CHAPTER VI
STUDIES ON (a) THE PHYSIOLOGICAL ELECTRON DONOR OF
A. fischeri NITRITE REDUCTASE AND (b) INTERMEDIATES
DURING ENZYMATIC REDUCTION OF NITRITE TO AMMONIA
CHAPTER VI

SECTION 1

ELECTRON PARAMAGNETIC RESONANCE STUDIES OF HEME C AND ITS NITROSYL DERIVATIVE IN A. fischeri NITRITE REDUCTASE AND MECHANISM OF NITRITE REDUCTION
SUMMARY

Low temperature EPR spectra of purified *A. fischeri* nitrite reductase have been presented in this Chapter. The spectrum of the oxidized enzyme is extremely complex and showed signals due to several species of heme. The main features, which were present in all samples of the enzyme, were at $g$-values 3.7, and 2.88, 2.26 and 1.51. The species at $g$-value 3.7 is attributed to the active heme species of the enzyme. The species giving this signal was unreactive towards the substrate, nitrite and ligands, CN$^-$ and azide in the oxidized state of the enzyme. Reduction of the enzyme in the presence of excess nitrite produced the spectrum of nitrosyl heme derivative detectable by EPR spectroscopy with a $g$-value at 2.01, and a $^{14}$N hyperfine splitting into three lines separated by 1.6 mT and $^{15}$N splitting into two lines separated by 2.3 mT. A similar derivative was observed on treatment of the enzyme with hydroxylamine. Exchange of nitric oxide was observed between the $^{15}$N-nitrosyl-heme derivative and $^{14}$NO gas.

It is proposed that the reaction cycle involves reduction of the enzyme followed by binding of nitrite to heme 1 and formation of the nitrosyl intermediate. The data suggest that the nitrosyl heme is an intermediate in the reduction of nitrite to ammonia. It seems likely that a bound form of hydroxylamine is also involved in the reduction of nitrite to ammonia.
GENERAL INTRODUCTION TO EPR THEORY

Electron paramagnetic resonance technique is based on the magnetic properties of the matter, more precisely of the electron. The EPR spectroscopic studies have been used in the area of enzyme chemistry concerned with electron transfer, the field of enzymic oxidation-reduction, where compounds with unpaired electrons or transition metals with unfilled electron shells may arise or disappear. However, the usefulness of EPR spectroscopy is not confined to oxidation-reduction; it can also furnish information on changes in the ligand environment of transition metal ions.

A paramagnetic substance may be defined as one that possesses no resultant magnetic moment in the absence of an external field but acquires a magnetic moment in the direction of an applied field. Paramagnetic substances are metal ions with incomplete 'd' shells like those of transition series, free radicals or certain molecules with unpaired electrons.

The electron has a negative charge and a quantum mechanical property that is analogous to spinning on its axis. For a single unpaired electron spin $S$ is $1/2$, so there are only two possible spin states, which we can consider as spins in opposite directions. The value that $S$ takes along a specified direction ($M_s$) is then $+1/2$ or $-1/2$. A moving charge gives rise to a magnetic moment, and hence the spin of each electron leads to two possible magnetic states. The two orientations have two different energy i.e. each energy state of the electron is split into two parts by the application of an external magnetic field, this is known as Zeeman splitting.
The amount of splitting depends on the strength of the magnetic field. The energy difference (ΔE) between the two spin states is proportional to the applied field H, the value for the magnetic moment of the electron $\beta$ and another constant $g$. Thus the basic equation for transition between the two energy levels for unpaired electron is $\Delta E = h\nu = g\beta H$. In principle, resonance absorption by an unpaired electron can occur whenever the ratio of the frequency of exciting energy to the magnetic field strength satisfies the condition, $\nu/H = \beta g/h$. It is this net absorption of electromagnetic energy at resonance that is detected and amplified in electron paramagnetic resonance spectroscopy.

The basic principles of EPR are thus analogous to those of NMR, both being magnetic resonance techniques. However, since the resonance frequency of an unpaired electron is about 658 times larger than that of the commonly used nuclei like the proton in NMR, the NMR experiments are carried out in the megahertz (MHz) range, while EPR experiments require frequencies in the gigahertz (GHz) range.

$g$-value

The total magnetic moment which is due to the coupling of the electron spin angular momentum and the orbital angular momentum of the unpaired electron gives rise to the tensor 'g'. The $g$-value is most basic parameter that characterizes an EPR spectrum. At resonance, the magnetic field at the unpaired electron will be resultant of the externally applied magnetic field and the internal magnetic field which it induces within the molecule. Thus in a molecule the value of 'g' will be
altered from that of the free electron spin. The $g$-value is partly dependent on the polarity of the solvent, but mainly on the orientation of the molecule in the magnetic field. There may be as many as three values for '$g$', one for each of the three mutually perpendicular molecular axes of the paramagnetic center in its molecular coordinating system (i.e. $g_{xx}$, $g_{yy}$, $g_{zz}$). In an isotropic system with fast molecular motion, the measured $g$-value is the average of the $g$-values along the three molecular axes.

Nuclear hyperfine splitting

Apart from the local fields induced due to the application of the external magnetic field, there are local fields that are permanent and generated by the magnetic moments of nuclei within the same molecule. The interaction between the unpaired electron and the nuclear magnetic moment is termed as the nuclear hyperfine splitting. The EPR spectrum is thus split into a number of lines separated by hyperfine splitting constant $A$. As in the case of $g$-values, the value of $A$ depends on the orientation of the molecule with respect to the applied magnetic field giving rise to $A_x$, $A_y$, $A_z$. In biological systems, nuclei with magnetic moments include nitrogen ($^{14}$N), hydrogen ($^{1}$H) and deuterium ($^{2}$H) and carbon ($^{13}$C) as well as nuclei of the transition metals. The number of possible values of magnetic moment is $2I + 1$ where $I$ is the spin state of nucleus. So if $I$ is 1 (as for $^{14}$N) there are three possible spin states ($M_i = 1, 0, -1$), which lead to three lines in the EPR spectrum. If the unpaired electron interacts with more than one nucleus
a more complex spectrum is obtained.

