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THE BIOCHEMICAL PATHWAY OF DISSIMILATORY NITRITE REDUCTION IN DENITRIFICATION

by

Els Weeg-Aerssens

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ABSTRACT

THE BIOCHEMICAL PATHWAY OF DISSIMILATORY NITRITE REDUCTION IN DENITRIFICATION

by

ELS WEEG-AERSSENS

Stable isotopes and purified nitrite reductase were used as two independent approaches to investigate the mechanism of nitrite conversion to nitrous oxide by Pseudomonas stutzeri JM300. Three independent experiments using ^{15}N and ^{18}O and involving isotope equilibration, competition and isotope dilution were supportive of a pathway of nitrite reduction in which there are two binding events of nitrite to the enzyme. The N-N bond in denitrification, at least for this organism, is most likely formed by nucleophilic addition of nitrite to an enzyme bound nitrosyl. In whole cells, given NO and hydroxylamine, the latter was able to channel all of the NO-nitrogen into the nitrosation product. However, no such competitive effect of hydroxylamine on nitrite reduction was found, suggesting that the main flux of nitrite nitrogen to N_2O in the cell did not involve a free NO intermediate. Our findings were inconsistent with an alternative pathway proposal in which chemical dimerization of two nitroxyl ions results in nitrous oxide formation.

The nitrite reductase was purified using anion exchange, gel filtration and hydroxyapatite chromatography. The highly purified enzyme reduced nitrite to both nitric oxide and nitrous oxide. The enzyme did not reduce nitric oxide when it was the only substrate nor

did it reduce nitric oxide when nitrite was added. The kinetic parameters for nitrite for NO and N_2O formation were estimated; the first nitrite binding had a $Km_{(app)}$ of 1.35 uM and a $Vmax_{(app)}$ of 1.6 umole N/mg.min, while second had a $Km_{(app)}$ of 59 uM and a $Vmax_{(app)}$ of 0.9 umole N/mg.min. These kinetic parameters are in a range that is consistent with the physiological conversion of NO_2^- to N_2O by two sequential nitrite binding events.

Nitrite reductase from which the heme $\underline{d}_1$ was extracted was devoid of nitrite reducing activity. Reconstitution with the synthetic heme $\underline{d}_1$ restored both NO and N_2O producing activities from NO_2^-. Taken together, these data show that nitrite is reduced to nitrous oxide by nitrite reductase and that free NO is not an intermediate in this conversion or that "NO reductase" does not play a significant role in denitrification in Pseudomonas stutzeri.

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CHAPTER ONE

THE BIOCHEMICAL MECHANISM OF DISSIMILATORY NITRITE REDUCTION IN DENITRIFICATION

Denitrification is the dissimilatory reduction of nitrate to nitrogen gases. It is carried out by a variety of bacteria that occupy a wide range of natural habitats including soil, water, foods and the digestive tract (1, 2, 3, 4). These organisms are facultative anaerobes: they prefer oxygen as terminal electron acceptor but will denitrify under anaerobic conditions when nitrogen oxides (NO_3^-, NO_2^-, NO, N_2O) are available. From a basic scientific point of view, denitrification is of interest because it is a unique energy generating process common to many bacteria.

Denitrification is also of considerable practical importance. It is one of the two major processes, the other being nitrogen fixation, which control the amount of nitrogen available to the biosphere. Bacterial denitrification is also the only type of anaerobic respiration which releases an essential element from the soil and makes it very expensive to recover. Sulfide oxidizers and chemical oxidation recapture sulfide released by sulfate and sulfite reducers. Carbon dioxide is recycled through photosynthesis and as a substrate for methanogens and acetogens. Methane set free by methanogens is recycled by methane oxidizers. The end product of denitrification, dinitrogen, can only be brought returned to biological circulation through an energy expensive processes, chemical or biological fixation. Denitrifiers are stated by Margulis and Lovelock (5) to be

responsible for generating most of the nitrogen in the earth's atmosphere. On land, 30% (15 million tons) of the nitrogen applied as fertilizer is lost through denitrification (6). Nitrogen flux from oceans accounts for another 36 million tons (1). Moreover, denitrifiers emit free nitrous oxide, which has been found to contribute to ozone destruction in the stratosphere (7, 8) and to projected increases in planet temperature (9). A process having such an enormous impact on our environment naturally has evoked intense curiosity and thorough study.

Man has dreamt of controlling denitrification for many decades, while hopefully being cautiously aware of the risk involved in tampering with a process of such global significance. The agronomist would like to reduce agricultural losses of nitrogen without causing a global imbalance in the nitrogen cycle. There are two basic approaches to controlling the process: one is at the soil management level in which NO_3^- excess, carbon supply and especially water are managed to minimize anaerobic sites with excess nitrite. The second is to devise a specific chemical inhibitor of the process. To evaluate the feasibility of the latter, it is important to understand the chemical mechanism of the key step in denitrification, the formation of the N-N bond. This is the subject of this thesis.

In many textbooks, the denitrification pathway is rudimentarily represented as follows:

$$NO_3^- \overset{(1)}{----} NO_2^- \overset{(2)}{----} (\ NO?\ ) \overset{(3)}{----} N_2O \overset{(4)}{----} N_2$$

It has not been established whether steps 2 and 3 are carried out by two different enzymes or by just one, nitrite reductase. The status of nitric oxide in the pathway is uncertain. From this simple scheme, it is obvious that, if we are going to prevent losses of nitrogen gases from soil, we should concentrate on steps 1 and 2. Once nitric oxide (NO) is formed, it is in our intrest to let the process continue to N_2O or N_2, because of its toxicity. We have devoted our attention to the elucidation of mechanism of reduction of nitrite to N_2O, which is the step in the sequence where "fixed" nitrogen becomes gaseous nitrogen - and is lost to the soil.

In chapter two, the state of the knowledge of the pathway of nitrite reduction in denitrification at the time when this work started is introduced. Three main "putative" pathways had been proposed: one presents nitric oxide as a free obligatory intermediate and thus requires two enzymes, nitrite reductase and nitric oxide reductase for steps 2 and 3 above (1). A second (10) and a third (11) proposed a pathway in which one enzyme, nitrite reductase, reduced nitrite to nitrous oxide: nitric oxide was not an obligatory intermediate but in equilibrium with an enzyme bound intermediate and could thus be called an "abortive" product of the pathway. These two schemes differed, however, in the mechanism of N-N bond formation by nitrite reductase: the second scheme postulated the chemical

dimerization of two equivalent NO^- (nitroxyl) anions (10) while the third postulated an enzymatic, nucleophilic addition of a nitrite anion to an enzyme bound nitrosyl (NO^+) resulting in the first dinitrogen intermediate (trioxodinitrate) (11). Stable isotope studies (^{18}O) with whole cells of Pseudomonas stutzeri provided us with the first test of these hypotheses; the data supported the third pathway. It was found that the nitrite concentration affected relative rates of individual steps within the NO_2^- to N_2O transformation, consistent with our hypothesis that nitrite enters the pathway again, before N-N bond formation, but after the first nitrite has bound to the enzyme (Ch. 2, this work).

The second phase of the work employed cell free extracts together with stable isotopes to conduct experiments in which any effect of limitation in transport across the cell membranes was eliminated. Azide, a nucleophile, could now be used as a nitroxyl trapping agent. At the same time, Kim and Hollocher (10) showed that nitrite reductases could also catalyze nitrosation reactions, in part analogous to what we believed to happen with nitrite (a nucleophile). Other nitrogenous nucleophiles apparently could also attack the enzyme bound nitrosyl intermediate and form N_2 and N_2O in redox reactions called "nitrosations", without use of external reductant (10). This was conclusive evidence for the existence of the postulated nitrosyl intermediate. Catalysis of nitrosation by the enzyme provided us with a new tool to study denitrification: if catalysis of nitrite reduction and nitrosation share a common intermediate (the nitrosyl), we ought to find competition between nitrite and other nucleophile(s) at the branchpoint. This competition was demonstrated through isotope experiments with ^{15}N labeled nitrite and unlabeled azide, by using a

constant azide concentration and varying the nitrite concentration. Denitrification was of a higher kinetic order than nitrosation with respect to the nitrite concentration; this was again indicative of a process of N-N bond formation in which nitrite enters the pathway after the nitrosyl intermediate. A third type of experiment, an isotope dilution experiment, was carried out with both stable isotopes, ^{18}O and ^{15}N. Denitrification and nitrosation occured simultaneously in the presence of ^{18}O labelled water. The ^{18}O content of nitrous oxide from denitrification was lower than the ^{18}O content of nitrous oxide from nitrosation; the latter was taken to be identical of the ^{18}O content of the nitrosyl. This finding indicated that a source of unlabelled or less labelled oxygen "diluted" the ^{18}O content of nitrosyl oxygen before it was converted into nitrous oxide from the denitrification branch. This unlabelled oxygen source is most likely free nitrite, since H_2O is even more highly labelled.

Thus, all three independent approaches provided strong albeit indirect evidence for scheme three as the pathway of nitrite reduction in P. stutzeri JM300. Direct evidence could only be obtained from a more defined system, especially from using a purified nitrite reductase. I purified the Pseudomonas stutzeri JM300 nitrite reductase and studied its kinetic properties. Chapter 4 describes this work. I found that the purified enzyme made nitrous oxide as well as nitric oxide from nitrite, but did not reduce nitric oxide when it was the only substrate, nor did it reduce nitric oxide when nitrite was present. The kinetic parameters for nitric oxide production (Km = 1.35 uM, Vmax = 1.6 umole N/mg.min) and nitrous oxide production (Km = 59 uM, Vmax = 0.9 umole N/mg.min) were comparable: these values are consistent with a physiologically feasible conversion of NO_2^- to N_2O

by a mechanism which involves sequential binding of two nitrite anions. The low Km of the enzyme for the second nitrite binding event (59 uM), when compared to the Km for nitrosation of the P. aeruginosa nitrite reductase by a nucleophiles such as azide (11 mM) and others (2 to 20 mM) (10), illustrates the strong specificity of the enzyme for nitrite as a substrate at the second binding event.

Nitric oxide accumulation in the whole cell, such as we found it in the headspace above the pure enzyme, would be a disadvantage to the cell because of the general toxicity of nitric oxide (12, 13). It is possible that, "in vivo", the nitrite reductase is membrane associated and therefore works more efficiently and does not result in as much of the abortive production of nitric oxide. It is also possible that the "membrane bound" nitric oxide reductase activity, found independently by myself and by many other laboratories, is not entirely due to nitrite reductase, but to a nitric oxide reductase: whether the latter exists, and if it does, whether it plays a role in respiratory denitrification, remains an open question.

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CHAPTER TWO

ISOTOPE LABELING STUDIES ON THE MECHANISM OF N-N BOND FORMATION IN DENITRIFICATION

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Isotope Labeling Studies on the Mechanism of N-N Bond Formation in Denitrification*

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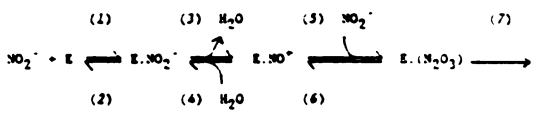
The mechanism of the denitrification and nitrosation reactions catalyzed by the heme *cd*-containing nitrite reductase from *Pseudomonas stutzeri* JM 300 has been studied with whole cell suspensions using $H_2{}^{18}O$, ^{15}NO, and $^{15}NO_2^-$. The extent of $H_2{}^{18}O$ exchange with the enzyme-bound nitrosyl intermediate, as determined by the ^{18}O content of product N_2O, decreased with increasing nitrite concentration, which is consistent with production of N_2O by sequential reaction of two nitrite ions with the enzyme. Reaction of NO with whole cells in $H_2{}^{18}O$ gave amounts of ^{18}O in the N_2O product consistent with equilibration of nitric oxide with a small pool of free nitrite. Using ^{15}NO and NH_2OH, competition between denitrification and nitrosation reactions was demonstrated, as is required if the enzyme-nitrosyl complex is an intermediate in both nitrosation and denitrification reactions. The first evidence for exchange of ^{18}O between $H_2{}^{18}O$ and a nitrosation intermediate occurring after the enzyme-nitrosyl complex, presumably an enzyme-bound nitrosamine, has been obtained. The collective results are most consistent with denitrification N_2O originating via attack of NO_2^- on a coordinated nitrosyl, as proposed earlier (Averill, B. A., and Tiedje, J. M. (1982) *FEBS Lett.* 138, 8–11).

The biochemical pathway by which nitrite is reductively converted to nitrous oxide in denitrifying bacteria is currently a matter of controversy (1). The main issues are the following. (i) Is nitric oxide or nitrous oxide the physiological product of nitrite reductase? (ii) Is N_2O formed by nucleophilic attack of NO_2^- on an enzyme-bound nitrosyl intermediate (2) (Scheme 1), or does it arise from reduction of the nitrosyl to free nitroxyl (HNO) with subsequent spontaneous dimerization? (3) (Scheme 2).



SCHEME 1. **Formation of the N-N bond by nucleophilic attack of nitrite on an enzyme-bound nitrosyl. *E*, nitrite reductase; (N_2O_3), the first dinitrogen intermediate. *1, 2, ... 7*, correspond to individual steps with rate constants k_1, k_2, ... k_7.**

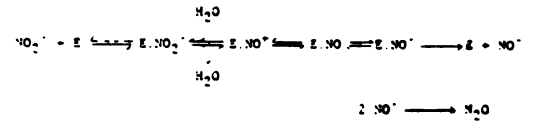
A variety of lines of evidence has been adduced in favor of NO as an obligatory intermediate (1, 4, 5), while evidence against the intermediacy of *trans*-hyponitrite ($N_2O_2^{2-}$) (6) and oxyhyponitrite ($N_2O_3^{2-}$) (7) has been presented. Perhaps most importantly, $H_2{}^{18}O$ exchange and trapping experiments have conclusively demonstrated the existence of an enzyme-bound nitrosyl intermediate (*E*-NO^+) during catalysis by whole cells (8) and by the purified heme *cd*-containing nitrite reductase of *Pseudomonas aeruginosa* (3). This nitrosyl intermediate is common to both Schemes 1 and 2 and is a plausible intermediate in NO formation as well. To date, no evidence has been reported that would serve to unambiguously distinguish between the postulated mechanisms, with the possible exception of the oxyhyponitrite study (7). Isotopic equivalence of nitrogen atoms in product N_2O was demonstrated (9) and interpreted as favoring HNO as an intermediate (Scheme 2), when in fact *cis*-hyponitrite is equally plausible (Scheme 1), if the nitrogen atom attached to the metal exchanges rapidly with the other via a 1,2 shift (as seems likely (10)).

The only experiments that could be interpreted as favoring either Scheme 1 or Scheme 2 are those of Bryan *et al.* (11) and Mariotti *et al.* (12), which demonstrated the existence of an isotope effect associated with denitrification of nitrite in *Pseudomonas stutzeri* cultures and in soils, respectively. In both studies, the magnitude of the isotope effect was found to vary with the concentration of both nitrite and reductant. The simplest interpretation of the observed increase in isotopic fractionation factor with increasing NO_2^- concentration is that it is due to sequential addition of two nitrite ions to the enzyme no later than the first irreversible step, although the results are also consistent with an indirect effect of nitrite on reductant concentration.

We report herein the results of isotope equilibration and isotope tracer experiments designed to provide a better understanding of the mechanism of N-N bond formation by *P. stutzeri*, which contains a heme *cd* nitrite reductase.

MATERIALS AND METHODS

Growth and Assay Conditions—The bacterial strain used was *P. stutzeri* JM 300. Cultures were grown in 3% tryptic soy broth (Sigma) containing 0.4% potassium nitrate and 0.2% sodium bicarbonate. A 1% inoculum from a refrigerated stock culture was routinely incu-



SCHEME 2. **Formation of nitrous oxide by dimerization of free nitroxyl anions.**

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¶ Alfred P. Sloan Foundation Fellow, 1981–85.

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bated at 37 °C and harvested 18 h later, during late exponential growth (A_{660} = 1.0). Cells were harvested by centrifugation at 12,000 × *g* for 15 min at 4 °C, washed three times in phosphate-buffered (100 mM, pH 6.8) growth medium without nitrate, and resuspended in this medium to achieve approximately 0.3 (S.D. 0.03) mg of protein/ml. $H_2{}^{18}O$ was added to the medium for ^{18}O exchange experiments. Cells were kept on ice throughout the harvesting procedure. The washed cell suspension was then used to prepare assay mixtures. An assay mixture contained the cell suspension in a serum vial sealed with a rubber stopper. The vial was made anaerobic by flushing with helium and then briefly flushed with acetylene to prevent N_2O consumption. Argon was added as an internal standard. Additions of reagents were made anaerobically by means of gas-tight syringes. Reagent solutions were made anaerobic by flushing with helium. Reactions were started by the addition of nitrite or nitric oxide and stopped by the addition of 1 ml of 20% v/v NaOH or KOH, except when nitric oxide and hydroxylamine were both present; under alkaline conditions these react chemically to form N_2O (13). In this case, reactions were stopped by immediate freezing of the sample in liquid nitrogen. Base was added to the frozen sample to remove CO_2, after which the sample was frozen again. These samples were not allowed to thaw before the gas phase was analyzed.

Hydroxylamine stock solutions were prepared from the hydrochloride salt (obtained from Sigma), neutralized with NaOH, and used within 4 h. Nitrite solutions were used the same day. Nitrite and hydroxylamine solutions were prepared in 100 mM phosphate buffer of pH 6.8. All chemicals used were reagent grade.

For each experiment, controls with autoclaved cells (200 °C, 20 min) were treated and analyzed the same way as the samples. If they contained significant amounts of N_2O, the experiment was redesigned until no chemical N_2O production interfered with the measurements.

Isotopes and Their Analysis—$Na^{15}NO_2$ and $H_2{}^{18}O$ were purchased from Monsanto Co., Dayton, OH. The isotopic purity of $^{15}NO_2^-$ was verified to be greater than 99.9%. ^{15}NO was prepared by mixing 1 ml of 200 mM H_2SO_4, 1 ml of 100 mM KI, and 1 ml of 290 mM $Na^{15}NO_2$ in a 5-ml helium-flushed vial; this yielded 52% (S.D. 10) ^{15}NO in the gas phase, which was used as the substrate for the NO experiments. Initial equilibrium concentrations of NO in the liquid phase were calculated using an Ostwald coefficient of 0.047 (25 °C) (14).

Gas samples were analyzed with a HP 5985 gas chromatography/mass spectrometry system equipped with a Porapak Q column. The mass spectrometer was operated using electron impact and selective ion monitoring. System temperatures were: injector, 60 °C; column, 55 °C; ion source: 60 °C. Under these conditions the fractionation of N_2O into NO^+ and N_2^+ inside the instrument was minimized. The electron multiplier voltage was in the range of 1400–3000 MeV. The autotune value was 2000 MeV.

Results are expressed as mass abundance ratios calculated directly from integrator counts. The percentage equilibration between N_2O and $H_2{}^{18}O$ is calculated as follows.

$$\frac{100(100(N_2{}^{18}O/N_2O + N_2{}^{18}O) - 0.204)}{\text{atom \% } H_2{}^{18}O \text{ in the water} - 0.204}$$

The natural abundance of ^{18}O is 0.204% (15).

To test the accuracy of isotope ratios obtained this way, the ratio $C^{18}O_2/(CO_2 + C^{18}O_2)$ in atmospheric CO_2 was determined for the range of electron multiplier voltage settings used and compared to the theoretical ratio (0.00408). The measured values ranged from 0.00403 to 0.00425.

RESULTS AND DISCUSSION

The specific activity of *P. stutzeri* for nitrite reduction was determined to be 0.3 (S.D. 0.001) μmol of NO_2^-/mg of protein·min, which corresponds to 0.09 (S.D. 0.01) μmol of NO_2^-/ml of cell suspension·min at harvest time. The reported K_m value of 0.1 mM for NO_2^- uptake (11) was assumed for calculations of the extent of reaction for the nitrite concentrations used. For the first experiment, it was important that samples were taken as early in the reaction as mass spectrometer sensitivity would allow, in order to minimize isotopic equilibration between the free nitrite pool and the $H_2{}^{18}O$. Samples were taken at 10% extent of reaction except for at the lowest nitrite concentration (0.09 mM), where the extent of reaction at sampling time had to be longer.

Effect of Nitrite Concentration on the Extent of Equilibration between N_2O from Nitrite and $H_2{}^{18}O$—If formation of the N-N bond involves nucleophilic attack of nitrite on an enzyme-bound nitrosyl, *E*-NO^+, an increase in nitrite concentration should make the reversible dehydration step leading to the nitrosyl relatively more rate limiting. Conversely, at low nitrite concentrations the dehydration step should become more reversible and allow the *E*-NO_2^- and *E*-NO^+ intermediates to equilibrate to a greater extent. We chose to monitor the equilibration between $H_2{}^{18}O$ and product N_2O as a function of nitrite concentration, since this should decrease as the dehydration step becomes more rate limiting. One assumption made was that the free nitrite pool did not become appreciably labeled with ^{18}O during the course of the experiment. This seems reasonable since measurements were taken over short periods of time, the nitrite pool was large and should dilute any labeled nitrite, and the equilibration values showed no time dependence (data not shown). Other workers have tried unsuccessfully to trap NO in the free nitrite pool (16), which suggests that *P. stutzeri* has a low dissociation constant for nitrite. The experimental results (Table I) show that there is a reproducible decrease in the extent of equilibration between N_2O and $H_2{}^{18}O$ as nitrite concentrations increase.

It is possible to predict theoretically how the ^{18}O content of the nitrous oxide pool should behave as a function of nitrite concentration for each of the proposed mechanisms. Let us consider Scheme 1 first. When the reaction is initiated, the enzyme-bound nitrosyl pool is not yet enriched with ^{18}O. The ^{18}O content of initially produced N_2O is equal to the ratio: net flux of ^{18}O into product N_2O/rate of N_2O production. Applying the theory of net rate constants (17), one can calculate that this ratio is,

$$k_3 \cdot k_4/(k_4 + (k_5 \cdot k_7 \cdot [NO_2^-]/(k_6 + k_7)))$$

where k stands for the rate constant corresponding to steps

TABLE I

Extent of ^{18}O equilibration between N_2O and $H_2{}^{18}O$ as a function of nitrite concentration

The assay mixtures contained 17.3 atom % $H_2{}^{18}O$. The reaction was initiated by addition of nitrite. Data shown are three analyses for each of two replicates.

