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SOME PROPERTIES OF NITRITE AND HYDROXYLAMINE REDUCTASES FROM *DERXIA GUMMOSA*

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Key Word Index—*Derxia gummosa*; nitrite reductase; hydroxylamine reductase; azaserine.

Abstract—NADH-nitrite and -hydroxylamine reductases were co-purified from *Derxia gummosa*. The stoichiometries for the reduction of nitrite and hydroxylamine to ammonia were 3 NADH:1 NO_2^-:1 NH_3 and 1 NADH:1 NO_2^-:1 NH_3. The K_m values for nitrite and hydroxylamine were 4.8 μM and 5.3 mM, respectively, and for NADH they were 6.3 μM for nitrite reductase and 150 μM for hydroxylamine reductase. The optimal pH value for both enzyme activities was 8.5. Both activities were inhibited by NADH in the absence of the appropriate substrate, namely nitrite or hydroxylamine. Studies with amino acid modifiers indicate that histidine, glutamate/aspartate, sulphydryl and tyrosine are essential components of the enzyme protein. Kinetic studies show that nitrite and hydroxylamine were competitive for the same binding site on the enzyme. The results indicate that although nitrite and hydroxylamine reductases are associated with the same enzyme, its main function is the reduction of nitrite to ammonia. Azaserine inhibited the induction of the enzyme.

INTRODUCTION

Assimilatory nitrite reductase in algae and higher plants is a ferredoxin-linked enzyme [1, 2]. This enzyme from *Chlorella* and higher plants contains two atoms of non-haem iron and has a molecular weight of 63 000 [3–6]. On the other hand, nitrite reductase from a fungus *Neurospora crassa* is NAD(P)H-dependent requiring FAD for maximal activity [7–9]. Similar results were also obtained with a nitrite reductase from *Azotobacter vinelandii* and *A. chroococcum* [10, 11]. An NADH-dependent hydroxylamine-reducing system was reported in extracts of *A. vinelandii* [10] but it was not detected in those of *A. chroococcum* [11]. Hydroxylamine reductase activity was found to be associated with nitrite reductase from *Achromobacter fisheri* [12], *Neurospora crassa* [9] and baker's yeast [13]. A nitrite reductase from baker's yeast had also a sulphite reductase activity.

In the present paper we describe some of the properties of an NADH-linked nitrite reductase from *Derxia gummosa* which also has hydroxylamine reductase activity.

RESULTS

Purification of enzyme

As shown in Table 1, nitrite and hydroxylamine reductases were co-purified and the ratio of their activities during purification remained constant, i.e. approximately 2.

Electron donors

As shown in Table 2, NADH was a more effective electron donor for both nitrite and hydroxylamine reductase activities than NADPH. Either FAD or FMN stimulated both activities about two-fold. Reduced forms of benzyl viologen (BVH) or methyl viologen (MVH) were

Table 1. Nitrite and hydroxylamine reductase activities during purification

Fraction	(A) Nitrite reductase* (units/mg protein)	(B) Hydroxylamine reductase* (units/mg protein)	Purification for nitrite reductase (fold)	Ratio (B/A)
I S_{20}	10	22	1	2.2
II S_{144}	22	46	2	2.1
III $(NH_4)_2SO_4$	88	170	9	1.9
IV DE-52	422	868	42	2.1

*1 unit = 1 nmol NO_2^- or NH_2OH utilized/min.
Enzyme activities were determined as described in the Experimental.

Table 2. Electron donors for nitrite and hydroxylamine reductase activities

Electron donor	Final concentration (mM)	% Activity	
		Nitrite reductase	Hydroxylamine reductase
NADH	1.0	100	100
NADPH	1.0	54	46
$FADH_2$*	2.0	94	117
$FMNH_2$*	2.0	95	105
BVH*	10	63	210
MVH*	10	70	220
$Na_2S_2O_4$	5	80	57
NADH + FAD	1 + 0.02	216	206
NADH + FMN	1 + 0.02	192	196

*Reduced by sodium dithionite.