**EPR spectroscopic studies on nitrite reductases**

Nitrite reductases from various sources contain transition metals, Fe and Cu. The EPR spectroscopy has been used to understand the mechanism of nitrite reduction to ammonia and to determine the prosthetic groups of the enzymes from various sources.

In the plant nitrite reductase the substrate nitrite and other nitrogenous intermediates of the reaction are presumed to bind to the siroheme and the function of the iron-sulfur center is presumed to be the transfer of electrons to them (Aparicio et al., 1975; Hucklesby et al., 1976; Vega and Kamin, 1977; Cammack et al., 1978). An electron paramagnetic resonance (EPR) signal assigned to nitrosyl-Fe (II) complex of siroheme (Aparicio et al., 1975; Fry et al., 1980) was observed in samples of the nitrite reductase enzymes frozen during turnover (Cammack et al., 1978). This complex was presumed to present the first stage in the reduction of nitrite to ammonia. EPR studies of spinach and *E. coli* nitrite reductases have shown the presence of a strong magnetic interaction between the siroheme and Fe₄S₄ centers (Janick and Siegel, 1983; Wilkerson et al., 1983). It has also been shown that heme - Fe₄S₄ interaction is maintained on ligation of the heme by a number of compounds. Evidence has also been presented which shows that potential weak field heme ligands can promote interconversion of the $S = 1/2$ and $S = 3/2$ type EPR signals characteristic of the exchange coupled heme - Fe₄S₄.
center in fully reduced NADPH-sulfite reductase hemo-flavo-protein complex subunit of *E. coli* (Janick and Siegel, 1983).

Nitrite reductase from *Desulfovibrio desulfuricans* has been purified and shown to contain six c-type heme groups per molecule of the enzyme rather than siroheme and Fe₄S₄ center which are the prosthetic groups of plants and *E. coli* nitrite reductases (Liu and Peck, 1981; Murphy et al., 1974b; Siegel, 1978; Lancaster et al., 1979). EPR studies have shown that nitrite reductase from *D. desulfuricans* also reacts with nitrite to form heme-NO complex, but only a fraction of the c-type hemes reacted with nitrite. In contrast, all the hemes reacted when exposed to NO (Liu et al., 1980). These observations suggested some specificity among the six hemes in their reactivity towards nitrite.

Nitrite reductase from *A. fischeri* catalyzes the six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). It contains two c-type hemes per molecule of the enzyme, but no non-heme iron or molybdenum (Prakash and Sadana, 1973). The intermediates in the reduction of nitrite to ammonia by nitrite reductase are unknown. The *A. fischeri* enzyme catalyzes the reduction of both nitrite and hydroxylamine to ammonia and the two substrates are reduced at the same catalytic site (Prakash and Sadana, 1972). The *Kₘ* for hydroxylamine, however, is approximately two orders of magnitude greater than that for nitrite (Prakash and Sadana, 1972). The high *Kₘ* value seems to preclude hydroxylamine as a free intermediate in the reaction. The same conclusion has also been drawn by us (Sadana et al., 1981), when we tried to trap hydroxylamine
in the form of oxime and were unable to detect it by GC/MS method.

In this Chapter EPR spectroscopic studies were carried out with A. fischeri nitrite reductase for detecting the intermediate and to understand the mechanism of nitrite reduction.

METHODS

Sample preparation for EPR spectroscopy

Except where otherwise stated the enzyme was used at a concentration of approximately 5 mg/ml in 30 mM potassium phosphate buffer, pH 7.5.

Samples for EPR spectroscopy were prepared in quartz tubes of 3 mm internal diameter (Varian Associates, Palo Alto, CA, USA). For experiments in reducing conditions the tubes were flushed with Ar gas through a stainless steel catheter. Reagents such as sodium nitrite, methyl viologen and dithionite were added with 10 μl syringes, fitted with 15 cm needles, while stirring vigorously with a stainless steel wire. Mixing, where it could be observed by mixing of coloured solutions, appeared to be complete within 3 seconds. The enzyme solution for preparation of the nitrosyl derivative and turnover experiments were prepared in oxygen-free 25 mM potassium phosphate buffer, pH 6.8 and kept in stoppered vials under a flow of Ar gas. The samples were frozen by immersing the tubes in a mixture of 2-methyl-butane and methylcyclohexane (6:1 v/v) cooled to approximately 100K with liquid nitrogen. The freezing time for tubes already at 0°C was approximately 2 sec.
EPR spectra were recorded on a E4 spectrometer (Varian) with an EPR 9 liquid-helium transfer system (Oxford Instrument Co., Osney Mead, Oxford, UK) for sample cooling. g-Values were referred to 1, 1-diphenyl-2-picrylhydrazyl as standard. Spectral subtraction was recorded with a Laborora averaging system.

RESULTS

Spectra of the oxidized enzyme

The EPR spectrum of the _A. fischeri_ nitrite reductase is extremely complex (Fig. 17) and shows signals due to several species of heme, as well as signals at $g = 4.3$ and $g = 2.1$ which probably represent minor contaminants of non-heme iron and copper, respectively. The signal at $g = 4.2 - 4.3$ was also observed by Bray et al. (1964) and was relatively sharp and easily detected. It is of ubiquitous occurrence in biological materials and is often ascribed to impurities. A $g \approx 4.3$ line is characteristic of several non-heme, high-spin, ferric iron complexes. For example, it is found in iron complexes of conalbumins and transferrins (Aasa et al., 1963; Windle et al., 1963), in rubredoxin (Lovenberg, 1967; Bachmayer et al., 1967). The signal at $g = 6$ is typical of high-spin ferric heme. Its intensity varied between samples and did not correlate with enzymic activity. This signal is not due to active enzyme but might be a denatured form. Morton and Bohan (1971) have observed that in the lyophilization process some of the molecules of horse heart ferricytochrome c were
FIG. 17 EPR spectra of the oxidized *A. fischeri* nitrite reductase, approximately 5 mg/ml in 25 mM potassium phosphate buffer, pH 6.5, recorded at (a) 34K (b) 10K

Instrument settings:

- microwave power, 2 mW
- frequency, 9.25 GHz
- modulation amplitude, 1 mT.
sufficiently distorted to convert the protein to a high-spin derivative giving a signal at $g = 6$. It can be presumed that some of the molecules of *A. fischeri* nitrite reductase might also have been distorted enough to give a high-spin derivative giving a signal at $g = 6$.

