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KINETIC ASPECTS OF NITRATE REDUCTASE FROM *CYANIDIUM CALDARIUM*

INHIBITION BY REDUCED PYRIDINE NUCLEOTIDES

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SUMMARY

Kinetic studies on nitrate reductase from *Cyanidium caldarium* have been performed and K_m values for nitrate, benzyl viologen and pyridine nucleotides have been measured. The Lineweaver–Burk plots were linear for nitrate or with benzyl viologen as electron donor, but showed an upward deflection with pyridine nucleotides, which is characteristic of inhibitory effects at concentrations above 0.1 mM. Extrapolation of the initial hyperbolic function for NADPH by corrections based on data derived from the Lineweaver–Burk plot revealed a cooperative behaviour by excess NADPH in the inhibitory range.

NADH inhibited non-competitively with benzyl viologen as electron donor and showed mixed inhibition with respect to nitrate in this system. The inhibitory effect was not eliminated by heating for 3 min at 55 °C or by 3 M urea, indicating that the inhibitory site is distinct from that at which NAD(P)H reacts as electron donor.

INTRODUCTION

In recent years it has been postulated that ADP exerts a possible allosteric control on nitrate reductase from tomato leaves[1] and spinach[2]; in spinach, competitive inhibition by carbamyl phosphate with respect to nitrate was also described[3].

Recently we have observed that pyridine nucleotides, which are usually employed for the *in vitro* reduction of nitrate, and which presumably represent *in vivo* the electron source for nitrate reductase, produce a marked inhibition on the enzyme from the unicellular alga *Cyanidium caldarium*.

In this article the kinetic aspects of this inhibition are described and the hypothesis that such an inhibition may be of regulative significance is postulated.

MATERIALS AND METHODS

C. caldarium, Strain 0206, was obtained from Professor T. D. Brook, Wisconsin University. The alga was grown in the light at 42 °C, on nitrate as the sole nitrogen source. Nitrate reductase activity was assayed at 30 °C and at pH 7.5, by measuring the nitrite formed, as previously described[4]. The crude extract, prepared as described in a previous paper[4], was centrifuged at high speed (150 000 × *g*) and all the activity was found in the supernatant to which $(NH_4)_2SO_4$ was added to give a 35% saturated solution. The precipitate, redissolved in 0.005 M phosphate buffer (pH 7.5) and desalted on Sephadex G-25, was used as enzyme source.

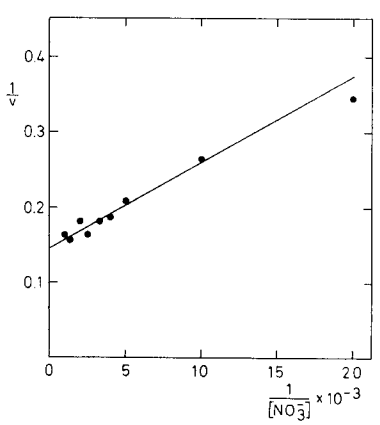
Nitrate reductase can occur in the extract of *Cyanidium* partially as a latent enzyme[4]. However, the latency is lost during the $(NH_4)_2SO_4$ precipitation and the enzyme utilised for the present experiments was present totally in the fully active form.

RESULTS AND DISCUSSION

Effect of substrate concentration on nitrate reductase activity

Nitrate reductase utilises *in vitro* a wide variety of electron donors to reduce nitrate, such as reduced pyridine nucleotides, flavins and viologens. Activity measurements of enzyme as a function of nitrate concentration have been performed utilising either reduced benzyl viologen (0.06 mM) or NAD(P)H (0.25 mM) as electron donor. It is apparent that a Lineweaver–Burk plot of the reciprocal of activity against the reciprocal of nitrate concentration gives a straight line in all cases (Figs 1 and 2). However, the apparent K_m value measured with benzyl viologen of $8.4 \cdot 10^{-4}$ M, is 10-fold higher than that calculated with NADH of $8.7 \cdot 10^{-5}$ M or with NADPH, $7.6 \cdot 10^{-5}$ M.

