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Electron-Paramagnetic-Resonance Studies of the Mechanism of Leaf Nitrite Reductase

SIGNALS FROM THE IRON–SULPHUR CENTRE AND HAEM UNDER TURNOVER CONDITIONS

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Low-temperature e.p.r. spectra are presented of nitrite reductase purified from leaves of vegetable marrow (*Cucurbita pepo*). The oxidized enzyme showed a spectrum at $g = 6.86$, 4.98 and 1.95 corresponding to high-spin Fe^{3+} in sirohaem, which disappeared slowly on treatment with nitrite. The midpoint potential of the sirohaem was estimated to be −120mV. On reduction with $Na_2S_2O_4$ or $Na_2S_2O_4$+Methyl Viologen a spectrum at $g = 2.038$, 1.944 and 1.922 was observed, due to a reduced iron–sulphur centre. The midpoint potential of this centre was very low, about −570mV at pH8.1, decreasing with increasing pH. On addition of cyanide, which binds to haem, and $Na_2S_2O_4$, the iron–sulphur centre became further reduced. We think that this is due to an increased midpoint potential of the iron–sulphur centre. Other ligands to haem, such as CO and the reaction product NH_3, had similar but less pronounced effects, and also changed the lineshape of the iron–sulphur signal. Samples were prepared of the enzyme frozen during the reaction with nitrite, Methyl Viologen and $Na_2S_2O_4$ in various proportions. Signals were interpreted as due to the reduced iron–sulphur centre (with slightly different g values), a haem–NO complex and reduced Methyl Viologen. In the presence of an excess of nitrite, the haem–NO spectrum was more intense, whereas in the presence of an excess of $Na_2S_2O_4$ it was weaker, and disappeared at the end of the reaction. A reaction sequence is proposed for the enzyme, in which the haem–NO complex is an intermediate, followed by other e.p.r.-silent states, leading to the production of NH_4^+.

Nitrite reductase [ferredoxin–nitrite oxidoreductase (EC 1.7.7.1), which should not be confused with NAD(P)H–nitrite oxidoreductase (EC 1.6.6.4) from *Neurospora* (Lafferty & Garrett, 1974)] of higher-plant leaves catalyses the six-electron reduction of nitrite to NH_4^+, with reduced ferredoxin as electron donor. The enzyme contains haem iron of a similar type to the sirohaem of bacterial sulphite reductase (Murphy *et al.*, 1974; Aparicio *et al.*, 1975; Hucklesby *et al.*, 1976) and labile sulphur (Zumft, 1972; Hucklesby *et al.*, 1974; Aparicio *et al.*, 1975; Vega & Kamin, 1977), indicative of an iron–sulphur centre, from which e.p.r. signals have been obtained (Aparicio *et al.*, 1975; Vega *et al.*, 1976). Optical absorption measurements indicate that nitrite, and inhibitors such as cyanide and CO, bind to the haem iron (Aparicio *et al.*, 1975; Hucklesby *et al.*, 1976; Vega & Kamin, 1977) and it has been proposed that the iron–sulphur centre serves to transfer electrons from ferredoxin to the haem.

Aparicio *et al.* (1975) reported two types of e.p.r. signal from the enzyme under different reaction conditions. When reduced with $Na_2S_2O_4$ in the presence of cyanide, a signal around $g = 1.94$ was observed, typical of a reduced iron–sulphur centre. When nitrite, $Na_2S_2O_4$ and Methyl Viologen were added to produce conditions under which the enzyme would catalyse its reaction, signals were observed at $g = 2.06$ and 2.00, which were assigned to a complex of haem iron with NO, and it was suggested that this compound was an intermediate in the reaction. Further, Vega *et al.* (1976) have briefly reported a spectrum from the oxidized enzyme corresponding to high-spin ferric haem, at $g = 6.72$, 5.21, 4.98 and 2.03. The spectrum disappeared on reduction or on treatment with nitrite or cyanide (Vega & Kamin, 1977).

We have previously described the isolation of nitrite reductase from vegetable marrow, *Cucurbita pepo* (Hucklesby *et al.*, 1976). The optical spectra of this enzyme are similar to those of the spinach enzyme (Vega & Kamin, 1977). In the present paper

we describe measurements of e.p.r. spectra from both the haem and iron–sulphur components of the enzyme in various states of oxidation and reduction. Spectra were obtained from samples frozen at intervals in various reaction systems, and these throw light on the probable course of the reaction mechanism.