Initial NO_2^- concentration	Incubation time	Calculated extent of reaction	Isotope abundance		Extent of ^{18}O equilibration between $H_2{}^{18}O$ and N_2O
			N_2O	$N_2{}^{18}O$	
mM	*min*	%			%
0.09	5	100	2,466	160	34
			4,456	268	32
			8,260	511	32
					$\bar{X}$ = 32.7
0.09	5	100	3,619	238	34
			5,831	395	35
			10,800	736	36
					$\bar{X}$ = 35.0
0.9	1	10	2,238	61	14
			2,291	75	17
			9,800	300	16
					$\bar{X}$ = 15.7
0.9	1	10	3,422	83	13
			4,174	102	13
					$\bar{X}$ = 13.0
9.0	10	10	9,932	138	7.7
			15,220	243	7.9
			19,110	311	8.1
					$\bar{X}$ = 7.9
9.0	10	10	19,330	307	7.9
			8,295	131	7.8
			14,750	241	8.1
					$\bar{X}$ = 7.9

3–7, in Scheme 1. We can simplify this ratio as: (constant$_1$/[NO_2^-]). The enzyme-bound nitrosyl pool should quickly become equilibrated with $H_2{}^{18}O$ and reach a steady ^{18}O content, the magnitude of which is directly dependent on the ratio: influx of $H_2{}^{18}O$ into the enzyme-bound nitrosyl pool/turnover rate of that pool. We can express this ratio as:

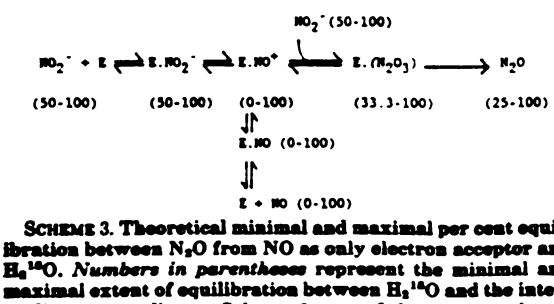
$$k_4 \cdot (k_6 + k_7)/k_6 \cdot k_7[NO_2^-]$$

or simplify as (constant$_2$/[NO_2^-]). During this time, N_2O of a constant ^{18}O content is produced. Eventually, the free nitrite pool must become enriched with ^{18}O. This will happen sooner if the nitrite reductase has a high K_d for nitrite. If this is the case, equilibration of the nitrite pool will perturb the patterns just described, since the N_2O formed will then reflect the isotopic composition of the free nitrite pool. A continuously increasing ^{18}O content of the product would then be observed.

If we apply the same analysis to the mechanism shown in Scheme 2, in which nitrite is not a second reactant and thus cannot change relative rates between single steps in the pathway, we find that both the initial appearance of ^{18}O in the product (before equilibration of the enzyme-bound nitrosyl pool) and the steady level of ^{18}O reached before the nitrite pool begins to equilibrate are constant and, thus, independent of the nitrite concentration. Our results with *P. stutzeri* are thus in agreement with Scheme 1.

Isotopic Equilibration between N_2O from Nitric Oxide and $H_2{}^{18}O$—If Scheme 1 is correct, whole cells should reduce nitric oxide to nitrous oxide, but in the process N_2O should equilibrate to some extent with the label in $H_2{}^{18}O$. It is possible to predict a theoretical minimal extent of equilibration of 25% for a situation where nitric oxide is the only substrate and the N-N bond formation requires free nitrite (Scheme 1). This is represented in Scheme 3, where numbers in parentheses stand for the minimal and maximal per cent equilibration expected for each intermediate shown. If Scheme 2 is correct, the equilibration between N_2O and $H_2{}^{18}O$ could range from 0 to 100% (assuming that nitrite reductase is the only enzyme reducing nitric oxide to nitrous oxide). Results presented in Table II show that the expected equilibration did indeed occur; since the average value is 23.3 (S.D. 7.6), Scheme 1 is not eliminated, and, thus, the data are consistent with either Scheme 1 or Scheme 2.

To check for chemical production of NO_2^- from NO reacting with traces of oxygen, which could affect the results, sterile controls were given the same amount of NO and reaction time as the experimental mixtures but were flushed with argon to remove NO. Then, a concentrated suspension of live cells was added anaerobically and allowed to react overnight. Any NO_2^- that was formed would show up in these controls as N_2O. Background levels of N_2O in the controls were low (±5% of



Scheme 3. Theoretical minimal and maximal per cent equilibration between N_2O from NO as only electron acceptor and $H_2{}^{18}O$. ***Numbers in parentheses*** **represent the minimal and maximal extent of equilibration between $H_2{}^{18}O$ and the intermediate. According to Scheme 1, one of the oxygens that is lost during formation of N_2O from N_2O_3 comes from the second nitrite, the other one from the nitrosyl or from the second nitrite.**

TABLE II

Extent of ^{18}O equilibration between $N_2{}^{18}O$ and N_2O with NO as substrate

The assay mixtures contained 14.6 atom % $H_2{}^{18}O$. The reaction was initiated by adding NO at a concentration of 42 (S.D. 4.2) μM. Analysis was after 15 min of incubation. Data are for three replicates with each value the mean of two analyses.

Isotope abundance		Extent of ^{18}O equilibration between $H_2{}^{18}O$ and N_2O
N_2O	$N_2{}^{18}O$	
		%
8,282	179	13
11,880	480	26
8,812	436	31
		$\bar{X}$ = 23.3 ± 7.6

TABLE III

Competition between nitrosation of hydroxylamine and denitrification when ^{15}NO was substrate

Assay mixtures contained 10.6 μM ^{15}NO (99.9 atom % ^{15}N). Analysis was after 15 min of incubation. Data are means of two analyses at each NH_2OH concentration.

NH_2OH concentration	Isotope abundance		N_2O from nitrosation
	$^{15}N_2O$	$^{14,15}N_2O$	
mM			%
0	ND[a]	178	0
5	44	71	38
10	80	ND	100
25	32	ND	100
50	[b]	[b]	[b]

[a] ND, not detected.

[b] At these high concentrations of hydroxylamine, there was chemical production of N_2O in sterile controls.

TABLE IV

Absence of competition between nitrosation of hydroxylamine and denitrification when $^{15}NO_2^-$ was substrate

Assay mixtures contained 0.1 mM $^{15}NO_2^-$ (99.9 atom % ^{15}N). Analysis was after 5 min of incubation. Data are means of two analyses at each NH_2OH concentration.

NH_2OH concentration	Isotope abundance		N_2O from nitrosation
	$^{15}N_2O$	$^{14,15}N_2O$	
mM			%
10	67	2735	2.4
40	51	3694	1.4
80	63	3909	1.6
160	53	2005	2.6
320	32	1119	2.8

N_2O levels in the experiment) and could be accounted for by incomplete removal of gases by flushing.

Competition Studies between Nitrosation and Denitrification Reactions—The heme *cd*-containing nitrite reductase is known to catalyze nitrosation reactions between nitrite and nucleophiles such as hydroxylamine and azide (3). The enzyme-bound nitrosyl intermediate in denitrification is presumed to be the species which undergoes nucleophilic attack. One would, therefore, expect to observe competition between nitrosation and denitrification of nitrite, since the nitrosyl intermediate is common to both processes. In Scheme 2, nitric oxide is reduced to nitrous oxide via an enzyme-nitroxyl complex and free nitroxyl. In Scheme 1, nitric oxide is reduced in essentially the same way as nitrite, although one would expect a very small free nitrite pool with nitric oxide as the only electron acceptor.

As shown in Table III, 10 mM hydroxylamine completely inhibited nitric oxide reduction to N_2O (initial NO concentration, 10.5 μM). Hydroxylamine concentrations as high as 320

mM, however, did not inhibit denitrification with 100 μM nitrite, as shown in Table IV. The amount of nitrosation product in the latter was low and apparently not dependent on the hydroxylamine concentration. The latter result is similar to observations by Kim and Hollocher (3) at higher nitrite concentrations and is presumably due to the very high affinity of the enzyme for nitrite. Only with NO as a source of the nitrosyl intermediate could the competition be demonstrated.

^{18}O Enrichment of Nitrous Oxide when Denitrification of Nitrite and Nitrosation of Hydroxylamine Occur Simultaneously in the Presence of $H_2{}^{18}O$—Previous experiments by other workers (8) with whole cells of *P. stutzeri*, *P. denitrificans*, and *Paracoccus denitrificans* were repeated on *P. stutzeri* JM 300. When denitrification occurs in the presence of hydroxylamine and ^{15}N-nitrite in medium containing a given atom % $H_2{}^{18}O$, N_2O species from denitrification ($^{15}N^{15}N^{16}O$ and $^{15}N^{14}N^{16}O$) and from nitrosation ($^{14}N^{15}N^{16}O$, $^{14}N^{15}N^{16}O$, and $^{15}N^{14}N^{16}O$) can be distinguished. Kim and Hollocher (3) found that $^{15}N^{14}N^{18}O$ was not produced and concluded that the only way in which ^{18}O could enter the nitrosation product was through the enzyme-nitrosyl intermediate. Their results show, for each of the three bacterial species studied, that the ^{18}O content of the $^{15,15}N_2O$ was consistently smaller than that of the $^{14,15}N_2O$, when one takes into account a correction factor of two for the latter (the loss of water from the nitrosated NH_2OH to give N_2O causes loss of one of the two oxygens, with equal probability). The authors suggested that the observed differences in ^{18}O content were insignificant and that the two pools of nitrous oxide species were similarly enriched.

As shown in Table V, an essentially fully ^{18}O equilibrated $^{14,15}N_2O$ pool and a $^{15,15}N_2O$ pool containing only a small amount of ^{18}O were observed when denitrification and nitrosation with NH_2OH were carried out simultaneously. The results in Table V are not in good agreement with existing views of catalysis of nitrosation reactions by nitrite reductase. If both denitrification and nitrosation produce N_2O containing oxygen derived from the nitrosyl group on the enzyme, a fully $H_2{}^{18}O$ equilibrated N_2O pool from nitrosation would lead us to expect a denitrification N_2O pool that is at least 50% equilibrated with $H_2{}^{18}O$. The most likely explanation is that the nonsymmetrical enzyme-bound nitrosation intermediate, O=N–NHOH (presumably coordinated to iron via the nitroso nitrogen), undergoes rapid $H_2{}^{18}O$ exchange at the nitroso nitrogen and that the hydroxylamine oxygen is lost exclusively upon conversion to N_2O. Although nitroso compounds undergo $H_2{}^{18}O$ exchange at only a modest rate (18), metal coordination to the nitroso nitrogen would be expected to increase the rate of $H_2{}^{18}O$ exchange significantly. Exclusive loss of the hydroxylamine oxygen does not, however, agree with earlier experiments indicating that nitrosation with hydroxylamine proceeds via an effectively symmetrical intermediate (3), as does denitrification (2). These results represent the first evidence that ^{18}O exchange with water can occur via an intermediate that lies beyond the enzyme-nitrosyl complex. All previous $H_2{}^{18}O$ exchange experiments have assumed that ^{18}O does not enter any intermediates except the nitrosyl; our results indicate that such assumptions must be viewed with caution, at least for the nitrosation reactions.

TABLE V

^{18}O enrichment of N_2O species from nitrosation of hydroxylamine and from denitrification of $^{15}NO_2^-$ occurring simultaneously in presence of $H_2{}^{18}O$

Assay mixtures contained 9.5 atom % $H_2{}^{18}O$ and 9 mM hydroxylamine. The reaction was initiated by addition of $^{15}NO_2^-$ to achieve a concentration of 9 mM. Analysis was after 10 min of incubation. Data are from means of two analyses for three (nitrosation) and four (denitrification) replicates.

Nitrosation				
$H_2{}^{18}O$ added		Controls, no $H_2{}^{18}O$		Corrected[a] extent of equilibration between $H_2{}^{18}O$ and N_2O
m/e 45	*m/e* 47	*m/e* 45	*m/e* 47	
				%
360	54	1,354	48	104
402	43	1,732	66	67
213	30	713	23	96
				$\bar{X}$ = 89
Denitrification				
$H_2{}^{18}O$ added		Controls, no $H_2{}^{18}O$		Corrected[a] extent of equilibration between $H_2{}^{18}O$ and N_2O
m/e 46	*m/e* 48	*m/e* 46	*m/e* 48	
				%
8,458	262	6,288	172	0
14,810	589	11,690	346	9
5,473	159	24,920	898	0
15,795	566	7,332	229	5
				$\bar{X}$ = 3.5

[a] Corrected values were obtained by subtraction of the average apparent per cent equilibration found in the controls to which no $H_2{}^{18}O$ had been added.

CONCLUSIONS

The decrease in ^{18}O equilibration between $H_2{}^{18}O$ and N_2O with increasing nitrite concentration indicates that a second nitrite ion traps the *E*-NO^+ intermediate more rapidly at higher nitrite concentrations, thereby decreasing ^{18}O incorporation into *E*-NO^+. This result is consistent with earlier isotopic fractionation studies (11) suggesting addition of two nitrite ions to nitrite reductase prior to the first irreversible step and constitutes the first direct chemical evidence favoring the mechanism of Scheme 1 (nucleophilic attack of NO_2^- on *E*-NO^+) over that of Scheme 2 (reduction of *E*-NO^+ to N_2O via free HNO). Use of NO to generate the nitrosyl intermediate resulted in equilibration of $H_2{}^{18}O$ with product N_2O that was quantitatively consistent with either mechanism and permitted observation of competition between denitrification and nitrosation reactions. Simultaneous denitrification and nitrosation in $H_2{}^{18}O$ gave amounts of ^{18}O incorporation that could only be interpreted in terms of ^{18}O exchange occurring with an enzyme-bound intermediate unique to nitrosation and occurring after the *E*-NO^+ species. Taken together these results are most consistent with denitrification occurring via nucleophilic attack of nitrite on an enzyme-bound nitrosyl intermediate rather than via free nitroxyl.

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CHAPTER THREE

EVIDENCE FROM ISOTOPE LABELLING STUDIES FOR A SEQUENTIAL MECHANISM FOR DISSIMILATORY NITRITE REDUCTION

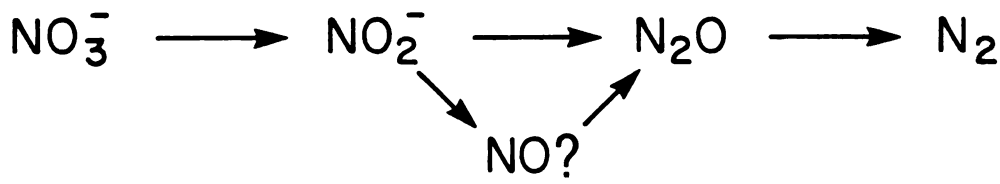
INTRODUCTION

The pathway by which denitrifying bacteria convert nitrite to N_2 consists of at least three distinct enzyme-catalyzed steps, as shown below (Scheme 1):

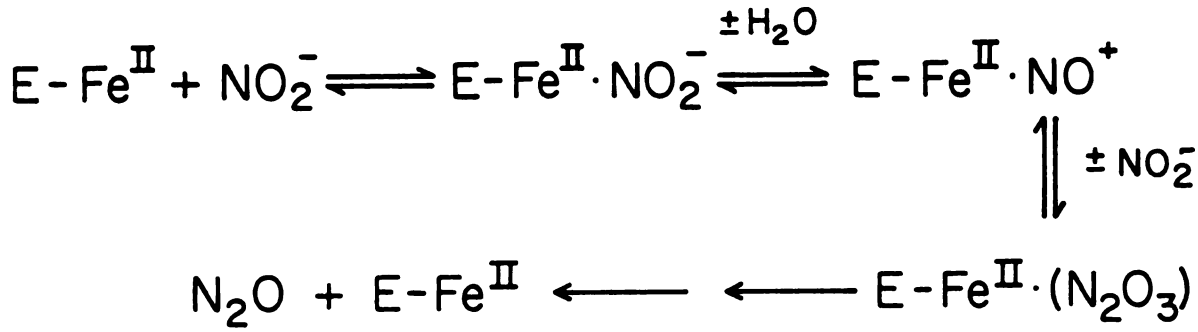
(1) $$NO_2^- \longrightarrow NO_2^- \longrightarrow N_2O \longrightarrow N_2.$$
$$\searrow (NO?) \nearrow$$

The first step is catalyzed by a molybdenum-containing nitrate reductase similar to that found in plants and bacteria capable of nitrate assimilation (1), while the last step is catalyzed by an unusual copper-containing reductase (2). In contrast, the second step has remained controversial, with disagreement as to whether it is carried out by a single enzyme or by two enzymes, with NO as a free obligatory intermediate. Even among workers who favor a single enzyme, there is substantial controversy (3) regarding the mechanism by which two nitrite ions are converted to N_2O: (i) is the N-N bond of N_2O formed by nucleophilic attack of a second NO_2^- upon a metal-coordinated nitrosyl species (4) (Scheme 2); or (ii) is the nitrosyl intermediate first reduced to free nitroxyl (HNO), which spontaneously dimerizes to N_2O (5) (Scheme 3)?

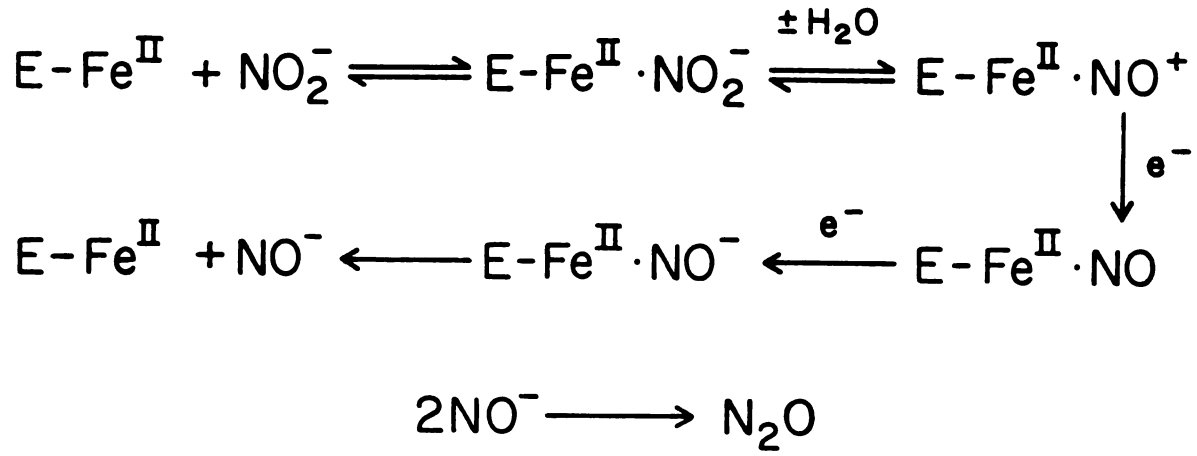
Although studies in a number of laboratories have been interpreted as favoring NO as a free obligatory intermediate (thus implying the existence of a separate NO reductase) (3,6,7), definitive evidence is still lacking. It has, however, been conclusively demonstrated by



Scheme 1. The pathway of denitrification, with identified or postulated intermediates indicated.



Scheme 2. Formation of the N-N bond by nucleophilic attack of nitrite on an enzyme-bound nitrosyl.



Scheme 3. Formation of nitrous oxide by dimerization of nitroxyl anions.

$H_2{}^{18}O$ exchange and trapping experiments that nitrite reduction by whole cells (8) and the purified heme cd_1-containing nitrite reductase (5) of Pseudomonas aeruginosa proceeds via an enzyme-bound nitrosyl intermediate ($E\text{-}NO^+$). This nitrosyl intermediate arises from dehydration of coordinated nitrite (5,8), and is common to Schemes 2 and 3 (and possibly to NO formation from NO_2^- as well (4)). Intermediates beyond the nitrosyl species remain nebulous, although evidence against the intermediacy of trans-hyponitrite ($N_2O_2^{2-}$) (9) and oxyhyponitrite ($N_2O_3^{2-}$) (10) has been presented. The demonstration of positional isotopic equivalence of nitrogen atoms in product N_2O (11) was interpreted as favoring HNO as an intermediate (Scheme 3), but in fact coordinated cis-hyponitrite is equally plausible if it undergoes a rapid intermolecular exchange of the coordinated and uncoordinated nitrogen atoms (12).

Thus, with the possible exception of the oxyhyponitrite study (10), no evidence that would unambiguously distinguish between the three possible mechanisms has been reported. Competition between denitrification and nitrosation reactions has been demonstrated only using ^{15}NO and NH_2OH, suggesting that an enzyme-nitrosyl intermediate is common to both reactions when NO, rather than NO_2^- is the substrate (13); the relevance of this finding to nitrite reduction is perhaps open to question. The only experiments that have provided evidence, albeit indirect, for Scheme 3 vs. Scheme 2, are studies on the ^{15}N isotope effect associated with denitrification of nitrite by P. stutzeri cells (14) and in soils (15), which found that the magnitude of the isotope effect increased with increasing nitrite concentration. The most obvious (but not unique) interpretation is that this result is

due to sequential addition of two nitrite ions to the enzyme prior to the first irreversible step.

In a previous paper (13), we presented our first systematic study of dissimilatory nitrite reduction. With whole cells of P. stutzeri, it was shown that the extent of isotopic equilibration between $H_2{}^{18}O$ and the product of denitrification, N_2O, decreased as the nitrite concentration increased, suggesting that $H_2{}^{18}O$ and NO_2^- compete for a common intermediate (i.e., favoring Scheme 2). In this work, we had to make the not unreasonable assumption that the ^{18}O-enrichment of the free nitrite pool was negligible during the time of our measurements; this has now been confirmed experimentally by Shearer and Kohl (16). P. stutzeri nitrite reductase is thus a "sticky" enzyme, meaning that nitrite, once bound to the enzyme, is committed to react and does not readily dissociate.

In this paper, we present the results of stable isotope studies relating to the pathway of nitrite reduction and to the effect of solubilization of the enzyme on the relative rates of the individual steps. We present evidence that strongly favors the pathway represented in Scheme 2: N_3^-, $H_2{}^{18}O$, and NO_2^- all compete for a common enzyme-bound nitrosyl intermediate. This implies that the N-N bond of N_2O must be formed via nucleophilic attack of a second nitrite ion on a coordinated nitrosyl derived from the first nitrite. Our results are inconsistent with a pathway such as the one represented in Scheme 3, in which two equivalent nitroxyl anions combine to form nitrous oxide (5), and also eliminate NO as a free intermediate.