Enzyme activities of fraction IV, Table 1 were determined as described in the Experimental. Nitrite reductase activity (100%): 195 nmol NO_2^- utilized/min/mg protein, and hydroxylamine reductase activity: 410 nmol NH_2OH utilized/min/mg protein.

more effective as electron donors for hydroxylamine reductase than for nitrite reductase. The ratios of nitrite reduced to ammonia produced and NADH oxidized were 1:1:3 (Table 3); and the ratios of hydroxylamine utilized to ammonia formed and NADH oxidized were 1:1:1 (Table 4).

Table 3. Stoichiometry of nitrite reduction

NO_2^- added (μmol)	NADH oxidized (μmol)	NH_3 formed (μmol)	Ratio	
			NH_3/NO_2^-	$NADH/NH_3$
0.1	0.35	0.11	1.1	3.18
0.5	1.55	0.51	1.02	3.04
1.0	2.91	0.96	0.96	3.03

The reaction mixture (2 ml) in 200 mM Tris–HCl buffer (pH 8.5) containing NADH, 4 μmol; FAD, 10 nmol and enzyme (fraction IV, Table 1), 0.2 mg and $NaNO_2$ as indicated was incubated at 30° for 30 min. Then residual NADH was determined in 0.5 ml aliquots of the reaction mixture diluted to 3 ml with 200 mM Tris–HCl buffer (pH 8.5) in 1 cm quartz cuvettes in a Varian spectrophotometer at 340 nm. Ammonia produced was determined as described in the Experimental.

K_m values and optimal pH for enzyme activities

The apparent K_m values for nitrite and hydroxylamine were 4.8 μM and 5.3 mM respectively. The K_m values for NADH were 6.3 μM for nitrite reductase and 150 μM for hydroxylamine reductase. The optimal pH for both enzyme activities was 8.5.

Inhibitor studies

The data in Table 5 indicate that both enzyme activities were inhibited by *p*-chloromercuribenzoate (pCMB), *N*-ethylmaleimide (NEM), rotenone, antimycin A, 8-hydroxyquinoline, 2,2′-dipyridyl, potassium cyanide and dinitrophenol (DNP). Potassium thiocyanate inhibited hydroxylamine reductase only. Amytal, sodium azide, sodium diethyldithiocarbamate and 2-heptyl 4-hydroxyquinoline *N*-oxide, however, were without effect.

Reversible inactivation of nitrite and hydroxylamine reductases by NADH

The results in Fig. 1A and B show that when aliquots of fraction IV (Table 1) were preincubated with NADH then nitrite and hydroxylamine reductases were inactivated within 1 min. Maximum inhibition (60%) was achieved with only 1 μM NADH. This inhibition of nitrite reductase was offset when nitrite was added to the enzyme at the same time as NADH (Fig. 1A). A similar result was obtained for hydroxylamine reductase but in this case hydroxylamine was added instead of nitrite (Fig. 1B). The addition of nitrite to a deactivated enzyme restored nitrite reductase activity (Fig. 1A) as did the addition of hydroxylamine for hydroxylamine reductase (Fig. 1B). Neither nitrate, ammonium salts nor NAD^+ was effective in reactivating NADH-inhibited nitrite or hydroxylamine reductases. Inhibition of the two enzyme activities by NADPH was about half that recorded for NADH each at 0.1 mM and these activities were also restored on adding the appropriate substrate.

Effects of amino acid modifiers on the enzyme

The results in Tables 6 and 7 show the effects of amino acid modifiers on nitrite and hydroxylamine reductase activities as well as substrate protection against inactivation by these reagents. Preincubation of aliquots of fraction IV (Table 1) with *p*-chloromercuribenzoate (pCMB), Rose Bengal dye, iodine and 1-cyclohexyl

Table 4. Stoichiometry of hydroxylamine reduction

Incubation time (min)	NH_2OH utilized (μmol)	NADH oxidized (μmol)	NH_3 produced (μmol)	Ratio	
				NH_3/NH_2OH	$NADH/NH_2OH$
5	1.10	1.24	1.15	1.05	1.08
10	2.08	2.26	2.11	1.01	1.07
20	3.96	4.10	3.86	0.97	1.06

