Biochem. J. (1973) **133**, 701–708
Printed in Great Britain

Reduced Nicotinamide–Adenine Dinucleotide–Nitrite Reductase from *Azotobacter chroococcum*

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(*Received* 12 *February* 1973)

1. The assimilatory nitrite reductase of the N_2-fixing bacterium *Azotobacter chroococcum* was prepared in a soluble form from cells grown aerobically with nitrate as the nitrogen source, and some of its properties have been studied. 2. The enzyme is a FAD-dependent metalloprotein (mol.wt. about 67000), which stoicheiometrically catalyses the direct reduction of nitrite to NH_3 with NADH as the electron donor. 3. NADH–nitrite reductase can exist in two either active or inactive interconvertible forms. Inactivation *in vitro* can be achieved by preincubation with NADH. Nitrite can specifically protect the enzyme against this inactivation and reverse the process once it has occurred. 4. *A. chroococcum* nitrite reductase is an adaptive enzyme whose formation depends on the presence of either nitrate or nitrite in the nutrient solution. 5. Tungstate inhibits growth of the micro-organism very efficiently, by competition with molybdate, when nitrate is the nitrogen source, but does not interfere when nitrite or NH_3 is substituted for nitrate. The addition of tungstate to the culture media results in the loss of nitrate reductase activity but does not affect nitrite reductase.

The enzyme involved in the assimilatory reduction of nitrite to NH_3 in algae and higher plants has been thoroughly characterized in recent years and classified as ferredoxin–nitrite reductase (Beevers & Hageman, 1969; Hewitt, 1970; Kessler, 1971; Losada, 1972). Nitrite reductase purified to homogeneity from green cells of different sources (*Chlorella*, spinach and squash leaves) contains 2 atoms of non-haem iron/molecule of 63000 daltons and does not seem to be a flavoprotein (Losada & Paneque, 1971; Cárdenas *et al.*, 1972*a*,*b*; Zumft, 1972). On the other hand, the enzyme isolated from the nitrate-assimilating fungi *Neurospora crassa* (Nason *et al.*, 1954; Nicholas *et al.*, 1960) and *Torulopsis nitratophila* (Rivas *et al.*, 1973) has been characterized as an NAD(P)H–nitrite reductase which specifically requires FAD and some metal component(s).

By contrast with nitrite reductases from the plant kingdom, information on nitrite reductase from bacteria of the assimilatory nitrate-reducing type is very scanty (Nason, 1962; Takahashi *et al.*, 1963; Hewitt & Nicholas, 1964). Spencer *et al.* (1957) found in extracts of *Azotobacter vinelandii* a soluble nitrite and hydroxylamine reductase system, which utilized reduced nicotinamide nucleotides as electron donors and required added flavin nucleotides for maximal activity. Inhibitor studies indicated that the system had an essential metal component. The product of the reduction of nitrite by the extracts was identified as NH_3, whereas that of hydroxylamine reduction was not established. Both nitrite reductase and hydroxylamine reductase were adaptive enzymes, their formation being stimulated by nitrate. *Escherichia coli* strain Bn grown in deep standing cultures with nitrate as the sole source of nitrogen has been shown to contain at least three nitrite reductases that reduced nitrite (and hydroxylamine) to NH_3 (Lazzarini & Atkinson, 1961), but only the enzyme specific for NADH appears to be responsible for physiological nitrite reduction (Kemp & Atkinson, 1966). Zarowny & Sanwal (1963) have also observed a NADH-specific nitrite reductase in extracts of *E. coli* K-12 grown with nitrate as nitrogen source. Prakash & Sadana (1972) have obtained from *Achromobacter fisheri* grown on nitrate under low O_2 partial pressure a homogenous haemoprotein that catalyses the reduction of nitrite (and hydroxylamine) to NH_3 and have pointed out that any biosynthetic function of the enzyme appears fortuitous.

