Biochimica et Biophysica Acta, 368 (1974) 54–60

BBA 46807

INACTIVATION OF NITRATE REDUCTASE BY NADH IN *NITROBACTER AGILIS*

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(Received April 4th, 1974)

SUMMARY

Nitrate reductase from *Nitrobacter agilis* was inactivated by NADH (but not by NADPH) in the absence of nitrate.

The inactivation of the enzyme by over-reduction with NADH was overcome by oxidizing the reduced enzyme with nitrate, ferricyanide, NAD^+ or $NADP^+$.

INTRODUCTION

Nitrobacter agilis is a chemoautotrophic nitrifying bacterium oxidizing nitrite to nitrate [1]. In addition, it has an NADH-linked nitrate reductase which mediates the opposing reaction, i.e. the reduction of nitrate to nitrite [2]. The latter enzyme has been solubilized, partially purified and characterized by Straat and Nason [3] and by Wallace and Nicholas [2]. Subsequently, Faull et al. [4] demonstrated that nitrite oxidase and nitrate reductase from *Nitrobacter* are separate enzymes.

Recently, Losada and his coworkers have found that nitrate reductase from bacteria [5], yeasts [6] and algae [7–12] can exist in either active or inactive interconvertible forms. They have also reported that interconversion of the active and inactive forms can be directly achieved in vitro by reducing and oxidizing the enzyme with physiological or artificial substrates [5, 6, 8, 9]. On the other hand, Vennesland and her associates have found that nitrate reductase in cell-free extracts of *Chlorella vulgaris* is largely in an inactive form, which can be extensively activated on adding nitrate and a phosphate buffer of low pH; the addition of NADH without nitrate resulted in a loss of enzyme activity [13, 14]. More recently, the same group has reported that the restoration of the inactive form required an oxidizing agent and that this effect was inhibited by CO [15].

The present work describes an inactivation by NADH of nitrate reductase in *Nitrobacter*. Evidence for the in vitro interconversion of the active and inactive forms of nitrate reductase, by reduction and oxidation of the enzyme, is also presented.

MATERIALS AND METHODS

Culture of the organism

The strain of *N. agilis* used was supplied by Dr M. I. H. Aleem, Department of Microbiology, University of Kentucky, Lexington, Ky., U. S.A. The bacterium was grown as described by Wallace and Nicholas [2]. The cells were collected in a continuous flow rotor at 30 000 $\times g$ in a Sorvall RC-2 refrigerated centrifuge.

Method for disrupting the cells

Freshly harvested cells were washed until free of nitrite and then disrupted by an ultrasonic probe (20 kcycles/s) for 20 min at 4 °C, as described by Wallace and Nicholas [2]. Cell debris and unbroken cells were removed by centrifuging at 10000 $\times g$ for 15 min and the supernatant fraction (Fraction S_{10}) was fractionated further.

Separation of the two enzymes

This fractionation of Fraction S_{10} with solid $(NH_4)_2SO_4$ was carried out at 2 °C. The $(NH_4)_2SO_4$ was added slowly to the extract (0–44 % saturation) with continuous stirring and it was left standing for a further 30 min. The supernatant fraction left after centrifuging at 10 000 $\times g$ for 15 min contained the nitrate reductase enzyme (90 %) while the nitrite oxidase was located in the pellet.

Enzyme assays

Nitrate reductase. The assay was carried out under aerobic conditions. The reaction mixture contained in a final volume of 2 ml: potassium phosphate buffer (pH 7.0), 115 μmoles; KNO_3, 2 μmoles; NADH, 2 μmoles; FMN, 12.5 nmoles, and an appropriate amount of enzyme preparation. The reaction mixture was incubated at 30 °C for 30 min, and then 0.1 ml of 1 M zinc acetate and 1.9 ml of 95 % ethanol (v/v) were added to remove residual NADH, which would otherwise interfere with the subsequent diazotization reaction for nitrite [16]. After standing for 10 min, the contents were centrifuged at 3000 $\times g$ for 5 min and a suitable portion of the supernatant solution was used for nitrite determination. The specific activity of the enzyme is expressed in nmoles nitrite produced/30 min per mg protein.

NADH cytochrome c reductase. The assay was carried out under aerobic conditions at 30 °C in a 1 cm cuvette. The reaction mixture contained in a final volume of 2 ml: potassium phosphate buffer (pH 7.0), 115 μmoles; NADH, 0.3 μmole; cytochrome *c*, 50 nmoles, and an appropriate amount of enzyme. NADH cytochrome *c* reductase was followed by monitoring the change in absorbance at 550 nm in a Unicam SP800 recording spectrophotometer.

Protein

This was determined by the method of Lowry et al. [18] after precipitating the protein with 5 % trichloroacetic acid (w/v). Bovine serum albumin was used as a standard.

Chemicals

NAD^+, $NADP^+$, NADH, NADPH, FMN and cytochrome *c* were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Sodium dithionite solution was

freshly prepared by dissolving crystals of sodium dithionite in 0.1 M $NaHCO_3$. All other chemicals were of analytical grade and were dispensed in double-distilled water.

