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SENSITIVITY TO DTNB OF NADH-GLUTAMATE DEHYDROGENASE FROM THE LEAVES OF BEAN SEEDLINGS

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; bean leaves; glutamate dehyrogenase, dithio*bis*nitrobenzoic acid (DTNB) sensitivity; enzyme regulation.

Abstract—Supply of 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB) to excised bean leaves or to enzyme preparations inhibited NADH-glutamate dehydrogenase activity. The inhibition was more pronounced for enzyme from light-grown leaves than from dark-grown leaves. Further, the inhibition increased with increase in NADH concentration in the assay mixture and was little affected by enzyme concentration. The enzyme from light-grown leaves is more sensitive to inhibition by DTNB than the enzyme from dark-grown leaves.

INTRODUCTION

Several light dependent enzymes are known to be regulated by thiol compounds [1, 2]. Glutamate dehydrogenase (GDH) from various plant species is also modulated by thiol binding agents [3–5] and among various agents tested DTNB appeared to be the most potent enzyme inhibitor in *Agave* [5]. In a preliminary investigation, we found that NADH-GDH from dark-grown leaves differed from the GDH from light-grown leaves in some regulatory aspects. Further experiments were performed to see if the two enzymes from the two leaf sources (light and dark) differed towards their sensitivity to DTNB.

RESULTS AND DISCUSSION

In vitro effects of some thiol modulating compounds on NADH-GDH activity in leaf samples from either light- or dark-grown seedlings were studied, to evaluate their possible involvement in light/dark effects (Table 1). Inclusion of 0.33–3.33 mM reduced glutathione (GSH) in the assay mixture inhibited enzyme activity slightly in both samples, the inhibition increasing with increase in GSH concentration. Dithiothreitol (DTT) had little effect on the activity of dark-enzyme while it slightly increased the activity of light-enzyme. DTNB, on the other hand, inhibited activity of both enzymes and the inhibition was complete at 0.33 mM DTNB. At concentrations lower than this, the inhibition of light-enzyme was higher than that of dark-enzyme.

Supply of 0.5 mM DTNB to excised leaf segments from dark or light grown seedlings for 1 hr inhibited enzyme activity (data not shown). Inhibition was more pronounced in leaf segments from light-grown seedlings than those from dark-grown seedlings.

The enzyme activity from light- or dark-grown samples increased linearly with increase in enzyme concentration (Fig. 1). Inclusion of 0.08 mM DTNB at each enzyme concentration in the assay mixture inhibited enzyme activity, the percent inhibition being little affected by concentration of enzyme. However, inhibition of light enzyme by DTNB was more severe than that of dark-one at each enzyme concentration.

The inhibition of enzyme activity by DTNB was strongly dependent upon NADH concentration in the assay mixture. As the concentration of NADH increased, the inhibition also increased (Table 2). At each concentration of NADH, the inhibition of light-enzyme was higher than that of dark-enzyme.

Table 1. Effect of thiol modulating compounds on *in vitro* NADH-GDH activity in leaf samples from light- or dark-grown seedlings

Thiol modulator, mM		Enzyme activity, relative to control	
		Dark	Light
None (control)		100	100
GSH	0.33	93	87
	1.66	81	77
	3.33	78	73
DTT	0.33	91	117
	1.66	97	123
	3.33	92	104
DTNB	0.08	95	66
	0.17	68	38
	0.33	0	0

NADH-GDH activity in the leaves from dark- and light-grown seedlings watered with nutrient solution containing 10 mM NH_4Cl was assayed in the presence of the desired concentration of the modulator as indicated. The control level of enzyme activity was 13.5 ± 1.4 units for dark and 7.2 ± 0.2 units/mg protein for light-enzyme.

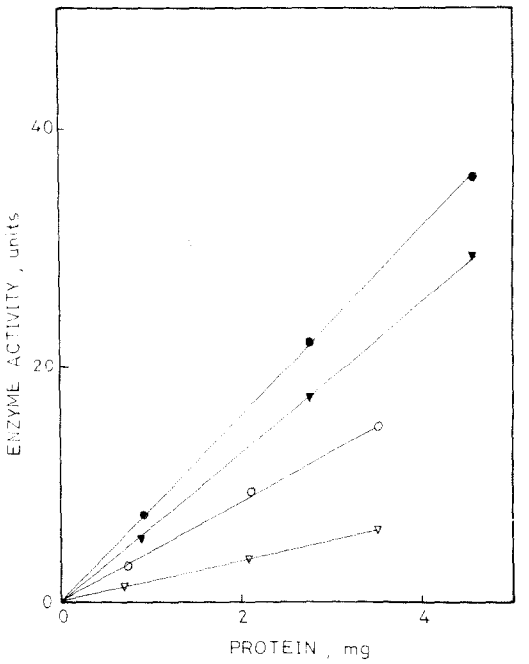

Fig. 1. Effect of protein concentration on enzyme activity from light- or dark-grown samples in the absence or presence of DTNB. NADH-GDH activity in preparation from light- or dark-grown samples was assayed at different enzyme concentrations either in the absence or presence of 0.08 mM DTNB added to the assay mixture. Open circles, light-enzyme minus DTNB; open triangles, light-enzyme plus DTNB; closed circles, dark-enzyme minus DTNB; closed triangles, dark-enzyme plus DTNB.