The present paper describes the preparation and characterization of a soluble NADH–nitrite reductase of the assimilatory type from *Azotobacter chroococcum* cells aerobically grown on nitrate as the nitrogen source. The enzyme is a FAD-dependent metalloprotein (mol.wt. about 67000) of adaptive nature that stoicheiometrically catalyses the direct reduction of nitrite to NH_3 and exists in either active or inactive interconvertible forms.

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Materials and Methods

Cell culture

A. chroococcum (strain A.T.C.C. 4412, from Salamanca University's culture collection) was grown under air with vigorous shaking at 27°C on a synthetic medium containing (mmol/litre): mannitol, 39; KNO_3, 8; $MgSO_4,7H_2O$, 0.8; $CaCl_2$, $2H_2O$, 0.34; $FeSO_4,7H_2O$, 0.19; NaCl, 3.4; Na_2MoO_4, 0.01; disodium EDTA, 0.02; potassium phosphate buffer, pH7.3, 10. When NH_3 or NH_4NO_3 replaced KNO_3 as the source of nitrogen, total concentration of nitrogen was also maintained at 8mM. Since nitrite was toxic at concentrations higher than 1mM, care was taken not to exceed this limit when $NaNO_2$ or $NaNO_2$ plus $(NH_4)_2SO_4$ was the nitrogen source. In experiments involving the effect of tungstate, molybdate was omitted from the standard nutrient solution and tungstate was added as indicated; the cells used for inoculation in these experiments were grown on nitrate media lacking added molybdate.

Cell-free extracts

Cells (10g fresh wt.) were harvested in the exponential phase of growth by low-speed centrifugation, washed with 50mM-potassium phosphate buffer, pH7.0, and resuspended in 3ml of the same buffer. The crude extract was prepared by breaking the cells at 2°C for 2min in a vibration homogenizer (Bühler) with 100ml of glass beads (0.1 mm diam.). The broken material was extracted with 50ml of the phosphate buffer and after centrifugation for 20min at 40000*g* the supernatant was used as enzyme source for the preparation of nitrite reductase.

Nitrite reductase

Since the crude extract possessed high NADH oxidase activity, it was centrifuged at 2°C in a L2-50B Spinco ultracentrifuge for 3h at 120000*g* and the resulting supernatant, which was practically free of oxidase activity, was used as the nitrite reductase preparation. The enzyme was unusually unstable when purification was attempted.

Enzyme assays

Two different methods were used for the assay of nitrite reductase activity. NADH–nitrite reductase was measured by following the nitrite-dependent oxidation of NADH. The reaction was carried out under air at room temperature and absorbance at 340nm was recorded with a Beckman DK-2A spectrophotometer, or exceptionally (for the measurement of the K_m value for NADH) fluorescence at 450nm was recorded with an Aminco–Bowman 4-8202 spectrophotofluorimeter. The reaction mixture included, in a final volume of 2ml: potassium phosphate buffer, pH7.0. 150μmol; NADH, 0.3μmol; $NaNO_2$, 2μmol; an appropriate amount of enzyme preparation. Although high-speed centrifugation practically removed the NADH oxidase activity originally present in the crude extracts, NADH–nitrite reductase activity was always corrected for the rate of NADH oxidation in the absence of nitrite. Reduced Methyl Viologen–nitrite reductase was determined by nitrite disappearance (Ramírez *et al.*, 1966), but with potassium phosphate buffer, pH7.0, instead of Tris–HCl buffer. One unit of activity corresponds to the oxidation of 1 μmol of NADH (or the stoicheiometric reduction of $\frac{1}{3}\mu$mol of nitrite)/min.

Nitrate reductase activity was measured by colorimetrically following the appearance of nitrite. The reaction mixture was incubated at 30°C for 2min and contained, in a final volume of 1ml: potassium phosphate buffer, pH7.0, 100μmol; KNO_3, 10μmol; Methyl Viologen, 0.15μmol; $Na_2S_2O_4$ in 0.1ml of 95mM-$NaHCO_3$, 0.8mg; an appropriate amount of enzyme preparation. The reaction was stopped by vigorously shaking the assay mixture. Enzyme activity units are expressed as μmol of nitrite formed/min.