A straight line occurs also when the reciprocal of the activity is plotted against



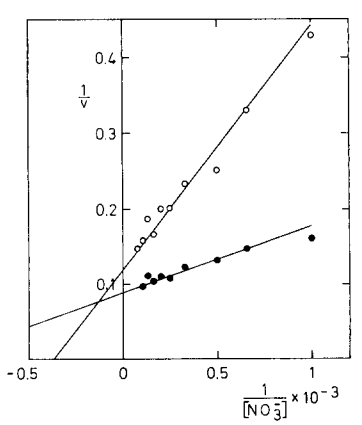
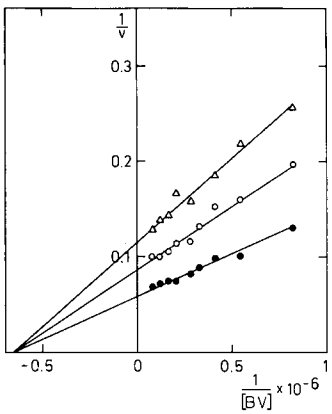


Fig. 1. Lineweaver–Burk plot of nitrate reductase activity *vs* nitrate concentration. NADH, $2.5 \cdot 10^{-4}$ M, is the electron donor. Activity: nmoles of nitrite produced per min per system.

Fig. 2. Lineweaver–Burk plot of nitrate reductase activity against nitrate concentration in the absence (●—●) or in the presence (○—○) of NADH $5 \cdot 10^{-4}$ M. Reduced benzyl viologen, at the fixed concentration $6 \cdot 10^{-5}$ M is the electron donor. Activity as in Fig. 1.

the reciprocal of benzyl viologen concentration, at a fixed $2.5 \cdot 10^{-2}$ M nitrate concentration, from which a K_m value for benzyl viologen of $1.3 \cdot 10^{-6}$ M was calculated; by contrast, when the reciprocal of nitrate reductase activity is plotted against the reciprocal of NADH or NAD(P)H concentrations, at the above reported concentration of nitrate, a straight line occurs only at the lower concentrations (Figs 4 and 5A); at the higher concentrations of pyridine nucleotides, in fact, above 0.1 mM, the plot deviates from the linearity and presents a marked upward deflection. The K_m values calculated from the straight segments of the plots are $8.6 \cdot 10^{-5}$ M and $3.6 \cdot 10^{-4}$ M for NADH and NADPH, respectively.



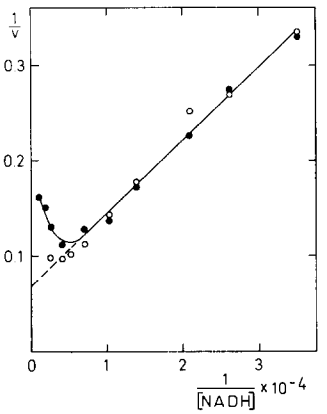


Fig. 3. Plot of the reciprocal of nitrate reductase activity *vs* benzyl viologen (Bv) concentration in the absence (●—●) or in the presence of NADH $5 \cdot 10^{-4}$ (△—△) and $2.5 \cdot 10^{-4}$ M (○—○). The nitrate concentration was $25 \cdot 10^{-3}$ M. Activity as in Fig. 1.

Fig. 4. Lineweaver–Burk plot of nitrate reductase activity *vs* NADH concentration in the absence (●—●) or in the presence (○—○) of NAD^+ $5 \cdot 10^{-4}$ M. Nitrate concentration was $25 \cdot 10^{-3}$ M. Activity as in Fig. 1.

The saturation curve of nitrate reductase with NADPH (Fig. 5B) or NADH (not shown), begins as an hyperbolic function followed by inhibition. Comparison of the experimental data with the extrapolated hyperbola in the Lineweaver–Burk plot, gives an indication that the inhibition may be cooperative.

It is relevant that pyridine nucleotides are inhibitory only in the reduced form; NAD^+, up to $5 \cdot 10^{-4}$ M, when added together with the variable amount of NADH or NADPH, does not increase or promote inhibition of the enzyme activity (Figs 4 and 5A).

The experiments reported above suggest that NAD(P)H may react at a regulatory site distinct from the catalytic site. This interpretation is supported also by the following experiments.

Inhibition of benzyl viologen nitrate reductase by NADH

NADH acts as an inhibitor of benzyl viologen nitrate reductase with respect to both nitrate and benzyl viologen. The NADH inhibition is noncompetitive with respect to benzyl viologen (Fig. 3) and of the mixed competitive–noncompetitive type with respect to nitrate (Fig. 2), when benzyl viologen serves as the electron donor.