MATERIALS AND METHODS

Cell growth and assay conditions. The bacterial strain used was P. stutzeri JM 300. Cultures were grown and harvested as described earlier (13), but cells were washed only once instead of three times. Cells were resuspended in a volume of 50 mM HEPES[1] buffer pH 7.3, equal to 1% of the harvested volume. Cell disruption was by French press (3 passages, 12,000 psi) or by sonication with a Branson sonifier (2.5 min. on ice, 5x30 s. at 40% of maximum output with 30 s. intervals) as specified under Results and Discussion. Cell-free crude extracts were obtained by filtration through a 0.22 μm filter. These extracts contained no whole cells, as they were unable to initiate growth when used as an inoculum into sterile medium, even after several weeks of monitoring for possible growth. Typical assay conditions are given here; variations are indicated in the text. Assays were carried out in 8 ml serum vials sealed with butyl rubber stoppers and aluminum crimps. They contained 1 or 3 ml liquid phase. The final buffer concentration in assay mixtures was 25 mM HEPES (pH 7.3). The reducing system used was sodium succinate and the natural electron transport components present in the cell-free crude extracts. The reaction was initiated by anaerobic addition of nitrite. Labeled water ($H_2{}^{18}O$) was added to the buffer. Addition of acetylene to inhibit nitrous oxide reductase proved unnecessary since this enzymatic activity virtually disappeared upon cell disruption. Vials were made anaerobic by flushing with argon. For each newly prepared extract, the specific activity was

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[1] The abbreviation used is: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

established by measurement of nitrous oxide production on the gas chromatograph.

The incubation periods were chosen such that the extent of reaction, measured by GC and expressed as N_2O produced as a fraction of the maximum N_2O possible, would never exceed 20%. This was crucial for the nitrosation experiments, where it was assumed that the nitrite and azide concentrations were roughly constant during the course of the experiment. Samples were analyzed by GC/MS immediately or stored frozen for later analysis. The rate of N_2O production from 1 mM nitrite plus 50 mM azide was about 0.01 mM N/hour. Assays contained 0.1 to 0.3 ml crude cell free extract. Denitrification rates in crude extracts were two orders of magnitude slower than in whole cells. All experiments were reproduced at least twice.

Reagents. All chemicals used were reagent grade. Azide stock solutions were prepared immediately before use. Stable isotopes were obtained from Monsanto (Mound, Ohio). ^{18}O- labelled water contained 15 atom% ^{18}O. Isotopic purity of ^{15}N- labelled nitrite was better than 99.9%.

Controls. Sterile controls (autoclaved for 20 min. at 200°C) and controls without isotope were routinely performed for each experiment. Experiments were designed so that chemical N_2O production was negligible.

GC/MS equipment and conditions. We used both an HP 5985 and an HP5995C GC/MS; the latter gave much better sensitivity. Source and mass analyzer temperatures were set at 150°C to prevent excessive decomposition of N_2O to N_2 and NO in the instrument. The electron multipliers were set in the range 200 to 1000 eV above autotune.

RESULTS AND DISCUSSION

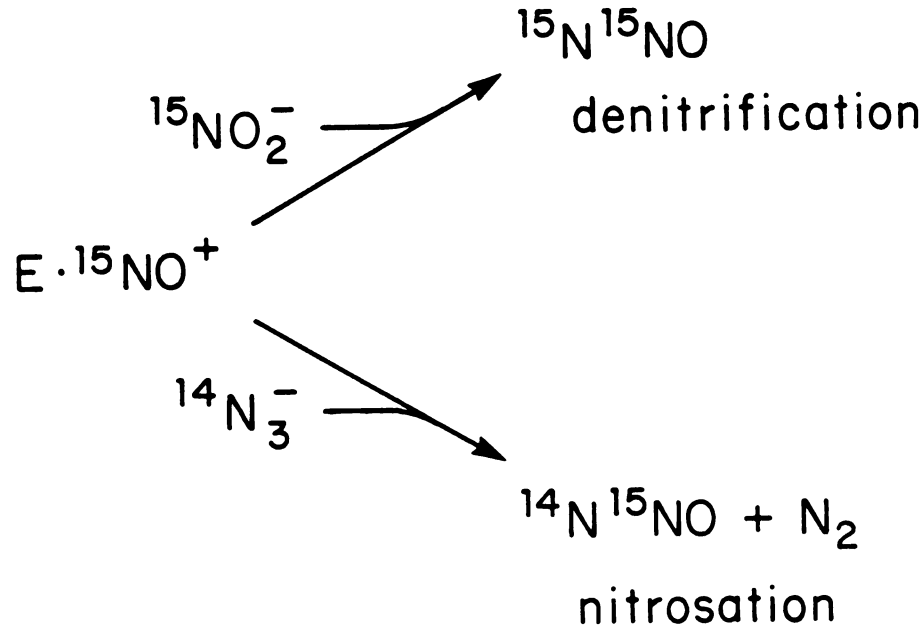
Competition between denitrification and nitrosation. In our earlier work, we examined the competition between denitrification and nitrosation reactions using whole cells of P. stutzeri and NH_2OH as a nucleophile to trap the nitrosyl intermediate. We found that hydroxylamine was relatively inefficient at trapping the nitrosyl species, giving only a few percent nitrosation product at 10 to 320 mM NH_2OH and 0.1 mM NO_2^- (13). More importantly, however, we found that there was evidence for ^{18}O exchange into an intermediate in the nitrosation reaction that is downstream from the enzyme-nitrosyl complex.

Accordingly, we have examined azide as the trapping nucleophile. Since it cannot form an N_2O_2 intermediate during nitrosation, it decreases the probability of ^{18}O exchange into subsequent nitrosation intermediates. Azide is, however, completely ineffective as a nitrosyl trapping agent with whole cells under the conditions examined (1mM $Na^{15}NO_2$, 1 to 10 mM N_3^-, fresh Luria Broth medium as reductant); insignificant amounts of nitrosation products ($\leq$ 1% of total N_2O) were observed. Since azide is known to trap the nitrosyl intermediate in crude cell-free extracts (8) and in the purified heme cd_1 enzymes (5), this suggests that azide is not readily transported through the cell membrane against a charge gradient. Indeed, we find that cell free extracts prepared by French press treatment show up to 30% nitrosation with 50 mM NaN_3 and 0.1 to 1.0 mM $Na^{15}NO_2$. This finding is consistent with proposed location of nitrite reductase on the cytoplasmic side of the cell membrane (17).

If nitrite and a nucleophile such as azide are indeed competing for the nitrosyl intermediate common to Schemes 2 and 3, then it is possible to quantitate the flux through denitrification vs. nitrosation by measuring the relative amounts of N_2O-46 and N_2O-45 respectively (Scheme 4). Thus, the competition can be examined as a function of nitrite or azide concentration. Systematically varying the N_3^- concentration at constant $[NO_2^-]$ will not distinguish the pathways of Schemes 2 and 3, since in either case the amount of nitrosation is expected to increase with increasing $[N_3^-]$. This type of experiment would simply confirm the existence of a trappable nitrosyl intermediate (5,8). In contrast, if the $[NO_2^-]$ is varied at constant $[N_3^-]$, the predicted results differ for the two schemes. For the pathway in Scheme 2, the nitrosation: denitrification product ratio should decrease with increasing $[NO_2^-]$, since the second NO_2^- will compete with N_3^- for the nitrosyl intermediate. For a pathway such as that in Scheme 3, the nitrosation denitrification: ratio should be independent of $[NO_2^-]$, since nitrite does not appear in the scheme after formation of the nitrosyl intermediate.

The data shown in Table 1 demonstrate that the N_2O-45/N_2O-46 ratio decreases with increasing $[NO_2^-]$, indicating that denitrification is indeed of a higher kinetic order with respect to nitrite than is nitrosation. At higher nitrite concentrations, a saturation effect is observed, as expected. Earlier attempts (5) to demonstrate this competition reaction failed, presumably because of the very high nitrite concentrations used ($\geq$ 10 mM NO_2^-).

Effect of reductant on the nitrosation/denitrification ratio. In order to examine a more chemically defined system than LB medium, in



Scheme 4. Nitrosation and denitrification products from $^{15}NO_2$ and N_3^-.

Table 1

The ratio of nitrosation (N_2O-45) to denitrification (N_2O-46) products as a function of nitrite concentration. Numbers in parentheses are standard deviations. Conditions: [NaN_3] = 50 mM, reductant = Luria broth; cell-free crude extracts of P. stutzeri prepared via French press; two or three replicates per nitrite concentration, all shown. Background N_2O (m/z 45) was 1% of total N_2O in control samples without azide. No significant amounts of N_2O were formed in sterile controls.

	Isotope Abundance		
$NaNO_2$ (mM)	N_2O-45	N_2O-46	ratio: $\frac{N_2O\text{-}45}{N_2O\text{-}46}$
0.1	11440	46380	0.25
	11080	48430	0.23
			av. 0.24 (± 0.01)
0.2	11650	48080	0.24
	8865	41700	0.21
			av. 0.22 (± 0.02)
0.5	3800	43820	0.087
	6295	73710	0.085
			av. 0.086 (± 0.001)
1.0	3565	60220	0.059
	2320	49740	0.047
	2680	54170	0.050
			av. 0.052 (± 0.005)

which the actual reductant concentration is unclear, we carried out the same competition experiment as described above utilizing 50 mM sodium succinate as the reductant. The results are given in Table 2. The same general trend is observed as with Luria Broth medium, namely that the ratio of N_2O-45 (arising from nitrosation) to N_2O-46 (arising from denitrification) decreases with increasing concentration of nitrite, at least for $[NO_2^-] \leq 1$ mM. The relative amount of nitrosation at a given nitrite concentration is, however, approximately a factor of 2-3 higher with succinate vs. LB medium, for reasons that are not clear.

The fact that the trend in the N_2O-45/N_2O-46 ratio reversed between 1 and 10 mM nitrite suggested that reductant had become rate-limiting for denitrification at the highest nitrite concentrations. Accordingly, we examined the effect of varying the succinate concentration at 0.05 and 10 mM NO_2^- (Table 3). The data clearly show that the nitrosation:denitrification product ratio decreases with increasing succinate concentration at 10 mM NO_2^-. The variability between repeated experiments is relatively large for less than saturating levels of succinate, because the concentration of residual reductant from the growth medium and the internal reductant from the cells themselves vary, depending on how long the cells were starved for carbon prior to harvesting. Denitrification rates were independent of reductant at very low NO_2^- concentrations, as expected.

Oxygen-18 content of nitrous oxide from denitrification vs. nitrosation. The major difference between the two proposed mechanisms of denitrification (Schemes 2 and 3) is the nature of the two nitrogen species that form the initial N-N bond. In Scheme 3, the N-N bond is

TABLE 2

Ratio of nitrosation (N_2O-45) to denitrification (N_2O-46) products as a function of nitrite concentration with succinate as reductant. Conditions as in Table 1 except for use of 50 mM sodium succinate in place of Luria Broth medium.

$NaNO_2$ (mM)	Isotope Abundance		ratio: $\frac{N_2O\text{-}45}{N_2O\text{-}46}$
	N_2O-45	N_2O-46	
0.05	7110	9834	0.72
	8710	10080	0.86
			av. 0.79 (± 0.07)
0.10	9555	21500	0.44
	10350	22900	0.45
			av. 0.44 (± 0.01)
1.0	11313	56575	0.20
	11075	72925	0.15
			av. 0.17 (± 0.03)
10.0	11450	41500	0.28
	8913	39750	0.25
			av. 0.27 (± 0.03)

TABLE 3

Effect of reductant on ratio of nitrosation to denitrification products. Conditions as in Table 2 except for indicated concentrations of succinate. Data from repeated experiments using different cell preparations are indicated by *.

[succinate] (mM)	N_2O-45 / N_2O-46	
	$[NO_2^-]$ = 10 mM	$[NO_2^-]$ = 0.05 mM
10	0.31 ± 0.03	0.57 ± 0.04
	0.59 ± 0.02*	
50	0.19 ± 0.02	0.49 ± 0.01
	0.25 ± 0.05*	
100	0.17 ± 0.01	0.54 ± 0.10
	0.17 ± 0.01*	

formed by reaction of two equivalent nitroxyl anions (NO^-). Therefore, if the reaction is carried out with $^{15}NO_2^-$ in the presence of $H_2{}^{18}O$, the ^{18}O content of the E-$^{15}NO^+$ (nitrosyl) intermediate will be reflected in the ^{18}O content of the denitrification product, $^{15}N_2O$, which can be measured directly. The ^{18}O content of the nitrosyl intermediate can be measured by trapping it with $^{14}N_3^-$ to produce $^{14}N^{15}NO$; the ^{18}O content of the nitrosation product will reflect that of the E-NO^+ intermediate. In Scheme 2, however, the two nitrogen species are not equivalent at the point at which the N-N bond is formed by attack of free NO_2^- on the E-NO^+ intermediate. Only the latter will contain appreciable amounts of ^{18}O. Free nitrite will be virtually unlabelled, since the rate for dissociation of NO_2^- from the enzyme is very low (16), and any ^{18}O-labelled nitrite formed is diluted by the nitrite pool. The results of experiments in which the ^{18}O content of N_2O originating from denitrification (N_2O-48/(N_2O-46 + N_2O-48)) and from nitrosation with N_3^- (N_2O-47/(N_2O-45 + N_2O-47)) was determined are shown in Table 4. We find that $^{15}N_2O$ from denitrification is 50%-60% equilibrated with the $H_2{}^{18}O$, while $^{14}N^{15}NO$ from nitrosation is 80-85% equilibrated with the $H_2{}^{18}O$. This is consistent only with Scheme 2, in which nitrite containing no ^{18}O dilutes the ^{18}O content of the E-NO^+ intermediate.

The ratios of the extent of equilibration are not exactly 1:2, as predicted by Scheme 2 and in more detail in Scheme 5 below, assuming that oxygen atoms are lost with equal probability from either nitrogen atom during conversion of the dinitrogen intermediates to N_2O. One possible explanation is the fact that, even at high [NO_2^-] in the absence of N_3^-, we always observe <u>ca.</u> 8% equilibration of N_2O with

TABLE 4

^{18}O isotopic enrichment of denitrification (N_2O-46 and N_2O-48) and nitrosation (N_2O-45 and N_2O-47) products. Conditions: $[NaN_3]$ = 50 mM, $[Na^{15}NO_2]$ = 1.0 mM, reductant = 100 mM sodium succinate; cell-free extracts prepared by sonication.

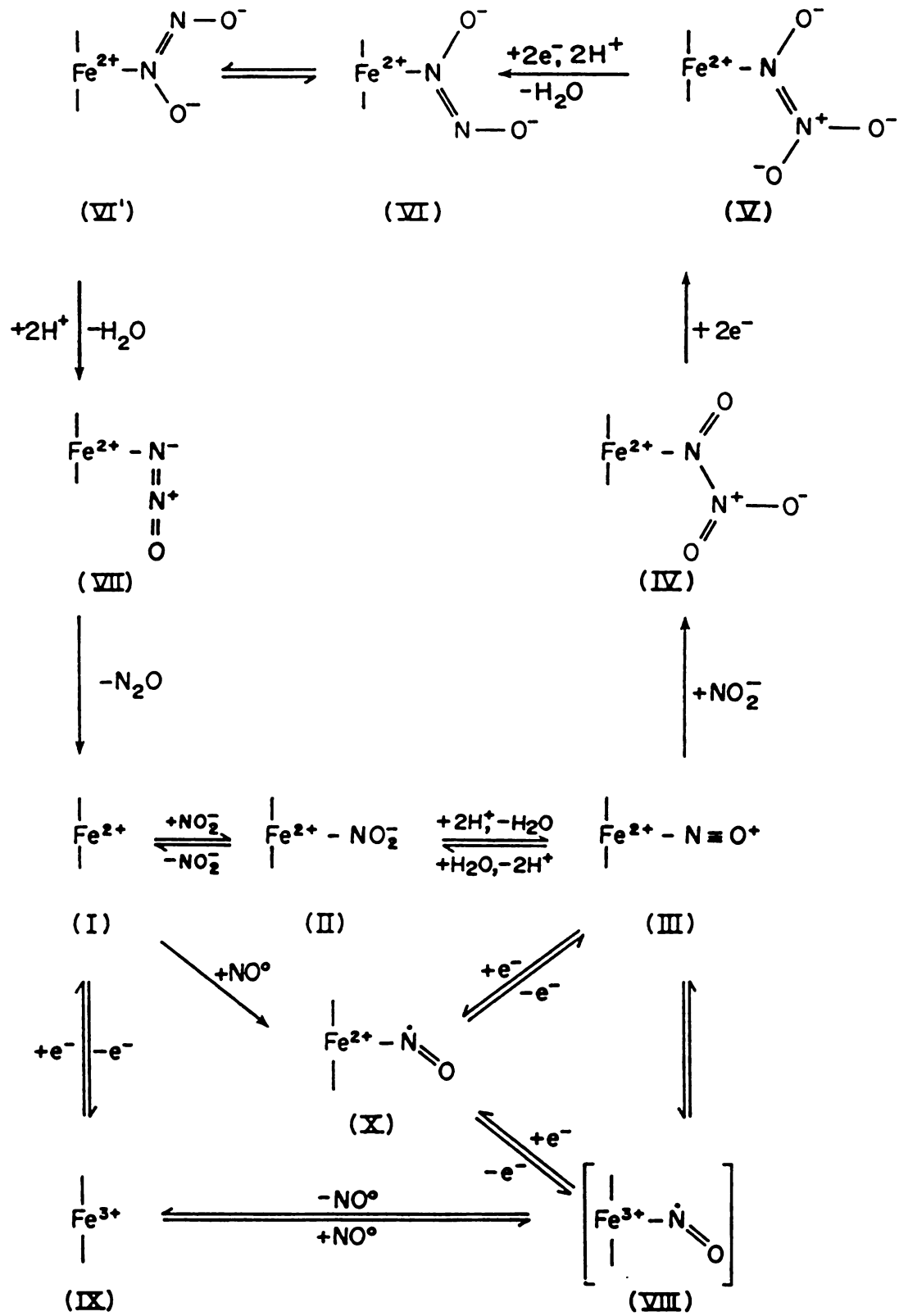
Isotope Abundance			Isotope Abundance		
N_2O-46	N_2O-48	% Equilib.	N_2O-45	N_2O-47	% Equilib.
11,807,400	620200	53.2	8,702,400	776,400	88.8[a]
13,823,800	838500	51.2	9,364,600	760,500	81.3[a]
		av. 57.2 (± 4.0)			av. 85.1 (± 3.8)

Isotope Abundance			Isotope Abundance		
N_2O-46	N_2O-48	% Equilib.	N_2O-45	N_2O-47	% Equilib.
28,000	1235	55.0	3110	210	83.9[b]
25,380	1085	53.3	2600	160	76.6[b]
17,930	730	50.7	2810	180	79.7[b]
16,200	670	51.6	2280	150	81.8[b]
		av. 52.7 (± 2.0)			av. 80.5 (± 2.4)

[a] Data obtained on HP 5995C using 9 atom % $H_2{}^{18}O$.

[b] Data obtained on HP 5985 using 7.3 atom % $H_2{}^{18}O$.

Scheme 5. A detailed representation of the pathway by which NO_2^- is reduced to N_2O via sequential reaction of two NO_2^- ions with the enzyme.


(VI')
(VI)
(V)
+2e⁻, 2H⁺
-H₂O
+2H⁺
-H₂O
+2e⁻
(VII)
(IV)
-N₂O
+NO₂⁻
(I)
(II)
(III)
+NO₂⁻
-NO₂⁻
+2H⁺,-H₂O
+H₂O,-2H⁺
+NO°
+e⁻
-e⁻
+e⁻
-e⁻
(X)
+e⁻
-e⁻
-NO°
+NO°
(IX)
(VIII)

$H_2{}^{18}O$ (13). This indicates either that NO_2^- is unable to completely suppress ^{18}O exchange into the E-NO^+ intermediate or that some ^{18}O exchange occurs via a denitrification intermediate containing an N-N bond, as we have observed for nitrosation with NH_2OH (13).

Mechanistic implications. The results presented above demonstrate that nitrite competes with azide for the E-NO^+ intermediate that is formed by dehydration of nitrite. Our earlier work (13) and the isotope dilution experiments described above demonstrate that nitrite competes with $H_2{}^{18}O$ (and with azide) for the same intermediate, presumably E·NO^+. These results are consistent only with a sequential mechanism for reaction of two nitrite ions to form N_2O (e.g., a mechanism such as that outlined in Scheme 2), and eliminate the nitroxyl mechanism (Scheme 3) from further consideration. Because the mechanism of denitrification has been the object of substantial controversy, it is worthwhile to briefly consider the major lines of evidence adduced previously in favor of other mechanisms and against the sequential mechanism indicated by the present data.

To facilitate this, a more detailed version of the mechanism shown in Scheme 2 is presented in Scheme 5; this is an expanded version of the hypothetical pathway presented in our original paper (4). This pathway (upper portion of Scheme 5) has several key features. Initially, nitrite binds to a ferrous heme (I) and is dehydrated to a reactive (18,19) ferrous-nitrosyl complex (III) via the ferrous-nitrite complex (II), as demonstrated by Hollocher and co-workers (5,8). Nucleophilic attack of a second nitrite on the coordinated NO^+ of III produces **IV**, containing bound N_2O_3. Reduction by two electrons produces **V**, containing coordinated oxyhyponitrite, $N_2O_3{}^{2-}$. Reduction

by a second two electrons and dehydration produces a species (VI) containing coordinated cis-hyponitrite, $N_2O_2^{2-}$, which upon further dehydration yields the ferrous-N_2O complex (VII). Loss of N_2O from VII regenerates the ferrous heme (I). The lower portion of this scheme shows how evidence previously interpreted as favoring NO as a free intermediate may be accounted for and is discussed below.