The results suggest that the enzyme contains free sulphydryl groups at the active centre [1], which are apparently more abundant in the light-enzyme than in dark-enzyme. The inhibition by DTNB does not seem to be time dependent, as addition of DTNB to the enzyme preparation inhibits the enzyme activity immediately. Significant inhibition during DTNB supply to excised leaves is also observed without any considerable lag period. Percent inhibition of both enzymes by DTNB is little affected by enzyme protein concentration. It is likely that the number of –SH residues likely to be affected by the presence of DTNB is limited and the concentration of DTNB used is able to bind with all of them even at higher concentrations of the protein. However, inhibition of both enzymes by DTNB increases with NADH concentration in the assay mixture. It may be postulated that enzyme first forms a complex with NADH and then this complex is acted upon by DTNB to block free sulphydryl groups. Obviously, the degree of inhibition will depend upon the availability of enzyme-NADH complex, which would increase with increase in NADH concentration for a given enzyme level.

EXPERIMENTAL

Seeds of *Phaseolus vulgaris* cv. Rajmah were surface sterilized with 0.1% $HgCl_2$ for 1 min and then washed thoroughly with H_2O. Seedlings were raised in small plastic pots containing acid washed sand, either in continuous darkness or in light of *ca* 65 W/m^2 radiant flux density, supplied by a mixture of fluorescent tubes and incandescent bulbs, at 25 ± 2°. They were watered daily with modified half strength Hoagland's soln containing 10 mM NH_4Cl as sole nitrogen source. Primary leaves from 7-day-old seedlings were used for the extraction of the enzyme.

Extraction and purification of enzyme. The enzyme was extracted using pestle and mortar from the freshly harvested leaves in the extraction medium containing 0.05 M NaPi buffer, 2 mM EDTA, 1 mM $CaCl_2$, 0.1% mercaptoethanol and 0.5% Triton-X 100. The ratio of the plant material to extraction medium was 1:4. The homogenate was centrifuged at 20000 *g* for 15 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to bring 30% satn. The protein precipitated was removed by centrifugation and more $(NH_4)_2SO_4$ was added to the supernatant to 55% satn. The ppt thus obtained was removed by centrifugation and dissolved in 1 ml of 0.05 M NaPi buffer (pH 7.4) containing 2 mM EDTA. The protein soln was then filtered through a column (25 × 1 cm) of Sephadex G-75 and eluted by the same buffer. The two fractions (4th and 5th) containing maximum enzyme activity were pooled and used as enzyme preparation.

Enzyme assay. NADH-GDH activity was assayed at 28 ± 2° by observing the decrease in absorbance at 340 nm for 5 min, using a double beam (Varian) spectrophotometer, by the method of ref. [6]. The unit of enzyme activity is the number of nmol of NADH oxidized/min.

Protein estimation. Protein in the enzyme preparation was

Table 2. *In vitro* inhibition of NADH-GDH from either dark or light grown seedlings by DTNB at different concentrations of NADH

	Enzyme activity, units/mg protein					
	Dark			Light		
NADH (μM)	−DTNB	+DTNB	Percent inhibition	−DTNB	+DTNB	Percent inhibition
16.5	6.5 ± 1.1	5.6 ± 1.0	14	4.4 ± 0.13	2.7 ± 0.77	39
33.0	9.9 ± 1.0	6.4 ± 1.5	35	7.3 ± 0.21	3.3 ± 1.4	55
66.0	15.8 ± 0.9	7.8 ± 1.8	51	9.2 ± 0.9	3.0 ± 1.6	67
132.0	17.9 ± 0.36	5.1 ± 2.9	72	9.7 ± 0.15	1.3 ± 0.6	87

NADH-GDH enzyme from light- and dark-grown seedlings was assayed in the absence and presence of 0.17 mM DTNB at various concentrations of NADH using saturating concentrations of ammonium sulphate (200 mM) and 2-oxoglutarate (6.66 mM).

estimated by the method of ref. [7] after precipitation with TCA.

The data presented are the averages of at least three replicate experiments with ± s.e. in the tables.

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