [16], algae [17–22] and higher plants [20] exists in two interconvertible forms; inactive, when reduced by NADH or dithionite and active, when oxidized by nitrate, oxygen or ferricyanide. According to these reports, pre-incubation with NADH inactivates the enzyme by reduction. Exactly contrary results were obtained by Gandhi *et al.* [24], who showed that nitrate reductase from rice seedlings is specifically activated by pre-incubation with NADH. Protection of nitrate reductases from its inactivating factor by pre-incubation with NADH has also been reported by Kadam *et al.* [5, 25], Yamaya and Ohira [6], Yamaya *et al.* [9] and Matsumoto *et al.* [13].

Table 4. Stability of Nitrate reductase activity in wheat leaf extracts at 0° and 30°

| Pre-incubation time (min) | Nitrate reductase activity at 0° | at 30° |
|---|---|---|
| 0 | 1.6 | 1.6 |
| 10 | 1.4 | 0.4 |
| 20 | 1.1 | 0.2 |
| 30 | 1.0 | nil |
| 40 | 1.0 | nil |
| 60 | 0.9 | nil |

Extracts from 20-day-old wheat leaves were incubated at 0° and 30° for different time intervals. *In vitro* nitrate reductase activity was determined as described in Experimental. Activity was expressed as μmol $NO_2^-$ formed/g fr. wt in 30 min.

Inactivation of nitrate reductase was observed during pre-incubation, both at 0° and 30° (Tables 2 and 3). The rate of inactivation at higher temperatures was extremely rapid and may involve chemical as well as enzymatic reactions. In rice leaf extracts made free of nitrate and NADH by dialysis, it was observed that NADH alone offered some protection against inactivation at 30° while nitrate alone was ineffective (Table 5). When NADH and nitrate were added together during preincubation at 0°, enzyme activity was considerably stimulated (Table 5).

Dunn-Coleman and Pateman [26] showed that nitrate reductase from *Asperigillus nidulans* which is NADPH specific, is regulated by the pentose phosphate pathway, which generates NADPH. *In vitro* studies with cell-free extracts also indicated that decreased levels of NADPH resulted in rapid loss of nitrate reductase activity, which could be reversed by the addition of NADPH. When NADPH level is low, the enzyme is inactivated by oxidation. According to the authors, regulation of activity of nitrate reductase by NADPH provides a fast acting and reversible control mechanism.

Dunn-Coleman and Pateman [27] further suggested that nitrate reductase activity is subject to redox control, the enzyme being inactivated when there is insufficient NADPH and reversibly activated when increased amounts of NADPH are generated. It was clearly demonstrated that inactivated nitrate reductase could be reactivated by the addition of NADPH [28]. Our results indicate that a similar mechanism is probably operating in plant enzyme which is NADH specific.

Ping-pong (bi-bi) reactions proposed by Maldonado *et al.* [29] for spinach nitrate reductase showed that NADH binds and $NAD^+$ is released, before nitrate binds to the enzyme. Thus inactivation of nitrate reductase by NADH

Table 5. Stability of nitrate reductase activity in dialysed rice leaf extracts

| | Extract | | Dialysed extract | | Dialysed extract | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | $+NO_3^-$ | | +NADH | | $+NO_3^- +$NADH |
| hr | 0° | 30° | 0° | 30° | 0° | 30° | 0° | 30° | 0° |
| 0 | 3.2 | 2.0 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 |
| 1 | 3.2 | 1.7 | 2.2 | 1.7 | 2.2 | 1.7 | 2.2 | 1.4 | 4.0 |
| 2 | 2.0 | 1.1 | 2.5 | 1.1 | 2.5 | 1.1 | 2.2 | 1.7 | 4.4 |
| 3 | 2.5 | nil | 2.5 | 0.7 | 2.2 | nil | 2.0 | 1.7 | 5.5 |
| 4 | 2.6 | nil | 2.3 | 0.4 | 2.2 | nil | 2.0 | 1.7 | 5.8 |

Rice leaf extracts were extensively dialysed with 0.01 M phosphate buffer, pH 7.5 at 0–4°. The dialysed extracts were tested to see that no nitrate remained and incubated at 0° and 30° either alone or in the presence of nitrate or NADH, or both, as shown. After various time intervals, the nitrate reductase activity was assayed as described in the Experimental. The activity was expressed as $\mu$mol $NO_2^-$ formed/g fr. wt in 30 min.

does not take place in the presence of nitrate. Tischler *et al.* [30] showed that nitrate reductase is more stable in the presence of both NADH and nitrate, the availability of which is a factor influencing the nitrate reductase activity. It appears that the enzyme is stable during turnover compared to the resting state with either NADH alone or $NO_3^-$ alone.

The observed stimulation of nitrate reductase by NADH and nitrate could be physiologically advantageous to the plant. Assimilation of nitrate is strongly stimulated in light when NADH supply is also abundant. If sufficient nitrate is available at the same time, nitrate reductase will be activated by the combined effect of NADH and nitrate, which will help in its rapid assimilation.

## EXPERIMENTAL

Rice (*Oryza sativa* L.) (CV.P 2-21) and wheat (*Triticum aestivum* L.) CVR-306 seedlings were grown in pots and periodically irrigated with 15 mM $KNO_3$. Nitrate reductase assay in frozen leaves was done as described by Subbalakshmi *et al.* [12]. Leaf discs from 15–20-day-old seedlings were placed in dry thin walled test tubes and immersed in liquid $N_2$ for 2 min. After thawing at room temp., activity was determined in 0.1 g leaf discs in the presence of 2 ml of 0.1 M Pi buffer, pH 7.5 containing 10 mM $KNO_3$ and 1.25 mM NADH. The tubes were incubated at 30° in the dark for 30 min. At the end of the incubation period, the reaction was stopped by 0.1 ml of 0.1 M zinc acetate and 1.9 ml of 70% EtOH. The contents were centrifuged and nitrite was determined in suitable aliquots by adding 1 ml each of 1% sulphanilamide in 1 M HCl and 0.01% *N*-(1-naphthyl)-ethylenediamine dihydrochloride. *In vivo* [31] and *in vitro* [32] activities of nitrate reductase were determined according to standard methods.

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