Three identical reaction mixtures (3 ml) in 200 mMδ Tris–HCl buffer (pH 8.5) containing 20 mM hydroxylamine hydrochloride (neutralized with NaOH), 5 mM NADH, 10 μM FAD and 0.15 mg enzyme (fraction IV, Table 1) were dispensed in test tubes (1 × 13 cm) and incubated at 30°. Aliquots (0.5 ml) were taken at the times indicated and assayed for hydroxylamine and ammonia as described in the Experimental. NADH was assayed in 1 cm quartz cuvettes at 340 nm.

Table 5. Effects of inhibitors on nitrite and hydroxylamine reductase activities

Inhibitor	Final concentration (mM)	Inhibition (%)	
		Nitrite reductase	Hydroxylamine reductase
pCMB	0.1	60	67
NEM	0.1	52	58
Rotenone	0.1	56	52
KCNS	0.1	0	90
KCN*	1.0	45	42
2,2'-Dipyridyl*	1.0	40	38
8-Hydroxyquinoline	1.0	96	50
Antimycin A	0.2	45	26
DNP	0.1	40	80

*No inhibition was observed when these compounds were added to the enzyme after incubating the enzyme with the appropriate substrate, either NO_2^- or NH_2OH.

The reaction mixture (3 ml) in a 1 cm quartz cuvette contained in μmol: 500 Tris–HCl buffer (pH 8.0), 0.01 FAD, 0.3 NADH and 20 μg enzyme (fraction IV, Table 1). After preincubating for 5 min at 30°, either 3 μmol of NO_2^- or 60 μmol NH_2OH was added to start the reaction. The rates of nitrite and hydroxylamine reductases determined by NADH oxidation were linear over a 10 min period. Enzyme activity (100%) was 1.12 μmol NADH oxidized/min/mg protein for nitrite reductase and 0.8 μmol NADH oxidized/min/mg protein for hydroxylamine reductase.

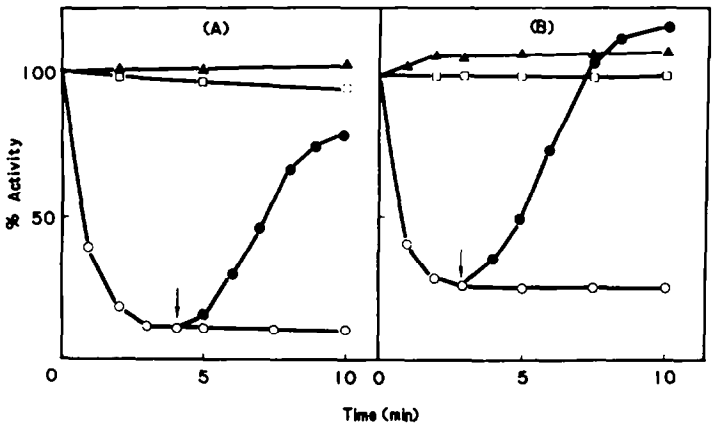

Fig. 1. Inactivation of nitrite reductase (A) and hydroxylamine reductase (B) (fraction IV, Table 1) by NADH and their reactivation by nitrite (A) and hydroxylamine (B). The enzyme preparation (0.4 mg protein) was preincubated in 1 ml 50 mM Tris–HCl buffer, pH 8.5 (□), containing 0.2 mM NADH (○), or (A), 0.2 mM NADH + 1 mM $NaNO_2$ (▲) and (B), 0.2 mM NADH + 20 mM NH_2OH (▲). The addition of NO_2^- (A) or NH_2OH (B) is indicated by arrows (●). Enzyme activities were determined as indicated by following the oxidation of NADH at 340 nm as described by Vega *et al.* [11]. Enzyme activities (100%) were 0.98 and 0.66 μmol NADH oxidized/min/mg protein for nitrite and hydroxylamine reductases, respectively.