RESULTS

Inactivation by NADH

Nitrate reductase was inactivated by incubating the fraction with NADH. The results shown in Table I indicate that only NADH was effective. NAD^+, $NADP^+$ or NADPH, however, did not inactivate the enzyme. NADH cytochrome *c* reductase was not affected by incubating the fraction with NADH.

Protection by nitrate and reactivation by ferricyanide

The results in Table II show that when the enzyme was preincubated with NADH plus nitrate, the original activity was maintained. The data also demonstrate the immediate reactivation of the NADH-inactivated enzyme by ferricyanide. The

TABLE I

EFFECT OF VARIOUS NUCLEOTIDES ON NITRATE REDUCTASE

The enzyme preparation (0.6 mg protein) was preincubated for 4 h at 0 °C in 1 ml 50 mM potassium phosphate buffer (pH 7.0), with the specified nucleotides each at 1 mM. NADH nitrate reductase activity was determined at 30 °C using 0.1-ml aliquots of the preincubation mixtures, which were made up to a final volume of 2 ml with reagents of the standard assay, as described in Materials and Methods. Activity of the control was 62 nmoles nitrite formed/30 min per mg protein.

Addition	NADH nitrate reductase relative activity (%)
None	100
NAD^+	107
$NADP^+$	97
NADH	20
NADPH	88

TABLE II

INACTIVATION OF NITRATE REDUCTASE BY NADH, PROTECTION BY NITRATE AND REACTIVATION BY FERRICYANIDE

The enzyme preparation (0.6 mg protein) was preincubated for 4 h at 0 °C in 1 ml 50 mM potassium phosphate buffer (pH 7.0), with 1 mM NADH or 1 mM NADH plus 2 mM nitrate. Nitrate reductase activity was determined (with and without 0.5 mM ferricyanide) as described in Table I. Activity of the control was 62 nmoles nitrite formed/30 min per mg protein.

Addition	Control, NADH nitrate reductase relative activity (%)	Addition of ferricyanide to NADH nitrate reductase relative activity (%)
None	100	104
NADH	22	109
NADH plus nitrate	96	102

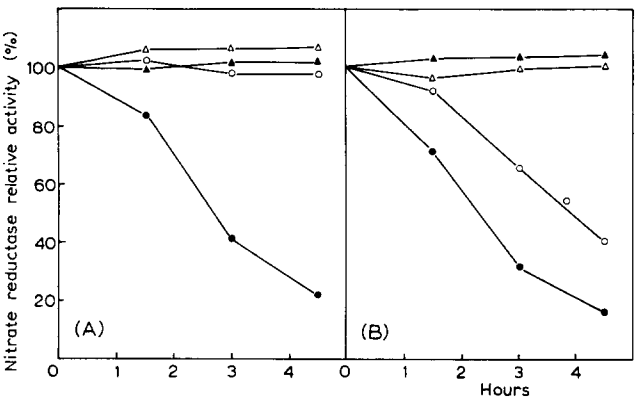

Fig. 1. Time course for the inactivation of nitrate reductase by NADH and its protection by nitrate. The enzyme preparation (0.8 mg protein) was preincubated at 0 °C (A) or 35 °C (B) in 1 ml 50 mM potassium phosphate buffer (pH 7.0) with 1 mM NADH, 2 mM nitrate and 1 mM NADH plus 2 mM nitrate. At the times indicated, nitrate reductase activity was determined as described in Table I. Activity of the control was 58 nmoles nitrite formed/30 min per mg protein. ○–○, control; ●–●, NADH; △–△, nitrate; ; ▲–▲, NADH+nitrate.

time course for the inactivation of nitrate reductase by NADH and its protection by nitrate at 0 °C and 35 °C, respectively, is presented in Figs 1A and 1B. NADH either at 0 or 35 °C converted the active nitrate reductase into its inactive form, but in both cases inactivation was prevented when nitrate was present. Nevertheless, there was an important difference between the two temperatures; thus at 0° C the activity of the untreated enzyme did not change during the experiment, whereas at 35 °C it was progressively inactivated with time. In the latter case, inactivation at 35 °C was also protected by the substrate, nitrate. In all cases, the inactive enzyme could be reactivated by ferricyanide.

Protection and reactivation by NAD^+ and $NADP^+$

Although NADH inactivated nitrate reductase, this effect was not observed in the presence of nitrate (Table II). The results in Table III show that either NAD^+

TABLE III

PROTECTIVE EFFECT OF NAD^+ AND $NADP^+$ AGAINST INACTIVATION OF NITRATE REDUCTASE BY NADH

The enzyme preparation (0.8 mg protein) was preincubated for 4 h at 0 °C in 1 ml 50 mM potassium phosphate buffer (pH 7.0), with NADH or NADH plus NAD^+ or $NADP^+$ at the concentrations shown. Nitrate reductase activity was determined as described in Table I. Activity of the control was 66 nmoles nitrite formed/30 min per mg protein.