The major argument presented in favor of nitroxyl as an intermediate (8,11) has been the positional isotopic equivalence of nitrogen in $^{14,15}N_2O$ produced by concomitant reduction of $^{15}NO_2^-$ and ^{14}NO observed by Garber and Hollocher (11). Their data argue for a symmetrical intermediate in the reaction, which could be either a free mononitrogen intermediate (e.g., HNO) or an effectively symmetrical dinitrogen intermediate. The latter is perfectly consistent with a sequential mechanism if the coordinated cis-hyponitrite intermediate (VI in Scheme 5) interconverts rapidly between the two isomers with different nitrogen atoms coordinated to iron (VI $\rightleftarrows$ VI'). Available chemical evidence suggests that this equilibration is likely to be very rapid. For example, variable temperature NMR studies have shown that substituted pyridazines (which also contain two sp^2 nitrogen atoms linked by a formal double bond) when bound to ruthenium porphyrins exchange nitrogen donor atoms in an intermolecular process at rates of 10^2-10^6 sec^{-1} (12). Since substitution reactions of ruthenium complexes are generally much slower than for the corresponding iron complexes, one would expect such reactions at iron to be very rapid indeed, much faster than the overall enzymatic reaction. Thus, both the data on positional isotopic equivalence (11) and the ^{18}O enrichments (8) reported by Garber and Hollocher are equally consistent with

either the nitroxyl or sequential mechanism.

Scheme 5 postulates that oxyhyponitrite, $N_2O_3^{2-}$, is an enzyme-bound intermediate. Since $Na_2N_2O_3$ is readily prepared (20), it is possible to examine whether $N_2O_3^{2-}$ is converted to N_2O by the enzyme. Experiments with several denitrifying bacteria and whole cell extracts have been reported by Garber, Wehrli, and Hollocher as evidence that $N_2O_3^{2-}$ "can be neither a free nor an enzyme-bound intermediate" in denitrification (10). This conclusion is open to question on two levels. First, the bacteria and extracts used showed very low denitrification activity (on the order of only 2-fold higher than controls with no cells, and in one case zero activity). The lability of $HN_2O_3^{2-}$, the decomposition of which to NO^- and NO_2^- is markedly catalyzed by metal ions (49a), is expected to lead to large background levels of gaseous products with whole cells or crude cell-free extracts. We have performed similar experiments with purified cd- type and Cu-containing nitrite reductases that have been extensively treated to minimize contamination by adventitious metal ions, and still find relatively high background levels of gaseous decomposition products (C. Hulse, E. Weeg-Aerssens, J. M. Tiedje, and B. A. Averill, unpublished results).

Even if the data of Garber, Wehrli, and Hollocher (10) are accepted at face value, their interpretation is open to question. Examination of the enzymological literature reveals no general answer to the question of what one should expect when an enzyme is confronted with a putative intermediate that does not normally dissociate from the enzyme. There are, however, several specific cases in which this phenomenon has been examined. For example, oxaloacetate and NADPH are

postulated as nondissociable intermediates in the reaction of malic enzyme, yet the conversion of oxaloacetate and NADPH to L-malate and $NADP^+$ is catalyzed by the enzyme at only 10% of the V_{max} with NADPH, CO_2, and pyruvate (21). Similarly, formyl phosphate is an enzyme-bound intermediate in the formyltetrahydrofolate synthetase reaction, yet is turned over by the enzyme at ca. 3% of the rate of the normal substrates (MgATP, H_4folate, and formate) (22). These results have been explained in terms of a sequential mechanism with a kinetically trapped intermediate (i.e., one with both a slow dissociation and a slow binding step) (22). Similar behavior for species V in Scheme 5 is not unreasonable, and would render detection of enzymatic activity difficult with a labile substrate such as $N_2O_3^{2-}$. In the case of formyltetrahydrofolate synthetase, the lability of formyl phosphate prevented detection of catalytic activity with it as a substrate for over 25 years (23,24).

The other major alternative mechanism for denitrification, proposed over a decade ago, postulates the existence of two enzymes, a nitrite reductase that produces NO as the sole product and a separate NO reductase that reduces 2 NO molecules to N_2O (25) (cf. Scheme 1). The evidence supporting the existence of two enzymes and NO as a free obligatory intermediate is: (i) the observation that most purified nitrite reductases produce only NO from NO_2^-, while at least small amounts of NO reductase activity are found in other fractions (6,7, 26-29); (ii) denitrifying bacteria and cell-free suspensions produce and consume NO during nitrite reduction (30-35); (iii) nitrite reductase catalyzes exchange of N between isotopically labelled nitrite and a pool of added NO during reduction of NO_2^- to N_2O (36,37); and (iv)

formation of EPR signals due to ferric heme-NO complexes upon addition of nitrite to purified nitrite reductase (38-40).

All of the above evidence, however, can be equally well explained in terms of a sequential mechanism catalyzed by a single enzyme (Scheme 5). Even though there is substantial evidence for the existence of two crude fractions in cell-free extracts of denitrifiers, this has not matured into proof, as a purified NO reductase has thus far eluded all investigations (7,25,34,41-44). The chemical reactivity of NO makes it reasonable to suggest that at least some conversion of NO to N_2O may be due to secondary or non-physiological activities of other cellular components, as shown recently by Zumft for ferrous iron-ascorbate mixtures (45). Arguments (ii) and (iii) above can be readily interpreted in terms of the reactivity of the ferrous-nitrosyl intermediate III, as shown in the bottom portion of Scheme 5. Studies with synthetic heme nitrosyls (18,19) indicate that, in contrast to ferrous heme-NO complexes, the NO of the one-electron oxidized species is labile (reaction III $\rightleftarrows$ IX), producing the ferric heme and free NO. This reaction would account for the production and consumption of NO by denitrifiers, for the small and relatively constant pool of NO observed during reduction of nitrite (30,35,37), for the exchange of labelled N between NO_2^- and added NO (36,37), and for N_2O production (25), cell growth (33), active transport (46), and proton translocation (7) with NO as sole electron acceptor, since the dehydration reaction (II $\rightleftarrows$ III) is known to be reversible (5,8,47). The traditional explanation for argument (iv) above has been the sequence NO_2^- → ferrous heme·NO → NO. Since the dissociation of NO from ferrous heme-NO complexes is extraordinarily slow (even slower than CO dissociation (48)), this

sequence seems unlikely. Indeed, there is no evidence that the ferrous heme-NO complex forms or decays within the turnover time of the enzyme (i.e., that it is kinetically competent). As indicated in Scheme 5, this species (X) can form in a variety of ways and is irrelevant to the catalytic mechanism.

Finally, an alternative sequential mechanism exists that is also consistent with the results reported herein. This involves a reaction of a coordinated nitroxyl anion ($Fe\text{-}NO^-$) with NO_2^- to produce coordinated oxyhyponitrite ($Fe\text{-}N_2O_3^{2-}$; species V in Scheme 5) directly, and has as precedent the known reaction of HNO with NO_2^- to produce $HN_2O_3^-$ (49). Recent electrochemical studies by Kadish (19) and Fajer (50) have shown, however, that it is very difficult to reduce ferrous heme nitrosyl complexes ($Fe^{2+}\text{-}NO^{\cdot}$). Reported reduction potentials for the $Fe^{2+}\text{-}NO^{\cdot}/Fe^{2+}\text{-}NO^-$ couple with porphyrin and related ligands are in the range of -0.8 to -0.9 V vs. Standard Hydrogen Electrode (19.50), and do not vary greatly with the nature of the macrocyclic ligand (porphyrin vs. chlorin vs. isobacteriochlorin (50)) or the axial ligand (19,50). Since the biological reductant is either ascorbate (E_o' = + 60mV) or succinate (E_o' = + 30mV) and E_o' for the NO_2^-/N_2O couple is +0.77V, it is difficult to accept the intermediacy of such a strongly reducing species as the coordinated nitroxyl anion in denitrification. (This argument is also relevant to the nitroxyl mechanism proposed earlier by Hollocher (5) and discussed above.)

Effect of enzyme solubilization on the fate of the nitrosyl intermediate. If, as seems plausible, a common mechanism applies for at least all heme cd_1-containing nitrate reductases, the finding of Garber and Hollocher (37) that different denitrifiers exhibit varying

degrees of ^{18}O exchange into product N_2O suggests that differences in active site environment may affect the partitioning of the nitrosyl intermediate among the three competing reactions shown in Scheme 5. A similar change in active site environment may also explain the apparent shift from N_2O production in whole cells or cell-free extracts to NO production in purified heme $\underline{cd_1}$ nitrite reductase. All that is required is that the relative rates of reactions III → IV and III → IX in Scheme 5 are reversed in the purified vs. membrane-bound enzyme. We have now obtained preliminary data using isotope labelling studies that suggest that the fate of the nitrosyl intermediate is indeed affected by the extent of solubilization of the enzyme.

We have shown previously (13) with whole cells of P. stutzeri that the extent of ^{18}O exchange between $H_2{}^{18}O$ and product N_2O is a function of the nitrite concentration. The extent of equilibration ranged from 35% at 90 μM nitrite to 7.9% at 9 mM nitrite. We have now repeated this experiment using cell-free crude extracts prepared by sonication. As shown in Table 5, the same trend toward increased ^{18}O equilibration at lower nitrite concentrations is observed with the sonicated extracts, but all values are 3-4 fold higher than the results with whole cells.

The competition experiments described in Tables 1-3 above employed cell-free crude extracts prepared by French press. For nitrite concentrations ranging from 50 μM to 1 mM and azide concentrations of 50 mM, we found that the fraction of total N_2O formed via denitrification ranged from 56 to 83%. Data obtained under the same conditions for cell-free extracts prepared by sonication are given in Table 6. Even though the general trend is again the same, with an increasing

TABLE 5

^{18}O isotopic enrichment of denitrification products as a function of nitrite concentration for sonicated crude cell-free extracts. Conditions: reductant = 100 mM sodium succinate; medium contained 9 atom % $H_2{}^{18}O$.

	Isotope Abundance		
$[NO_2^-]$ mM	N_2O-44	N_2O-46	% Equilibration
10	20,131,177	465,193	23.4
	20,881,770	621,293	30.5
			av. 27.0 (± 3.6)
1	21,923,078	1,254,134	59.2
	28,666,146	1,692,224	61.1
			av. 60.2 (± 1.1)

TABLE 6

Competition between nitrosation and denitrification in crude cell free extracts prepared by sonication. Conditions: as in Table 1 except for reductant = 100 mM sodium succinate; crude extracts prepared by sonication.

	Isotope Abundance		
$[NaNO_2^-]$ (mM)	N_2O-45	N_2O-46	Ratio: $\frac{N_2O\text{-}45}{N_2O\text{-}46}$
0.05	20,020	164,100	0.122
	21,380	168,200	0.127
			av. 0.125 (± 0.003)
0.10	18,220	375,750	0.049
	26,080	369,900	0.071
			av. 0.060 (± 0.011)
1.0	21,270	405,950	0.052
	18,990	420,780	0.045
			av. 0.049 (± 0.004)
10.0	82,330	449,140	0.155
	76,840	462,700	0.142
			av. 0.149 (± 0.007)

fraction of N_2O due to denitrification with increasing nitrite concentration, the actual figures are very different, with nitrosation accounting for only 5 to 10% of total N_2O. (Once again reductant apparently becomes rate limiting at high $[NO_2^-]$, as noted previously in Table 2.)

These observations, although preliminary in nature, suggest that isotope labelling studies can be used to probe alterations in relative rates of individual steps within the catalytic mechanism as the enzyme is purified. More detailed studies will be required to correlate observed changes in relative rates with the physical state of the enzyme (e.g., lipid content of the preparation, degree of aggregation) and are in progress. Nonetheless, these results suggest that it is not unreasonable to look to perturbations in active site environment to explain the shift in product from N_2O to NO as the enzyme is purified, rather than invoking the existence of separate NO_2^- and NO reductases.

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CHAPTER FOUR

PURIFICATION, MOLECULAR CHARACTERIZATION AND KINETIC PROPERTIES OF *PSEUDOMONAS STUTZERI* CYTOCHROME CD_1 TYPE NITRITE REDUCTASE

INTRODUCTION

Two types of dissimilatory nitrite reductases are known to play a role in bacterial denitrification: one is more predominant among denitrifiers isolated to date and contains two kinds of hemes (c,d_1). The other one is not a cytochrome, but contains copper and was first found in *Alcaligenes faecalis* (formerly *Pseudomonas denitrificans*; 1). Our work has been limited to nitrite reductases of the first type. The cytochrome c.d_1 type dissimilatory nitrite reductase (ferrocytochrome c:nitrite oxidoreductase) has been purified in the past from several denitrifying bacteria. Original purification procedures are the following: *Pseudomonas aeruginosa* (2,3,4,5,6,7,8), *Thiobacillus denitrificans* (9,10), *Alcaligenes faecalis* (formerly *Pseudomonas denitrificans* (5,11,12), *Paracoccus denitrificans* (formerly *Micrococcus denitrificans*) (13,14,15) and *Paracoccus halodenitrificans* (16). They are all dimers of two equivalent subunits with a total molecular weight in the 120 to 130 kDa range and with two hemes (1 heme c and 1 heme d_1) per subunit. Purification methods which gave high yields were the one for *Thiobacillus denitrificans* (10), with 100 grams of cell paste yielding 45 mg pure enzyme and the *Alcaligenes faecalis* nitrite reductase preparation (11), yielding 56 mg enzyme

from 4650 mg total protein. The most popular contemporary method gave a 25% recovery (7). A comprehensive review on denitrification and nitrite reduction is available (17).

Over the last few years we have studied the pathway of dissimilatory nitrite reduction in Pseudomonas stutzeri JM 300. In 1982, Averill and Tiedje (18) proposed a pathway in which nitrite is reduced by the enzyme to both nitric oxide and nitrous oxide, with nitric oxide postulated to be being an "abortive" product, i.e. in equilibrium with enzyme-bound nitric oxide (nitrosyl). We do not believe that nitric oxide is an obligatory intermediate in the conversion of nitrite to nitrous oxide by this enzyme. Isotope studies have provided conclusive evidence that, at least in this organism, the N-N bond in nitrite reduction is formed by nucleophilic addition of a second nitrite molecule to an enzyme bound intermediate (19,20; Ch. 3, this work). We believe this intermediate is most likely a heme-nitrosyl; there is also chemical precedence for N-N bond formation by nitrosation of metal stabilized nitrosyls, catalyzed by this enzyme (21).

There has been much controversy in the past about the identity of the product(s) of nitrite reductases. Not only were there differences in behaviour between nitrite reductases from different bacterial species, but also between different preparations of the nitrite reductase from the same species. Most often, nitric oxide was reported as the only product of nitrite reduction (3,10,11,14,16,22,23). Occasionally, nitrous oxide was detected along with nitric oxide, but as a minor product (5,9,12,21,24,25,26). Nitrous oxide as major product has only been reported twice (27,28): both found transient formation of nitric oxide in the process of nitrite reduction to N_2O.

Nitrite reductase, as a consequence of its involvement in a respiratory pathway, is generally accepted to be membrane associated in vivo. A pool of soluble nitrite reductase is always present after cell disruption, but a fraction of the nitrite reductase pool is more tightly membrane associated (16,29,24). At least a significant portion of the enzyme is soluble and is most efficiently released from the membrane by sonication. There may be a chemical equilibrium in the cell between a membrane-bound and a soluble pool, or the soluble pool may be an artifact from cell breakage. In Pseudomonas perfectomarinus, 15% of the nitrite reductase activity was more tightly bound to the membrane than the rest, which led to the speculation that there were two "kinds" of nitrite reductases in the cell (24). The membrane bound nitrite reductase of Paracoccus halodenitrificans was extracted with detergent, purified and shown to be identical to the cytoplasmic nitrite reductase (16).

Membranes from denitrifiers have been shown to possess nitric oxide reducing activity (24,26,30) and to reduce nitrite to nitrous oxide without detection of free nitric oxide (16,24,30). NO reduction generates energy through proton translocation (31) and thus it seems that nitric oxide reduction, like nitrite reduction, is part of a respiratory pathway. Nitrite reducing activity and nitric oxide reducing activity have been differentially extracted from membranes (30,32) and have been physically separated from each other.

Reports on partially purified nitric oxide reductases are scarce and poorly characterized: an iron-flavoprotein in P. aeruginosa (33) and in P. stutzeri (34); a cytochrome c in P. perfectomarinus (23,35). Nitrite reductases have been reported to reduce exogenous nitric oxide (27) and one purified "nitric oxide reductase" later

turned out to be the cytochrome $\underline{c.d_1}$ nitrite reductase (5). Nitric oxide reduction is typically much slower than nitrite conversion to nitrous oxide (21,24,36). Whole cells both produce and consume nitric oxide (5,11,33,36,37,38,39,40).

This work is focused on the purification, molecular properties and reactivities with nitrite and nitric oxide of the purified nitrite reductase from Pseudomonas stutzeri JM300. Growth and partial purification of the nitrite reductase from Pseudomonas stutzeri (Van Niel strain) has been described by Kodama (41).

We have obtained a highly purified nitrite reductase from Pseudomonas stutzeri and used it address the following questions: (i) What are the substrates and products of the pure enzyme ? (ii) If significant N_2O production from nitrite occurs, is this process kinetically competent (are Km values low and Vmax values high enough) to have potential ecological significance? (iii) Is there any effect of purification on the apparent kinetic parameters of the enzyme? (iv) How similar is this nitrite reductase to previously characterized nitrite reductases?

MATERIALS AND METHODS

Growth of P. stutzeri. P. stutzeri JM300 was grown in the following medium: 30 g/l Tryptic Soy Broth (Sigma) supplemented with KNO_3 (5 g/l), $NaHCO_3$ (2 g/l),10 uM $CuSO_4$ and 10 mg/l $FeSO_4.7H_2O$. Here we describe the purification of nitrite reductase from 15 l of this medium, which yielded 65.3 g of wet cell paste. Cultures were routinely transferred as 0.5% inocula to 100 ml fresh medium, in 155 ml glass serum bottles with butyl rubber stoppers crimped with aluminum seals. The headspace above fresh medium was not made anaerobic, as the bacteria used the oxygen present initially and create their own anaerobic, denitrifying growth conditions. Larger quantities of bacterial cells were prepared by inoculating 100 ml of actively growing bacteria in late exponential phase (18 to 20 h after inoculation) into 15 l of medium in a large glass container closed with a rubber stopper and equipped with a sterile gas outlet: a 0.22 um filter connected to a hypodermic needle, inserted into the rubber stopper. Cultures in small serum bottles were grown while shaking at 100 rpm in a 37 C incubator. The 15 l culture flasks were shaken at 50 rpm.

The cells were harvested by centrifugation (12,000 g x 15 min) during late exponential growth when the gas (N_2) formation rate has reached its maximum and has begun to decline: this was monitored by subsampling and measuring the rate of N_2 evolution from a 1 ml subsample of cells assayed by gas chromatography in an argon-flushed 8 ml serum vial.

Crude extract preparation. The cell paste was resuspended in 30 mM Hepes buffer, pH 7.3. The cells were lysed by sonication (Heat Systems - Ultrasonic W-225) at 40 % of maximum output for a total of 5 min in intervals of 30 s, followed by cooling in an ice bath. Longer sonication time did not result in any significant additional release of nitrite reductase activity in the supernatant.

Activity assay. Enzyme activity was measured by gas evolution (NO + N_2O) with NADH/phenazine methosulfate (PMS) as the electron donating system and nitrite as substrate (42). The assay contained 2 umol NADH, 0.12 umol PMS and 5 umol $NaNO_2$ in a total volume of 1 ml. All stock solutions were made in Hepes buffer (50 mM, pH 7.3). NADH and nitrite were added anaerobically to argon flushed serum bottles (8 or 25 ml) which contained the PMS and the buffer. The reaction was started by anaerobic addition of an appropriate amount of enzyme. NADH stock solution was made fresh daily. Gas formation was monitored on a Perkin Elmer 910 gas chromatograph equipped with a 63Ni electron capture detector (ECD). The carrier gas was 85 % argon, 15 % methane. The oven temperature was 55 C and contained a 1.83 m stainless steel Porapak Q column. Carrier flow was adjusted so that the approximate retention times for nitric oxide and nitrous oxide were 1 min and 2.2 min, respectively. Under these conditions, the retention times of nitric oxide and oxygen are extremely close which makes it necessary to use a strictly anaerobic technique for sampling and injecting the gas phase of assay vials. We used gas-tight syringes equipped with a gas lock. For most experiments the enzyme activity was expressed as the initial rate of gas evolution; this is the most sensitive assay available. Recovery of activity during the enzyme purification was expressed as

the rate of NADH oxidation, measured as the rate of decrease in absorbance at the reduced NADH absorption maximum of 340 nm. The total volume of the assay was 0.6 ml, contained in 2 mm quartz anaerobic cuvettes (Precision Glass) stoppered with rubber Venoject stoppers. The amounts of assay reagents used (above) were adjusted accordingly.

Purification of nitrite reductase. We modified the purification method as described by Parr et al.(7) for P. aeruginosa. The most important modification was the last column; Parr et al. (7) used carboxymethyl cellulose. Since we found the isoelectric point of the P. stutzeri enzyme to be quite low (<6), we would have to expose the enzyme to a pH value below the physiological pH range (pH 6 to 8) in order to successfully use a cation exchange column. Instead, we used a hydroxyapatite column, since it had been successfully used by Newton (13) for Paracoccus denitrificans cytochrome $c.d_1$.

Ammonium sulfate precipitation:

RNAase (0.1 mg) and 0.1 mg DNAse (both from Sigma) were added to the crude extract to reduce viscosity. Solid ammonium sulfate was added to bring the extract to 45% saturation. The pH was adjusted to 7.5. After centrifugation (12,000 g x 30 min) to remove cell debris, the supernatant was centrifuged for 2 h at 100,000 x g to remove small membrane fragments. The supernatant was dialyzed against 10 mM Tris pH 7.

First anion exchange (DEAE-I):

A DEAE-52 column (Whatman, preswollen, 2.5 x 20 cm) was equilibrated with 10 mM Tris pH 7 and loaded. A near linear gradient of 0 to 400 mM KCl was created as follows: 200 ml of 10 mM Tris pH 7 and 200 ml of 400 mM KCl in 10 mM Tris pH 7 were connected by a salt bridge. The low salt solution was stirred and pumped to the top of the

column at a flow rate of 3 ml/min. Fractions of 3 ml were collected. This was a fast though effective clean-up before the sizing column. The green nitrite reductase eluted before a red heme containing fraction. All green fractions were combined (up to the first slightly pink fraction), concentrated to about 6 ml and dialyzed against 50 mM Tris pH 7.