There remain two species in the spectrum of Fig. 17 which were present in all samples of the enzyme. The first, which we will refer to as heme 1, has a feature with a peak at $g = 3.7$, the rest of the spectrum was too broad to be detected. This feature was most prominent at 10K (Fig. 17 b). The second, which we refer to as heme 2, has a rhombic spectrum with $g$-values at 2.88, 2.26 and 1.51. These are most clearly seen at 34K at the microwave power used (Fig. 17 a); all of them changed together on reduction and other treatments. Both of these are consistent with low-spin ferric heme species, which is typical of many cytochromes (Brautigan et al., 1977). Since there are two hemes per molecule of the enzyme it is possible that these are dissimilar and give rise to the two species. The alternative explanation is that one of the low-spin heme signals corresponds to the native enzyme, and the other to a denatured form. On treatment with dithionite-reduced methyl viologen or deazaflavin the signal at $g = 3.7$ was found to disappear rapidly while that at $g = 2.88$, 2.26 and 1.51 remained for up to a minute. This evidence indicates that the signal at $g = 3.7$ is more likely to represent the active heme species of the *A. fischeri* enzyme.

The $g$-value of 3.7 implies a large anisotropy (Palmer, 1979) for heme 1, but is within the range for low-spin ferric
cytochromes. For example, cytochrome $b$ of the mitochondrial respiratory chain has $g_z = 3.78$ (DerVartanian et al., 1973).

It is difficult to draw further conclusions about the ligation to the iron in the *A. fischeri* enzyme because only the $g_z$ feature was observed, the other $g$-value features being presumably too broad to be detected. The occurrence of only one prominent $g$-value feature has also been observed for heme $d$ in nitrite reductase of *Pseudomonas aeruginosa* (Walsh et al., 1979).

The EPR spectrum of the oxidized *Achromobacter* enzyme showed the same features at pH values 4.8, 7.0 and 9.0, although there were quantitative differences (Fig. 18). At pH 4 the linewidths of all the features were broader. At pH 9 the $g = 6$ signal grew while the $g = 2.88, 2.26$ and 1.51 spectrum was smaller suggesting a low-to high-spin transition. Effect of pH has been studied in a number of hemoproteins (Brautigan et al., 1977). It was shown that EPR spectra of these hemoproteins change when pH is varied from acidic to basic range. At extremes of pH, the horse and bakers' yeast iso-1 cytochrome display several high- and low-spin forms which have been identified, showing that a variety of protein-derived ligands will coordinate to the heme-iron including methionine- and cysteine-sulfur, histidine imidazole and lysine $\varepsilon$-amine.

The addition of 5 mM nitrite for 10 min at 20°C had no effect on the spectrum of the oxidized enzyme. It has been observed with other nitrite reductases, such as that from *C. pepo* and spinach. The reaction of the oxidized enzyme with nitrite is very slow (Cammack et al., 1978; Vega et al., 1976).
FIG. 18  EPR spectra of the nitrite reductase
at (a) pH 9.0 (b) pH 7.0 (c) pH 4.8
Protein concentration approximately 5 mg/ml
in 25 mM potassium phosphate buffer.
Conditions of measurement:
temperature, 8K
microwave power, 20 mW
frequency, 9.25 GHz
modulation amplitude, 1 mT.
ESR Absorption derivative vs. Magnetic field (T)

- (a)
- (b)
- (c)
Addition of cyanide, azide or fluoride at concentrations up to 50 mM to the A. fischeri enzyme also had no effect on the spectrum although the signal at $g = 6$ disappeared in the presence of cyanide. This is consistent with a heme in which a low field high-spin ligand such as $H_2O$ is displaced by high field low-spin ligand such as $CN^-$. The oxidized protein appears to be unreactive to these ligands, which suggests that in the first step of the enzyme reaction, the heme is reduced before nitrite binds to the enzyme.

The enzyme is known to be inactivated by exposure to dithionite, so an attempt was made to reduce it with methyl viologen reduced by limiting quantities of dithionite. On treating the concentrated enzyme in deoxygenated buffer with 2 mM methyl viologen and 0.5 mM additions of dithionite, it was found to be impossible to keep methyl viologen in the reduced state. The blue colour of the reduced viologen disappeared within one or two seconds. Even when excess dithionite was added the colour disappeared within a short time.

**EPR spectra of nitrosyl heme**

Reduction of the enzyme with limiting amounts of dithionite-reduced methyl viologen in the presence of excess nitrite (14N) induced the EPR spectrum shown in Fig. 19 (a) with a sharply-defined hyperfine splitting into three lines separated by 1.65 mT. This type of spectrum has been seen in a number of nitrosyl Fe (II) heme proteins (Palmer, 1979). The splitting is due to the nuclear spin ($I = 1$) of 14N. A similar spectrum (not shown) was observed on treatment with nitric oxide gas.
FIG. 19  EPR spectra of nitrosyl-heme nitrite reductase (a) approximately 5 mg/ml enzyme in 25 mM potassium phosphate buffer, pH 6.8 was treated for 1 min at 20°C with 5 mM Na\textsuperscript{14}NO\textsubscript{2}, 2 mM methyl viologen and 1 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4}. (b) as (a) but with Na\textsubscript{15}NO\textsubscript{2} instead of Na\textsubscript{14}NO\textsubscript{2}. (c) The \textsuperscript{15}N nitrosyl derivative, which was prepared as for (b), then passed through a small Sephadex G-25 column as described by Fry et al. (1980) and treated with \textsuperscript{14}NO gas before freezing.

