ACTIVATION OF NITRATE REDUCTASE FROM RICE SEEDLINGS BY NADH

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SUMMARY

Rice leaf nitrate reductase was specifically activated by preincubation both at 0° and 25°, with low concentrations of NADH. The nucleotide acted as a positive effector of the enzyme after a time lag of 20 min. NADPH, $FMNH_2$ and NAD were without any effect.

INTRODUCTION

Nitrate reductase (E.C.1.6.6.1) from Chlorella vulgaris has been shown to be inactivated by reduction with NADH (1). The inactive enzyme could be activated by oxidation with ferricyanide (1,2). Similarly the active form of Chlorella fusca nitrate reductase could be converted into its inactive form by reduction with 0.3mM NADH in the presence of 0.3mM ADP and the inactivated enzyme could be completely reactivated by oxidation with ferricyanide (3). In this paper we report exactly contrary behaviour of nitrate reductase from rice seedlings, which is significantly activated by preincubation with NADH both in the presence or absence of ADP. It is suggested that specific activation of the enzyme by NADH is involved.

MATERIALS AND METHODS

Fifteen day old rice seedlings (variety Improved Sabarmati) grown in water were provided with Hoagland solution containing nitrate. After 24 h further growth in the presence of the inducer, nitrate reductase was extracted from the leaves as described earlier (4,5). The enzyme extract was dialysed for 24 h at 0° with frequent

Table 1. Effect of preincubation with different nucleotides on the activity of nitrate reductase in green and etiolated seedlings.

Nucleotide added during preincubation	Nitrate reductase		
	Green Seedlings		Etiolated Seedlings
	0°	25°	0°
Control	5.6	2.5	0.2
NADH	9.0	9.6	0.2
ADP	5.7	-	0.2
NADH + ADP	7.9	-	0.2
NADPH	5.5	4.0	0.2

Enzyme extracts from 15 day old green and etiolated seedlings supplied with nitrate for 24 h, were preincubated with 0.4mM concentrations of different nucleotides for 1 h at 0°. Subsequently NADH - nitrate reductase activity was assayed at 30°. In the green seedlings effect of preincubation at 25° with NADH and NADPH was also studied.

changes of 0.01M potassium phosphate buffer pH 7.0. The dialysed extract was preincubated with NADH, ADP or other nucleotides and nitrate reductase activity was subsequently assayed with NADH as the electron donor by standard methods (4). Nitrate reductase activity represents umoles of NO_2^- formed/g tissue in 20 min.

RESULTS

Activity of nitrate reductase from green seedlings was stimulated to the extent of 60% by preincubation at 0° with NADH. ADP at 0.4mM had no effect when added alone. Preincubation with NADH and ADP together enhanced the activity by 42% (Table 1). It was shown earlier that ADP at much higher concentrations of 5 and 20mM when added to the assay mixture, inhibited rice leaf nitrate reductase by 20 and 50% respectively (6). In etiolated seedlings nitrate reductase activity was negligible and preincubation with NADH had no effect,

Table 2. Effect of NADH concentration on the activation of nitrate reductase.

NADH in the preincubation mixture (mM)	Nitrate reductase
Control	4.4
0.04	8.8
0.20	10.9
0.40	13.5

Enzyme from green seedlings was preincubated at 25° with different concentrations of NADH for 40 min. NADH - nitrate reductase was then assayed as described in the text.

thus indicating that an inactive precursor of the enzyme which could be activated by NADH, was not present in the dark grown seedlings (Table 1).

When the enzyme extract was preincubated without any additions at 25°, about 60% activity was lost. However, in the presence of NADH, the activity was enhanced four fold and was even considerably higher than in the original enzyme kept at 0° (Table 1). At 0°, NADH was specifically required for stimulation and NADPH was ineffective. At 25°, however, NADPH also exhibited a slight effect. This could very well be attributed to the activity of NADPH phosphatase, which could function at 25° but not at 0°. The presence of this enzyme which generates NADH from NADPH in leaves had earlier been reported (7).