3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene-sulphonate (mopho CDI) respectively resulted in significant losses of both nitrite and hydroxylamine reductase activities. Preincubation with either NO_2^- or FAD protected nitrite reductase from inhibition by Rose Bengal (Table 6) and similar results were obtained for hydroxylamine reductase (Table 7) except that hydroxylamine was used instead of nitrite. Nitrite and hydroxylamine also offset the inhibitory effects of morpho CDI on nitrite and hydroxylamine reductases respectively. Preincubation of the enzyme (fraction IV, Table 1) with 20 μM NO_2^- resulted in a complete protection of nitrite reductase activity against either 0.5 mM Rose Bengal or 15 mM morpho CDI (Fig. 2A). A similar result was obtained for hydroxylamine reductase when 10 mM NH_2OH was used instead of nitrite (Fig. 2B).

Table 6. Effects of amino acid modifiers on nitrite reductase activity and enzyme protection by substrates

Modifier	Final concentration (mM)	Inhibition of enzyme activity (%) Preincubation of enzyme: No addition	NO_2^-	FAD	NADH
Sulphydryl					
pCMB	1.0	95	95	95	90
Histidine					
Rose Bengal	0.5	85	13	35	80
Tyrosine					
Iodine	0.5	92	90	94	88
Carboxyl					
Morpho CDI	15	65	24	52	58

Aliquots (0.5 ml containing 0.45 mg protein, fraction IV, Table 1) in glass tubes (1 × 13 cm) were preincubated with either NO_2^- (0.1 mM), FAD (25 μM) or NADH (0.1 mM) for 10 min at 30° in a final volume of 0.9 ml and then the appropriate modifier (0.1 ml) was added and incubation continued for 15 min. Then further aliquots were taken from these reaction mixtures and assayed for nitrite reductase as described in the Experimental. When NADH was included in the preincubation treatment, the enzyme was first reactivated by adding 0.1 mM NO_2^- followed by a further incubation at 30° for 5 min and then nitrite reductase activity was determined in aliquots of the reaction mixture. Enzyme activity (100%) was 380 nmol NO_2^- utilized/min/mg protein.

Table 7. Effects of amino acid modifiers on hydroxylamine reductase activity and enzyme protection by substrates

Modifier	Final concentration	Inhibition of enzyme activity (%) Preincubation of enzyme: No addition	NH_2OH	FAD	NADH
pCMB	1.0	94	90	88	90
Rose Bengal	0.5	80	36	45	78
Iodine	0.5	85	80	86	90
Morpho CDI	1.5	68	36	58	60

The experimental procedures were similar to those described in Table 6, except that hydroxylamine (20 mM) was used instead of nitrite. Enzyme activity was assayed as described in Experimental. Enzyme activity (100%) was 720 nmol NH_2OH utilized/min/mg protein.

Competitive effects of nitrite and hydroxylamine

Nitrite inhibited hydroxylamine reductase (K_i 0.52 mM) and this inhibition was competitive for hydroxylamine (Fig. 3); similarly, hydroxylamine competitively inhibited nitrite reductase (K_i 24 mM).

Induction of nitrite and hydroxylamine reductases

Maximum activities of nitrite and hydroxylamine reductases were recorded in cells (grown for 20 hr with 1 mM NH_4Cl at 30°) within 30 min of adding 0.1 mM $NaNO_2$ at 30° *in vivo*. However, the addition of 0.05 or 0.1 mM $NH_2OH \cdot HCl$ instead of nitrite did not induce either nitrite or hydroxylamine reductase activities. Chloramphenicol (40 μg/ml) and rifampicin (25 μg/ml) completely inhibited the induction of these enzymes. Azaserine at a final concentration of 0.5 mM in the culture medium almost completely inhibited the induction of the two enzyme activities (Table 8). Similar effects of azaserine were observed with *Azospirillum brasilence* ATCC 29145 (Table 8). The induction of nitrite reductase in *Azotobacter vinelandii* (OP), however, was not affected by this compound. Azaserine did not inhibit the enzymes *in vitro*.