Conditions of measurement:

- temperature, 60K
- microwave power, 20 mW
- frequency, 9.25 GHz
- modulation amplitude, 1 mT.
ESR Absorption derivative

Magnetic field (T)

(a)

$^{14}\text{NO}_2$

(b)

$^{15}\text{NO}_2$

(c)

$^{14}\text{NO}$

$g$-Value

0.31 0.32 0.33 0.34
An enzyme sample similarly reduced in the presence of excess Na\textsuperscript{15}NO\textsubscript{2} (Fig. 19 b) showed a splitting into two lines separated by 2.3 mT, as expected for the nuclear spin (I = 1/2) of $^{15}$N.

The $^{15}$N-nitrosyl derivative, prepared as for Fig. 19 (b), was passed through a column of Sephadex G-25 to remove excess nitrite, concentrated on an amicon concentrator B 15 (Amicon Corp. Lexington, MA 02773) and then treated with $^{14}$NO gas as described by Fry et al. (1980). The EPR spectrum changed to a three-line hyperfine splitting due to $^{14}$NO (Fig. 19 c). This indicates chemical exchange of NO in the nitrosyl complex. By contrast the nitrosyl complex of \textit{C. pepo} nitrite reductase did not show this exchange indicating that it is kinetically more stable than in the \textit{A. fischeri} enzyme (Fry et al., 1980). Exchange was not observed when the $^{15}$N-nitrosyl \textit{A. fischeri} nitrite reductase was treated, under non-reducing conditions, with Na$^{14}$NO\textsubscript{2} demonstrating that nitric oxide binds more tightly than nitrite.

When the nitrosyl derivative, prepared with nitrite and reduced methyl viologen, was treated with excess dithionite (5 mM) and the sample reoxidized by exposure to air, the spectrum with the characteristic three-line hyperfine splitting disappeared. This result suggests that the nitrosyl group was reduced, presumably to ammonia. This observation is consistent with the interpretation (though it does not prove) that nitrosyl derivative is an intermediate in the reaction cycle of the enzyme.
The nitrosyl heme spectrum was also observed, with decreased intensity, on treatment of the oxidized enzyme with hydroxylamine (Fig. 20). This could be explained by disproportionation of the hydroxylamine to ammonia and the enzyme-bound form of nitric oxide. Lancaster et al. (1979) and Hirasawa-Soga et al. (1982, 1983) have reported, with spinach nitrite reductase, the formation of nitrosyl heme complex on addition of hydroxylamine to the enzyme.

Spectra observed under turnover conditions

As previously noted, the EPR spectrum of the oxidized *Achromobacter* enzyme did not change when the substrate nitrite was added. However, it changed immediately when a reductant was also added so that nitrite reduction could proceed. In the experiment of Fig. 21, the reaction was started by addition of 10 mM dithionite to the enzyme solution containing 10 mM nitrite and 5 mM methyl viologen at 0°C and frozen within 10 seconds. All of the signals due to oxidized heme were greatly diminished (Fig. 21 b), and the signal near $g = 2$ appeared. This latter signal was saturated with microwave power when measured at 15K, and this part of the spectrum is shown in Fig. 22 (a) recorded on an expanded scale at 60K. It is clearly similar to the nitrosyl heme signal of Fig. 19(a). Therefore, under turnover conditions the heme $c$ in the enzyme are reduced, and some nitrosyl heme is present. No such signals were observed with boiled enzyme under similar assay conditions.

In this experiment, the oxidizing substrate nitrite was
FIG. 20  EPR spectrum of *A. fischeri* nitrite reductase treated with 10 mM hydroxylamine for 10 min before freezing. Protein concentration, approximately 5 mg/ml in 25 mM potassium phosphate buffer, pH 6.8.

Conditions of measurement:

- temperature, 60K
- microwave power, 20 mW
- frequency, 9.25 GHz
- modulation amplitude, 1 mT.
ESR ABSORPTION DERIVATIVE

$g$-VALUE

MAGNETIC FIELD (T)

0.31 0.32 0.33 0.34
FIG. 21 EPR spectra of *A. fischeri* nitrite reductase before, during and after reaction.

(a) Oxidized enzyme approximately 5 mg/ml in 25 mM potassium phosphate buffer, pH 6.8.
(b) A sample at 0°C containing enzyme, approximately 5 mg/ml, 10 mM NaNO₂, 5 mM methyl viologen, was frozen 10 seconds after addition of 10 mM Na₂S₂O₄.
(c) A similar sample at 20°C frozen after 5 min.

Conditions of measurement:

- temperature, 15K
- microwave power, 20 mW
- frequency, 9.25 GHz
- modulation amplitude, 1 mT.
FIG. 22 (a) and (b) EPR spectra of the same samples as Fig. 21 (a) and 21 (b) respectively.
Recorded at an expanded scale and at 60K.
present in excess over the reductant, dithionite. After incubating for 5 min and 20°C to ensure that reaction was complete, another sample was frozen for EPR spectroscopy. There was partial reappearance of the signals from oxidized heme 2 but not heme 1 (Fig. 21 c). The signal around $g = 2$ was also more prominent (Fig. 21 c and 22 b). This result indicates that it is heme 1 that forms the nitrosyl derivative, and it is therefore likely to be the active site of nitrite reductase.

**DISCUSSION**

An unusual feature of the spectra of oxidized *A. fischeri* nitrite reductase is that the heme iron is low spin. This implies that the heme iron is fully coordinated by the heme and two ligands from the protein. Presumably nitrite displaces one of them when it binds. By contrast the iron in the siro-heme-containing nitrite reductases is high-spin in the oxidized state (Cammack et al., 1978; Lancaster et al., 1979; Cammack et al., 1982; Hirasawa-Soga et al., 1982; 1983). The hemes c in *D. desulfuricans* nitrite reductase show a combination of high- and low-spin EPR signals (Liu et al., 1980).

Hemoproteins in situ and in isolated form can exist in a variety of oxidation- and spin-states, each dependent upon immediate environment of the heme (Peisach et al., 1968). The complex nature of EPR spectra of oxidized *A. fischeri* nitrite reductase may also arise due to change in the heme environment of some of the molecules of the enzyme. It has been observed that conformational changes of the protein
moiety give rise to changes in the EPR spectra (Peisach and Blumberg, 1969; 1971). The effect of pH on hemoproteins has been studied by Brautigan et al. (1977). They have reported that each of the hemoproteins displays up to four low-spin EPR forms that are in pH-dependent equilibrium and can all be seen at near neutral pH. As the pH is raised the predominant pH form is converted into two forms with $g = 3.4$ and $g = 3.6$. *A. fischeri* enzyme did not clearly show the conversion of one form to the another in the range of the pH studied.

