# THE EFFECT OF REDUCING AGENTS ON IN VITRO NITRATE REDUCTASE ACTIVITY IN *ASPERGILLUS NIDULANS*

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## 1. Introduction

Nitrate reductase can have either NADH or NADPH as a cofactor. In most organisms studied [1–5] the enzyme is inactivated when it binds to reduced pyridine nucleotide in the absence of substrate. The nitrate reductase of *Aspergillus nidulans* is unusual in that reduced NADP is needed to keep the enzyme active both in vivo and in vitro [6–8].

The purpose of the experiments described here was to see if reducing agents, such as Cleland's reagent and cysteine, protect *Aspergillus nidulans* nitrate reductase, or whether the effect is specific to NADPH. The findings presented here indicate that it is the redox state of nitrate reductase cofactor which determines the level of the enzyme activity. These results provide further evidence to suggest that nitrate reductase activity is subject to redox control.

## 2. Methods and materials

The strain of *Aspergillus nidulans* used was a translocation free biotin auxotroph *biA*1 (Glasgow no. 051). Analytical grade chemicals were used throughout. Cysteine and Cleland's reagent were obtained from British Drug Houses, Poole. NADPH was obtained from Boehringer, London. Media and supplements were as in [9]. Nitrogen-less minimal medium used was as in [10]. The mycelium used for nitrate reductase assays was grown in shaken culture at 30°C for 17 h with 10 mM $NaNO_3$, harvested and extracted as in [10]. The extracts were centrifuged at 60 000 × *g* for 30 min at 5°C. NADPH-nitrate oxidoreductase (EC 1.6.6.3) was assayed as in [10].

## 3. Results

Figure 1 shows the in vitro inactivation of nitrate reductase from cell-free extracts at 30°C in the presence or absence of varying concentrations of cysteine. The addition of 1 mM and 3 mM cysteine to cell-free extracts initially caused some inactivation of nitrate reductase; 7% and 15%, respectively. Cell-free extracts incubated in the absence of cysteine showed a 90% inactivation of the initial nitrate reductase activity during the first 30 min incubation. Thereafter, there was little further inactivation of the enzyme. Incubation of cell-free extracts with 1 mM cysteine caused no significant inactivation of nitrate reductase activity during 1 h incubation. However, increasing the cysteine concentration to 3 mM caused rapid inactivation of nitrate reductase. After 1 h incubation only approx. 20% initial activity remained.

Figure 2 shows the effect of NADPH and Cleland's reagent on the level of nitrate reductase activity in cell-free extracts. NADPH prevented any significant inactivation of nitrate reductase in cell-free extracts incubated at 25°C (results not shown). By increasing the incubation temperature to 30°C, approx. 50% inactivation of nitrate reductase resulted when incubated with NADPH for 1 h. The addition of 1 mM Cleland's reagent to a cell-free extract inactivated approx. 50% initial nitrate reductase activity. However, subsequent incubation at 30°C resulted in a steady recovery of enzyme activity. The addition of 3 mM Cleland's reagent to a cell-free extract inactivated approx. 75% enzyme. There was no significant change in enzyme activity level during 1 h incubation.

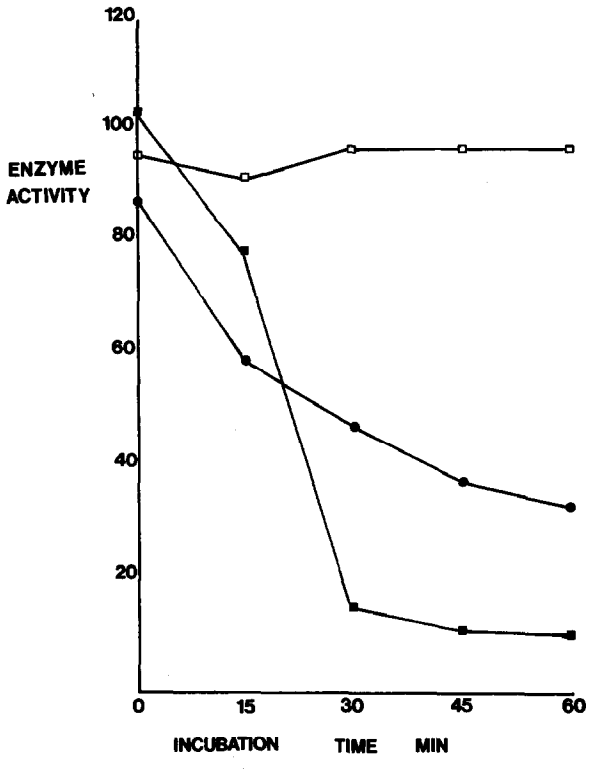


Fig.1. Results of in vitro inactivation of nitrate reductase in the presence of cysteine. Cells were pregrown for 17 h at 30°C. The enzyme preparations from the cells were incubated in 2.65 ml orthophosphate buffer, pH 7.75, with 3.5 μg FAD at 30°C. At the incubation times indicated, nitrate reductase activity was determined as in [10]. (■——■) No cysteine; (□——□) 1 mM cysteine; (●——●) 3 mM cysteine.