Gel Filtration:

A Sephacryl column (S-300, Pharmacia, 2.5 x 75 cm) was run in the upflow mode. Half of the sample (3 ml) was loaded at one time in order to optimize resolution and prevent column overload. Fractions of 2 to 3 ml were collected. All green fractions were combined for loading onto the next column, without dialysis.

Second anion exchange (DEAE-II):

After loading, the column (Whatman DE-52, 2.5 x 15 cm) was washed with 50 mM Tris pH 7 for about 2 h at 3 ml/min. An olive-green band (nitrite reductase) bound tightly to the top of the column. The column was eluted with a gradient of KCl in 50 mM Tris pH 7. The gradient was from 50 to 200 mM KCl (400 ml of each). Fractions of 3 ml were collected. Nitrite reductase eluted in two clearly distinguishable peaks, an early darker green fraction and a late lighter green fraction. The valley separating the two fractions in the gradient was around 100 mM KCl. The two green fractions were collected separately (termed FR.1 and FR.2) and each dialyzed against 10 mM potassium phosphate buffer, pH 7.

Hydroxyapatite:

The column (Biorad, Biogel HT, 1.5 x 20 cm) was equilibrated with 10 mM potassium phosphate buffer at pH 7 and loaded at 100 ml/h. Each fraction was loaded separately and washed with 150 mM potassium

phosphate buffer, pH 7 for 1 to 1.5 h. Fraction 1 nitrite reductase was eluted with 250 mM potassium phosphate buffer, pH 7. Fraction 2 bound more tightly to the column and was eluted with 500 mM potassium phosphate buffer, pH 7. For each of the two fractions that were loaded, the eluted green fractions were combined and dialyzed against 25 mM Hepes pH 7.3.

Kinetic measurements. Kinetic parameters were estimated from initial rate measurements using N_2O and NO production as the assay. Approximately 1 ug enzyme was used for N_2O production and 0.1 ug enzyme for NO production to stay within the linear range of the detector and of the assay for at least 20 min. The data are replicates of three replicate measurements.

Native molecular weight. The native molecular weight was estimated on a Superose 6 (1 cm x 30 cm) sizing column by FPLC (Pharmacia). The flow rate was 0.4 ml per min of 25 mM Hepes pH 7.3 and 0.1 M KCl. Molecular weight standards were from Biorad. Approximately 20 ug of each standard and of nitrite reductase were loaded onto the column.

Subunit molecular weight. The subunit molecular weight was estimated by SDS PAGE using an SE 300 vertical slab unit (Biorad). Gels of 0.8 mm thickness (7.5 % T, 2.7 % C acrylamide separating gel and 4 % T, 2.7 % C acrylamide stacking gel) were prepared by the method of Laemmli (43). Electrophoresis was for 10 h at 35 mA for two gels. Gels were stained by silver staining (44,45). The molecular weight was estimated by the method of Weber & Osborn (46). Molecular weight standards were obtained from Sigma.

Isoelectric focusing. The apparatus was the same as above. The method used was by Gary Giulan, Dept. of Physiology, University of Wisconsin, Madison, WI as described in the Hoefer Scientific manual

(1987). Urea (6 M) was used in the gel and in the sample buffer; this was necessary to prevent precipitation in the wells. The pH gradient in the gel was 3.5 to 10 (ampholytes Pharmalyte, Sigma). Isoelectric focusing standards were from Sigma.

Protein concentration. Protein was determined by the bicinchoninic (BCA) acid method (47) with bovine serum albumin (Sigma) as the standard; the reagents were obtained from Pierce.

Spectroscopy. Visible spectra were recorded on a Perkin-Elmer (model lambda 5) spectrophotometer. Sodium dithionite was added directly to the cuvette to obtain the reduced spectra. The enzyme is normally oxidized under air.

RESULTS

Purification of nitrite reductase. Purification data are given in Table 1. The overall yield was high (86%); the amount of pure enzyme recovered was 37.5 mg, which indicated that roughly 0.5% of the total protein in the cells was nitrite reductase. Fig. 1 shows the different steps in the purification on an SDS-gel. Newton (13) used a hydroxyapatite column for Paracoccus denitrificans nitrite reductase purification and obtained three fractions of nitrite reductase, which could be eluted by step gradients. Our findings are similar but not identical: a first red heme containing fraction eluted in 150 mM potassium phosphate buffer. Its visible spectrum was identical to the one from nitrite reductase apoprotein (devoid of heme d_1, Fig. 2) and thus could be the apoprotein. The second and third heme containing fractions were two forms of active, highly purified nitrite reductase; both migrated as one band on SDS gels; the second one has a higher specific activity than the first one (Table 1). These two bands also were separated on ion exchange columns. From their visible reduced spectra, however, they showed no differences in apparent heme d_1 content.

The total yield of purified nitrite reductase decreased proportionally to the total purification time and to the amount of time the enzyme spent in diluted form.

Spectral characteristics. Reduced and oxidized visible spectra of P. stutzeri nitrite reductase are shown in Fig. 3. They are very similar to the ones shown for Pseudomonas aeruginosa nitrite reductase (22). In the oxidized spectrum, one peak characteristic for

TABLE 1

Purification scheme, Pseudomonas stutzeri nitrite reductase.

Treatment	Protein (mg)	Specific Activity umole NADH/mg.min	Activity Units umole NADH/min	Recovery (%)	Purif. Factor
(NH4)3(SO4)	3808	0.097	369	100	1
DEAE-I	880	0.47	414	112	3
S-300	180	2.46	442	120	23
DEAE-II					
FR. 1	85	2.89	246	67*	30
FR. 2	33	8.05	267	72*	83
Hydroxyapatite					
FR. 1	30.3	7.68	233	63*	74
FR. 2	7.2	11.63	84	23*	121

*: Recoveries for DEAE-II and hydroxyapatite columns were calculated separately for FR.1 and FR.2 and should be summed to obtain total recovery.

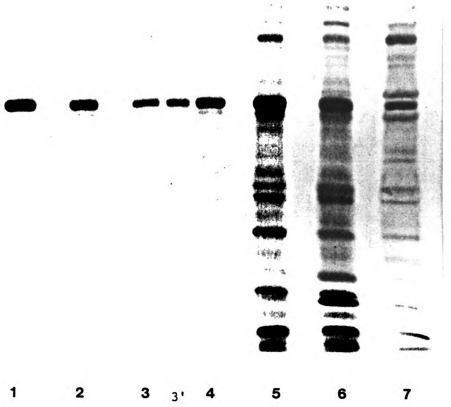


Fig 1. SDS - PAGE of P. stutzeri nitrite reductase purification steps.
1: hydroxyapatite, 500 mM phosphate buffer fraction
2: hydroxyapatite, 250 mM phosphate buffer fraction
3: DEAE-II, early green fractions
4: DEAE-II, late green fractions
5: Sephacryl S-300, combined green fractions
6: DEAE-I, combined green fractions
7: Ammonium sulfate precipitation, supernatant

3': same as 3

Fig. 2. The absorption spectrum of the apoprotein (-heme $\underline{d}_1$) of the nitrite reductase from *P. stutzeri*. ------, oxidized; ————, reduced.

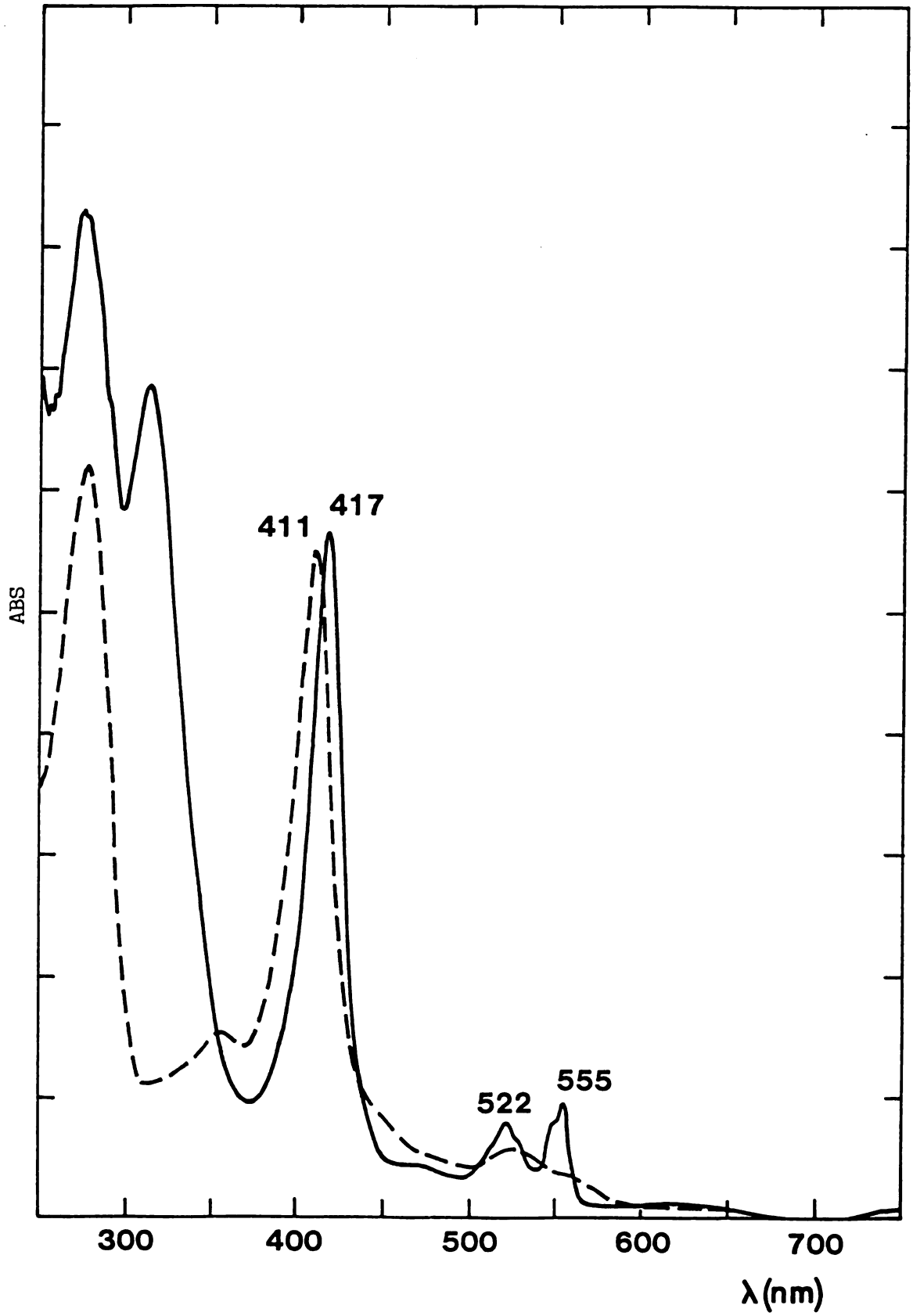
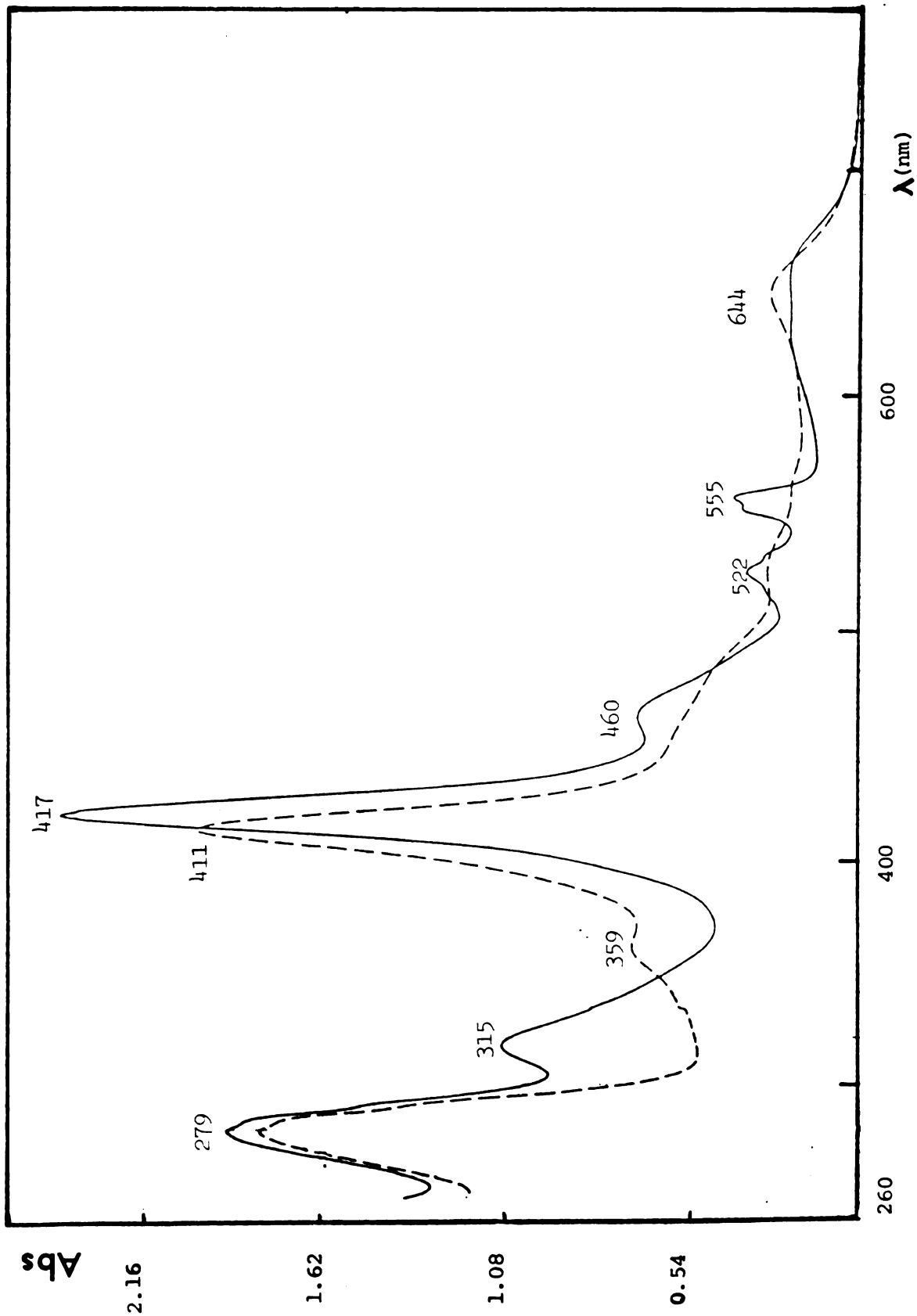

ABS
411
417
522
555
300
400
500
600
700
λ(nm)

Fig. 3. The absorption spectrum of nitrite reductase from Pseudomonas stutzeri. The enzyme was dissolved in 0.25 M phosphate buffer pH 7.3. The enzyme concentration was 2 mg/ml. ------, oxidized; ———— , reduced.


Abs
2.16
1.62
1.08
0.54
260
400
600
λ(nm)
279
315
359
411
417
460
522
555
644

the c-heme is found at 411 (Soret) nm. Heme $\underline{d}_1$ peaks are at 460 (shallow shoulder) and 644 nm. In the sodium ditihionite reduced spectrum, heme c peaks are at 417 (Soret), 522 and 555(split alpha) nm, heme $\underline{d}_1$ peaks at 460 (higher shoulder) and an absorption band in the 600-675 nm area. The apoprotein (minus heme $\underline{d}_1$) spectrum is essentially the same, however, it shows none of the characteristic heme $\underline{d}_1$ absorption peaks (Fig. 3). Apoprotein can be prepared by acid acetone extraction of nitrite reductase (48,49,50); in its reduced visible spectrum, peaks characteristic for heme $\underline{d}_1$ (460, 650 nm) are missing (compare Figs. 2 and 3).

Molecular properties. The native molecular weight as determined by gel filtration was 76,500 (Fig. 4). The subunit molecular weight as determined by SDS-PAGE was 67,800 (Fig. 5). The isoelectric point as determined by isoelectric focusing was 5.4 (Fig. 6).

Kinetics of nitrite reduction. The purified P. stutzeri nitrite reductase always produced N_2O as well as NO. Progress curve data illustrate that the N_2O/NO ratio increased when the nitrite concentration was higher. This suggested that the Km for a second binding of nitrite resulting in the dinitrogen product (N_2O) was higher than the Km for the first nitrite binding which led to NO production (Fig. 7). Progress curves typically showed an initial accumulation of NO, which then leveled off while N_2O continued to accumulate. Fig. 7 also shows a lag in production of both gases which we believe was due to inadequate removal of oxygen since the lag was longer when bottles were insufficiently flushed and shorter when more enzyme was used. In the same bottle the lag was consistently longer for N_2O appearance than for NO appearance.

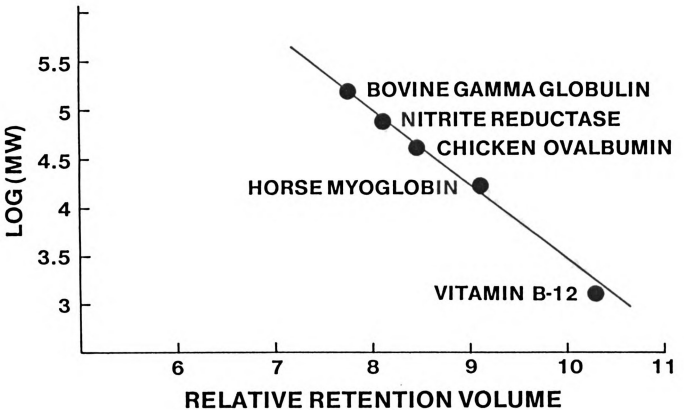


Fig. 4. Determination of the native molecular weight of Pseudomonas stutzeri nitrite reductase by gel exclusion chromatography.

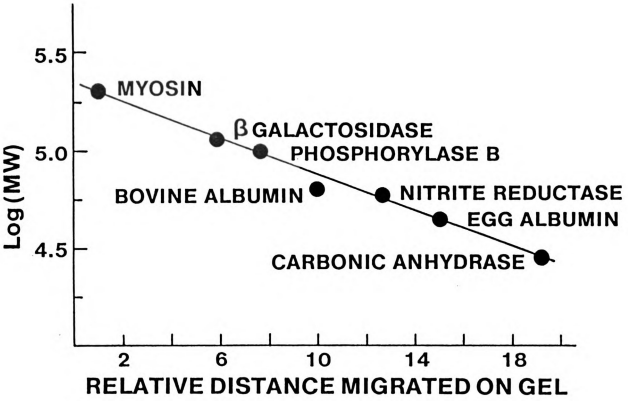


Fig. 5. Determination of the subunit molecular weight of Pseudomonas stutzeri nitrite reductase by SDS - PAGE

Fig. 6. Determination of the isoelectric point of P. stutzeri nitrite reductase by isoelectric focusing.