Cammack et al. (1978) have reported that *C. pepo* nitrite reductase reacts with nitrite in the oxidized state which is detected by EPR detectable signals, but reaction with the oxidized enzyme is very slow, requiring several minutes at 0°C for complete disappearance of the signal at $g = 6.8$ and $5.0$. It was thought that such a slow reaction could not be a part of the enzyme reaction under turnover conditions, and suggested that the heme must be first reduced to ferrous state, probably directly by ferredoxin. The EPR spectrum of *A. fischeri* enzyme in oxidized state did not change when nitrite was added. The enzyme was also unreactive towards CN$^-$, azide and fluoride. These studies indicated that in the first step of the enzyme reaction, the heme is reduced before nitrite binds. It seems that nitrite binds to the reduced form of the heme which is undetectable by EPR. This supports the hypothesis of Cammack et al. (1978) that heme must be reduced first before nitrite binds to the heme.

The spectrum of the nitrosyl derivative of *A. fischeri* nitrite reductase is different from that observed in the
siroheme-containing plant nitrite reductase, which shows rather clearly defined $g$-values at 2.073, 2.060, 2.007 with hyperfine splitting on the latter two $g$-values (Cammack and Fry, 1980). The spectrum of Fig. 19 (a) shows a prominent triplet splitting at $g = 2.01$. The other $g$-values are broad and poorly defined. It may be pointed out that with plants siroheme-nitrite reductase evidence for nitrogen hyperfine splitting was obtained by the use of $^{14}$NO-$^{15}$NO difference spectrum (Fry et al., 1980) or by third harmonic EPR spectroscopy (Cammack and Fry, 1980). The spectrum of nitrosyl derivative of A. fischeri nitrite reductase (Fig. 19 a) shows a prominent triplet splitting at $g = 2.01$. The other $g$-values are broad and poorly defined. The unusual broadening of the EPR signals are tentatively attributed to an interaction between the low-spin ferric heme (spin = 1/2) and the ferrous-nitric oxide complexes as spin coupled induced broadening. Broadened metal-nitric oxide complexes arising from such postulated interactions have been reported by Uiterkamp and Mason (1973) and Uiterkamp et al. (1974).

According to the interpretation of Kon and Kataoka (1969) the feature at $g = 2.01$ corresponds to $g_z$, normal to the heme plane. The effect of $^{15}$N substitution demonstrates that the splitting is due to binding of NO to heme iron. No superhyperfine lines in the $g_z$ region related to the interaction of the iron with the proximal histidine are detected, suggesting a large distance between the metal and $N_c$ of the imidazole. Similar spectra have been observed in nitrosyl cytochrome P-420 (O'Keef et al., 1978) and nitrosyl hemoglobin in the presence of inositol-hexaphosphate (Rein et al., 1973; Hill et al., 1979).
as well as a number of nitrosyl-heme model complexes (Kon, 1975). It has generally been concluded in these cases that the bond between the iron and the proximal ligand (opposite the NO) is either weak or broken.

The nitrosyl heme spectrum observed after reaction with a relatively large amount of Na$_2$S$_2$O$_4$ (Fig. 22 b) is different from the others in having an additional broad component. Such heterogeneity in the spectra of nitrosyl derivative has been observed with other hemoproteins. For example the spectrum of nitrosyl hemoglobin consists of a broad featureless component and a component with a narrow hyperfine splitting. The ratio of these components depends on the pH and concentration of allosteric effectors (Rein et al., 1973). Therefore, the spectrum of Fig. 22 (b) might be due to one nitrosyl heme which can exist in two forms, or alternatively it might be due to two different states of the enzyme.

Although the composition of _A. fischeri_ nitrite reductase is different from those from other sources, it is possible to propose a similar mechanism. Nitrite does not bind to the oxidized enzyme; so presumably the active site heme must be reduced first, which is probably heme 1. The nitrite appears to be reduced in several stages while it is bound to the heme. With the plant enzymes, the first stage appears to be a nitrosyl form (Aparicio et al., 1975; Cammack et al., 1978) and the data presented here are consistent with this possibility for the _A. fischeri_ enzyme. On this interpretation, the observation that the nitrosyl form remains after reaction with reductant and excess nitrite, implies that the nitrosyl form
is a stable intermediate in the reaction cycle or precedes a rate-limiting step.

Hydroxylamine is a weak substrate for the enzyme, and treatment of the oxidized protein with it resulted in the formation of the stable nitrosyl derivative. Lancaster et al. (1979) and Hirasawa-Soga et al. (1982; 1983) have also reported the formation of nitrosyl heme complex on addition of hydroxylamine to the oxidized spinach nitrite reductase. As proposed for the plant nitrite reductase (Vega and Kamin, 1977) it seems likely that hydroxylamine is an intermediate in the reaction, in an enzyme-bound but not a free form. Prakash and Sadana (1972); Sadana et al. (1981) have also reported that hydroxylamine is an enzyme-bound intermediate but not a free form.
CHAPTER VI

SECTION 2

STATUS OF HYDROXYLAMINE AS AN INTERMEDIATE IN THE REDUCTION OF NITRITE TO AMMONIA BY A. fischeri

NITRITE REDUCTASE
INTRODUCTION

The intermediates during the enzymatic reduction of nitrite to ammonia have not been identified unequivocally. Meyer and Schultze (1894) proposed that nitrite reduction proceeds through a sequence of three 2-electron steps via two intermediates, the first at the oxidation level of hyponitrite and the second being hydroxylamine. The occurrence of hydroxylamine in tissues of higher plants has been reported occasionally. Wood (1953) concluded that free hydroxylamine, which is highly toxic, is unlikely to be present in the appreciable concentration. The presence of oximes was, however, regarded as likely and consistent with the supposed production of hydroxylamine as an intermediate in nitrite reduction by green plants and microorganisms. Nason et al. (1954) postulated that nitrite reduction to ammonia probably proceeds through a sequence of two electron steps via an unknown intermediate and hydroxylamine. Nicholas (1959) concluded that a series of enzymes in plants mediate the reduction of nitrite to ammonia via hyponitrite and hydroxylamine as the physiologically important root. McNall and Atkinson (1957) have reported that E. coli strain Bn could utilize hyponitrite, hydroxylamine, or nitrous oxide as its sole nitrogen source and provided independent support for the involvement of these compounds as intermediates in nitrite reduction.