Analytical methods

Growth was determined by measuring absorbance changes at 660nm. Nitrite was measured by the method of Snell & Snell (1949), and nitrate with nitrate reductase from spinach leaves as described by Relimpio *et al.* (1972). NH_3 was determined by treatment with Nessler's reagent after diffusion and absorption of the gas in 5mM-H_2SO_4 in Conway units (Conway, 1957). Protein was measured by the method of Lowry *et al.* (1951) with ovalbumin as a standard.

The molecular weight of NADH–nitrite reductase was estimated by sucrose-density-gradient centrifugation (Martin & Ames, 1961). Samples (0.2ml) of nitrite reductase and several other proteins of known molecular weight (catalase, 240000; glucose 6-phosphate dehydrogenase, 110000; serum albumin, 67000) were layered on the top of a 3.4ml linear 5–20% (w/v) sucrose gradient layered over 0.15ml of 50% (w/v) sucrose. Sucrose was dissolved in 50mM-potassium phosphate buffer, pH7.0. Centrifugation was carried out at 45000 rev./min for 11h at 2°C in a Spinco L2-50B ultracentrifuge with a SW-56 Ti rotor. Gradients were fractioned from the bottom of the tube with a Densi-Flow apparatus from Buchler Instruments Inc., Fort Lee, N.J., U.S.A. Fractions (3 drops) were collected with a LKB fraction collector. Protein and enzyme activities were then determined in samples of each fraction.

Results

Electron donors and cofactors

Table 1 shows that NADH was an effective electron donor for the reduction of nitrite catalysed by *A.*

Table 1. *Electron donors and cofactors for nitrite reductase from A. chroococcum*

In the experiments with reduced nicotinamide nucleotides as electron donors, the reaction mixture contained, in a final volume of 2ml: potassium phosphate buffer, pH 7.0, 150 μmol; enzyme preparation, 0.4 mg; $NaNO_2$, 2 μmol; NAD(P)H, 0.3 μmol; where indicated FAD, 5 nmol. NAD(P)H oxidation was followed by the change in absorbance at 340 nm. Other experimental conditions were the same as in the standard assay for NADH–nitrite reductase activity. In the experiments with dithionite as reductant the reaction mixture contained in a final volume of 2 ml: potassium phosphate buffer, pH 7.0, 150 μmol; enzyme preparation, 2 mg; sodium dithionite, 7.5 mg in 0.3 ml of a 2.5% (w/v) $NaHCO_3$ solution; $NaNO_2$, 4 μmol; where indicated Methyl or Benzyl Viologen, 1.5 μmol; FAD or FMN, 1.5 μmol. The reaction was colorimetrically followed by nitrite disappearance. Other experimental conditions were the same as in the standard assay for Methyl Viologen–nitrite reductase activity.

Electron donors and cofactors	NAD(P)H oxidized or nitrite reduced (nmol/min per mg of protein)
NADH	357
NADH, FAD	705
NADPH	66
NADPH, FAD	66
$S_2O_4^{2-}$, Methyl Viologen	192
$S_2O_4^{2-}$, Benzyl Viologen	120
$S_2O_4^{2-}$, FAD	124
$S_2O_4^{2-}$, FMN	120
$S_2O_4^{2-}$	124
None	0

chroococcum nitrite reductase. The reaction was specifically stimulated about twofold by the addition of FAD; FMN was without effect. NADPH was a relatively ineffective electron donor compared with NADH and the addition of FAD gave no additional stimulation. The reducing agent sodium dithionite was also operative as electron donor for the enzymic reduction of nitrite. The reaction was stimulated about twofold by the addition of Methyl Viologen as an artificial electron carrier, but Benzyl Viologen and flavin nucleotides were without effect.