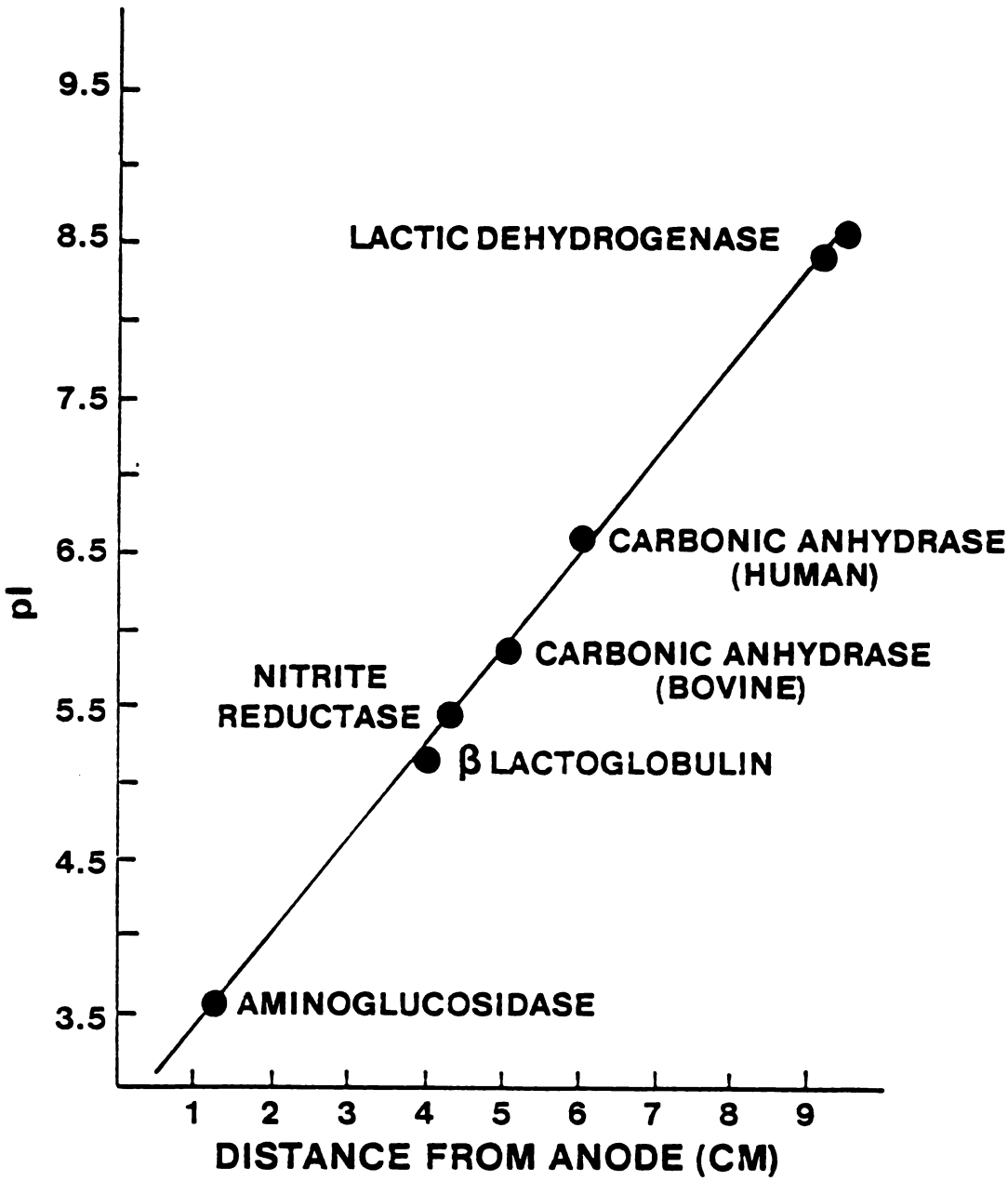
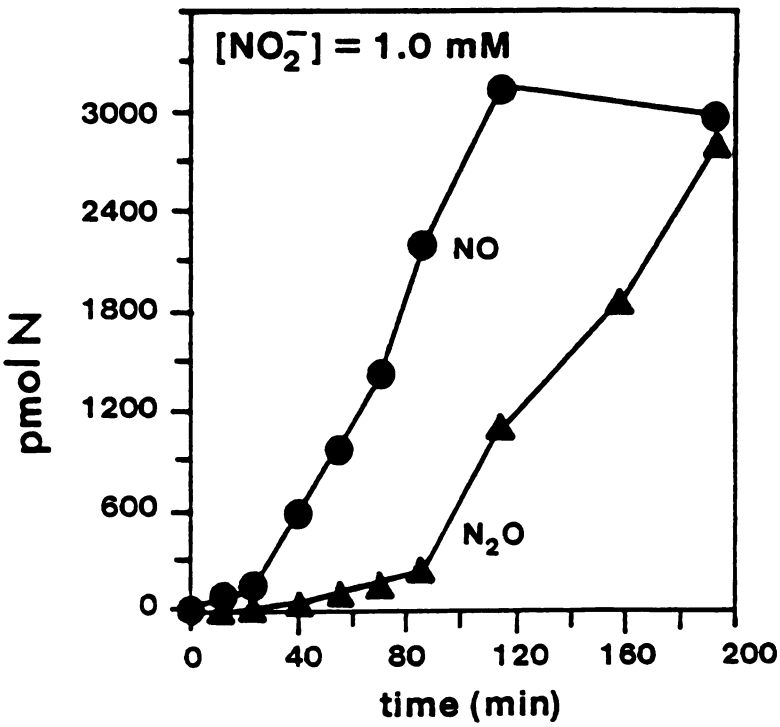
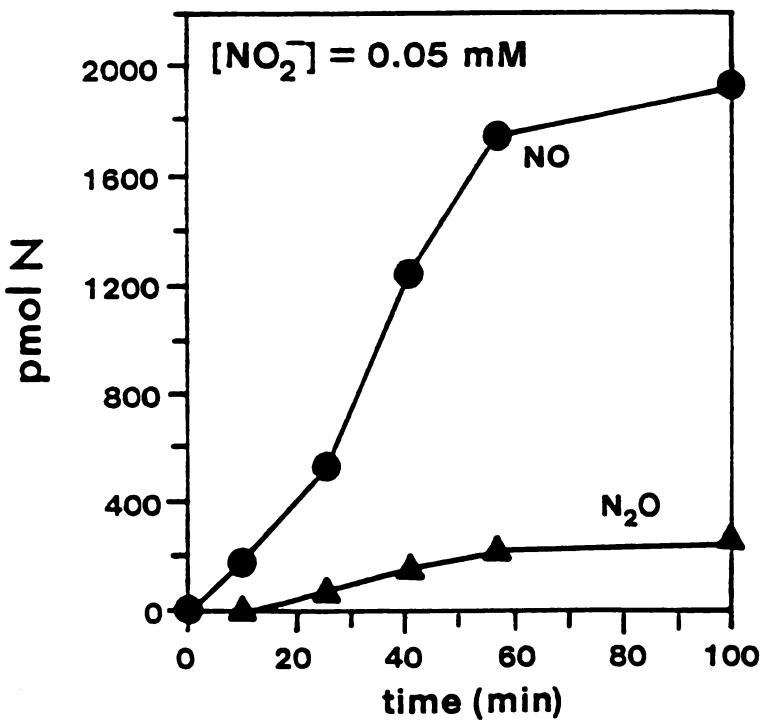

9.5
8.5
7.5
6.5
5.5
4.5
3.5
pI
LACTIC DEHYDROGENASE
CARBONIC ANHYDRASE
(HUMAN)
CARBONIC ANHYDRASE
(BOVINE)
NITRITE
REDUCTASE
β LACTOGLOBULIN
AMINOGLUCOSIDASE
1
2
3
4
5
6
7
8
9
DISTANCE FROM ANODE (CM)

Fig. 7. Progress curves of nitric oxide and nitrous oxide production from nitrite by Pseudomonas stutzeri nitrite reductase.
A: High initial nitrite concentration (1 mM)
B: Low initial nitrite concentration (0.05 mM)


[NO2-] = 1.0 mM
3000
2400
1800
1200
600
0
pmol N
NO
N2O
0
40
80
120
160
200
time (min)


[NO2-] = 0.05 mM
2000
1600
1200
800
400
0
pmol N
NO
N2O
0
20
40
60
80
100
time (min)

The kinetic parameters for the two reactions were determined by measuring initial rates of NO production at low nitrite concentrations (1 to 25 uM), the second one by measuring initial rates of N_2O production at higher nitrite concentrations (50 to 1000 uM). In this experiment the bottles were more exhaustively flushed to reduce the lag. Initial rates were calculated from the first three time points which were linear (approx. 3 to 20 min) The two Km values were different enough that measurement of one did not interfere with measurement of the other. In the nitrite concentration range where NO production rates were increasing, N_2O production was still so slow that it did not interfere with the initial rate measurements. In the range where N_2O production became apparent, NO production was at its Vmax. For NO production, the apparent Km was 1.35 uM NO_2^- with an apparent Vmax of 1.6 umole NO_2^-/mg.min (Fig. 8). For N_2O production, the apparent Km was of 59 uM NO_2^- with an apparent Vmax of 0.93 umole NO_2^-/mg.min (Fig. 9).

We compared these kinetic parameters with the ones for crude but ultracentrifuged extract, in order to determine whether the purification treatments between ultracentrifugation and pure enzyme from the hydroxyapatite column had any deleterious effect on the enzyme's kinetic properties. In the crude extract, the first Km (for NO production) was essentially the same as for the pure enzyme: 1.10 uM NO_2^- (data not shown). The second Km (for N_2O production, Fig. 10) was higher for the purified enzyme: 59 uM vs. 34 uM for the crude enzyme. While the factor of two difference is statistically significant, it is not clear whether this difference has any importance to understanding the behaviour of this enzyme.

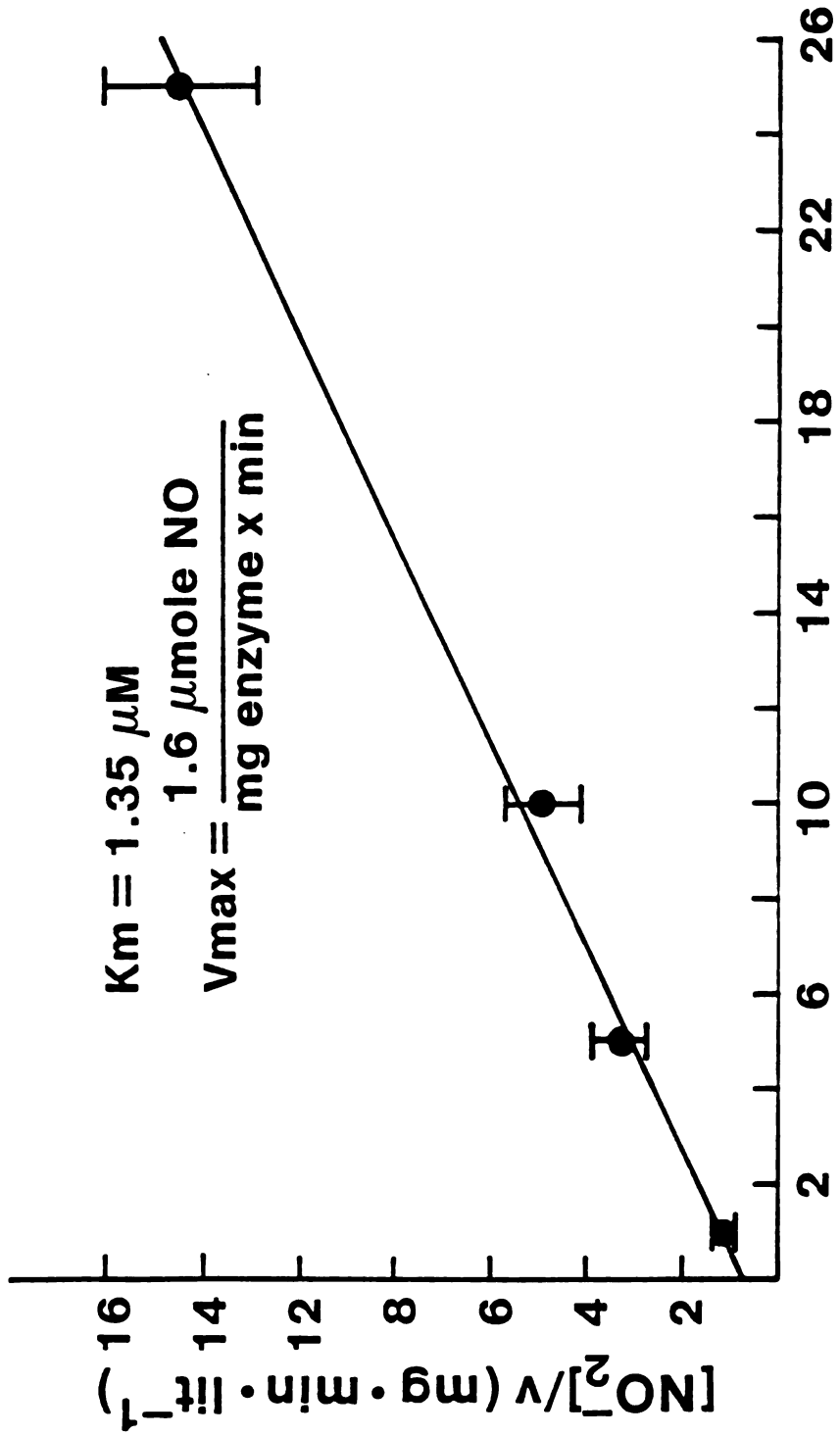


Fig. 8. Kinetic parameters of nitric oxide production by Pseudomonas stutzeri nitrite reductase.

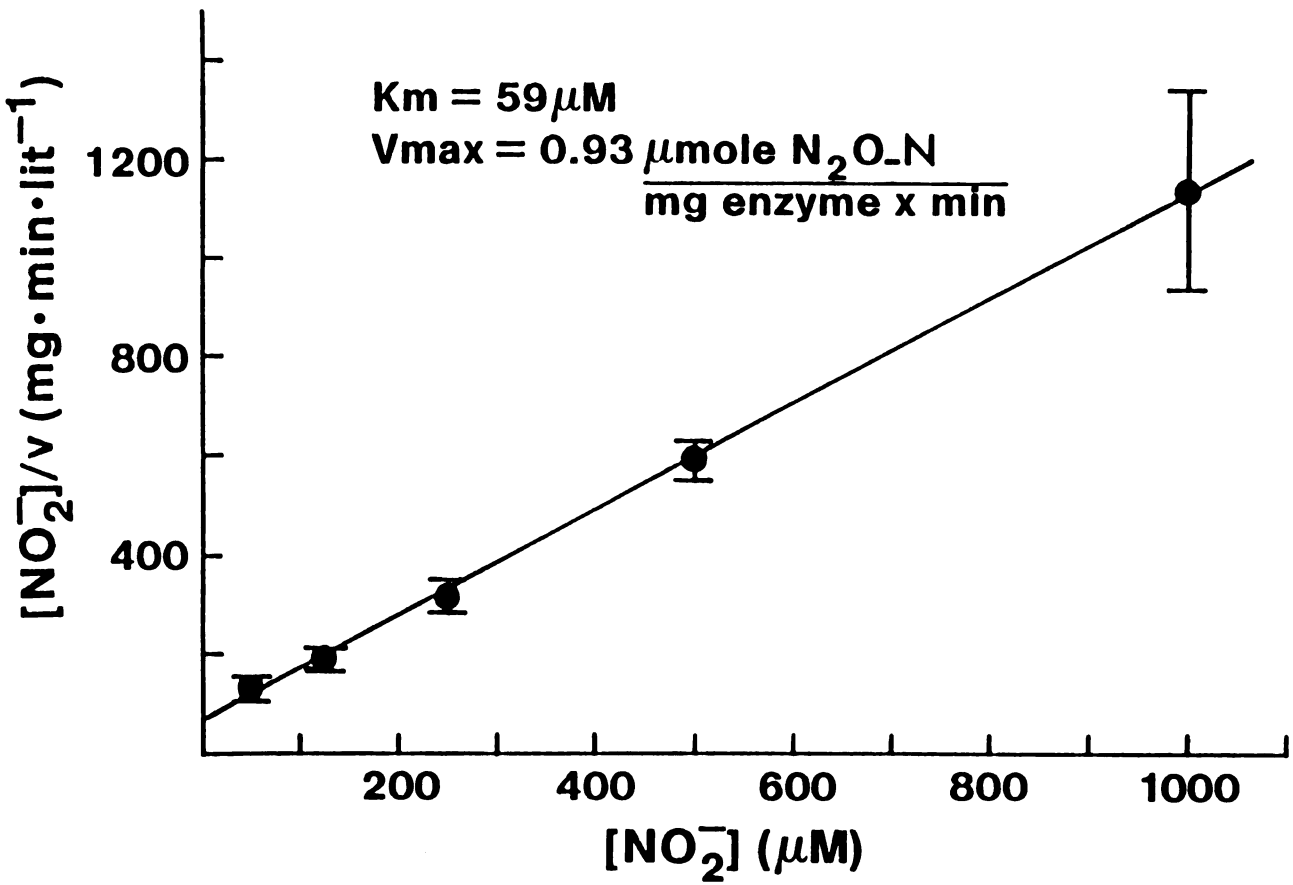
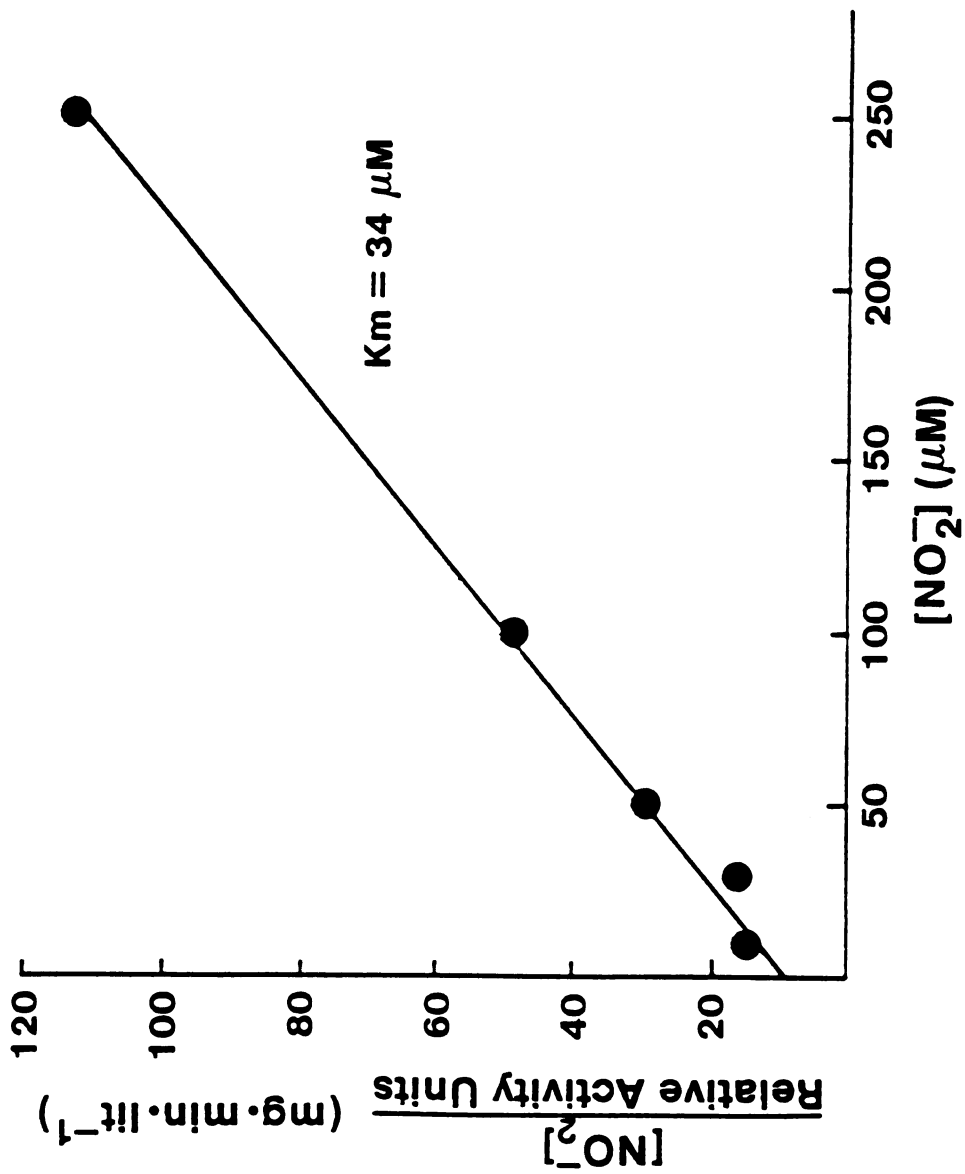


Fig. 9. Kinetic parameters of nitrous oxide production by Pseudomonas stutzeri nitrite reductase.

Fig. 10. Kinetic parameters of nitrous oxide production by crude, ultracentrifuged extract of P. stutzeri cells, containing nitrite reductase.


Km = 34 μM
Relative Activity Units
[NO2-]
(mg·min·lit-1)
120
100
80
60
40
20
50
100
150
200
250
[NO2-] (μM)

Control experiments with autoclaved enzyme showed no chemical production of NO or N_2O from nitrite, and no chemical N_2O production from NO (at NO concentrations representative for those seen in a typical progress curve). Also, the NADH/PMS reducing system did not sustain any significant chemical N_2O production from mixtures of NO and NO_2^- under our experimental conditions.

Nitric oxide as substrate. Neither the purified enzyme nor the crude ultracentrifuged extracts consumed nitric oxide when it was the only substrate. With the NADH/PMS reducing system, when the purified enzyme was given 1 mM $^{15}NO_2^-$ and 100 Pa NO in the headspace, the only product formed was $^{15,15}N_2O$: apparently, free NO is not reduced under these conditions, since neither $^{15,14}N_2O$ nor $^{14,14}N_2O$ were detected. The products were analyzed by mass spectrometry as described previously (19). In earlier work with whole cells (19) and with non-ultracentrifuged crude extracts we did find nitric oxide reduction to nitrous oxide. Washed membrane fragments which were virtually devoid of all nitrite reducing activity still had much NO reducing activity (unpublished data). The NO reducing activity was apparently associated with the membrane fraction removed during the ultracentrifugation.

DISCUSSION

Over the past few years we have obtained indirect evidence, from isotope studies in whole cells (19) and crude cell-free (but not ultracentrifuged) extracts (20; Ch. 3, this work) of P. stutzeri JM 300, for a pathway of nitrite reduction in this organism in which the first dinitrogen bond is made by nucleophilic addition of a nitrite anion to an enzyme bound nitrosyl intermediate.

Earlier we investigated the effect of hydroxylamine on denitrification by whole cells with $^{15}NO_2^-$, resp. ^{15}NO as substrates (19). With NO as a substrate, at hydroxylamine concentrations of 10 mM, all of the N_2O formed was from nitrosation of hydroxylamine (Table 2), indicating effective interception of a mononitrogen intermediate in the NO reduction pathway by hydroxylamine. When $^{15}NO_2^-$ was a substrate, however, hydroxylamine at a concentration of 320 mM did not result in any significant formation of nitrosation product ($^{14,15}N_2O$; Table 3), which indicates that hydroxylamine was too weak a nucleophile to intercept any mononitrogen intermediates in the nitrite reduction pathway in the whole cell. We believe this to mean that the main flux of nitrite nitrogen in the whole cell does not involve a free NO intermediate.

In crude extracts, possible interference by an often postulated, but so far never purified, "nitric oxide reductase" may be a problem, therefore we purified the nitrite reductase and studied its kinetic properties. In a pathway in which there are two binding events, it should be possible to measure apparent Km values for each of the two nitrite binding events. We determined a Km of 1.4 uM for NO production

TABLE 2

Competition between nitrosation and denitrification when ^{15}NO was substrate.

NH_2OH (mM)	N_2O from nitrosation (%)
0	0
5	38
10	100
25	100

TABLE 3

Absence of competition between nitrosation of hydroxylamine and denitrification when $^{15}NO_2^-$ was substrate.

NH_2OH (mM)	N_2O from nitrosation (%)
10	2.4
40	1.4
80	1.6
160	2.6
320	2.8

(first nitrite binding) and a Km of 59 uM for N_2O production (second nitrite binding).

Biphasic Lineweaver-Burk plots for Pseudomonas aeruginosa nitrite reductase have been presented by Saraste and Kuronen (29). These authors stated that:

" The biphasic kinetics observed ... cannot be easily interpreted at present. Possible explanations are the presence of two isoenzymes with different affinities to nitrite,or an equilibrium between dimeric and monomeric forms, or interaction of the two nitrite binding sites in the dimeric enzyme ". We estimated two Km (NO_2^-) values from their biphasic plot: a low Km of approximately 6 uM, and a high Km of approximately 68 uM for the purified enzyme, values comparable to ours for P. stutzeri nitrite reductase.

A Km for the second nitrite binding of this order of magnitude (around 60 uM) is low enough for this reaction to be potentially significant in nature. However, nitric oxide accumulates to a larger extent in the headspace above the pure enzyme or crude extracts (several 100 ppm) than it does above whole cells (10 ppm, for Flavobacterium; 51) or in soils (1 to 100 ppb). This could be attributed to physical factors: nitric oxide released inside the cell may quickly become bound again by an unknown component. Rupturing the cells may set it free instead (52). This is one possibility. The "component" may be a "nitric oxide reductase". We find, however, there is also reason to believe that the membrane bound nitrite reductase "in vivo" may show a different kinetic behaviour than the purified enzyme. Saraste (53) found a 20-fold stimulation of the specific activity of the Pseudomonas aeruginosa nitrite reductase cytochrome oxidase activity when the enzyme was incorporated into artificial

membrane vesicles. Unfortunately the nitrite reductase activity was not investigated. For solubilized but not purified Pseudomonas stutzeri nitrite reductase we found a low Km for NO production of 1.10 uM NO_2^-, close to the value for the pure enzyme, and a high Km for N_2O production of 34 uM NO_2^-, about half of the value in the pure enzyme. A factor of about two was also found by Saraste and Kuronen (29) between the high Km values for nitrite in whole cells vs. pure enzyme. It seems that the Km for N_2O production may be affected by solubilization and/or purification.