The significance of hyponitrite in nitrite metabolism of higher plants was rejected by Frear and Burrel (1958). They pointed out its great instability at physiological pH values and concluded that when introduced into plant leaves it was
first oxidized to nitrite before reappearing as $^{15}$N-labelled ammonia.

The purified nitrite reductases from marrow or spinach were unable to reduce hyponitrite or nitric oxide (Hucklesby and Hewitt, 1970; Pickard and Hewitt, 1972) indicating that hyponitrite and nitric oxide are not the potential intermediate during the reduction of nitrite to ammonia. *E. coli* nitrite reductase catalyzes the reduction of nitrite as well as hydroxylamine to ammonia; the $K_m$ for hydroxylamine being 150 times greater than that for nitrite. The high $K_m$ for hydroxylamine excludes it as a free intermediate (Kemp and Atkinson, 1966). Similar conclusions were reached with ferredoxin-nitrite reductase from *Chlorella*, spinach, and squash leaves (Beevers and Hageman, 1969; Losada, 1972). Vega et al. (1973) reported that *A. chroococcum* nitrite reductase catalyzes the stoichiometric reduction of nitrite to ammonia without the formation of hydroxylamine as a free intermediate.

*A. fischeri* nitrite reductase catalyzes the six-electron reduction of nitrite to ammonia (Prakash and Sadana, 1972). The enzyme also catalyzes the reduction of hydroxylamine, and both the substrates (nitrite and hydroxylamine) are reduced at the same active site. No free intermediate could be determined during the six-electron reduction of nitrite. The $K_m$ for hydroxylamine was approximately two orders of magnitude greater than that for nitrite and hence excluding hydroxylamine as a free intermediate during the reduction of nitrite to ammonia.
In the present studies an attempt has been made to determine whether hydroxylamine is an intermediate during nitrite reduction by \textit{A. fischeri} nitrite reductase. The reduction of Na$^{15}$NO$_2$ to ammonia is carried out in the presence of hydrazine, which has been shown to inhibit the utilization of hydroxylamine in \textit{Nitrosomonas} (Hollocher et al., 1981). The hydroxylamine is converted to oxime by cyclohexanone, and estimated by using GC/mass spectrometer which can monitor 0.01 nmol hydroxylamine oxime.

**METHODS**

**Preparation and analysis of oxime**

For the preparation of standard oxime: hydroxylamine hydrochloride (100 mg, 1.44 mmol) was added to 160 µl of cyclohexanone (1.6 mmol) in 0.15 M potassium phosphate buffer, pH 6.8, in a test tube and shaken by a vortex stirrer at room temperature (30°C) for 90 min. The oxime was extracted from the buffer with 25, 15 and 15 ml of diethyl ether, respectively. The ether extracts were combined and dried over anhydrous Na$_2$SO$_4$ for 3 h and then evaporated to dryness. The residue was recrystallized from petroleum ether (b.p. 30 - 40°C), m.p. 90°C. The purity of the oxime was checked by thin layer chromatography on silica gel G using ethyl acetate-benzene (20:80, v/v) as a solvent system.

The oxime was analyzed in a Hewlett-Packard GC/MS model 5992B mass spectrometer fitted with a column (740 mm x 20 mm) containing 2% OV-101 plus 0.2% Carbowax (20 M) on 100 - 200 mesh Chromosorb W. Helium flow was 3.5 ml/min and electron multiplier voltage was 1800 V. Samples, 0.5 to 1.0 µl in
petroleum ether, were introduced by direct injection through a septum. A solvent control was run between each sample to assure that the injection port and column were free of the previous sample. Cyclohexanone eluted with a retention time of approximately 1.4 min at a column temperature of 40°C and appeared shortly after the solvent front. MS data were obtained by means of both the Peak finder programme and the Selective Ion Monitor Programme.

Estimation of $^{15}\text{NH}_3$ produced from $^{15}\text{NO}_2^-$ by nitrite reductase

The production of $^{15}\text{NH}_3$ from $^{15}\text{NO}_2^-$ by nitrite reductase was determined by $^{15}\text{N}$ enrichment technique after alkaline hypobromite oxidation of $^{15}\text{NH}_3$ to $^{15}\text{N}_2$ according to the procedure of Dua et al. (1979).

The ammonia produced was distilled under alkaline conditions into boric acid and concentrated to 2 ml after addition of one drop of 1 N $\text{H}_2\text{SO}_4$. The sample was transferred into one of the limbs of a Rittenberg tube and alkaline hypobromite added to the other limb. The tube was affixed to a mass spectrometer vacuum system and rigorously evacuated to $10^{-7}$ mm Hg. The contents of the Rittenberg tube were then mixed to generate $\text{N}_2$ gas from ammonia. The tube was immersed in liquid nitrogen to freeze out water vapour and nitrogen oxides. The gas was then introduced into an evacuated expansion flask and passed into the mass spectrometer.

The $^{15}\text{N}$ enrichment was determined by measuring mass 28, 29, 30 representing $^{14}\text{N} - ^{14}\text{N}$, $^{14}\text{N} - ^{15}\text{N}$, $^{15}\text{N} - ^{15}\text{N}$, respectively, using the Micromass 602C mass spectrometer, AEI Manchester.
RESULTS AND DISCUSSION

There are a number of reagents which can be used for converting hydroxylamine to oxime. Ketones and aldehydes usually need aqueous alcohol to complete the reaction but the recovery of the oxime from the reaction mixture is incomplete. Keto acids also react with hydroxylamine in aqueous solution to form the corresponding oximes, but these compounds are too soluble in water to give a good recovery on extraction into hydrophobic solvents. However, cyclohexanone reacts with hydroxylamine in micro quantities in aqueous solution at pH 6.8 to form oxime with a reasonably good recovery in ether.