Enzymic reduction of nitrite with NADH

The characterization of the reaction catalysed by nitrite reductase with NADH as electron donor is shown in Table 2. The oxidation of NADH was dependent on the presence of nitrite and no reaction took place when the enzyme preparation was omitted or boiled for 5 min. In the absence of nitrite, the addition of either 1 mM-hydroxylamine or 1 mM-sulphite to the reaction mixture did not bring about any oxidation of NADH, even in the presence of FAD.

Table 2. *Reduction of nitrite by A. chroococcum nitrite reductase with NADH as electron donor*

Experimental conditions were the same as in the standard assay for NADH–nitrite reductase activity. Enzyme preparation, 0.6 mg.

System	NADH oxidized (nmol/min)
Complete	95
Minus NADH	0
Minus NO_2^-	8
Minus nitrite reductase	0
Complete, nitrite reductase heated (5 min at 100°C)	0

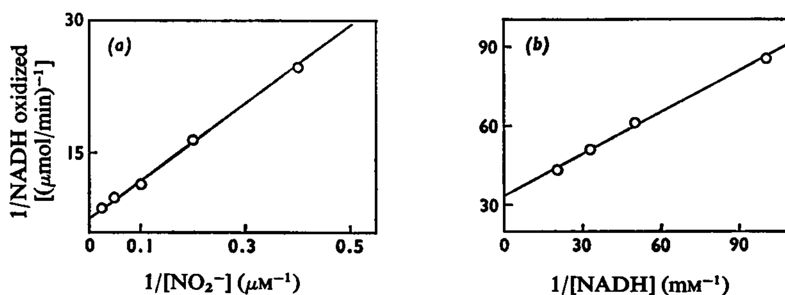

Fig. 1. *Reciprocal plots of the effect of (a) nitrite and (b) NADH concentrations on A. chroococcum nitrite reductase activity*

Experimental conditions were as in the standard assay for NADH–nitrite reductase activity, except that nitrite (*a*) or NADH (*b*) were added as indicated. Enzyme preparation, 0.6 and 0.2 mg respectively.

K_m values for nitrite and NADH

The effect of nitrite and NADH concentration on NADH–nitrite reductase activity is shown in Fig. 1 in the form of Lineweaver–Burk plots. From these results a K_m of 5.5μM for nitrite and of 15μM for NADH was calculated.

Molecular weight

The molecular weight of NADH–nitrite reductase was determined by sucrose-density-gradient centrifugation to be 67000.

Inhibitors

Table 3 shows the effect of several compounds on the enzymic reduction of nitrite. *p*-Hydroxymercuribenzoate and cyanide were potent inhibitors of nitrite reductase activity, whereas azide, cyanate and carbamoyl phosphate did not exert any inhibitory effect. Cyanide inhibition was of the competitive type with respect to nitrite, and the K_i value was 32nM (Fig. 2).

Table 3. *Effect of different inhibitors on the activity of A. chroococcum nitrite reductase*

Experimental conditions were the same as in the standard assay for NADH–nitrite reductase activity, except that different inhibitors were added at the final concentrations indicated. Enzyme preparation, 1.6mg.

Inhibitor	Concentration (μM)	Inhibition (%)
None		0
p-Hydroxymercuribenzoate	10	42
p-Hydroxymercuribenzoate	100	100
KCN	10	40
KCN	100	93
NaN_3	1000	0
KCNO	1000	0
Carbamoyl phosphate	1000	0

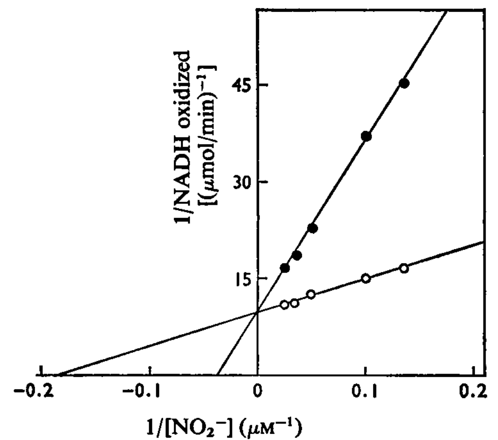

Fig. 2. *Competitive inhibition by cyanide with respect to nitrite of A. chroococcum nitrite reductase activity*

Experimental conditions were as in the standard assay for NADH–nitrite reductase activity, except that nitrite was added as indicated. •, + Cyanide (0.125μM); ○, control, no cyanide. Enzyme preparation, 0.6mg.