Results in Table 2 show that 0.4mM NADH in the preincubation mixture produced the maximum stimulation but even as little as 0.04mM NADH enhanced the enzyme activity two fold. The latter quantity of NADH is extremely small as compared to the quantity

used for the assay of nitrate reductase which is 0.4mM. Physiological concentrations of NADH in plant cells may be 0.006mM as NADH or 0.02 to 0.06mM as NAD + NADH (8).

The time course of stimulation of nitrate reductase by preincubation with NADH indicated a lag phase of about 20 min, after which a rapid reaction was observed (Fig 1). $FMNH_2$ was without any effect. Similarly it was seen that NAD alone was without any effect. At constant NADH concentration of 0.2mM, decreasing the NADH/NAD ratio from 2.5 to 0.25 did not alter the stimulation of nitrate reductase observed with preincubation with NADH alone.

DISCUSSION

Rice leaf nitrate reductase responds to preincubation with NADH in a manner exactly contrary to that reported for the Chlorella enzyme. In the latter, the enzyme is stated to be inactivated by reduction with NADH and the inactive enzyme is rapidly activated by oxidation with ferricyanide or slowly with oxygen (2). The rice enzyme was specifically activated by NADH preincubation both at 0° and 25°. NADPH, $FMNH_2$ and NAD were without any effect. Slight stimulation by NADPH at 25° could be an indirect consequence of the formation of NADH by an active phosphatase.

NADH-nitrate reductase complex consists of two moeities, one of which functions as NADH - cytochrome c reductase or diaphorase and the other containing molybdenum which reacts with nitrate. The specificity of the first site for NADH as the electron donor and the involvement of -SH groups are now well established (9). Since the Chlorella enzyme is also NADH specific, the physiological significance of its inactivation by preincubation with NADH is not clear. In fact, it has been reported that the NADH diaphorase from

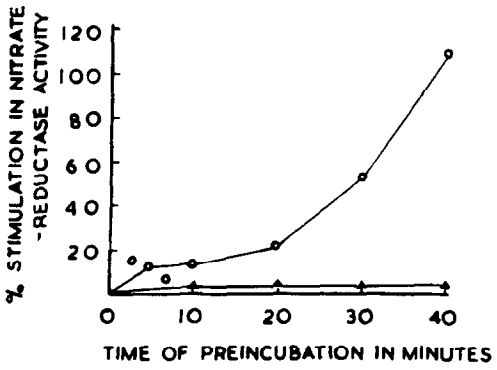

Fig. 1. Time course of activation of nitrate reductase by preincubation with NADH or $FMNH_2$. The enzyme was preincubated with 0.2mM NADH or 0.35mM $FMNH_2$ at 25° for different time intervals and then assayed after addition of 0.4mM NADH and 10mM NO_3^-. Per cent stimulation over control is indicated in the figure. In the control the enzyme alone was preincubated at 25° for 40 min without NADH.

O—O NADH

▲—▲ $FMNH_2$

Chlorella is protected from thermal inactivation unspecifically by a variety of nucleotides including NADH, NADP, NAD, ADP, AMP and GDP but the nitrate reductase is specifically inactivated by NADH (3). On physiological consideration, the activation of the rice enzyme by NADH makes sense. It has been reported that inhibition of spinach nitrate reductase by 10mM ADP is reversed by thiol and NADH concentration in the system (8). Nitrate reductase is extremely unstable both *in vitro* and *in vivo*. Its protection and activation by NADH is expected to enhance the rate of nitrate assimilation. In the light reaction of photosynthesis, reduced pyridine nucleotides are generated. Thus in addition to the direct role of light in the induction of the enzyme (4), the photosynthetic reactions also further activate it by generating NADH. In etiolated seedlings, since the enzyme is not synthesized, the question of its activation by NADH does not arise.

The mechanism by which NADH acts as a positive effector of

nitrate reductase is not clear (Fig 1). It appears likely that the site of the enzyme complex which accepts electrons from NADH and in which -SH group is involved, is specifically activated by NADH.

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