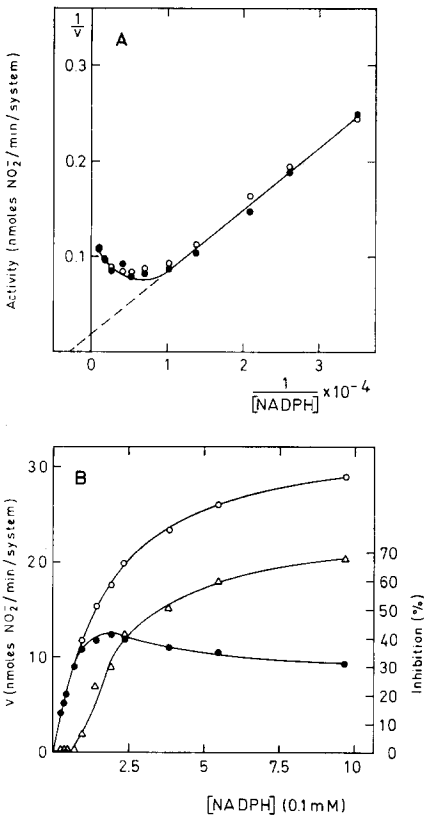


Fig. 5. A and B. (A) Double reciprocal plot of nitrate reductase activity as a function of NADPH concentration in the absence (●—●) or in the presence (○—○) of NAD^+ $5 \cdot 10^{-4}$ M. Nitrate concentration was 25 mM. (B) Nitrate reductase activity against NADPH concentration. ●—●, experimental results; ○—○, extrapolated hyperbola; △—△, % inhibition calculated from double reciprocal plot shown in A.

Action of mild denaturing agents of proteins

There are reports that many allosteric enzymes may be desensitized to the action of effectors when treated with denaturing agents of proteins such as mild heating, urea and *p*-chloromercuribenzoate, and this is the main indication of distinct regulatory site and catalytic sites[5,6].

The nitrate reductase system exhibits two activities which participate sequentially in the transfer of electrons from NAD(P)H to nitrate[7]. The first, a diaphorase activity, is more sensitive to mild denaturing agents of proteins than the second, the nitrate reductase proper activity. However, the integrity of both activities, probably supported by two different moieties of the nitrate reductase molecule[7], is necessary for the reduction of nitrate by pyridine nucleotides. When the enzyme is heated for 5 min at 55 °C or treated with 3 M urea for 3 h or with 0.1 mM *p*-chloromercuribenzoate, it loses the diaphorase activity and, as a consequence, the NAD(P)H activity, while retaining almost all the activity depending on benzyl viologen. We have thus a means to test whether pyridine nucleotides continue to be inhibitors of nitrate reductase under conditions where the enzyme, after treatment

with denaturing agents, is unable to utilise them as substrates, by assaying the inhibitory effect on the benzyl viologen activity.

Treatment with 3 M urea or heating at 55 °C for 3 min does not desensitize the benzyl viologen nitrate reductase to the NAD(P)H inhibition (not shown). These results suggest that the diaphorase and inhibitory site for NAD(P)H are distinct, which corroborates the above postulated hypothesis and furthermore indicates that the inhibitory site is apparently located on the nitrate reductase moiety of the nitrate reductase complex.

Noteworthy is the fact that nitrate reductase is inhibited *in vitro* by reduced pyridine nucleotides, which are substrates of the enzyme, and not by ammonia (unpublished observations) which is the final product of nitrate reduction. However, it was demonstrated recently in our laboratory that, in *Cyanidium*, ammonia promotes an alternative mechanism which supports *in vivo* inactivation of nitrate reductase, and whose action consists in the reversible conversion of the active enzyme into an inactive form[8,9]. So far we have not been able to reconstitute *in vitro* the mechanism of this ammonia-dependent inactivation.

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REFERENCES

1 Nelson, N. and Ilan, I. (1969) *Plant Cell Physiol.* 10, 143–148
2 Eaglesham, A. R. J. and Hewitt, E. J. (1971) *FEBS Lett.* 16, 315–318
3 Relimpio, A. Ma., Aparicio, P. J., Paneque, A. and Losada, M. (1971) *FEBS Lett.* 17, 226–230
4 Rigano, C. (1971) *Arch. Mikrobiol.* 76, 265–276
5 Woolfolk, C. A. and Stadtman, E. R. (1967) *Arch. Biochem. Biophys.* 118, 736–755
6 Whitehead, E. (1970) in *Progress in Biophysics and Molecular Biology* (Butler, J. A. V. and Noble, D., eds), Vol. 21, pp. 321–396, Pergamon, Oxford and New York
7 Losada, M., Aparicio, P. J. and Paneque, A. (1969) in *Progress in Photosynthesis Research* (Metzner, H., ed.), Vol. 3, pp. 1504–1509, Laupp, Tubingen
8 Rigano, C. and Violante, U. (1972) *Biochem. Biophys. Res. Commun.* 47, 372–379
9 Rigano, C. and Violante, U. (1973) *Arch. Mikrobiol.* 90, 27–33