DISCUSSION

Nitrite reductase from *Derxia gummosa* is similar to those found in fungi [7, 8, 14–16] and bacteria [10, 11] assimilating nitrate with respect to requirements for NAD(P)H as an electron donor and FAD as a cofactor. The stoichiometry of nitrite reduction was 1 NO_2^-: 1 NH_3, in agreement with the data for *A. vinelandii* [10], *A. chroococcum* [11] and *Achromobacter fisheri* [12].

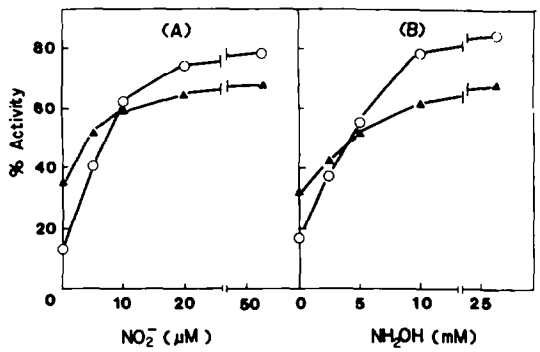

Fig. 2. Effects of various concentrations of nitrite (A) and hydroxylamine (B) on the protection of nitrite and hydroxylamine reductases respectively from inhibition by Rose Bengal and morpho CDI. Partially purified enzyme (0.2 mg protein, fraction IV, Table 1) in 1 ml 50 mM Tris–HCl buffer (pH 7.5), was preincubated with various concentrations of $NaNO_2$ (A) or NH_2OH (B) for 10 min at 30°. Then 0.1 ml of 5 mM Rose Bengal (○) or 0.1 ml of 150 mM morpho CDI (▲) was added and the incubation continued for 15 min. Enzyme activities were determined by following the utilization of NO_2^- or NH_2OH as described in the Experimental. Enzyme activity (100%) for nitrite reductase was 0.36 μmol NO_2^- utilized/min/mg protein and for hydroxylamine reductase, 0.82 μmol NH_2OH utilized/min/mg protein.

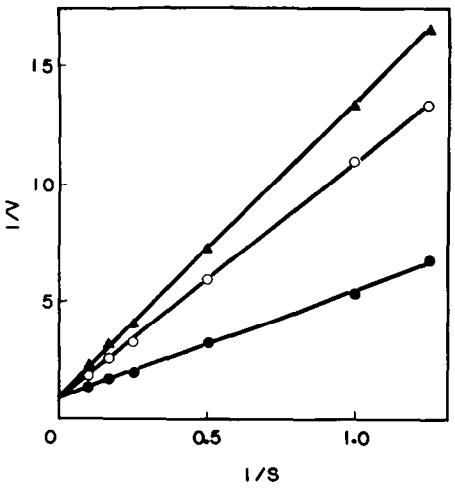

Fig. 3. Lineweaver–Burk plots of the effects of various concentrations of nitrite on hydroxylamine reductase activity. Enzyme was assayed by following the utilization of NH_2OH with NADH as the electron donor as described in Experimental, with 1 mM NO_2^- (▲); with 0.5 mM NO_2^- (○) and without NO_2^- (●). $1/S$ (Hydroxylamine mM)$^{-1}$; $1/V$ (μmol NH_2OH utilized/min mg protein)$^{-1}$.

Similar results have also been obtained with a ferredoxin-dependent nitrite reductase from algae and higher plants [1, 3, 17].

The nitrite reductase from *Derxia gummosa* was inactivated by NADH or NADPH in the absence of nitrite and this substrate specifically protected the enzyme from this effect. Similar results have been reported for NADH and nitrite for the enzyme from *A. chroococcum* and *E. coli* [11, 19]. The NADH inhibition of nitrite reductase was not however reversed by nitrite in *Neurospora* [18]. The NADH inactivation of nitrite reductase is different from that reported for nitrate reductase from higher plants [20] because FAD, EDTA or superoxide dismutase which reactivated nitrate reductase has no effect on the inactivated nitrite reductase in *Derxia*.

