# A LATENT NITRATE REDUCTASE FROM A THERMOPHILIC ALGA

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Summary

Treatment with phosphate and urea of a latent nitrate reductase obtained from the unicellular alga Cyanidium caldarium, causes a time- and concentration-dependent activation(about 4 fold)of both benzyl viologen- and $NADPH_2$-dependent activities of the enzyme. pCMB, $Hg^{2+}$ and $Ag^{+}$ also activate the benzyl viologen activity, while are strong inhibitors of the $NADPH_2$ activity.

The nitrate reductase in the acidophilic thermophilic unicellular alga Cyanidium caldarium can exist in two different metabolic forms: a fully active form, which utilises $NAD(P)H_2$ and reduced viologens or flavins for nitrate reduction in vitro, and a latent form which cannot reduce nitrate unless it has been activated(1).

There is evidence that in vivo the two forms are interconvertible. In fact, the active nitrate reductase is transformed into the inactive species when fully induced cells are supplied with ammonium ions, whereas the removal of these ions, the cells remaining in the presence of nitrate only, leads to a reactivation of the latent enzyme.

The latent nitrate reductase could be activated in vitro simply by heating, but this heat activated enzyme lacks the ability to utilise $NAD(P)H_2$ as electron donor, and is functional only with the benzyl viologen and flavins(1, 2). It could be



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activated also by phosphate, arsenate, urea, pCMB, $Hg^{2+}$ and $Ag^{+}$. The present paper describes the features of the latent enzyme activation by these chemical agents.

## Material and Methods

All the experiments reported in this article were performed with cell-free extract of Cyanidium prepared in phosphate buffer 0.005 M, pH 7.5, with a French-press and subsequent centrifugation at 27,000 x g to remove cellular debris. The nitrate reductase was assayed at 30°C and pH 7.5 by measuring the nitrite formed. $NADPH_2$ or benzyl viologen were used as electron donor. The latent enzyme was obtained by supplying the fully induced cells with $NH_4^+$ as previously described(2). Full details on the growth of the alga, preparation of cell-free extract and enzyme assay are given in a previous paper(1).

## Results

Effect of phosphate concentration on the latency of nitrate reductase. When separate aliquots of crude extract of Cyanidium

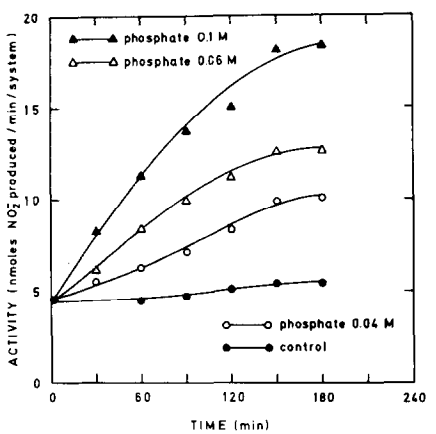


Figure 1. Time course of the latent benzyl viologen nitrate reductase activation by phosphate of various molarity(supplied as buffer pH 7.5). The crude extract was incubated at 0°C with the reported concentrations of phosphate and, at regular intervals, samples of 0.05 ml were removed and assayed for activity at 30°C. The assay mixture contained in a final volume of 2 ml: phosphate buffer, pH 7.5, 0.08 M; $KNO_3$, 50 µmoles; benzyl viologen, 0.1 µmole; dithionite, 0.8 mg.

containing latent nitrate reductase are incubated at 0°C in the presence of varying concentrations of phosphate(supplied as buffer, pH 7.5), and assayed at regular interval of time, an increase of about 4 fold in both benzyl viologen and $NADPH_2$ activities is observed, which is dependent on the time of incubation and on the concentration of phosphate(Figs. 1 and 2). It should be noted that the control(extract in 0.005 M buffer)does not show appreciable enhancement of activity even after 3 hours.

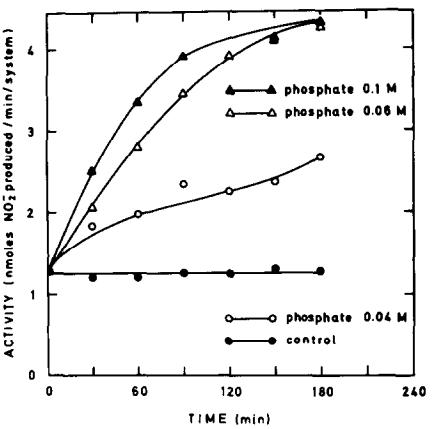


Figure 2. Time course of the latent $NADPH_2$ nitrate reductase activation by phosphate. Procedure and enzyme assay as in Fg. 1, except that the samples utilised for enzyme assay were 0.2 ml, and that the benzyl viologen and dithionite were replaced by $NADPH_2$ 0.25 mM.

