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NADH: NITRATE REDUCTASE ACTIVITY RESTORATION BY RHODANESE

UMBERTO TOMATI*, GIOVANNI GIOVANNOZZI-SERMANNI*, SILVESTRO DUPRÈ† and CARLO CANNELLA†

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* Laboratory of Radiobiochemistry and Plant Ecophysiology, National Research Council, Rome, Italy;
† Institute of Biological Chemistry, University of Rome, Rome, Italy.

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Abstract—The NADH: nitrate reductase from durum wheat leaves was inactivated by cyanide and its activity restored by thiosulphate and beef kidney rhodanese. Rhodanese and thiosulphate, added to NADH–nitrate reductase before cyanide treatment protected NADH–nitrate reductase activity. No oxidizing agent was required for the protection or restoration of cyanide treated NADH–nitrate reductase.

INTRODUCTION

NADH–nitrate reductase (EC 1.6.6.1) is a cytosol molybdoenzyme [1] whose activity seems to be under metabolic control [2–4]. The enzyme may exist in an inactive form, which is slowly converted to the active one by oxidizing agents [5–7]. Cyanide is a strong inhibitor of NADH–nitrate reductase in the presence of reduced pyridinenucleotide [8–10], the inhibition being removed by ferricyanide [2,4,11].

Rhodanese (thiosulphate: cyanide sulphurtransferase EC 2.8.1.1), a widely distributed enzyme present also in plants, [12–14] transfers sulphur from thiosulphate to nucleophilic acceptors, like cyanide. This paper presents data about the ability of thiosulphate and mammalian rhodanese to protect NADH–nitrate reductase and to restore activity after cyanide inactivation.

RESULTS

NADH–nitrate reductase was extracted from wheat leaves either with phosphate buffer containing EDTA and cysteine or in the presence of 4 mM $NiCl_2$: for both preparations, no differences were found in reductase activity. Nitrate was not added during the purification procedure and the endogenous nitrate concentration in leaves was *ca* 30 μg/g fr. wt.

By plotting NADH–nitrate reductase activity against log of cyanide concentration, straight lines were obtained. With 10^{-4}M KCN, 83% inactivation was observed; when, in addition rhodanese and excess thiosulphate are present, inactivation was reduced to 52%. When rhodanese was added with excess thiosulphate to the NADH–nitrate reductase–cyanide complex at different levels of inactivation, activity was restored within a few minutes to the extent observed when rhodanese was present at t_0. Thiosulphate or sulphide in the concentration range 10^{-2}–10^{-4}M, did not show any restorative or protective effects when added to the enzyme–cyanide complex in the absence of rhodanese.

Inactivation by cyanide was not eliminated by gel filtration on Sephadex G-25 of the enzyme–cyanide complex or by exhaustive dialysis against 0.1 M Pi buffer pH 7.6. Neither thiocyanate, at the same molar concentration as the cyanide, nor crystalline rhodanese in absence of thiosulphate, or in the sulphur-free form, restored or protected the nitrate reductase against cyanide inactivation (Table 1).

DISCUSSION

Cyanide is a well known metallo-enzyme inhibitor and its action on molybdo-enzymes has been studied [15–18]. In some cases, like milk xanthine oxidase, it is known that cyanide inhibition is due to cyanolysis of persulphide groups, with free thiocyanate formation. Inactivated enzyme can be largely reactivated by incorporation of sulphide in the protein [17]. For other molybdoenzymes, however, EPR studies suggest that cyanide forms complexes with the molybdo moiety [15–17].

According to Stiefel [19] the reduction of nitrate to nitrite is due to the molybdo moiety of NADH–nitrate reductase, and Mo (IV) is oxidized to Mo (VI). Reduction

Table 1. Protective effect of rhodanese against inactivation of NADH–nitrate reductase (NR) in the presence of cyanide

[KCN]	10^{-6}M	10^{-5}M	10^{-4}M
NR	75	38	17
NR + rhod. + $S_2O_3^=$ 5 × 10^{-4}M	95	75	48
NR + rhod.	78	42	20
NR + sulphur-free rhod.	76	40	18
Restorative effect of rhodanese on NADH–nitrate reductase–cyanide complex (NR–CN) prepared after inactivation of NR with the same [KCN] reported above			
NR-CN	75	40	20
NR-CN + rhod. + $S_2O_3^=$ 5 × 10^{-4}M	96	75	50
NR-CN + rhod	75	38	22
NR-CN + sulphur-free rhod	74	41	18

* % Activity is related to the untreated enzyme

to Mo (IV) would presumably be the action of the electron donor. The formation of the Mo–cyanide complex should change the properties of the molybdo moiety: in which case electron donors could allow the reduction to Mo (III), which is more stable with cyanide, and unable to reduce nitrate, because it does not participate in *O*-atom reactions [20].