Addition	Concentration (mM)		NADH nitrate reductase relative activity (%)
	NADH	NAD^+ or $NADP^+$	
None	—	—	100
NADH	1	—	26
NADH plus NAD^+	1	1.5	104
NADH plus $NADP^+$	1	1.5	102

TABLE IV

EFFECT OF NAD^+, $NADP^+$ AND NITRATE ON THE REACTIVATION OF NITRATE REDUCTASE INACTIVATED BY NADH

The enzyme preparation (1.6 mg protein) was preincubated for 4 h at 0 °C in 3 ml 50 mM potassium phosphate buffer (pH 7.0) with 1 mM NADH. Then the enzyme was incubated for 10 h at 0 °C with the specified nucleotides or nitrate at the concentrations shown. Activities are expressed as percentages of the control at zero time. Activity of the control was 66 nmoles nitrite formed/30 min per mg protein.

Addition	Concentration (mM)		NADH nitrate reductase relative activity (%)
	Nitrate	NAD^+ or $NADP^+$	
None	—	—	20
Nitrate	5	—	67
NAD^+	—	2	76
$NADP^+$	—	2	72

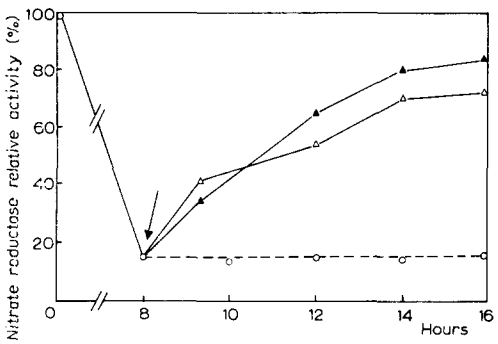

Fig. 2. Time course for the reactivation of NADH-inactivated nitrate reductase by NAD^+ or $NADP^+$. The enzyme preparation (1 mg protein) was preincubated at 0 °C for 8 h in 2 ml 50 mM potassium phosphate buffer (pH 7.0) with 1 mM NADH. At the time shown by the arrow, 2 mM NAD^+ or 2 mM $NADP^+$ was added to aliquots of the preincubation mixture. Nitrate reductase activity was determined at the times indicated, as described in Materials and Methods. Activity of the control was 54 nmoles nitrite formed/30 min per mg protein. △–△, NAD^+; ▲–▲, $NADP^+$. The reaction without NAD^+ or $NADP^+$ is also included(○- - -○).

or $NADP^+$ protected the enzyme against inactivation by NADH. Nitrate, NAD^+ or $NADP^+$ not only protected the enzyme against inactivation but also reactivated it almost completely (Table IV). The data in Fig. 2 show the time course for the reactivation by NAD^+ and $NADP^+$ of the NADH-inactivated enzyme. After an 8 h incubation with either NAD^+ or $NADP^+$, the enzyme was almost completely reactivated, and NAD^+ was found to be as effective as $NADP^+$.

DISCUSSION

Since chemoautotrophic bacteria such as *Nitrobacter* do not utilize sugars or organic acids as primary energy sources and do not use light energy as do photosynthetic organisms, there has been much speculation about the source of reducing

power and how it is generated in these bacteria. It is well established that NADH and ATP are required for the endergonic reduction reactions associated with the fixation of CO_2 in chemoautotrophic bacteria. Aleem et al. [19] demonstrated in *Nitrobacter*, as well as in other chemoautotrophic bacteria, a reversal of the electron transfer system, from reduced cytochrome *c* to NAD^+, thus producing NADH. Subsequently, this mechanism was confirmed by Aleem [20].

Wallace and Nicholas [2] described an enzyme system in *N. agilis* that mediates the reduction of nitrate to nitrite. This enzyme system operates in the reverse direction to the main energy-yielding reaction of this bacterium.

Inactivation of nitrate reductase by reduced pyridine nucleotides in algae has been reported by several workers, Moreno et al. [9], Solomonson et al. [21] and Herrera et al. [8]. More recently, evidence was presented that NAD(P)H results in an in vitro conversion of the active to an inactive form of the enzyme in algae, associated with the reduction of the molybdoprotein moiety. This transformation was found to be reversible and the inactive enzyme was reactivated upon reoxidation [8, 9, 15]. It has also been shown that nitrate reductase from *Chlorella* can be reversibly and specifically inactivated by reduction with NADH in the presence of ADP [12].

The present work shows that, in *Nitrobacter*, a nitrate reductase can be inactivated by incubating with NADH but not with NADPH. Both reductants, however, inactivated the enzyme in algae [11, 12]. Although NADH and NADPH are electron donors for nitrate reductase in *Nitrobacter* [1, 2], only NADH inactivated the enzyme. This inactivation was prevented by nitrate and reversed by ferricyanide. These latter results are in agreement with the data for algae [8, 9, 21]. In our studies not only did nitrate and ferricyanide protect and reactivate the *Nitrobacter* enzyme, as in the algae, but NAD^+ and $NADP^+$ were also effective.

ACKNOWLEDGEMENTS

One of us (J.H.) is grateful to the University of Adelaide for a post-doctoral award. The work was generously supported by a grant from the Australian Research Grants Committee.

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