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Effect of urea. Incubation at room temperature of fully active nitrate reductase with urea 3 M, results within 3 hours in a loss of $NADPH_2$-dependent activity; by contrast, the benzyl viologen activity is not, or very little affected by this treatment. The complete loss of the latter activity occurs on incubation with urea 5.5 M(umpublished observations).

The addition of urea to the latent nitrate reductase results in a definite increase in activity(measured both with $NADPH_2$ and reduced benzyl viologen)which is dependent on the time of incubation and on the concentration of urea(Figs. 3 and 4). It should

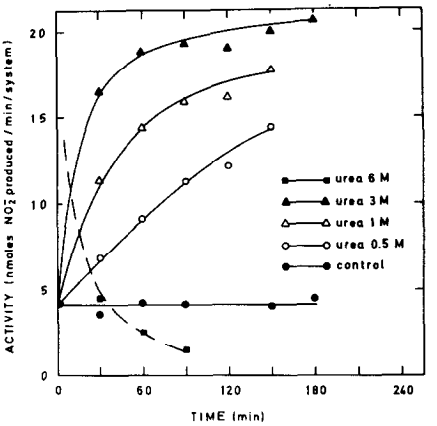


Figure 3. Time course of the latent benzyl viologen nitrate reductase activation by urea. The crude extract was incubated with varying concentration of urea at 0°C and, at regular intervals, samples of 0.05 ml were assayed for activity as in Fig. 1.

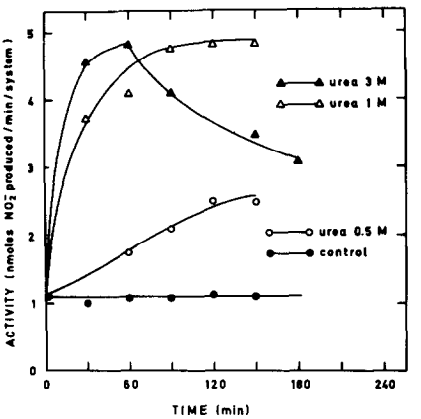


Figure 4. Time course of the latent $NADPH_2$ nitrate reductase activation by urea. Procedure as in Fig. 3. For the enzyme assay (as in Fig. 2) samples of 0.2 ml were used.

be noted also that, with urea 3 M, after the maximum of activation has been reached, whereas the benzyl viologen-dependent activity remains essentially constant, the $NADPH_2$-dependent activity exhibits a progressive loss of activity.

Effect of pCMB. The fully active nitrate reductase of Cyanidium, as with other nitrate reductase(3, 4), is differentially

inhibited by pCMB according to the electron donor utilised. The mercurial is a strong inhibitor of the $NADPH_2$ activity, but is almost without effect on the benzyl viologen activity. The latter is destroyed only by very high concentration of pCMB($10^{-2}$ M). The inhibition of $NADPH_2$ activity is reversed by cysteine(unpublished observations).

Addition of pCMB to a preparation of latent nitrate reductase of Cyanidium results in an immediate increase of benzyl viologen-dependent activity(Fig. 5). By contrast, the $NADPH_2$ activity is totally lost. As above reported, in fact, the mercurial is a strong inhibitor of the latter activity.

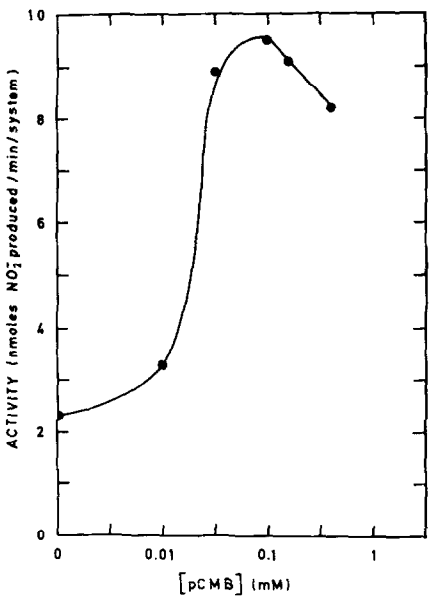


Figure 5. Activation of the latent benzyl viologen nitrate reductase with pCMB. Aliquots(0.05 ml)of extract were incubated for 5 min at 0°C with the reported amounts of mercurial, then assayed with benzyl viologen as in Fig. 1.