The design of the experiment for detection of the possible formation of hydroxylamine oxime during the enzymatic reduction of nitrite was similar to that described by Hollocher et al. (1981). Thus hydrazine (150 μmol) was used in an attempt to inhibit the further utilization of hydroxylamine and cyclohexanone (1 mmol) was used to convert hydroxylamine to the oxime. In another experiment cyclohexanone (1 mmol) was used with the enzyme in the reaction mixture in an attempt to trap any hydroxylamine as an oxime during nitrite reduction. The oxime formed was extracted and purified in a similar manner as given for the standard oxime. A pH of 7.0 was chosen to maximize the mole fraction of unprotonated NH₂OH (pK = 6.0) and minimize the mole fraction of unprotonated N₂H₂ (pK = 9.1). This was expected to promote oxime formation from cyclohexanone and minimize azine formation.
Using the procedure described above, a reaction mixture containing Na$^{15}$NO$_2$, cyclohexanone, benzyl viologen and sodium dithionite was incubated under anaerobic conditions for 10 min before extracting in ether. The ether extracts were blown to dryness with nitrogen at room temperature. The residue was suspended in 50 µl of diethyl ether and one µl aliquots were injected into GC/MS. It was observed that cyclohexanone stimulated the reduction of nitrite and was fully accounted for ammonia production.

There was no evidence for the production of $^{15}$NH$_2$OH oxime by the GC/MS method which can detect 0.01 nmol of the compound.

In another set of experiments upto 90 mmol hydrazine (NH$_2$ NH$_2$) was added to the reaction mixture and after terminating the reaction by exposure to air, cyclohexanone was added in an attempt to form the oxime and then a similar extraction procedure adopted. In these experiments, too, there was no indication that hydroxylamine oxime was produced during nitrite reduction. Indeed, hydrazine did not appear to inhibit the reduction of $^{15}$NO$_2^-$ to ammonia.

The EPR studies have shown that a nitrosyl heme compound is produced during the turnover of the enzyme indicating that a nitrosyl heme may be an intermediate in the reduction of nitrite to ammonia. The nitrosyl heme spectrum was also observed with hydroxylamine in the absence of reductant (benzyl viologen) which may result from disproportionation of the enzyme bound form of hydroxylamine to free ammonia.
and bound nitric oxide. A similar observation for the spinach nitrite reductase was interpreted as indicating that hydroxylamine is enzyme-bound and not a free intermediate in the reduction of nitrite to ammonia (Vega and Kamin, 1977). Our results also indicate that free hydroxylamine does not accumulate. Should a bound form of hydroxylamine be produced then it must be below 0.01 nmol which is the limit of its detection by the GC/MS method employed here.
CHAPTER VI

SECTION 3

FLAVODOXIN AS LIKELY PHYSIOLOGICAL ELECTRON DONOR FOR A. fischeri NITRITE REDUCTASE
INTRODUCTION

Plant and algal nitrite reductases are practically specific for single electron donors: ferredoxin and MVH or BVII with certain exceptions and do not utilize NAD(P)H directly (Losada et al. 1963; Paneque et al. 1964; Betts and Hewitt, 1966). Reduced flavins are generally ineffective (Zumft, 1972). However, the enzyme from marrow has been shown to reduce nitrite in presence of FMNH$_2$ but the activity was very low as compared with that when BVH or ferredoxin was used as electron donor (Hucklesby and Hewitt, 1970). The Chlorella enzyme was shown to function with illuminated chloroplast and flavodoxin (Zumft, 1972).

The ferredoxins are a group of iron-sulfur proteins present in photosynthetic organisms and in non-photosynthetic anaerobic bacteria. They participate in a number of electron transfer reactions including photosynthetic electron transport and nitrogen fixation. The ferredoxins from higher plants, eukaryotic algae and blue green algae, possess the 2Fe-2S active center, have a relative molecular mass of approximately 11,000 and accept a single electron on reduction. In general bacterial ferredoxins contain two 4Fe-4S active clusters, accept two electrons on reduction and have molecular weight between 6,000 to 15,000 depending on the source (Takruri et al. 1978). The amino acid sequence of C. pasteurianum ferredoxin and the nucleotide sequence of the C. pasteurianum Fd gene have been determined. The amino acid sequence deduced from the DNA analysis agrees exactly with that determined for the protein, with the sole exception of the encoded initiator methionine (Graves et al., 1985).

Flavodoxins are low molecular weight electron transfer proteins which have been shown to substitute for ferredoxins in many of the wide range of reactions in which ferredoxins
can function as electron carrier (Yoch and Valentine, 1972; Mayhew and Ludwig, 1975). The first such protein was isolated from the cyanobacterium _A. nidulans_ (Smillie, 1963) and its physiological role as a low-potential electron transfer agent was indicated by its ability to replace ferredoxin in light-dependent NADP+ reduction by higher plant chloroplasts (Smillie, 1965). One mol of FMN is present per mol of protein. Reduction of flavoprotein proceeds via a blue-flavosemiquinone radical. Flavoprotein carriers can yield reduced flavin semiquinone couple of relatively low potential (- 0.46V) which do not readily equilibrate with the high potential oxidized flavin semiquinone couple and acts effectively as one electron donor (Yoch, 1972).

Flavodoxins have been isolated from a variety of organisms from anaerobic fermentative bacteria to eukaryotic algae. They appear to fall into two groups, one having _M_r_ approximately 15,000 and the other 22,000 (Tanaka et al., 1975).

