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EFFECTS OF NADH AND THIOL COMPOUNDS ON WHEAT LEAF NITRATE REDUCTASE

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Abstract—The initial activity of wheat leaf nitrate reductase was depressed on inclusion of the following thiol compounds; dithiothreitol, dithioerythreitol or mercaptoethanol, but not cysteine and glutathione. This thiol effect simply resulted from an interference with the chemical determination of nitrite. Preincubation of the enzyme with NAD^+ and these thiols enhanced the inhibition of nitrate reductase activity. This effect was mediated by NADH production by the thiol reduction of NAD^+. The inactivation by NAD^+ in the presence of thiol compounds which was enhanced by cyanide ions could be reversed by ferricyanide, as has been observed previously for NADH-mediated inactivation of nitrate reductase.

INTRODUCTION

Nitrate reductase (NR, EC 1.6.6.1) from higher plants and micro-organisms can be reversibly inactivated by NAD(P)H in the absence of nitrate [1, 2]. The inactivation is mediated by the binding of cyanide to molybdenum (Mo) of the reduced NR complex. The inactive enzyme can be readily reactivated by either ferricyanide or exposure to blue light [1, 2]. A similar type of NR inactivation by thiols, viz. dithioerythreitol (DTE) and mercaptoethanol (at relatively higher concentrations, 20 mM and 50 mM respectively) has been reported for the enzyme from *Chlorella* [3] and spinach [4]. This thiol inactivation was enhanced by NAD^+. It has been proposed that the reduction of a specific regulatory site on the enzyme, by either NAD(P)H or thiols results in its inactivation [4]. We have re-investigated this proposed inactivation of NR by thiol compounds, based on the observations that very low levels ($\simeq 10\ \mu M$) of NADH mediate the inactivation of NR [2] and thiol compounds, viz. DTE and DTT reduce NAD^+ [5]. Because relatively higher concentrations of thiols were used in the earlier studies [3, 4], it was necessary to check whether they interfered with the chemical test for nitrite [6].

Table 1. Effects of thiol compounds on nitrite determination by the diazotization method

Concentration of thiols (mM)	Percentage yield of nitrite		
	DTT	DTE	Mercapto-ethanol
0.0	100	100	100
0.1	97	98	97
0.2	95	94	95
0.5	80	80	86
1.0	68	71	78
2.0	52	53	61

Nitrite (50 nmol) in 1 ml 0.1 M K-phosphate buffer (pH 7.5) containing various levels of the thiol compounds, was tested for colour production at 540 nm after adding 1 ml of 1% (w/v) sulphanilamide (in 1.5 N HCl) followed by 1 ml of 0.01% (w/v) aqueous solution of *N*-1-naphthylethylenediamine dihydrochloride.

RESULTS AND DISCUSSION

Inhibitory effects of thiols on NR activity and nitrite test

When a range of thiol compounds were tested for their effects on the NR activity of wheat leaves, it was found that the enzyme activity was decreased in the presence of either dithiothreitol (DTT), dithioerythritol (DTE) or mercaptoethanol (depending upon their concentrations used), whereas cysteine and glutathione were without effect. On testing the same levels of thiols on the chemical test for nitrite, a similar loss of colour was observed for DTT, DTE and mercaptoethanol (Table 1) whereas cysteine and glutathione were without any effect. With 2 mM final concentration (10 mM during preincubation with enzyme) of either DTT or DTE in the reaction mixture, the NR activity (apparent) was reduced by approximately half. Thus the inhibitory effect of thiols is due to an interference with the nitrite test and not the nitrate reductase enzyme itself, as reported earlier [3, 4]. The inhibition of NR activity of the spinach enzyme by mercaptoethanol [6] was claimed to be associated with a further chemical reduction of nitrite by the thiol compound, resulting in a loss of nitrite. We find, however, that this effect is simply due to the interference of thiols with the diazotization reaction for nitrite. This inhibitory effect was overcome by sparging the thiol nitrite mixture with air for 30 min before adding the diazotization reagents.

Moreover, on increasing the concentration of the sulphanilamide reagent (from 1% to 20% w/v) the inhibitory effect of DTT or mercaptoethanol (1 mM) on the chemical test for nitrite was also reversed.

Effects of thiol compounds on NR activity in the presence of NAD^+

The partially purified enzyme from wheat leaves was relatively stable after a 1 hr incubation at 25° in the absence of thiols (only 15% loss) (Table 2). It should be noted that DTT, DTE and mercaptoethanol (each at 10 mM) inhibited the initial activity of NR (zero time) because of interference with the nitrite test as discussed earlier. On preincubation for 1 hr, these thiols consistently stabilized the enzyme (Table 2). These results are contrary to the previous findings with *Chlorella* and spinach enzymes [3, 4] where on incubation with DTE and mercaptoethanol there was a significant loss of NR activity, and this was associated with the inactivation of enzyme by thiols. This discrepancy may be due to the inhibitory effects of thiols on the nitrite test.

When the enzyme was preincubated for 1 hr with NAD^+ along with the appropriate thiol compound (2 mM), a substantial loss in NR activity occurred (Table 2). Similar results have been reported by Moreno and Palacian [3] and Palacian *et al.* [4]. This enhanced inactivation of NR by NAD^+ in the presence of these thiols was observed only after preincubation (1 hr) with the enzyme. It had no effect, however, on the initial NR activity which confirms that NAD^+ in the presence of these thiols resulted in some inactivation of the enzyme during preincubation, as has been reported before [3, 4].