The effects of amino acid modifiers on nitrite and hydroxylamine reductase activities in *Derxia* and the protection afforded by nitrite and hydroxylamine respectively indicate that histidine and carboxyl groups may be required for the binding of nitrite and hydroxylamine to the enzyme. Histidine is also involved at the FAD-binding site since FAD protected both enzyme activities from inhibition by Rose Bengal. The fact that substrates do not prevent the inhibition by either pCMB or iodine (tyrosine modifier) [21] suggests that sulphydryl and tyrosine do not operate at the substrate site of the enzyme.

Assimilatory nitrite reductases are produced in the presence of its substrate in *A. vinelandii* [10], *A. chroococcum* [11] and *E. coli* [19]. Similar results were obtained with *Derxia gummosa*. The inhibition of this induction by chloramphenicol and rifampicin indicates that transcription as well as translation are involved in this process. Azaserine, an analogue of glutamine, restricted the induction of this enzyme in both *Derxia gummosa* and *Azospirillum brasilence* (Table 8). Glutamine had little effect on enzyme production; the glutamine inhibition of the induction in *Azospirillum in vivo* is less than that for azaserine (Table 8).

Our results show that hydroxylamine reductase activity is also associated with the enzyme because (a) the ratios of nitrite and hydroxylamine reductases remained constant throughout purification (Table 1); (b) both activities have similar requirements for a variety of electron donors (Table 2); (c) similar effects were recorded for both activities with a variety of inhibitors (Table 5), amino acid modifiers (Tables 6 and 7 and Fig. 2A, B) and by NADH inhibition (Fig. 1A, B); (d) hydroxylamine reductase activity was also induced by nitrite and this induction was also inhibited by azaserine (Table 8) and (e) kinetic studies show that nitrite and hydroxylamine are competitive for the same site on the enzyme (Fig. 3).

Nitrite reductase with hydroxylamine reductase activity from baker's yeast can also reduce sulphite to sulphide [13]; but the enzyme from *Derxia* did not utilize sulphite thus resembling nitrite reductases from *Achromobacter fisheri* [11], *E. coli* [22] and *Neurospora* [23].

The much lower K_m value for nitrite than for hydroxylamine, indicates that the main function of this enzyme is to reduce nitrite to ammonia. In this respect it is similar to the nitrite/hydroxylamine reductase enzyme from *Achromobacter fisheri* [12] and *Torulopsis nitratophila* [16].

EXPERIMENTAL

Materials. NADH, NADPH, FAD, FMN, benzyl viologen, methyl viologen, various inhibitors and amino acid modifiers were purchased from Sigma. DE-52 cellulose was obtained from Whatman. All other chemicals used were of analytical grade.

Bacteria and growth conditions. A culture of *Derxia gummosa* was kindly supplied by Professor Y. T. Tchan, Department of Microbiology, University of Sydney, Australia. The culture

Table 8. Effects of azaserine and glutamine on the induction of nitrite and hydroxylamine reductase in *Derxia gummosa* and *Azospirillum brasilence*

Inhibitor added to culture solution (final concn)	% Inhibition			
	Derxia		*Azospirillum*	
	Nitrite reductase	Hydroxylamine reductase	Nitrite reductase	Hydroxylamine reductase
None	0	0	0	0
Azaserine (0.5 mM)	92	90	81	85
Glutamine (2 mM)	10	13	38	34

Derxia gummosa was grown as described in the Experimental except that 1 mM NH_4Cl was the sole source of nitrogen. The culture medium for *Azospirillum brasilence* was as described by Albecht and Okon [27] except that 1 mM NH_4Cl was the sole nitrogen source. Both bacteria were grown at 30° for 24 hr on a gyratory shaker (120 rpm). Aliquots (20 ml) of the culture solution were incubated in 50 ml flasks with either 0.5 mM azaserine or 2.0 mM glutamine for 30 min on a reciprocating shaker at 30°. Then 0.5 mM $NaNO_2$ was added to each aliquot and incubation continued for 30 min. Nitrite and hydroxylamine reductase activities were then determined in cell-free extracts as described in the Experimental.

medium and growth conditions were as described previously [24] except that 10 mM KNO_3 was used as the nitrogen source. Cells harvested by continuous flow centrifugation were washed twice with 50 mM Tris–HCl buffer (pH 7.5).