A preliminary report of this work was presented at the 1977 Long Ashton Symposium on Nitrite Assimilation of Plants (Hucklesby *et al.*, 1978).

Experimental

Chemicals

DEAE-cellulose (standard exchange capacity) was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Gel-filtration materials were from Pharmacia Fine Chemicals, Uppsala, Sweden. Argon was supplied by British Oxygen Co., London SW19 3UF, U.K. $(NH_4)_2SO_4$ was A.R. grade from Fisons, Loughborough, Leics., U.K. $Na_2S_2O_4$ and Methyl Viologen (*NN'*-dimethyl-4,4'-dipyridylium dichloride) were obtained from British Drug Houses, Poole, Dorset, U.K. Mediators for redox titrations were obtained from the sources listed by Cammack *et al.* (1976).

Plant material

Vegetable-marrow plants, *Cucurbita pepo* L. (Sutton's Green Bush), were grown in sand culture in a greenhouse under natural light. Plants were irrigated 1–2 times each day with nitrate-containing Long Ashton nutrient solution (Hewitt, 1966). Partially expanded leaves from plants 3–5 weeks old were harvested and stored immediately at −15°C for periods of up to 6 months before use.

Purification of nitrite reductase

The enzyme was purified from batches of 1–2kg of frozen marrow leaves by a shortened version of the method described previously (Hucklesby *et al.*, 1976), which was identical with it to the end of the first $(NH_4)_2SO_4$ fractionation. All operations were at 0–4°C. The final precipitate from this fractionation, containing the enzyme, was dissolved in 0.02M-potassium phosphate buffer, adjusted to pH7.7 with KOH at 3°C, giving a final volume of 50ml/kg of leaf material used. This buffer, and all subsequent solutions used in the purification, also contained 0.1mM-EDTA and 1mM-cysteine hydrochloride. The redissolved enzyme was fractionated on a column of Sephadex G-100 (bed vol. = 4 litres) equilibrated against the same buffer. Active fractions from this column were combined giving a volume of 200–250ml. This fraction was adsorbed on a DEAE-cellulose column (bed vol. 40ml, height 14cm) equilibrated with 20mM-Tris/HCl, pH7.7, and the enzyme was eluted by a linear gradient of KCl (0.1–0.225M in a volume of 400ml) in the same buffer. Active fractions from this column were combined and diluted with 2vol. of 20mM-Tris/HCl, pH7.7, and adsorbed on a second DEAE-cellulose column identical with the first. The enzyme was purified on this column in the same way as with the first DEAE-cellulose column. The procedure was repeated on a third DEAE-cellulose column. The active fractions from this column were combined, and the enzyme solution was concentrated to 3ml by ultrafiltration through an Amicon PM30 membrane. A final purification on a column (150ml bed vol.) of Sephadex G-100, equilibrated in 0.02M-potassium phosphate buffer, pH7.7, containing 0.1M-KCl, gave a single peak of protein corresponding to nitrite reductase activity. Electrophoresis (Hucklesby *et al.*, 1976) gave a single band of protein. The enzyme used for the e.p.r. studies was taken from different stages of the purification and the specific activities of preparations used ranged from 30 to 40μmol/min per mg of protein at 27°C.

Assays

Nitrite reductase was assayed with $Na_2S_2O_4$ and a Methyl Viologen carrier. Two methods were used (Hucklesby *et al.*, 1972), the second of which was a quick method reserved for column eluates. Protein was determined as previously described (Hucklesby *et al.*, 1976).

Electron-paramagnetic-resonance studies

Except where otherwise stated the enzyme was used at a concentration of approx. 5.0mg/ml (actual enzyme concentration) in 30mM-potassium phosphate buffer (pH7.5)/180mM-NaCl.

Samples for e.p.r. spectroscopy were prepared in quartz tubes of known internal diameter (about 3.0mm) (Varian Associates, Palo Alto, CA, U.S.A.). For experiments in reducing conditions the tubes were purged continuously with Ar gas through a stainless-steel catheter. Reagents such as $NaNO_2$, Methyl Viologen and $Na_2S_2O_4$ (0.1M in 0.2M-Tris/HCl, pH9.0) were added with 10μl syringes fitted with 15cm needles, and stirred vigorously with the needle. Mixing, where it could be observed by mixing of coloured solutions, appeared to be complete within 3s. Samples were frozen by immersing in a bath of isopentane and methylcyclohexane (5:1, v/v) at 100K. The freezing time for tubes already at 0°C was approx. 2s.