Reduction of NAD^+ by DTT and mercaptoethanol

The inactivation of NR by the more electronegative thiols, viz. DTT, DTE [5, 7] and mercaptoethanol [6], in the presence of NAD^+ could result from NADH generated by thiol oxidation. The data in Table 3 indicate that DTT and mercaptoethanol can support the NADH dependent nitrite production (detected only after overcoming the interfering effect of thiols by adding higher concentrations of sulphanilamide; viz. 20%, instead of 1%) by NR when NAD^+ was included in the reaction mixture. Thus the inactivation of NR by DTT and mercaptoethanol in the presence of NAD^+ is probably mediated by NADH.

Comparison of thiol and NAD^+-mediated NR inactivation with that of NADH

The inactivation of NR by NADH is greatly enhanced by cyanide (10^{-6} M) and the inactive enzyme complex can be reactivated by oxidation with ferricyanide [1, 2]. The complete reactivation is achieved by brief treatment (5 min) with ferricyanide [2]. The inactivation of NR by NAD^+ in the presence of DTT was also enhanced by cyanide ions during a pre-incubation period (1 hr), and the inactive enzyme could then be reactivated by ferricyanide (Table 4), as has been reported for NADH-mediated inactive NR [2]. This provides further proof that NAD^+-mediated inactivation of NR in the presence

Table 2. Effects of thiol-compounds on the activity and stability of wheat leaf nitrate reductase

Thiol compounds	NR Activity (nmol NO_2^- produced/min/g fr. wt.) Initial	Percentage loss over initial activity on preincubation $-NAD^+$	$+NAD^+$
Nil	326	15	4
Dithiothreitol	156	8	94
Dithioerythritol	161	10	89
Cysteine	328	3	12
Glutathione	321	3	2
Mercaptoethanol	179	11	54

A wheat leaf NR sample (0–40% ammonium sulphate fraction, see Experimental) was tested with the thiol compounds shown and NAD^+ as indicated. The initial activity (without preincubation) and percentage loss after preincubation with and without NAD^+ for 60 min at 25° are shown. During preincubation with enzyme, the concentrations of thiols and NAD^+ were 10 mM and 1 mM respectively, while in the assay mixture the concentration of these compounds were 2 mM and 0.2 mM respectively.

Table 3. Effects of NAD^+ on nitrate reduction in the presence of dithiothreitol and mercaptoethanol

Additions	nmol NO_2^- produced
NAD^+ (1 mM)	0.00
DTT (10 mM)	0.20
Mercaptoethanol (20 mM)	1.20
DTT (10 mM) + NAD^+ (1 mM)	8.5
Mercaptoethanol (20 mM) + NAD^+ (1 mM)	8.5

The reagents were incubated with a partially purified NR (0–40% ammonium sulphate fraction) and NO_3^- (5 mM) for 3 hr at 25°. A 20% sulphanilamide in 1.5 N HCl was used for determination of nitrate.

Table 4. Inactivation of wheat leaf nitrate reductase by DTT ± NAD and its reactivation by ferricyanide

Treatment	NR Activity (%) No ferricyanide	Ferricyanide (0.6 mM)
Control	100	68
DTT (5 mM)	100	78
DTT (5 mM) + CN^- (1 μM)	95	73
DTT (5 mM) + NAD^+ (5 mM)	25	71
DTT (5 mM) + NAD^+ (5 mM) + CN^- (1 μM)	13	71

The NR enzyme was preincubated for 1 hr at 25° with the reagents shown. After passing through a Sephadex G-25 column to remove thiols, the enzyme samples were treated with ferricyanide (0.6 mM) for 5 min before assay of NR activity at 25° for 15 min. 100% ≡ 320 n mol NO_2^- produced/min/g fr. wt.

of thiols is due to the generation of NADH. The effect of DTT alone on apparent NR activity was not affected by cyanide ions (Table 4).

Thus, we conclude that there is no evidence for a thiol-mediated inactivation of nitrate reductase. The thiol interference with the nitrite test resulted in an apparent loss of NR activity. The further inactivation associated with NAD^+ in the presence of electronegative thiol compounds is mediated by NADH produced by the enzymatic reduction of NAD^+ by thiol compounds.

EXPERIMENTAL

Plant material and enzyme extraction. Wheat seeds (*Triticum aestivum* L. cv. Bindawarra) were grown as described previously [2] and a leaf sample taken at 12 days for enzyme extraction. The NR sample (pptd with 40% $(NH_4)_2SO_4$ satn) was prepared in 50 mM K-phosphate (pH 7.5) containing 0.1 mM DTT and 10 μM FAD as described in ref. [2].

Enzyme assay. NADH-NR assay was done as described in ref. [2]. The inactive enzyme was treated with ferricyanide [2] prior to assay.

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REFERENCES

1. Vennesland, B. and Guerrero, M. G. (1979) in *Encyclopedia of Plant Physiology* (Pirson, A. and Zimmerman, M. H., eds) Vol. IV, p. 425. Springer, Berlin.
2. Aryan, A. P., Batt, R. G. and Wallace, W. (1983) *Plant Physiol.* **71**, 582.
3. Moreno, C. G. and Palacian, E. (1974) *Arch. Biochem. Biophys.* **160**, 269.
4. Palacian, E., De la Rosa, F., and Moreno, C. G. (1974) *Arch. Biochem. Biophys.* **161**, 441.
5. Cleland, W. W. (1964) *Biochemistry* **3**, 480.
6. Walender, M. (1978) *Physiol. Plant.* **43**, 242.
7. Holmgren, A. (1981) *Trends Biochem. Sci.* **1**, 26.
8. Hoagland, D. R. and Arnon, D. I. (1950) *Calif. Agric. Expt. Stn. Circ.* 347.