At this time it is not possible to conclude whether, in the whole cell, the main nitric oxide sink is nitrite reductase or a putative nitric oxide reductase. In the membrane, nitrite reductase accepts electrons from its physiological electron donor - a process which may be many times faster than reduction of the solubilized enzyme with natural or physiological electron donors: typically, maximum rates of N_2O production are two orders of magnitude slower in extracts than in whole cells (20). This is most probably due to a more efficient electron transfer process to membrane bound nitrite reductase as a terminal component in the respiratory chain. It is feasible that, in whole cells, nitrite reduction maximum rates are fast enough to keep NO accumulation low.

Membrane associated nitric oxide reductases may may be hard to purify - several lines of evidence indicate that they certainly may exist: detection of membrane-bound enzymatic nitric oxide reducing capacity (26), separation of nitric oxide and nitrite reducing activities in different cellular fractions (30,32,35), purification of NO reductase in progress (54); we found differential extraction of nitrite reductase activity and nitric oxide reductase activity

possible in our own laboratory: virtually all of the nitrite reductase activity can be removed from membranes by repeated washes with buffers, but NO reductase activity remains membrane bound. Until a pure nitric oxide becomes available and the "in vivo" kinetics for both NO and nitrite reductases known, it will not be possible to say whether nitric oxide reductase or nitrite reductase is the main NO consumer in nature. Existence of nitrix oxide reducase (other than nitrite reductase) would not necessarily imply a role for it in denitrification. It may be an "NO scavenging" activity with a detoxifying function.

The nitrite reductase of Pseudomonas stutzeri is apparently very "sticky": nitrite is virtually not released from the enzyme, once bound. Absence of isotopic exchange (^{15}N) between NO and nitrite (39) with whole cells, a very low isotopic exchange rate between a free unlabelled nitrite pool and ^{18}O labelled water with whole cells (55) and the virtually zero rate of NO turnover by the pure enzyme (this work) all substantiate that statement. This explains why NO turnover by this enzyme can occur when nitrite is present (isotopic scrambling of $^{15}NO_2^-$ and ^{14}NO nitrogen in product N_2O in absence of isotopic exchange between the NO and NO_2^- pools; 39) but not when nitrite is absent - the sticky enzyme does not release nitrite which is needed to drive the reaction forward according to the pathway (20). For probably the same reason, we have never seen stoichiometric turnover of nitrite to nitrous oxide with the pure enzyme. For Pseudomonas aeruginosa PAO1, the rate of N_2O production with NO as only substrate was reported to be virtually zero (21). For the same organism (strain not reported; 27), measurable rates of NO reduction to N_2O and stoichiometric conversion of nitrite to N_2O. The degree of isotopic

scrambling between the NO and NO_2^- pools was very different for various denitrifiers (38,39).

The pathway of dissimilatory nitrite reduction in denitrifiers is, at this point, quite well illustrated, at least in P. stutzeri JM300: evidence from previous isotope studies (19,20) taken together with the kinetic characterization of the enzyme in this work support the mechanism first proposed by Averill and Tiedje (18). It is likely that this pathway common to all c,d_1 type nitrite reductases but that, for different denitrifier species and maybe even for different strains within the same species, the rate constants for individual reactions in the pathway may differ.

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CHAPTER FIVE

RECONSTITUTION OF THE DISSIMILATORY CYTOCHROME $\underline{cd_1}$ TYPE NITRITE REDUCTASE FROM *PSEUDOMONAS STUTZERI* WITH THE SYNTHETIC HEME $\underline{D_1}$

INTRODUCTION

Dissimilatory cytochrome $\underline{cd_1}$ type nitrite reductases from denitrifying bacteria are typically dimers of 120,000 to 130,000 Da molecular weight. The subunits are structurally identical and contain one heme c and one heme $\underline{d_1}$ each. Heme c is covalently bound to the enzyme by means of two thioether linkages and noncovalently through coordination of its iron, presumably by two protein ligands (1). Heme $\underline{d_1}$ is not covalently bound and is linked to the enzyme by coordination of its iron to one ligand, probably an imidazole (1). In vivo, nitrite reductase is believed to be membrane associated: its physiological function is as a terminal oxidase (i.e. nitrite reductase) in the respiratory chain under anaerobic, denitrifying conditions. Its physiological electron donors are cytochrome c 551 and/or azurin in the case of *Pseudomonas aeruginosa* (ferrocytochrome c-551:oxido-reductase, EC 1.9.3.2) (1). The enzyme from *Pseudomonas stutzeri* may be a monomer (Ch. 4, this work).

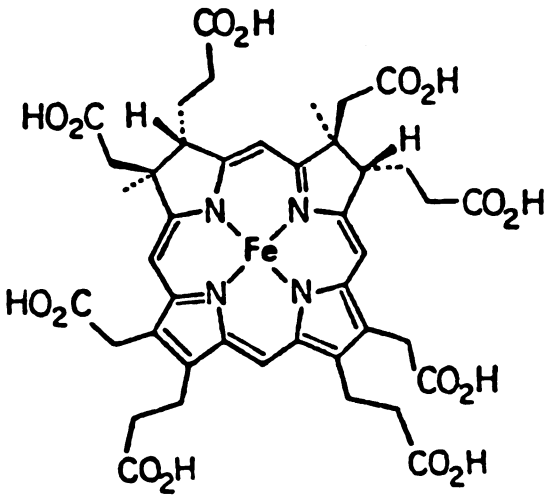
These dissimilatory nitrite reductases reduce nitrite to nitric oxide and nitrous oxide (Ch. 4, this work). Heme $\underline{d_1}$, thought to be the nitrite binding site (1), is easily removed from the enzyme by acidified acetone extraction (2-5). It was reported that 90 to 100% of

the original activity was recovered when the apoenzyme from P. aeruginosa was reconstituted with its own native heme d_1 and that 5% of the activity could be recovered when it was reconstituted with heme a from beef heart cytochrome oxidase. Activity assays, in this case, were done spectrophotometrically by the rate of reoxidation of cytochrome c-551 with O_2 as oxidant (5).

The structure of heme d_1 was first suggested to be a chlorin type structure with two carboxylic acid groups on an unsaturated pyrrole and two methyl alcohol groups on a saturated pyrrole (6,a,b). More recent evidence shows the chromophore to be a dioxo-isobacteriochlorin with two ketone groups on adjacent pyrrole rings (Fig. 1) (7,a,b). So far, this novel structure has been found only in bacterial nitrite reductases. The heme d_1 has recently been synthesized (8).

In this chapter we report on the biochemical activity and spectral properties of nitrite reductase apoprotein reconstituted with the synthetic heme d_1.

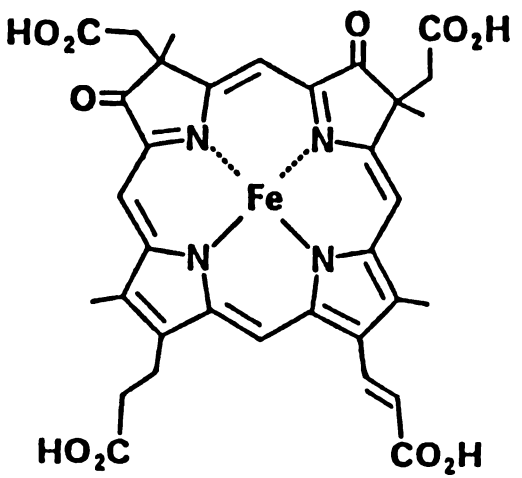
I acknowledge the collaboration of Weishih Wu who worked jointly with me in the experiments reported in this chapter.



SIROHEME

assimilatory nitrite reduction

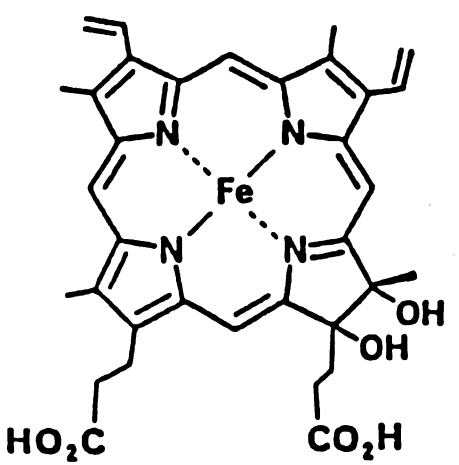
$NO_2^- \longrightarrow NH_3$



DIONEHEME (d_1)

dissimilatory nitrite reduction

$NO_2^- \longrightarrow N_2O$



HEME d

bacterial cytochrome oxidase

$O_2 \longrightarrow H_2O$

Fig. 1. The structure of siroheme, dionoheme, heme d

MATERIALS AND METHODS

The organism. The nitrite reductase was purified from Pseudomonas stutzeri strain JM300. The purification and some properties of this enzyme are described (Ch. 4, this work). Nitrite reductase had a spectroscopic absorbance ratio (A^{ox}_{411}/A_{280}) of 1.1 to 1.2, as do purified preparations of Pseudomonas aeruginosa nitrite reductase (9).

The extraction and reconstitution procedure. The procedure used was a modification of the procedure of Hill and Wharton (5). All enzyme manipulations were carried out at 4 C. Typically, 2 mg purified nitrite reductase ($A^{ox}_{410}/A_{280} > 1.1$) in 1 ml of buffer (25 mM Hepes, pH 7.3) was added to 3 mg bovine serum albumin (Sigma); the latter was used as a "carrier" protein to aid in the precipitation. Cold acidified (0.024 N HCl) acetone (8 ml) was added; the protein precipitated and the heme $\underline{d}_1$ was extracted into the overlying acetone solution. The protein was separated from the acetone by centrifugation and extracted once more to ensure complete removal of heme $\underline{d}_1$. The protein pellet was washed once with phosphate buffer (0.25 M, pH 7) and redissolved in phosphate buffer with urea (0.25 M phosphate buffer pH 7, 6M urea). An excess amount of (10:1 molar ratio) heme $\underline{d}_1$ (native or synthetic) was added to the apoprotein solution and the mixture was incubated with gentle stirring for 30 min. The apoprotein + heme $\underline{d}_1$ solution was dialyzed overnight against 50 mM Tris Buffer pH 7. The reconstituted enzyme was separated from the excess heme $\underline{d}_1$ by passing the solution over a short DEAE (diethyl aminoethyl cellulose) column (DE-23, Whatman, 0.5 x 5 cm): heme $\underline{d}_1$, which has a pK_a of 4.5, bound

to the top of the column; the enzyme was eluted with 100 mM phosphate buffer, pH 7.

Activity assay. Activity was measured by gas evolution (NO and N_2O) from nitrite with NADH/phenazine methosulfate as the electron donor system. The assay contained 6 umol NADH, 0.36 umol PMS and 3 umol $NaNO_2$ in a total volume of 3 ml. All stock solutions were made in Hepes buffer (50 mM, pH 7.3). An appropriate amount of enzyme solution was added, usually containing about 1 ug of enzyme. NADH and nitrite stock solutions were made oxygen free by repeated evacuating and filling with argon and were added anaerobically to a 25 ml serum bottle which contained the buffer and PMS and had been flushed with argon. The reaction was initiated by addition of enzyme. The nitrite concentration (0.5 mM) was saturating for both NO and N_2O production by the original enzyme (Ch. 4, this work). Enzyme activity was expressed as the initial rate of gas evolution (NO or N_2O).

Gas evolution was monitored on a Perkin Elmer 910 gas chromatograph equipped with a ^{13}Ni electron capture detector. The carrier gas was 85 % argon, 15 % methane. The column was a 6 foot stainless steel Porapak Q column operated at 55 C. Carrier flow rate was adjusted so that the approximate retention times for NO and N_2O were 1 min and 2.2 min, respectively. Under these conditions the retention times of nitric oxide and oxygen were extremely close and it was necessary to use strict anaerobic techniques for sampling and injecting the gas phase of the assay vials. We used gas-tight syringes equipped with a gas lock.

Visible spectra were recorded on a Perkin Elmer (model lambda 5) spectrophotometer.

Preparation of the heme d_1. The native heme d_1 was extracted from the enzyme by the procedure described above. The acetone solution which contained the heme was evaporated to near dryness in the dark at room temperature under a stream of argon. The residue was dissolved in phosphate buffer (0.25 M, pH 7) and centrifuged to remove any remaining protein precipitate. The solution of heme d_1 was adjusted to pH 7 with NaOH and stored in the dark under argon. Extraction of native heme d_1 by the method of Hill & Wharton (5) was tried but did not work in our hands: addition of 1 ml of 1.2 N NaOH to the 8 ml acidified acetone extract under air resulted in rapid decoloration (decomposition) of the heme d_1 concentrated in the NaOH layer. Heme d_1 is very labile in acetone solution (6,a) therefore it was important to minimize the time it spent in the acidified acetone.

The synthetic heme d_1 was prepared as described by Wu & Chang (8). Heme d_1 was prepared by insertion of iron into the free base tetramethylester of the tetrapyrrole (10). The hydrolysis of the methyl esters was done as follows: iron tetramethylester is dissolved in 40 ml tetrahydrofuran (THF) followed by addition of 2 ml of 1 N KOH. The solution was stirred in the dark under nitrogen gas for 12 hours until the organic layer was almost colorless. The THF was evaporated and the pH of the basic aqueous solution (in ice bath) was adjusted to 7 with concentrated HCl.

The enzyme concentration was estimated by the bicinchoninic acid method (11). For estimation of relative protein concentrations, the absorbance at 411 nm (oxidized enzyme) was used. The concentration of heme d_1 was estimated by using the published extinction coefficient of 32,100 $M^{-1}.cm^{-1}$ for oxidized imidazole-ferriheme d_1 (4).

RESULTS

Spectral characterization. The visible (oxidized and reduced) spectra of Pseudomonas stutzeri JM300 nitrite reductase are shown in Fig. 2. The spectral characteristics are very similar to the ones of Pseudomonas aeruginosa nitrite reductase (12). The Soret band at 417 nm in the reduced spectrum is attributed to to heme c and so are the split alpha band at 522 nm and the beta band at 555 nm. The heme d_1 is responsible for the weak shoulder (Soret) at 460 nm and the broad absorbance in the 644 nm area. The visible spectrum of the apoprotein (Fig. 3) lost the spectral characteristics typical of heme d_1. The spectra of the enzyme reconstituted with the synthetic and the native heme d_1 are shown in Figs. 4 and 5, respectively. They regained the features typical of heme d_1; the 460 nm shoulder is less prominent for spectra taken at lower protein concentrations such as the one in Fig. 5. The visible spectrum of the heme d_1 extracted from P. stutzeri nitrite reductase is shown in Fig. 6. It is identical to the visible spectrum of heme d_1 from P. aeruginosa (12).

Recovery of activity. The NO and N_2O producing activities for both the apoprotein and the reconstituted enzyme preparations are summarized in Table 1. The apoprotein had no activity but when reconstituted with the native and synthetic heme d_1 substantial activity was recovered. The data were corrected for loss of protein in the course of the reconstitution. Long term progress curves of nitrite reductase, apoprotein and apoprotein reconstituted with synthetic heme d_1 are shown in Fig. 7. Apparently, N_2O producing activity was never entirely recovered, whether the native or the synthetic heme d_1 was used. The N_2O producing activity of this enzyme was a more labile

Fig. 2. The absorption spectrum of nitrite reductase from Pseudomonas stutzeri. The enzyme was dissolved in 0.25 M phosphate buffer pH 7.3; the enzyme concentration was 2 mg/ml. ___ oxidized, _____ , reduced with $Na_2S_2O_4$

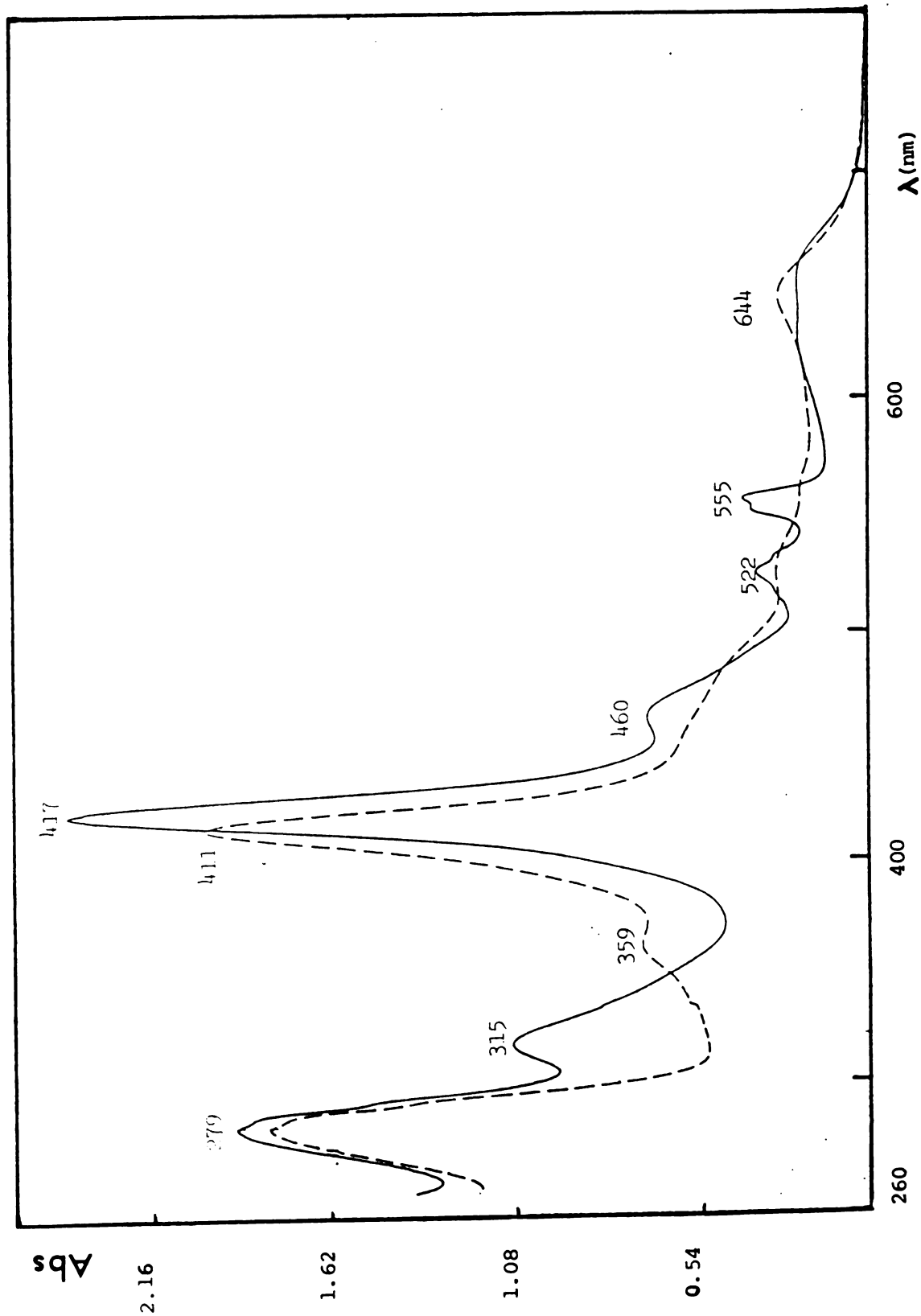

Abs
2.16
1.62
1.08
0.54
260
400
600
λ(nm)
417
411
379
460
359
315
555
522
644

Fig. 3. The absorption spectrum of the apoprotein (-heme d_1) of the nitrite reductase from Pseudomonas stutzeri

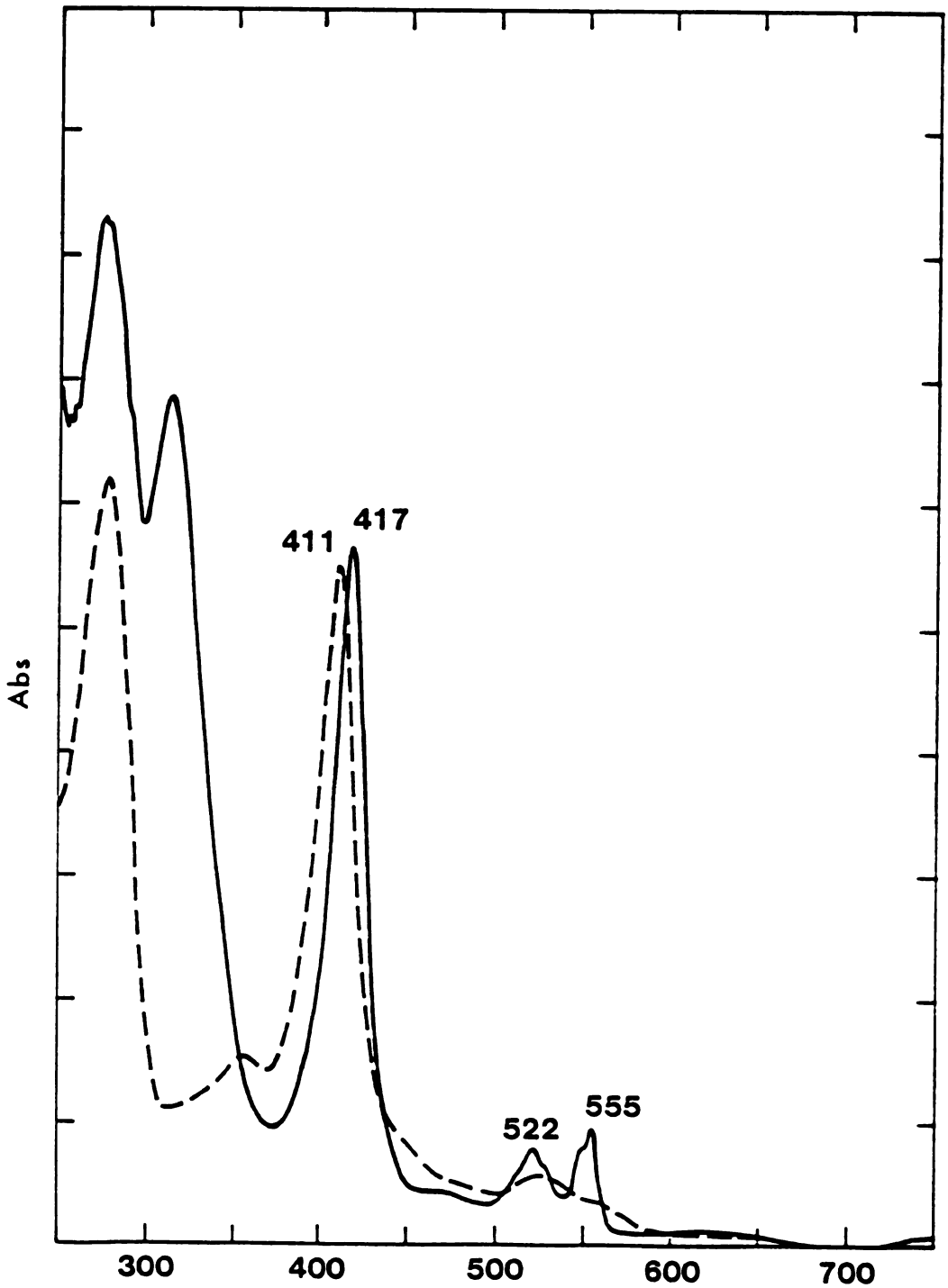

Abs
411
417
522
555
300
400
500
600
700

Fig. 4. The absorption spectrum of nitrite reductase from Pseudomonas stutzeri reconstituted with synthetic heme d_1. The enzyme was dissolved in 0.25 M phosphate buffer, pH 7.3. - - - - , oxidized; ———— , reduced with $Na_2S_2O_4$