E.p.r. spectra were recorded on an E4 spectrometer (Varian) with an ESR 9 liquid-helium transfer system (Oxford Instrument Co., Osney Mead, Oxford, U.K.) for sample cooling. *g* values were referred to 1,1-diphenyl-2-picrylhydrazyl as standard. Spectral subtraction was recorded with a Nicolet signal-averaging system.

Measurements of midpoint potentials

Redox titrations were carried out as previously

described (Cammack *et al.*, 1976). A quantity of the enzyme, in the presence of the mixed dye mediators (below), under Ar atmosphere, was adjusted with successive small additions of 0.1 M-$Na_2S_2O_4$ to fixed redox potentials that were measured with platinum and calomel electrodes. After equilibration for 5 min a sample was withdrawn into a quartz tube and frozen for e.p.r. spectroscopy. The process was repeated over relevant ranges of redox potentials. The size of the e.p.r. signal of the centre to be observed was plotted against the applied redox potential and the midpoint potential of the centre was determined by fitting to a theoretical curve calculated from the Nernst equation (see Cammack *et al.*, 1976).

For titration of the haem signal, the mediators used were: phenazine methosulphate ($E_0' = 80$ mV), Methylene Blue ($E_0' = 11$ mV), Indigotetrasulphonate ($E_0' = -46$ mV), 2-hydroxy-1,4-naphthoquinone ($E_0' = -145$ mV) and phenosafranine ($E_0' = -255$ mV), all at a concentration of 40 μM. For titration of the iron–sulphur centre, the mediators used were: phenosafranine ($E_0' = -255$ mV), Benzyl Viologen ($E_0' = -345$ mV), Methyl Viologen ($E_0' = -440$ mV), triquat (1,1′-trimethylene-2,2′-bipyridylium dibromide) ($E_0' = -544$ mV), tetraquat (1,1′-tetramethylene-2,2′-bipyridylium dibromide) ($E_0' = -640$ mV) and *NN*′-3-trimethyl-4,4′-bipyridylium dibromide ($E_0' = -617$ mV), all at a concentration of 50 μM.

Results

Spectra of haem iron

Fig. 1(*a*) shows the spectrum of an unreduced sample of nitrite reductase, and Fig. 1(*b*) shows the effect of nitrite. The intensity of the major feature around $g = 4.3$, and a minor feature at $g = 5.9$, varied depending on the batch of enzyme, and did not correlate with the enzyme activity. The spectrum was unchanged if nitrite was added to the oxidized enzyme at 0°C and the solution frozen after a few seconds. However over the course of 1 min at 20°C, certain spectral features disappeared. Fig. 1(*c*), the difference produced by adding nitrite, shows the features that we believe represent the spectrum of nitrite reductase itself. They consist of a peak at $g = 6.86$, a crossover at $g = 4.98$ and a small downward feature at $g \simeq 1.95$. These values correspond to g_z, g_y and g_x for high-spin Fe^{3+} in haem; the first two g values would both be 6.0 for axial symmetry, but are split by tetragonal distortion. These g values are slightly different from those reported by Vega *et al.* (1976) for the spinach enzyme, although these workers did not define which features the g values represented. The spectrum is similar to that observed in sulphite reductase of *Escherichia coli* (Peisach *et al.*, 1971), another enzyme that contains sirohaem.