Activation of apoferredoxin with thiosulphate and rhodanese from mammals or cabbage leaves, in the presence of ferric nitrate and a reducing agent, apparently proceeds through the transfer of sulphur from thiosulphate to the apoprotein [14, 21]. A similar sulphur transfer may take part also in the reactivation mechanism of durum wheat nitrate reductase inhibited by cyanide. In fact the *Micrococcus* nitrate reductase has been shown to contain "labile sulphide" [22]. However, such a structure has not yet been demonstrated for the durum wheat enzyme.

The protective effect of rhodanese against cyanide inactivation of nitrate reductase could be easily explained by a rapid transformation of cyanide to thiocyanate, which has no inhibitory effect on the nitrate reductase. Other more complex mechanisms, taking into account "labile sulphide" structures, as in apoferredoxin, would also be compatible with our results.

Cyanogenic glycosides, which occur widely in the plant kingdom [23], are likely to be *in vivo* potential inhibitors of NADH–nitrate reductase. Reductase activity in durum wheat did not depend on the presence of $NiCl_2$ in the extraction medium. On the basis of the Eck and Hageman hypothesis [27], we can conclude that the mean concentration of cyanogenic glycosides in durum wheat leaves is very low. Despite this, the hypothesis that the physiological role of rhodanese in plants could be the preservation of nitrate reductase activity is still tenable.

It is important to note that, in the presence of rhodanese, no oxidizing agent is required for the reactivation of cyanide treated NADH–nitrate reductase.

EXPERIMENTAL

Crystalline rhodanese was obtained from beef kidney according to ref. [24] and the activity measured according to ref. [25]. S-free rhodanese was prepared according to ref. [26]. NADH–nitrate reductase was extracted from durum wheat. Leaves were ground with glass beads in 25 mM Pi buffer pH 8.8 containing 1 mM EDTA and 10 mM cysteine (1 g fr. wt/6 ml). After centrifugation at 20000 *g* for 15 min at 4°, the enzyme was purified by gel filtration on a Sephadex G-200 column (40 × 4 cm), equilibrated and eluted at 20 ml/hr, with the extraction buffer. The active fraction was precipitated with $(NH_4)_2\ SO_4$ at 25–42% saturation. This preparation had an activity of 2 U/mg protein, the unit being defined as the amount of protein which reduces 1 μmol KNO_3/min NADH–nitrate reductase was also extracted from wheat leaves in the presence of 4 mM $NiCl_2$ [27]. Enzyme activity was measured by incubation of the enzyme for 15 min at 30° in a soln containing 50 μmol Pi buffer pH 7.6, 20 μmol KNO_3 and 0.4 μmol NADH in a final vol of 2 ml. The reaction was stopped by adding 0.2 ml of 1 M Zn acetate. Nitrite was estimated after addition of 1 ml of 1% sulphanilamide and 1 ml of 0.02% *N*-1-(naphthyl-ethylene diamine) (2 HCl), by reading the *A* at 540 nm after 30 min. A standard curve was determined with $NaNO_2$. Protein was estimated according to [28]. Endogenous nitrate in leaves was assayed according to the method of ref. [29]. The NADH–nitrate reductase–cyanide complex was prepared by adding cyanide to a soln containing 2 U/mg of protein and 1 mM NADH [30]. In these experiments the final concn of cyanide varied between 10^{-6}–10^{-4} M. After 5 min incubation at room temp. 0.5 ml of the complex soln was filtered through a Sephadex G-25 fine column (25 × 1.5 cm) equilibrated and eluted with 0.1 M Pi buffer pH 7.6. The effluent was monitored at 280 nm and tested for cyanide according to the method of ref. [31]. Restorative effects were studied by adding to 0.1 ml of the enzyme–cyanide complex, 0.1 ml of crystalline rhodanese (1 nmol) dissolved in 10 mM Pi buffer pH 7.6 containing 1 mM thiosulphate. The protective effect was studied by adding cyanide (final concn 10^{-6}–10^{-4}M), to 0.1 ml of NADH–nitrate reductase previously incubated for 5 min with 1 nmol of crystalline rhodanese dissolved in 0.1 ml of 10 mM Pi buffer pH 7.6 containing 1 mM thiosulphate.

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