Table 4. *Stoicheiometry of nitrite reduction and NADH oxidation in the reaction catalysed by A. chroococcum nitrite reductase*

The reaction mixture contained in a final volume of 2ml: potassium phosphate buffer, pH 7.0, 150μmol; enzyme preparation, 0.7mg; NADH, 0.45μmol; $NaNO_2$ as indicated. The reaction time was 15min. Other experimental conditions were the same as in the standard assay for NADH–nitrite reductase activity.

NO_2^- added (nmol)	NADH oxidized (nmol)	Ratio of NADH oxidized/NO_2^- added
20	66	3.30
40	113	2.83
60	170	2.84

Table 5. *Stoicheiometry of nitrite reduction and NH_3 formation in the reaction catalysed by A. chroococcum nitrite reductase*

The reaction mixture contained in a final volume of 2ml: potassium phosphate buffer, pH 7.0, 150μmol; enzyme preparation, 3.3mg; NADH, 12μmol; $NaNO_2$, 4μmol. The reaction was carried out at 30°C in open tubes and 0.2 and 0.5ml samples were taken at the times indicated for measurement of nitrite and NH_3 respectively.

Time (min)	NO_2^- disappeared (μmol)	NH_4^+ formed (μmol)	Ratio of NO_2^- disappeared/ NH_4^+ formed
10	1.51	1.64	0.92
15	2.40	2.44	0.99
20	3.35	3.14	1.16

Stoicheiometry of NADH oxidation, nitrite reduction and NH_3 formation

As shown in Table 4, the reduction of nitrite by nitrite reductase was concomitant with the oxidation of stoicheiometric amounts of NADH: 3mol of the nucleotide was oxidized/mol of nitrite reduced when the reaction was complete. The product of the enzymic reduction of nitrite was identified as NH_3 and the stoicheiometry of nitrite reduction and NH_3 formation was established: 1 mol of nitrite was consumed/mol of NH_3 formed (Table 5).

Interconvertible active and inactive forms of nitrite reductase

The rate of NADH oxidation by nitrite was linear with time for at least 15min under the conditions described for the enzyme assay. However, when nitrite reductase was preincubated at room temperature with NADH in the absence of nitrite a very rapid inactivation of the enzyme was observed. Fig. 3 shows the time-course of nitrite reductase inactivation by NADH. It shows also that when the enzyme was preincubated with NADH plus nitrite or with nitrite alone, the original activity was maintained. Nitrite not only protected nitrite reductase against inactivation by NADH, but also reactivated almost completely the inactive enzyme in a short period of time if added to the preincubation mixture after inactivation

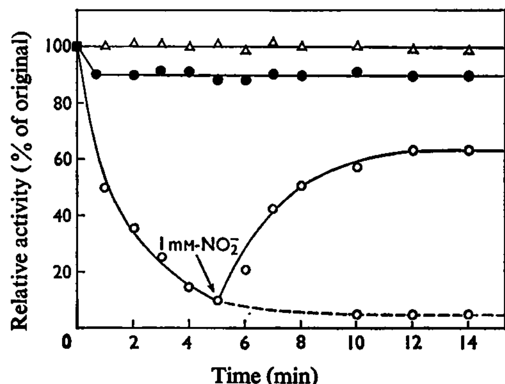

Fig. 3. *Time-course of A. chroococcum nitrite reductase reversible inactivation by NADH and its protection by nitrite*

The enzyme preparation (0.4mg/1.8ml) was preincubated at room temperature with 80mM-potassium phosphate buffer, pH7.0 (△), and 0.15mM-NADH (○) or 0.15mM-NADH plus 1mM-$NaNO_2$ (●). Reactivation by nitrite of the enzyme inactivated by NADH was initiated at the time indicated by the arrow. ○--○ shows reaction if no nitrite is added. NADH–nitrite reductase activity was measured at the times indicated after the mixture had been supplemented, when necessary, with the reagents of the standard assay.