No signals corresponding to high- or low-spin Fe^{3+} were observed in the nitrite-bound form,

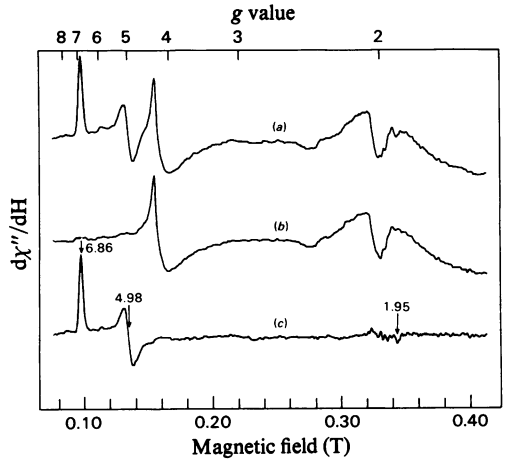

Fig. 1. *E.p.r. spectrum of nitrite reductase*
Spectra are shown for nitrite reductase (*a*) as prepared and (*b*) after treatment with 5 mM-$NaNO_2$ at 20°C for 2 min. The invariant features around $g = 2.1$ are due to a contaminant in the sample holder. Spectrum (*c*) is the difference between (*a*) and (*b*). The features marked are the three principal apparent g values. Conditions of measurement were: temperature, 11 K; microwave power, 20 mW; frequency, 9.25 GHz; modulation amplitude, 1 mT.

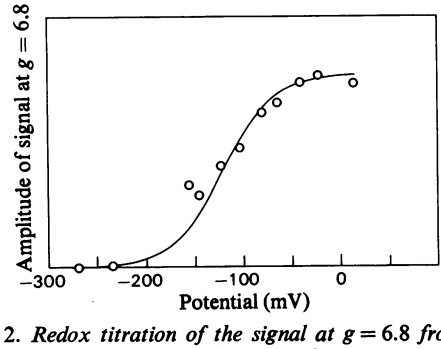

Fig. 2. *Redox titration of the signal at $g = 6.8$ from the ferric haem of nitrite reductase*
Enzyme was in 0.1 M-Tris/HCl buffer, pH 8.5. For details of the titration see the Experimental section. Spectra were recorded as described in the caption to Fig. 1. The curve fitted to the points is for a one-electron process with a midpoint potential of −120 mV.

although it is possible that Fe^{3+} might have been present but not detectable under the conditions of examination. Similarly, the signals disappeared when cyanide or sulphite were added. With either, a signal corresponding to low-spin Fe^{3+} would be expected, but none was observed between 6.2 and 77 K.

Oxidation–reduction-potential titrations of the signal at $g = 6.8$ indicated that the haem in nitrite reductase has a midpoint potential around $-120\pm$ 20mV at pH8.5 (Fig. 2).

After 2min reaction of the enzyme in the presence of nitrite with a limiting amount of $Na_2S_2O_4$, a signal was observed with axial symmetry, at $g_\perp = 2.06$, $g_{\parallel} = 2.00$ (Fig. 3). A similar spectrum in the spinach enzyme was assigned by Aparicio *et al.* (1975) to a complex of ferrous haem with NO. This seems the most likely explanation for this spectrum on present evidence, but it is noteworthy that there is little trace of the ^{14}N hyperfine splitting that would be expected, except for the slightly non-gaussian lineshape of the $g_{\parallel}$ feature around $g = 2$. The hyperfine structure in such compounds is not always well resolved, however, as in haemoglobin–NO in the presence of sodium dodecyl sulphonate (Kon, 1968). The maximum signal was associated with completion of the reaction with $Na_2S_2O_4$, Methyl Viologen and an excess of nitrite (i.e. more than 1mol of NO_2^-/ 3mol of $Na_2S_2O_4$) (see Fig. 5*c*).

Spectra of the iron–sulphur centre

On reduction with $Na_2S_2O_4$, the signals of ferric haem disappeared, and a signal around $g = 1.94$, typical of a reduced iron–sulphur centre, appeared. This signal was extremely weak, but could be made somewhat larger by adjusting to pH10.0, at which the potential of $Na_2S_2O_4$ is more negative (Fig. 4*a*), or by adding low-potential redox mediators such as Methyl Viologen (Fig. 4*b*). The signal from the

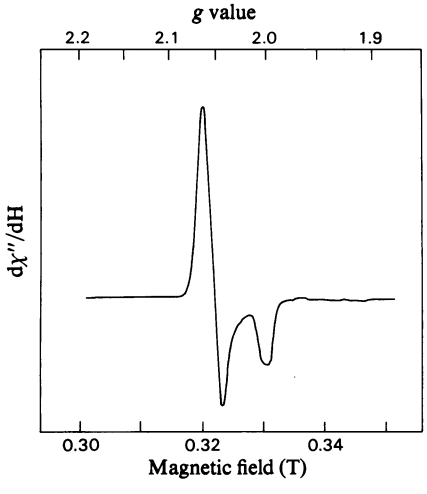

Fig. 3. *Spectrum of nitrite reductase after reaction with nitrite in excess*

Nitrite reductase was frozen after reaction with 2mM-$NaNO_2$, 0.1mM-Methyl Viologen and 4mM-$Na_2S_2O_4$ at 20°C for 2min. Conditions of measurement were: temperature, 30K; microwave power, 1mW; frequency, 9.25GHz; modulation amplitude, 0.5mT.

