THE REGULATION OF NITRATE REDUCTASE ACTIVITY FROM SPINACH (*SPINACEA OLERACEA* L.) LEAVES BY THIOL COMPOUNDS IN THE PRESENCE OF ADENOSINE-5'-DIPHOSPHATE

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1. Introduction

The concept of a carbon-nitrogen balance in plants stimulated speculation [1–3] that nitrate reductase (EC 1.6.6.1) may be controlled by kinetic mechanisms associated with photosynthesis or carbohydrate metabolism. Kinetic regulation is indicated by the discovery [4] that nitrate reductase activity *in vivo* may be less than that found after extraction. ADP is a negative effector of nitrate reductases from tomato [5] and spinach [6] leaves. The mechanism involves competition with NADH and non-competitive inhibition both in a non-linear manner [6]. Inhibition of the tomato enzyme was erratic [5] and ageing of the spinach enzyme caused desensitization [6]. We report that inhibition by ADP is reversibly determined by the thiol and NADH concentrations in the system.

2. Materials and methods

NADH (sodium salt) was obtained from C.F. Boehringer GmbH, Mannheim, ADP (sodium salt) and glutathione (GSH) from Sigma Chemical Co. Ltd, London; mercaptoethanol from Koch-Light, Calne, Wilts.; cysteine hydrochloride (CSH) and thioglycollic acid from B.D.H., Poole, Dorset; G-50 Sephadex from Pharmacia (G.B.) Ltd, London. Other reagents were analytical reagent grade. Thiols, NADH and ADP were dissolved freshly in 0.075 M Tris-HCl buffer, pH 7.5, using demineralized water shown to contain less than 0.01 ppm heavy metals [7].

Spinach was grown in sand culture with a 16 hr photoperiod and a nitrate nutrient [7]. Chilled leaves (200 g) were blended 1 min at 0° in 1:1 w/v 0.1 M Tris-HCl, pH 7.5, containing 1 mM EDTA and 1 mM cysteine hydrochloride (CSH). The extract was centrifuged 30 min at 50,000 *g*. Protein was precipitated by 45% saturation ammonium sulphate, centrifuged 20 min at 6000 *g* and redissolved in extracting buffer. The solution (30 ml) was freed from CSH and EDTA on a column of G-50 Sephadex (250 ml) equilibrated in 0.1 M Tris-HCl, pH7.5. Activity was assayed at 27° as nitrate-dependent oxidation of NADH measured in a Unicam SP800 recording spectrophotometer with a programmed four-cell changer. Velocity and NADH concentrations were determined from tangential measurements and changes in absorbance using the extinction coefficient 6.22 mM^{-1} cm^2 at 340 nm. Nitrite production was stoichiometrically equivalent to NADH oxidation (±5%) and was determined at intervals in a duplicate system using the alkaline iodine procedure [8] to prevent interference by NADH. Endogenous NADH oxidase activity was less than 3% of normal nitrate reductase activity. The reaction mixture at pH 7.5 contained, in addition to enzyme, the following (mM) in 4 ml; sodium nitrate, 6; NADH, 0.12; Tris-HCl, 75; ADP, 0 or 10; and thiols as indicated.

3. Results and discussion

In the absence of ADP the initial velocity with saturating NADH remained practically constant until NADH (K_m 2.5 μM) was almost completely oxidized, and was essentially independent of GSH up to 1 mM; fig. 1. In the presence of ADP but without GSH the

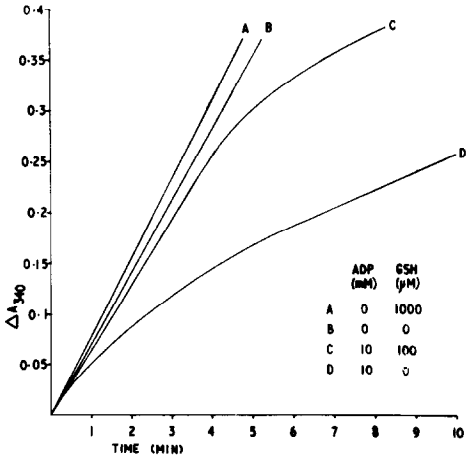

Fig. 1. Characteristic patterns of change in absorbance at 340 nm with time during oxidation of NADH by nitrate and nitrate reductase in normal and ADP-inhibited systems with or without GSH. Reaction conditions as described in the text. A: Uninhibited + 1000 μM GSH. B: Uninhibited with GSH. C: Inhibited by 10 mM ADP + 100 μM GSH. D: Inhibited by 10 mM ADP without GSH.

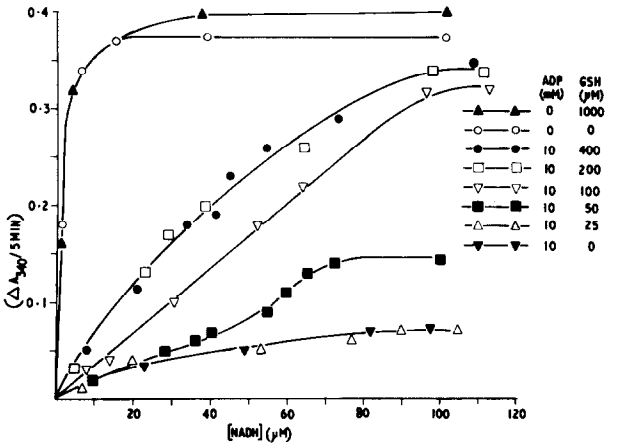

Fig. 2. Michaelis Menten plots of velocity against NADH concentration (uncorrected for slight 1st order denaturation with reaction time) for different GSH concentrations in inhibited and uninhibited nitrate reductase systems. Reaction conditions as described in the text and concentrations of ADP and GSH as shown.