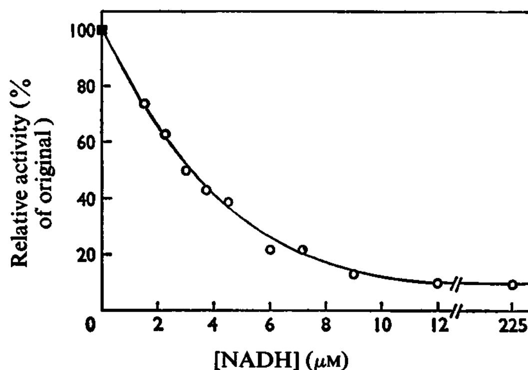

Fig. 4. *Effect of NADH concentration on A. chroococcum nitrite reductase inactivation*

The enzyme preparation (0.4mg/1.8ml) was preincubated at room temperature for 5min with 80 mM-potassium phosphate buffer, pH 7.0, and the indicated concentrations of NADH. NADH–nitrite reductase activity was then measured after the mixture had been supplemented with the reagents of the standard assay.

Table 6. *Inactivation by NAD(P)H and protection by nitrite of A. chroococcum nitrite reductase*

The preincubation mixture contained, in a final volume of 1.8ml: potassium phosphate buffer, pH7.0, 150μmol; nitrite reductase, 0.4mg; where indicated NAD(P)H, 0.3μmol; $NAD(P)^+$, 0.3μmol; $NaNO_2$, 2μmol; KNO_3, 10μmol; $(NH_4)_2SO_4$, 1μmol. After 5min at room temperature NADH–nitrite reductase activity was measured; if necessary the reaction mixture was supplemented with the reagents for the standard assay.

Addition	Relative activity (% of control)
None	100
NADH	5
NAD^+	110
NO_2^-	100
NADH+NO_2^-	90
NADH+NO_3^-	4
NADH+NH_4^+	5
NADPH	2
$NADP^+$	100
NADPH+NO_2^-	6

Table 7. *Effect of the inorganic nitrogen source on the cellular activity of nitrite reductase in A. chroococcum*

Cells were grown on media with the indicated nitrogen sources. After 12h cell-free extracts were prepared and centrifuged at high speed and NADH–nitrite reductase activity was measured in the resulting supernatants. Other experimental conditions are described in the Materials and Methods section.

Nitrogen source	Nitrite reductase (munits/mg of protein)
N_2	0
$N_2+(NH_4)_2SO_4$	0
N_2+KNO_3	12.7
$N_2+NH_4NO_3$	9.8
N_2+NaNO_2	7.3
$N_2+NaNO_2+(NH_4)_2SO_4$	12.4

by NADH had taken place. Fig. 4 shows nitrite reductase inactivation as a function of NADH concentration: total loss of activity was observed after preincubation with 10μM-NADH for 5min.

Only NADH was effective in promoting the inactivation process; NAD^+ was inert. NADPH was also a very effective inactivator, but nitrite only exhibited its protective effect against inactivation by NAD(P)H when NADH was the reducing agent; preincubation with NADPH in the presence of nitrite resulted in total inactivation. Protection by nitrite was specific, nitrate and NH_3 being ineffective in this respect (Table 6).