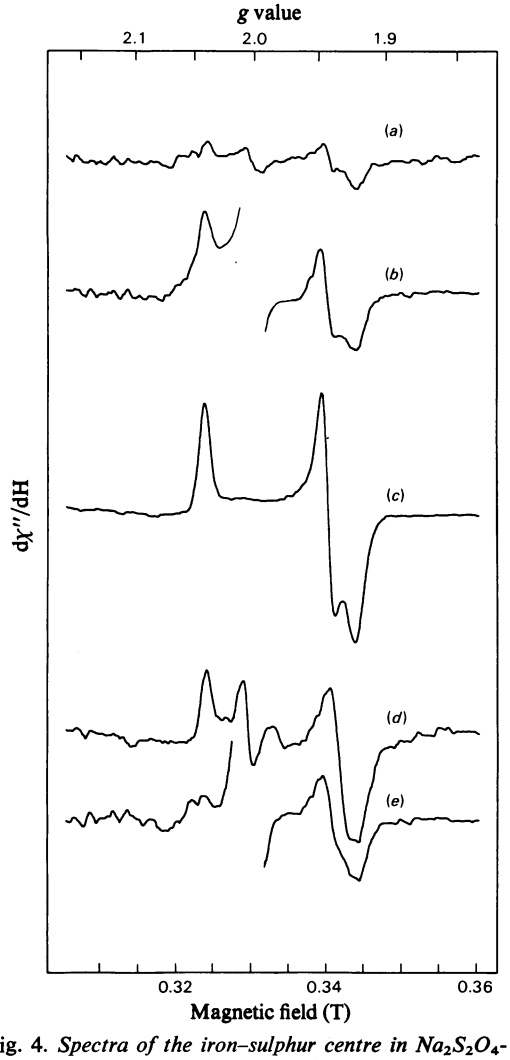

Fig. 4. *Spectra of the iron–sulphur centre in $Na_2S_2O_4$-reduced nitrite reductase*

Nitrite reductase was reduced with 10mM-$Na_2S_2O_4$ at 25°C (*a*) in 0.1M-glycine/NaOH buffer, pH10.0, (*b*) with 0.1mM-Methyl Viologen, (*c*) with 0.5mM-KCN, (*d*) bubbled with CO gas, (*e*) with 2mM-NH_4Cl+0.1mM-Methyl Viologen. The iron–sulphur centre gives rise to the signals around $g = 2.04$ and 1.94. The features around $g = 2$ in spectra (*b*) and (*e*) are due to reduced Methyl Viologen; those in spectra (*a*) and (*d*) probably originate from the $Na_2S_2O_4$. Conditions of measurement were: temperature, 20K; microwave power, 20mW; frequency, 9.25GHz; modulation amplitude, 1mT. The gain for spectrum (*c*) is 0.5 times that for the other spectra.

$Na_2S_2O_4$-reduced enzyme might be small for various reasons. The reduction by $Na_2S_2O_4$ was found to be slow, complete reduction requiring several minutes at 25°C. However, a more important factor was found to be the very low reduction potential of the iron–sulphur centre. Redox titrations were difficult, because it was not possible to obtain a sufficiently low potential with $Na_2S_2O_4$ to reduce the centre completely. Moreover the potential appeared to be pH-dependent, so that little improvement could be achieved by using a higher pH. The maximum size of the signal of reduced iron–sulphur centre could only be estimated by adding cyanide (see below). The midpoint potential was estimated to be −570mV at pH8.1, −615mV at pH8.7 and −660mV at pH9.8, assuming a one-electron transfer process.