**METHODS**

**NADPH-flavodoxin linked nitrite reductase assay system**

The reaction mixture contained 200 μmol of potassium phosphate buffer, pH 7.5, 75 μmol of glucose-6-phosphate, glucose-6-phosphate dehydrogenase 0.1 ml (2 units), NADP+ 0.05 μmol, spinach NADPH-flavin diaphorase 0.08 mg, flavodoxin (_C. crispus_) in the range of 1 nmol to 2.5 nmol, and distilled water to give a final volume of 0.9 ml in the main arm of the Thunberg tubes. After 30 min, when flavodoxin was reduced, 3 - 5 μg of nitrite reductase and 1 μmol of NaNO₂ were added
under a stream of O₂-free nitrogen. After 90 min further incubation, the reaction was terminated by adding 0.1 ml of 1 M barium acetate and 2.5 ml of 95% ethanol v/v to precipitate NADPH. The contents of the assay mixture were centrifuged and nitrite estimated in suitable aliquots.

The concentration of *C. crispus* flavodoxin was determined from its molar extinction coefficient, 10700 M⁻¹ cm⁻¹ (Fitzgerald et al., 1978).

RESULTS

*A. fischeri* nitrite reductase has been reported to utilize various electron donors, BVH, FMNH₂, FADH₂ (Frakash and Sadana, 1972). Reduced ferredoxin from either spinach or *C. pasteurianum* were unable to donate electron to *A. fischeri* nitrite reductase although these ferredoxins have been shown to serve as electron donor for assimilatory nitrite reductase (Betts and Hewitt, 1966).

Table 7: *Kₘ* values of *A. fischeri* nitrite reductase for various electron donors.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Electron donor</th>
<th><em>Kₘ</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BVH</td>
<td>2.8 - 4.1 x 10⁻⁵ M</td>
</tr>
<tr>
<td>2.</td>
<td>FMNH₂</td>
<td>2 x 10⁻⁴ M</td>
</tr>
<tr>
<td>3.</td>
<td>FADH₂</td>
<td>2 x 10⁻⁴ M</td>
</tr>
<tr>
<td>4.</td>
<td>Flavodoxin (<em>C. crispus</em>)</td>
<td>2.2 x 10⁻⁶ M</td>
</tr>
</tbody>
</table>
The \( K_m \) values for FMNH\(_2\) and FADH\(_2\) are much higher than for BVH (Table 7). An attempt was then made to determine the likely physiological electron donor for \textit{A. fischeri} nitrite reductase. Flavodoxin (Fld) from \textit{C. crispus} was tried to determine whether it would work as an electron donor for nitrite reduction by \textit{A. fischeri} nitrite reductase. Nitrite reductase activity was estimated at various concentrations of flavodoxin. The \( K_m \) value for flavodoxin, calculated from double reciprocal plot of \( 1/v \) versus \( 1/s \) (Fig. 23), was approximately \( 2.2 \times 10^{-6} \text{M} \) (Table 7). This is much less than that of BVH (\( K_m = 2.8 - 4.1 \times 10^{-5} \text{M} \)). Thus, reduced flavodoxin from \textit{C. crispus} appears to be the likely physiological electron donor for \textit{A. fischeri} nitrite reductase.

**DISCUSSION**

Nitrite reductases isolated from various sources show well-defined electron specificity. The assimilatory nitrite reductases from nonphotosynthetic organisms present a marked specificity for reduced pyridine nucleotides as electron donors and require FAD for maximal activity. Three types of enzymes with different specificity for reduced pyridine nucleotide can be distinguished: NAD(P)H- nitrite reductase, which can use either NADH or NADPH as electron donor and is characteristic of the fungus \textit{Neurospora} (Garrett and Amy, 1978); NADPH- nitrite reductase, with a marked specificity for NADPH as electron donor, characteristic of yeast \textit{Torulopsis nitratophila} (Rivas et al., 1973); and NADH- nitrite reductase, specific for NADH as electron donor found in
FIG. 23 Lineweaver-Burk plot of the effect of flavodoxin concentration on *A. fischeri* nitrite reductase activity. The assay conditions are as described in Methods.
prokaryotic organisms (Coleman et al., 1978; Vega et al., 1973).

Photosynthetic nitrite reductases show a marked specificity for ferredoxin as electron donor (Vega et al., 1980). Flavodoxin can substitute for ferredoxin as the immediate electron donor for different nitrite reductases (Manzano, 1977; Vega et al., 1980; Zumft, 1972). Among the artificial substitutes examined for ferredoxin, methyl viologen is the most effective. Reduced flavins are generally ineffective (Zumft, 1972; Hattori and Uesugi, 1968 b). However, marrow preparations showed 5 - 30% activity with flavins as compared to benzyl viologen (Hucklesby and Hewitt, 1970).

*A. fischeri* nitrite reductase can accept electrons directly from benzyl viologen, methyl viologen and flavins (PMNH$_2$, FADH$_2$). Pyridine nucleotides could not serve as electron donor, and flavins, FMN and FAD, showed 20% activity as compared to that obtained with benzyl viologen (Prakash and Sadana, 1972). The $K_m$ values for flavins are very high and therefore not likely to be the physiological electron donor. Ferredoxins from either spinach or C. pasteurianum were unable to donate electrons to *Achromobacter* nitrite reductase. Flavodoxin from C. crispus, on the other hand, acted as an efficient electron donor and its $K_m$ value was an order of magnitude lower than benzyl viologen. Flavodoxin thus appears to be the natural electron donor for *A. fischeri* nitrite reductase.

Fitzgerald et al. (1980) assessed the efficiencies of ferredoxins and flavodoxins from various sources as electron
mediators in systems for hydrogen evolution. They proposed that there was no apparent correlation of efficiency with mid point redox potential (Em) of the mediator. Activity of the mediators therefore primarily reflects differences in their tertiary structure conferring different affinities.

The same reasoning can be proposed here also when ferredoxins from either spinach or C. pasteurianum did not serve as electron donor but flavodoxin from C. crispus served as an efficient electron donor for A. fischeri nitrite reductase. Flavodoxins and ferredoxins are known to substitute each other in many biological reactions (Yoch and Valentine, 1972; Mayhew and Ludwig, 1975). In A. nidulans the flavodoxin was equally or more effective in vitro than ferredoxin in supporting nitrite reduction (Bothe, 1969). Flavodoxin has also been shown to replace ferredoxin as electron donor for Chlorella nitrite reductase (Zumft, 1972).