Induction of nitrite reductase

The nitrite reductase activities of cell-free extracts from *A. chroococcum* were greatly influenced by the nature of the inorganic nitrogen source in the culture medium. As shown in Table 7, the cellular nitrite reductase activities were very low when the source of nitrogen was exclusively air (i.e., 80% N_2 gas) or NH_3. In contrast, the nitrite reductase content was high in cells grown aerobically with nitrate or nitrite either with or without NH_3. When growing on media containing a mixture of different forms of inorganic nitrogen, the cells could utilize nitrate or nitrite even in the presence of NH_3.

Effect of tungstate on nitrate reduction

In the absence of added molybdenum, the addition of tungstate (0.1mM) to the culture medium completely prevented the aerobic growth of *A. chroococcum* when nitrate served as the sole nitrogen source, but this inhibition did not occur when inorganic nitrogen was supplied as nitrite or NH_3, even at tungstate concentrations that had totally suppressed growth with nitrate. The molybdenum requirement for growth was particularly marked when N_2 was the sole nitrogen source, and in this case the addition of only 1μM-tungstate to the nutrient solution without molybdenum was enough to prevent growth completely. The activities of nitrate reductase and nitrite reductase in *A. chroococcum* cells as a function of the tungstate concentration are shown in Table 8. The nitrate reductase specific activity of the cell-free extracts decreased markedly as the concentration of tungstate in the growth medium increased, whereas the nitrite reductase activity remained constant.

Table 8. *Effect of tungstate on the enzymic activities of the nitrate-reducing system in A. chroococcum*

Cells were grown on nitrate media lacking added molybdate and containing the indicated amounts of tungstate. After 14h cell-free extracts were prepared and centrifuged at high speed and the activities of nitrate reductase and nitrite reductase were measured in the resulting supernatants. Other experimental conditions are described in the Materials and Methods section.

	Enzyme activity (munits/mg of protein)	
WO_4^{2-} added (μM)	Nitrate reductase	NADH–nitrite reductase
0	23.0	20
10	3.3	35
100	3.1	25
1000	2.3	25

Discussion

Nitrite reductase from *A. chroococcum* closely resembles that from nitrate-assimilating fungi (Nason *et al.*, 1954; Nicholas *et al.*, 1960; Rivas *et al.*, 1973) with respect to NAD(P)H as electron donor and FAD as cofactor. It thus differs essentially from the ferredoxin–nitrite reductase of green plants, which cannot use nicotinamide nucleotides as electron donors and seems not to be a flavoprotein (Losada & Paneque, 1971; Cárdenas *et al.*, 1972*a,b*; Zumft, 1972).

The marked inhibition by *p*-hydroxymercuribenzoate of *A. chroococcum* NADH–nitrite reductase indicates the involvement of thiol groups in enzyme activity, and the sensitivity to cyanide, a metal-binding reagent, suggests that a metal component is essential. Since the inhibition by cyanide was competitive with nitrite, it seems likely that this chelating agent inhibits by reversibly binding at an essential site on the enzyme. On the other hand, azide at a concentration as high as 1mM did not show any inhibitory

effect. Therefore with respect to these metal inhibitors both NAD(P)H–nitrite reductase (Nason *et al.*, 1954; Nicholas *et al.*, 1960; Rivas *et al.*, 1973) and ferredoxin–nitrite reductase (Losada & Paneque, 1971; Cárdenas *et al.*, 1972*a*,*b*; Zumft, 1972) behave similarly.

It has been frequently claimed that the reduction of nitrite to NH_3 proceeds by a series of two-electron transfers, each catalysed by a different enzyme (Nason, 1962; Takahashi *et al.*, 1963; Hewitt & Nicholas, 1964). However, present evidence obtained with ferredoxin–nitrite reductase from *Chlorella* cells and spinach and squash leaves has demonstrated that nitrite is completely reduced to NH_3 with no obligate free intermediates (Beevers & Hageman, 1969; Losada & Paneque, 1971; Losada, 1972). Similar conclusions have been reached for the bacterial enzyme (Lazzarini & Atkinson, 1961; Kemp & Atkinson, 1966; Prakash & Sadana, 1972). The results reported here corroborate this view and show that in *A. chroococcum* nitrite is also directly and stoicheiometrically reduced to NH_3 without the formation of hydroxylamine as a free intermediate.