Aparicio *et al.* (1975) found that the iron–sulphur centre could readily be reduced by $Na_2S_2O_4$ if cyanide, an inhibitor of the enzyme, was also present. Fig. 4(*c*) confirms that the extent of reduction in the presence of cyanide is much greater than with $Na_2S_2O_4$ alone. The optical absorption spectrum indicates that cyanide binds to the haem in the enzyme (Aparicio *et al.*, 1975; Hucklesby *et al.*, 1976; Vega & Kamin, 1977), but clearly this binding is affecting the iron–sulphur centre in such a way that its midpoint is less negative. The g values of the signal were similar to those of the enzyme reduced by $Na_2S_2O_4$ and Methyl Viologen, with $g_x = 1.922$, $g_y = 1.944$, $g_z = 2.038$.

Reduction of the iron–sulphur centre was also enhanced by CO, another inhibitor of the enzyme, and by NH_4^+, the reaction product (Figs. 4*d* and 4*e*). These agents caused slight changes in the lineshape of the signal around $g = 1.94$, even though they were presumably all binding to the haem. The line shape of the signal in the presence of NH_4^+ is distinct from that produced by $Na_2S_2O_4$+Methyl Viologen, in that the downward features around $g = 1.93$ are less well separated. This observation is relevant to our interpretation of the spectra obtained under turnover conditions, described below.

The shift in potential of the iron–sulphur centre is of considerable significance to the mechanism of the enzyme, since it probably also happens when substrate binds to the enzyme. Otherwise the iron–sulphur centre could not be significantly reduced by the electron donor ferredoxin ($E_0' = -420$mV) or by less negative systems, with single-electron mediators (Hewitt *et al.*, 1968).

Spectra of the enzyme under turnover conditions

By running the reaction at 0°C, and freezing the sample tubes in a cold mixture of isopentane and methylcyclohexane at −173°C, it was possible to measure spectra of samples prepared under steady-state conditions while the reaction was taking place. The minimum time required for mixing the reagents and freezing the samples was approx. 5s. Under these conditions the reaction took approx. 25s to complete. Methyl Viologen was used as electron donor instead of ferredoxin because the narrow e.p.r. signal

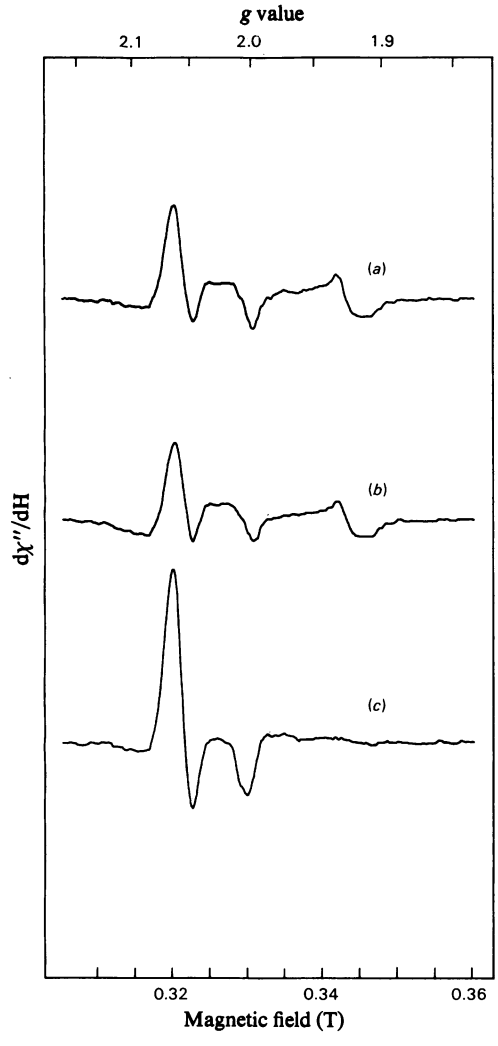

Fig. 5. *Spectra of nitrite reductase frozen during reaction with 2mM-$NaNO_2$, 0.1mM-Methyl Viologen and 4mM-$Na_2S_2O_4$*

The reaction was carried out at 0°C and pH7.5. Samples were frozen after (*a*) 5s, (*b*) 10s, and (*c*) 85s. The order of addition of reagents to the enzyme was: Methyl Viologen, $NaNO_2$ and, within a few seconds, $Na_2S_2O_4$. Time intervals quoted were the times between the start of mixing the $Na_2S_2O_4$ and the start of freezing the sample. Conditions of measurement were: temperature, 20K; microwave power, 20mW; frequency, 9.25GHz; modulation amplitude, 1mT.

of the reduced radical cation did not greatly interfere with signals from the enzyme under the conditions used for these measurements.