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The activation of benzyl viologen activity with pCMB could be totally reversed by cysteine(Table 1), the level of activity of the activated enzyme returning to the control level. After the enzyme has regained its latency by action of cysteine, it could still be reactivated by heat, urea and phosphate(Table 1). The

Table I

LOSS OF THE LATENCY BY PCMB ACTION AND ITS REVERSIBILITY BY CYSTEINE OF THE LATENT BENZYL VIOLOGEN NITRATE REDUCTASE

| System | Activity(nmoles $NO_2^-$ produced/min/system) |
|---|---|
| Extract | 35.5 |
| Extract + pCMB | 96.0 |
| Extract + pCMB + cysteine | 35.5 |
| Extract + pCMB + cysteine + phosphate | 118.0 |
| Extract + pCMB + cysteine heated | 105.0 |

The latent nitrate reductase was firstly incubated with pCMB (0.5 mM)for 5 min, and cysteine(5 mM)then added. After 5 min aliquots of the mixture(extract + pCMB + cysteine)were incubated with phosphate 0.1 M for 3 hours, or heated for 3 min at 60°C.

Activity was determined with benzyl viologen after each addition using samples of 0.05 ml, as reported in Fig. 1.

addition of cysteine has no effect on the activity of the enzyme activated by phosphate.

Other activators of the latent benzyl viologen nitrate reductase are $Hg^{2+}$ and $Ag^+$ at a molarity $5 \times 10^{-5}$ : at the same concentration these heavy metals are strong inhibitors of the $NADPH_2$ nitrate reductase. Among other chemical agents tested, NaCl, KCl, NaBr are without effect on the latent nitrate reductase; $KNO_3$, $KClO_3$, $MgSO_4$, $Na_2SO_4$, $(NH_4)_2SO_4$ exhibit a slight activating effect whereas arsenate and pyrophosphate activate the enzyme as the phosphate does(not shown). These compounds were employed at a final concentration 0.1 M, and the enzyme activity was measured 4 hours after their addition.

## Discussion

Of the two native forms of nitrate reductase which are obtained from Cyanidium, the fully active one and the latent, only the second may be activated by the reported agents. The first one,

which is formed as the majority of enzyme in the nitrate grown cells, is not activated by any of these treatments.

As in Cyanidium, inactivation *in vivo* of the nitrate reducing system by ammonia was found in Chlorella fusca by Losada et al.(5). These authors have also demonstrated that of the two functional moieties which constitute the nitrate reducing system, the diaphorase and the nitrate reductase proper, only the latter undergoes the inactivation by ammonia. The diaphorase and the nitrate reductase proper, however, are very closely associated and participate sequentially in the transport of electron from $NAD(P)H_2$ to nitrate(4).

The loss of $NAD(P)H_2$ nitrate reductase activity by treatments which do not affect the benzyl viologen-dependent activity seems to be due to the greater sensitivity to these treatments(mild heat, pCMB $10^{-4}$ M)of the diaphorase activity with respect to the nitrate reductase proper activity(the latter, however, is functional with benzyl viologen even when the diaphorase activity is destroyed). To a similar mechanism may be referred the inactivation of $NADPH_2$ nitrate reductase here studied by urea 3 M.

In Cyanidium at the moment we are not able to explain the mechanism which control the *in vivo* inactivation of nitrate reducing system and the phenomena which occur during the *in vitro* reactivation of enzyme. Concerning the latter, however, our results seem to indicate that the most likely mechanism is that the activation occurs through a conformational change of enzyme. The activation with pCMB indicates that the integrity of some SH group(s) is necessary for the mantainance of a peculiar structure of enzyme on which depends the latency. This explanation agrees well also with the fact that heat and urea, both of which are strong denaturing agents of proteins, activate the latent enzyme. The heat treatment, however, is so severe as to cause a loss of $NADPH_2$-dependent activity at the same time as the benzyl viologen-dependent activity is enhanced several fold(2). By contrast, the action of urea may be graduated by varying the molarity. Firstly the enzyme

loses the latency, then the $NADPH_2$ activity and finally, at higher concentration of urea, it loses the benzyl viologen activity. It may be supposed that there exist parts of the protein which are more delicate in structure than the part bearing the active site for nitrate, and which are progressively destroyed. One may imagine that the integrity of the first one to be destroyed is necessary for the latency of the enzyme because it bears an inhibitory site to which is bound an unknown inhibitor formed *in vivo* in the presence of ammonia.

In this connection it might be relevant that ATC-ase, an allosteric enzyme, is activated several fold by urea, heat, pCMB and heavy metals(6), losing at the same time its allosteric properties(7).

Acknowledgements

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