With regard to the existence of two either active or inactive interconvertible forms of *A. chroococcum* NADH–nitrite reductase, it is important to consider together the relevant roles of the two enzymes of the nitrate-reducing system (nitrate reductase and nitrite reductase) in the overall regulation of the assimilatory pathway of nitrate reduction in bacteria, fungi, algae and higher plants.

We have recently shown that NH_3, the end-product of this pathway, promotes *in vivo* the reversible inactivation of the second moiety of the NADH–nitrate reductase complex in green algae (Losada *et al.*, 1970; Herrera *et al.*, 1972). Apparently, NH_3 acts as an uncoupler of photophosphorylation thus leading to an increase in the cellular concentrations of NAD(P)H and ADP (Losada *et al.*, 1973). Lack of O_2 leads to the same effect (Losada *et al.*, 1973). Conversion *in vitro* of active *Chlorella* nitrate reductase into its inactive form depends on its reduction by NADH in the presence of ADP (Maldonado *et al.*, 1973). The transformation *in vitro* is also reversible and on reoxidation the enzyme again becomes active (Jetschmann *et al.*, 1972; Moreno *et al.*, 1972; Maldonado *et al.*, 1973). The phenomenon seems to be a general property of plant nitrate reductase rather than a peculiarity of the enzyme from photosynthetic cells, since it has been also observed with the enzymes from the yeasts *Hansenula anomala* (Pichinoty & Méténier, 1966) and *T. nitratophila* (Rivas *et al.*, 1973).

In prokaryotic organisms, it has been reported that the nitrate-reducing system of *A. vinelandii* and other bacteria requires O_2 for both the reduction of nitrate to nitrite and the reduction of nitrite to NH_3 (Takahashi *et al.*, 1963). We have recently shown that *A. chroococcum* nitrate reductase can exist in two interconvertible either active or inactive forms (M. G. Guerrero, J. M. Vega, E. Leadbetter & M. Losada, unpublished work). The results presented in the present paper demonstrate that *A. chroococcum* NADH–nitrite reductase can be inactivated by preincubation with NADH in the absence of nitrite and that the latter substrate specifically prevents and reverses such inactivation. These reversible inactivation processes seem again to be of general metabolic significance in bacteria and are probably related to redox changes in the enzyme proteins. Kemp & Atkinson (1966) had previously observed that activity of *E. coli* nitrite reductase *in vitro* was enhanced by preincubation with nitrite and decreased by preincubation with NADH.

Nitrite reductase is absent from extracts of *A. chroococcum* cells grown on N_2 or NH_3 as sole nitrogen source, but is formed by cells growing on nitrate or nitrite even in the presence of N_2 or NH_3, thus confirming the inducible nature of the assimilatory bacterial enzyme (Spencer *et al.*, 1957; Kemp & Atkinson, 1966).

The experiments with tungstate as a competitive inhibitor of molybdate have corroborated in *A. chroococcum* the results obtained previously with this bacterium (M. G. Guerrero, J. M. Vega, E. Leadbetter & M. Losada, unpublished work) and with *Chlorella fusca* (Cárdenas *et al.*, 1971; Vega *et al.*, 1971), that the site of molybdenum action in the assimilatory metabolic pathway leading from nitrate to NH_3 is exclusively in the reduction of nitrate to nitrite and that molybdenum is an essential component of nitrate reductase but does not play any role in the reaction catalysed by nitrite reductase.

This work was carried out with the aid of a research grant from the Instituto de Estudios Nucleares, Spain. We thank Professor A. Paneque, Professor E. Palacián and Professor J. Cárdenas for helpful discussion and Miss M. J. Pérez de León, Miss I. Fernández, Mr. J. Moreno and Mr. J. F. Alonso for skilled technical assistance.

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