During the reaction, signals ascribed to the haem–NO complex and to the reduced iron–sulphur centre were observed. The latter was different from the spectrum of the enzyme reduced with $Na_2S_2O_4$ alone, being more nearly axial, with $g_x = 1.911$, $g_y = 1.929$, $g_z = 2.018$ (see Fig. 7*c*). The reduced Methyl Viologen free radical gave a sharp signal at $g = 2.00$ (e.g. Fig. 6*c*) that was strongly saturated with microwave power under the conditions of measurement. The relative intensities of the signals depended on the relative concentrations of nitrite and $Na_2S_2O_4$ in the reaction mixture.

Figs. 5 and 6 show spectra of the enzyme frozen under normal reaction conditions, with nitrite, $Na_2S_2O_4$ and Methyl Viologen. In spectra 5(*a*)–(*c*), nitrite (a six-electron acceptor) was in excess of $Na_2S_2O_4$ (a two-electron donor). While the reaction was taking place the iron–sulphur centre was reduced, but only to a small extent, as shown by the signals at $g = 1.93$ in Figs. 5(*a*) and 5(*b*). The radical signal from reduced Methyl Viologen was relatively small, indicating that it was being reoxidized rapidly, and that its reduction by $Na_2S_2O_4$ was a rate-limiting process. At the completion of the reaction (Fig. 5*c*) the centre was reoxidized. The signal corresponding to the haem–NO complex was prominent throughout the reaction (this signal is somewhat saturated under the conditions of measurement), and became maximal at the end of the reaction.

These results suggest that the haem–NO complex is an intermediate in the reaction, but do not exclude the possibility that it is a by-product. Further evidence on this point was provided by measurements made when $Na_2S_2O_4$ was in excess, when the haem–NO signal disappeared in a time commensurate with the completion of the reaction, indicating that it is an intermediate in the reaction. Figs. 6(*a*)–(*c*) show a time course in the presence of nitrite, Methyl Viologen and an excess of $Na_2S_2O_4$. The smaller haem–NO signal, most easily seen in the upward feature at $g = 2.06$, disappeared when the reaction was complete (Fig. 6*c*). During the reaction the signals from reduced Methyl Viologen at $g = 2.00$ and the reduced iron–sulphur centre at $g = 1.93$ were more prominent than in Figs. 5(*a*) and 5(*b*). After the reaction was complete (Fig. 6*c*) the spectrum changed to that of the reduced enzyme–NH_4^+ complex (cf. Fig. 4*e*).

In Fig. 7, $Na_2S_2O_4$ and nitrite were present, but no Methyl Viologen. Under these conditions the rate of reduction of nitrite to NH_3 is decreased greatly. At the shortest time interval, 5s (Fig. 7*a*), a large haem–NO signal was observed. The iron–sulphur centre was only slightly reduced at 5s, but was quite considerably reduced at 10s. As noted above, the iron–sulphur centre is not readily reduced