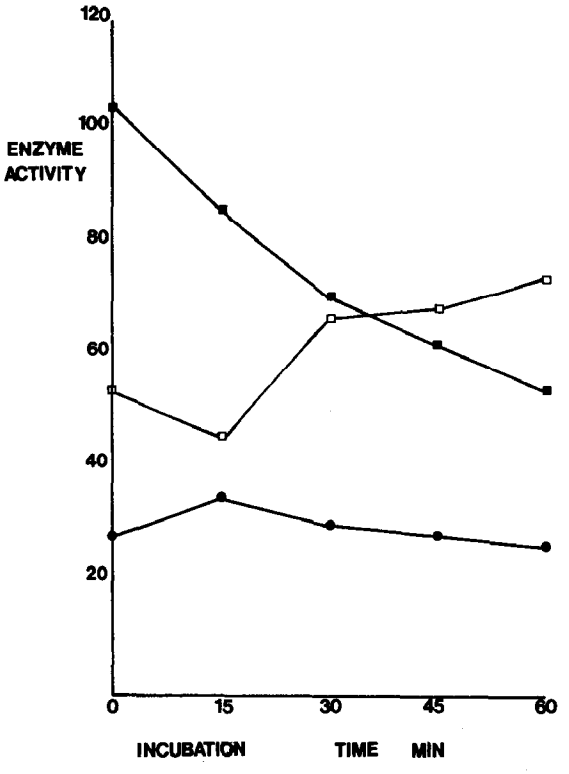


Fig.2. Results of in vitro inactivation of nitrate reductase in the presence of NADPH and Cleland's reagent. Cells were pregrown for 17 h at 30°C. The enzyme preparations from the cells were incubated on 2.65 ml orthophosphate buffer, pH 7.75, with 3.5 μg FAD at 30°C. At the incubation times indicated, nitrate reductase activity was determined as in [10]. (■——■) 1 mM NADPH; (□——□) 1 mM Cleland's reagent; (●——●) 3 mM Cleland's reagent.

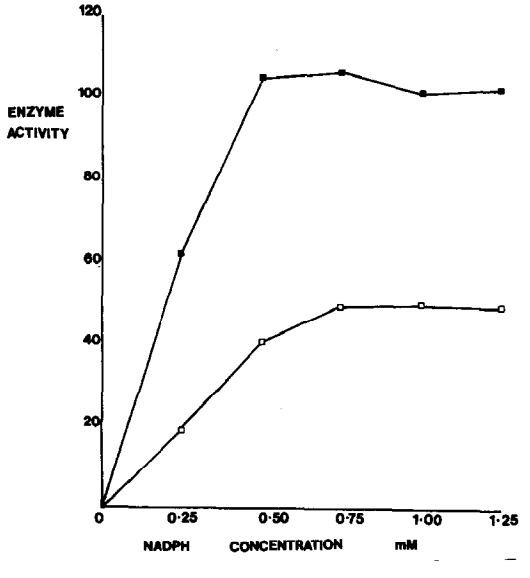


Fig.3. The effect of Cleland's reagent on nitrate reductase activity with varying concentrations of NADPH. Cells were pregrown for 17 h at 30°C. The enzyme preparations from the cells were incubated in 2.65 ml orthophosphate buffer, pH 7.75, with 3.5 μg FAD. Cell-free extracts were assayed for nitrate reductase activity in the presence or absence of Cleland's reagent with varying concentrations of NADPH. (■——■) No Cleland's reagent; (□——□) 1 mM Cleland's reagent.

Figure 3 shows the effect of Cleland's reagent on in vitro nitrate reductase activity over a range of NADPH concentrations. Cell-free extracts have no detectable nitrate reductase activity in the absence of the cofactor NADPH. Cell-free extracts had fully

active nitrate reductase with NADPH at conc. 0.5 mM. There was no significant change in the level of nitrate reductase activity when the NADPH concentration was increased to 1.25 mM. The presence of Cleland's reagent in a cell-free extract prevented full activation of nitrate reductase to the maximum level. Only approx. 50% enzyme became activated when Cleland's reagent was present, even with high NADPH concentration.

## 4. Discussion

The results presented in this paper indicate that cysteine, and to a lesser extent Cleland's reagent, can mimic NADPH in protecting nitrate reductase activity from inactivation. These results suggest that the protection of nitrate reductase activity by NADPH is not due to the properties of the pyridine nucleotide itself, but to its redox state. It seems likely that the inactivation of nitrate reductase is the result of insufficient reduced NADP. Nitrate reductase activity is therefore probably subject to redox control. Inactivation of nitrate reductase occurs when there is insufficient NADPH; activation of the enzyme is achieved by the generation of increased amounts of NADPH.

Recently it has been shown in *A. nidulans* that the NADPH generating enzymes of the Pentose Phosphate Pathway (PPP) are elevated in the presence of nitrate. This elevation of the PPP enzymes is under the control of the nitrate reductase regulatory gene [11]. This PPP enzyme elevation is probably to provide increased amounts of NADPH to reduce nitrate to ammonia. We have found that subjecting *A. nidulans* to carbon starvation results in a lowering of the NADPH/NADP ratio, concurrent with a reduction in the level of nitrate reductase activity [12]. The decreased generation of NADPH would result in increasingly more nitrate reductase becoming inactivated. This would explain the in vivo loss of nitrate reductase activity. The reduction of nitrate to ammonia constitutes a considerable drain to the cells reducing power. Inactivation of nitrate reductase when there is lowered NADPH generation would provide a rapid means of conserving the remaining NADPH.

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