initial velocity declined over a lag period of 2 to 7 min before inhibition was fully established in a way resembling the effects of cyanide on ceruloplasmin [9] and on spinach nitrate reductase [10]. It then remained nearly constant until NADH concentration approached the K_m (38 μM approx.) of the inhibited system (fig. 1). The lag occurred at all NADH concentrations tested between 20 and 200 μM. It was shortened when enzyme and ADP were pre-incubated between 10 and 100 min at 22° before starting the reaction with NADH and was prolonged when ADP was added to an already functioning enzyme. In the presence of ADP and sufficient GSH ($\geq$100 μM in fig. 2) the kinetics changed in several respects. The inhibition by 10 mM ADP when NADH was initially saturating (120 μM) was substantially reversed but as shown by steadily decreasing velocity (fig. 1) it developed progressively as NADH concentration (K_m 40 μM approx.) decreased (fig. 2). The obvious lag observed with ADP alone before inhibition was stabilized (fig. 1) disappeared. Recorder traces coincided when regions corresponding to similar NADH concentrations attained after different periods of time from various initial concentrations were superimposed. The lag was therefore abolished and not prolonged by GSH and the steady fall in velocity when GSH and ADP were both present was a function of decreasing NADH. Decreased ADP was less inhibitory but relationships with GSH were similar.

The effect of thiol concentration was complex (figs. 2 and 3). At 25 μM GSH the inhibition remained

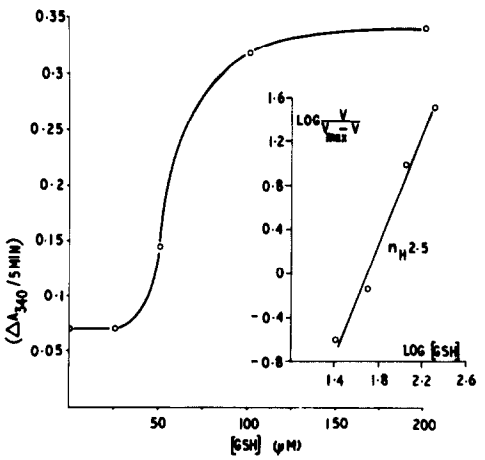

Fig. 3. Relationship between velocity at 100 μM NADH of inhibited system (with 10 mM ADP) and concentration of GSH. Inset: Hill plot for GSH; n = 2.5. Reaction conditions as described in the text.

severe. At 200 μM GSH the non-competitive inhibition was largely reversed. The Michaelis and Menten type kinetics with a high K_m showed that the competitive aspect of ADP inhibition [6] remained. At intermediate GSH concentrations biphasic (quasi sigmoidal) kinetics prevailed fo NADH especially when this exceeded 40 μM. The relationship between GSH concentration and velocity at 100 μM NADH (fig. 3) was also sigmoidal with a Hill plot coefficient of 2.5. In our opinion these results are not consistent with the action of separate isoenzymes having different kinetics. Mercaptoethanol, thioglycolate and CSH had similar effects but concentrations producing pronounced reversal of inhibition were between 300 and 700 μM. Maximal reversal (nearly 100%) occurred with CSH. This alone, of the thiols used, also caused oxygen depletion in the cuvette, by a non-enzymic reaction involving ADP and NADH oxidation without nitrite formation. This was allowed to terminate before starting the assay by adding enzyme. Although GSH did not promote non-enzymic NADH oxidation, the concentrations needed for substantial reversal of inhibition varied in different experiments in a manner related to dissolved oxygen. Effective concentrations ranged from > 200 μM for fully aerated solutions to about 100 μM after purging with argon. The thiol effect was specific to the inhibitory mechanism and was reversible. Enzyme still containing CSH added during extraction was not appreciably inhibited but Sephadex treatment restored the sensitivity to ADP. The reversal of inhibition occurred on adding CSH to enzyme extracted in its absence. The activity of Sephadex-treated preparations in the absence of ADP was not significantly influenced by GSH (figs. 1 and 2), or by CSH. Omission of CSH during extraction had relatively little effect (5 to 20%) on uninhibited activity of spinach preparations compared with its importance for several species [2, 3, 11, 12].

Two forms of control might be inferred from fig. 2, namely thiol-independent competitive inhibition by ADP when NADH concentrations approximate to the normal K_m, and thiol-dependent release mainly of the non-competitive aspect [6] of ADP inhibition when NADH is nearly saturating. Concentrations of NADH in cells or chloroplasts may be 6 μM as NADH [13] or 20 to 60 μM as NAD + NADH [14, 15]. Concentrations of ADP in spinach attain 40 to 250 μM in dark and decrease only about 40% in light [15, 16]. For physiological control by ADP for which inhibition is non-linear (K_i 0.1 mM at 10 mM) [6] compartmentation, different kinetics or additional mechanisms seem necessary. Chloroplasts prepared by methods which preserve the outer double membrane have nitrate reductase [17, 18]. We find [10] that Class 1 chloroplasts [19] prepared in Tris buffers which are known to destroy the outer envelope [20] retain nitrite reductase (60,000 daltons) [21] and ferredoxin (11,000 daltons) but have no nitrate reductase (150,000 daltons) [12]. The activity of our preparations can be partially sedimented after 20 min at 200,000 *g*, suggesting membrane-bound enzyme located possibly in the chloroplast outer envelope. Similar location [22] of an ATPase might facilitate the concentration of ADP in the compartment. A thiol-dependent reversible activation of nitrate reductase when compartmented relative to ADP as negative effector would have features analogous to the probable control of the dark-inactivated oxygen sensitive pyruvate-phosphate dikinase [23].

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