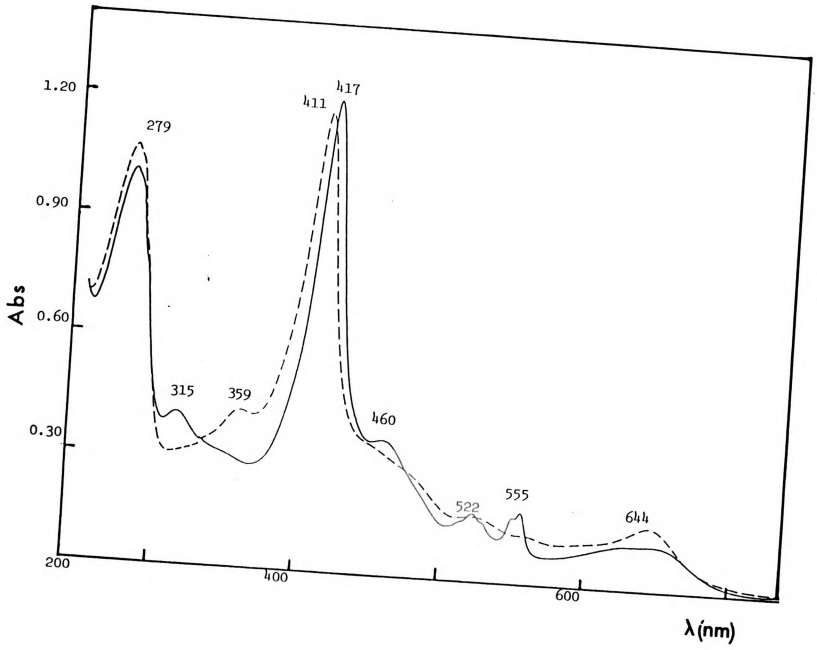

1.20
0.90
0.60
0.30
Abs
279
315
359
411
417
460
522
555
644
200
400
600
λ(nm)

Fig. 5. The absorption spectrum of nitrite reductase from Pseudomonas stutzeri reconstituted with native heme d_1. The enzyme was dissolved in 0.25 M phosphate buffer, pH 7.3. – – – – , oxidized; ——— reduced with $Na_2S_2O_4$

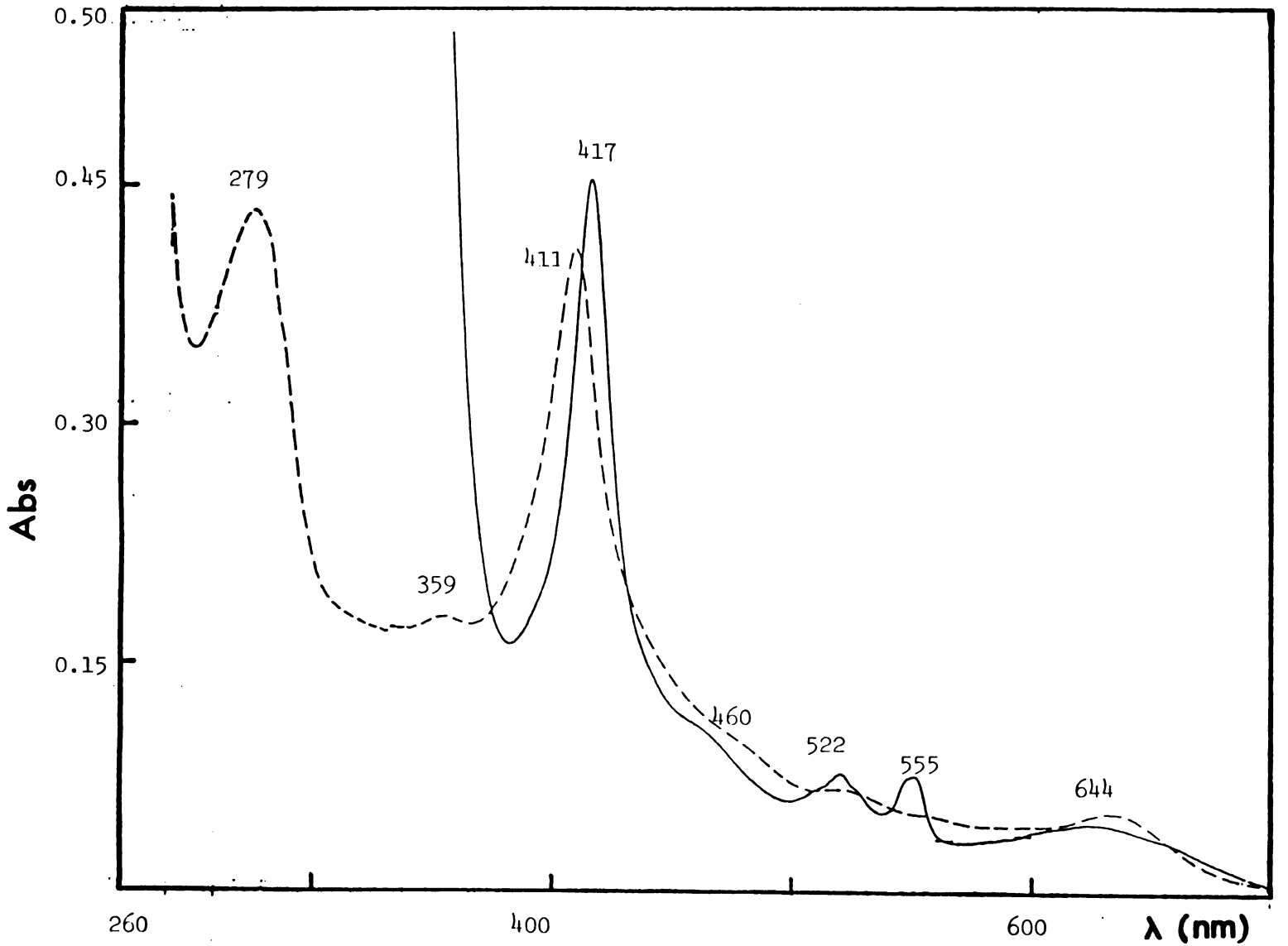
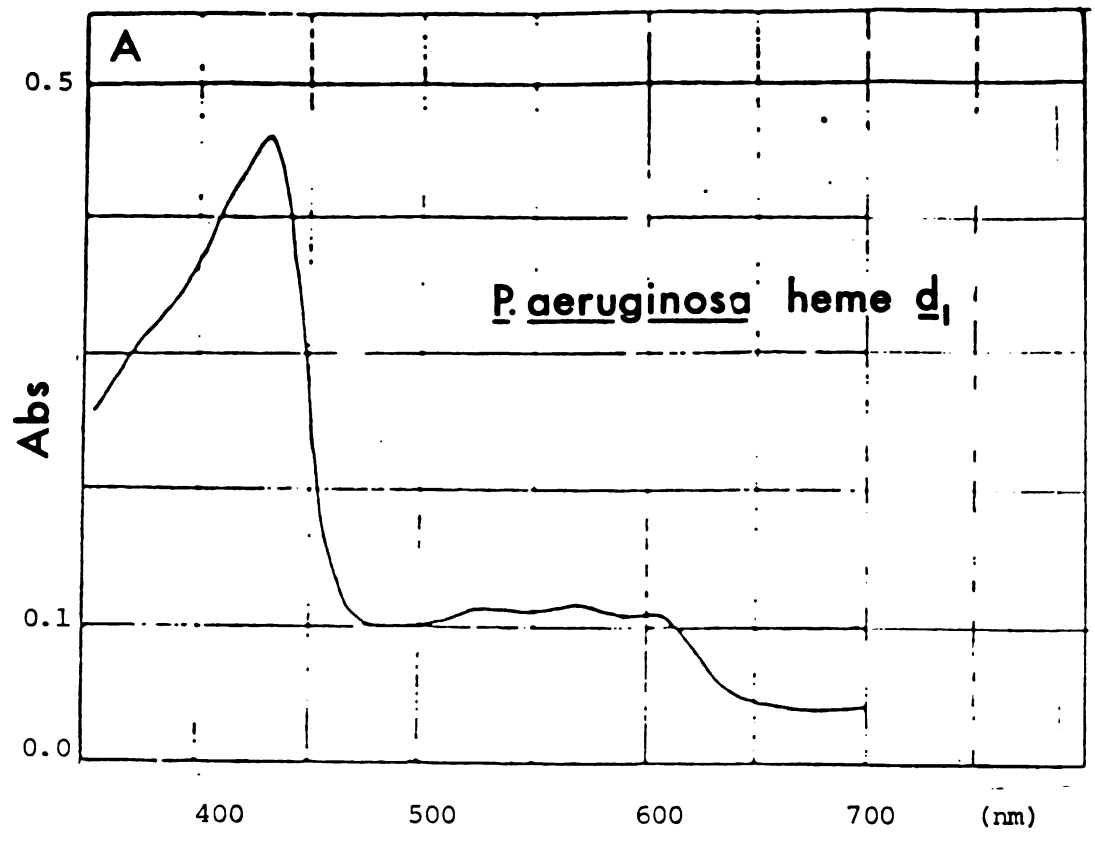
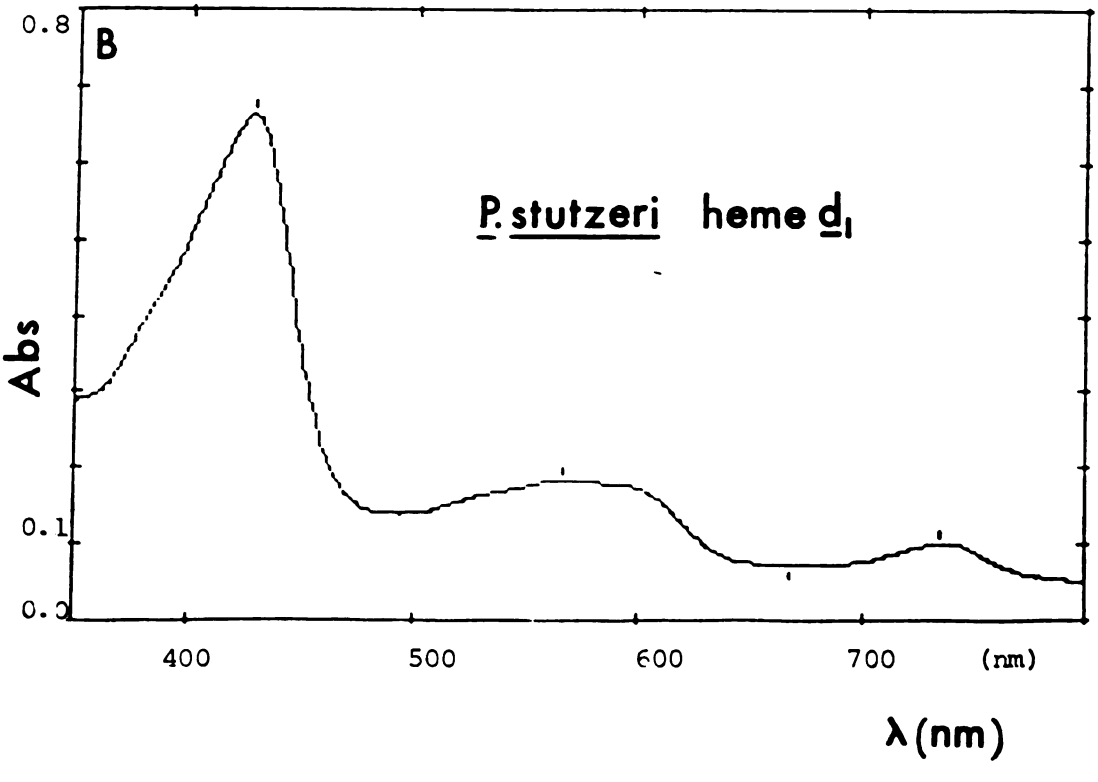

0.50
0.45
0.30
0.15
Abs
279
417
411
359
460
522
555
644
260
400
600
λ (nm)

Fig. 6. A: The absorption spectrum of heme $\underline{d}_1$ from Pseudomonas aeruginosa. The heme $\underline{d}_1$ was dissolved in acetone containing 0.12 N HCl.
Yamanaka, T. and K. Okonuki. Biochim. Biophys. Acta. 67 (1963), p. 497.

Fig. 6. B: The absorption spectrum of heme $\underline{d}_1$ from Pseudomonas stutzeri. The heme $\underline{d}_1$ was dissolved in acetone containing 0.24 N HCl.


A
0.5
0.1
0.0
Abs
P. aeruginosa heme d1
400
500
600
700
(nm)


B
0.8
0.1
0.0
Abs
P. stutzeri heme d1
400
500
600
700
(nm)
λ(nm)

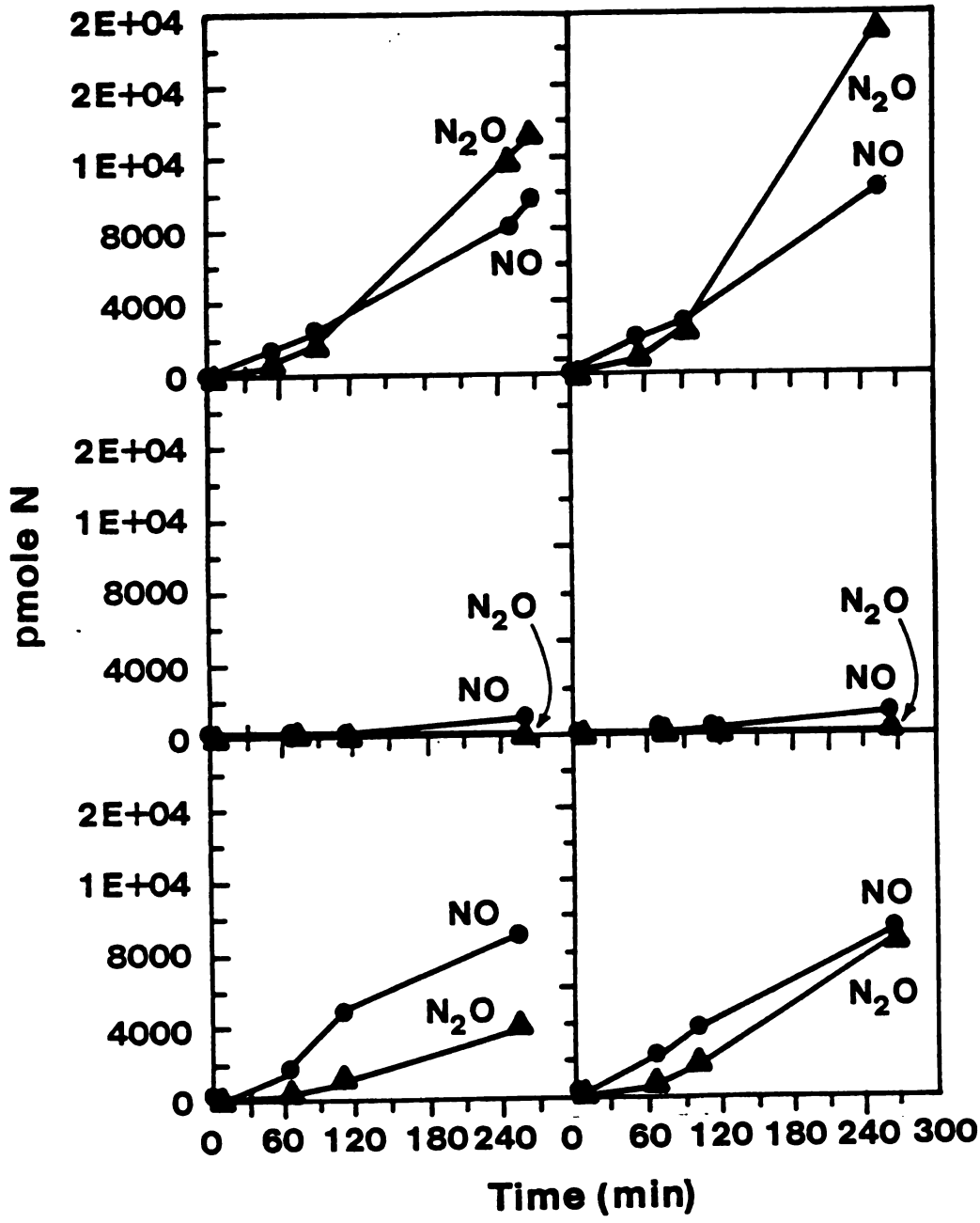


Fig. 7. Progress curves of nitric oxide and nitrous oxide production from 1 mM nitrite by
A: P. stutzeri nitrite reductase
B: apoprotein (- heme d_1)
C: nitrite reductase, reconstituted with synthetic heme d_1

activity than the NO producing activity. Possibly the latter was less affected by partial denaturation of the enzyme.

TABLE 1

Recovery of activity of nitrite reductase after reconstitution of the apoprotein with native and synthetic heme $\underline{d}_1$.

Treatment of nitrite reductase	Enzymatic activity measured	
	NO_2^- to NO[b]	NO_2^- to N_2O[b]
Intact, original enzyme	100	100
Apoprotein[a]	0	0
Reconstituted, native $\underline{d}_1$	50.9(6.5)	37.9(2.2)
Reconstituted, synthetic $\underline{d}_1$	81.1(4.4)	40.1(3.8)

a: Apoprotein remained soluble after dialysis to remove the urea and had no detectable activity during the time course of an initial rate experiment. Numbers between parentheses represent the deviation from the mean value of two initial rate determinations within the same experiment.

b: Activities are expressed in relative units. The activity of the intact, original enzyme represents 100 %.

DISCUSSION

The recovery of gas producing activity after reconstitution was substantial for both the synthetic and the native heme $\underline{d}_1$, but lower than the 90 to 100% recovery which was reported for the terminal oxidase activity after reconstitution of Pseudomonas aeruginosa nitrite reductase with its own heme $\underline{d}_1$ (6). In our case, however, activity was determined by measuring the products of the physiological reaction of the enzyme. The NO/N_2O product ratio of the enzyme reconstituted with synthetic heme $\underline{d}_1$ was somewhat lower than for the enzyme reconstituted with native heme $\underline{d}_1$; the latter ratio is more like the intact nitrite reductase. The synthetic heme $\underline{d}_1$ solution we used was a mixture of two enantiomers. This may account for the difference in NO/N_2O ratio if there was no distinction by the enzyme for the correct stereoisomer in the reassembly. As a consequence of the reconstitution procedure, whether synthetic or native heme $\underline{d}_1$ was used, the N_2O producing activity was less effectively restored. We suspect this may be due to permanent denaturing effects caused by the precipitation - resolubilization, affecting the second nitrite binding to the enzyme. The second nitrite binding seems to generally be more sensitive to in vitro treatments (Ch. 4, this work).

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APPENDIX

APPENDIX

ERRATUM IN CHAPTER TWO

Equation (1) in chapter two should be:

$$k_3.k_4/(k_2 + (k_3.k_5.k_7.[NO_2^-]/(k_6 + k_7)))$$

instead of

$$k_3.k_4/(k_4 + (k_5.k_7.[NO_2^-]/(k_6 + k_7)))$$

How to derive the formula. Using the "analysis of partitioning" for calculating net rate constants for transfer of label, by Cleland, W. W. (1) First we need the net forward rate constants for the involved steps in the pathway; these are the rate constants which would produce the same flux through the step if this step were irreversible:

$$NO_2^- + E \xrightarrow[1']{} E.NO_2^- \xrightarrow[3']{} E.NO^+ \xrightarrow[5']{NO_2^-} E.N_2O_3 \xrightarrow[7']{} N_2O$$

Net rate constants: $k_7' = k_7$

$$k_5' = (k_5.k_7.[NO_2^-])/(k_6 + k_7)$$

$$k_3' = (k_3.k_5')/(k_2 + k_3.k_5')$$

We calculate the initial rate of transfer of label to product (in our case of ^{18}O to N_2O). This is the rate of addition of the labelled

compound, multiplied by the net forward partitioning of the thus formed labelled enzyme bound intermediate. In other terms:

$$k_4.[E.NO^+] \quad x \quad (k_3.k_5,)/(k_2 + k_3.k_5,) \quad (1)$$

The rate of N_2O production can be expressed as the product of any of the enzyme bound intermediates multiplied by its net forward rate constant; if we do this for $E.NO^+$ we get:

$$([E.NO^+].k_5.k_7.[NO_2^-])/(k_6 + k_7) \quad (2)$$

The initial ^{18}O content of N_2O is expression (1) divided by expression (2); after some reorganizing we get:

$$k_3.k_4/(k_2 + (k_3.k_5.k_7.[NO_2^-]/(k_6 + k_7)))$$

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