Enzyme assays. Nitrite reductase was assayed by determining either the utilization of nitrite or NADH oxidation, the latter in 1 cm quartz cuvettes at 340 mm. For the NO_2^- utilization assay, the reaction mixture (1 ml) in 1 × 13 cm tubes contained: Tris–HCl buffer (pH 8.5), 50 mM; $NaNO_2$, 0.12 mM; NADH, 1 mM; FAD, 10 μM and an appropriate aliquot of enzyme (utilizing 40–100 nmol NO_2^- in 10 min at 30°). The reaction was started by adding NADH [25] and terminated by adding 0.1 ml 1.0 M $Zn(OAc)_2$ followed by 2.9 ml EtOH. After mixing on a Vortex shaker for 1 min the reaction mixtures were centrifuged at 5000 *g* for 5 min. Aliquots of the supernatants were assayed for nitrite by the method of Bhandari and Nicholas [25].

Hydroxylamine reductase was determined by following either the utilization of hydroxylamine or the oxidation of NADH at 340 nm. The reaction mixture (0.3 ml) contained $NH_2OH \cdot HCl$ (neutralized by NaOH to pH 7.0), 15 mM; NADH, 4 mM; EtOH, 100 mM; alcohol dehydrogenase (Sigma) to regenerate NADH, 5 units and appropriate amount of enzyme so that 5–10 μmol NH_2OH was reduced in 20 min. The reaction was terminated after a 20 min incubation at 30°, by adding 10 mM $NaNO_2$. Residual hydroxylamine in the reaction mixture was assayed immediately with a purified glutamine synthetase (from *Derxia*) by the transferase reaction which has been described previously [24].

When the K_m values for NADH were determined for both enzyme activities, the following NADH-regenerating system was included in the reaction mixtures: EtOH, 0.1 M and alcohol dehydrogenase (Sigma), 5 units/ml as well as NADH at various concentrations.

Preparation of enzyme. All enzyme preparations were carried out at 4°. Washed cells suspended in 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM dithiothreitol and 1 mM EDTA (subsequently referred to as the buffer), were disrupted by passage through a French pressure cell at 15000 psi. The homogenate was centrifuged at 20000 *g* for 20 min in a Sorvall RC-5B centrifuge (SS-34 rotor) and the supernatant (fraction I, Table 1) was then centrifuged at 144000 *g* for 90 min in a Beckman L8-70 ultracentrifuge (70.1 Ti rotor). The supernatant (fraction II, Table 1) was treated with satd $(NH_4)_2SO_4$ soln (adjusted to pH 7.0 with 1.0 M Tris at 4°) to give a 40–60% satn. After 30 min, the ppt was centrifuged at 20000 *g* for 15 min and the pellet was dissolved in 7 ml of the buffer (~30 mg protein/ml). This preparation (fraction III, Table 1) desalted by passing through a Sephadex G-25 column (2 × 20 cm, previously equilibrated with the buffer), was then loaded onto a DE-52 anion exchange column (2 × 15 cm) pre-equilibrated with the same buffer. After washing the column with fresh buffer until the A_{280} of the eluent was close to zero, the enzyme was eluted with a linear gradient of 0–0.5 N NaCl in 250 ml of the buffer. Active fractions (0.15–0.22 N NaCl) were pooled and desalted on a Sephadex G-25 column as described previously. This partially purified enzyme was very labile but when stored at −15° under argon it was stable for about 2 weeks.

Determination of ammonia. Ammonia was determined by the method of Russell [26] after first removing protein from the reaction mixtures by adding 0.1 ml 10% w/v trichloroacetic acid/ml and then centrifuging at 5000 *g* for 5 min.

Protein assay. Protein was determined by the method of Bradford [27] using bovine serum albumin as a standard.

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