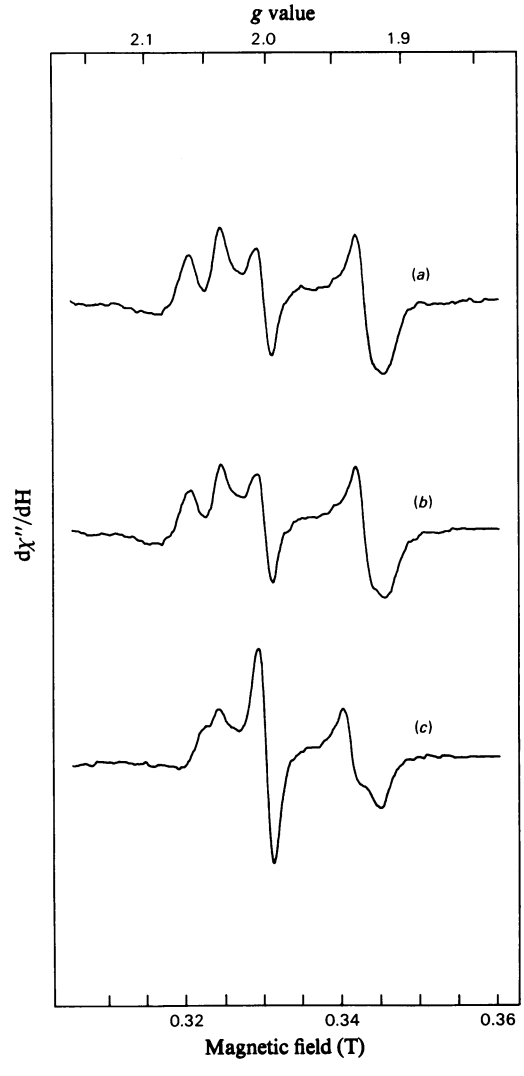

Fig. 6. *Spectra of nitrite reductase frozen during reaction with* 2 mM-$NaNO_2$, 0.1 mM-*Methyl Viologen and* 10 mM-$Na_2S_2O_4$

Samples were frozen after (*a*) 5s, (*b*) 10s and (*c*) 85s. Conditions were as for Fig. 5.

by $Na_2S_2O_4$ alone, so the reduction observed in the presence of nitrite was presumably a consequence of the binding of a ligand to the haem, by analogy with the effects of cyanide and CO. The signal corresponding to the reduced iron–sulphur centre remained large after 5min (Fig. 7*c*), whereas the haem–NO signal gradually diminished. Presumably the NO bound to haem was becoming further reduced to give an e.p.r.-silent state, without being

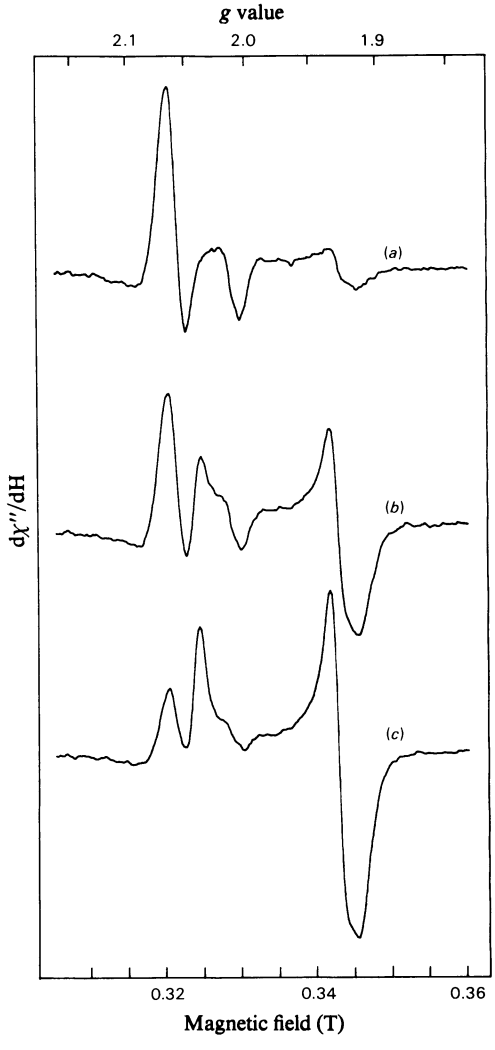

Fig. 7. *Spectra of nitrite reductase frozen during reaction with 2mM-$NaNO_2$ and 10mM-$Na_2S_2O_4$*
Samples were frozen after (*a*) 5s, (*b*) 10s and (*c*) 300s. Conditions were as for Fig. 5.

completely reduced to NH_4^+ as in Fig. 6(*c*). This leads to an apparent paradox, since although the iron–sulphur centre was in the reduced state it did not reduce the nitrogenous ligand, as it would if Methyl Viologen was present.

Discussion

The detailed mechanism of the reduction of nitrite to NH_3 is inevitably complex, since it involves sequential reduction by six electrons from ferredoxin. Studies of the e.p.r.-detectable intermediates enable us to draw conclusions about the early steps of the reaction cycle.

Step 1

Spectrophotometric measurements indicate that nitrite (formal oxidation state +3) binds to the haem. The reaction of nitrite with the oxidized form of the enzyme is slow, requiring several minutes at 0°C for complete disappearance of the signals around $g = 6.8$ and 5.0. Such a reaction could not be part of the enzyme reaction under turnover conditions, and therefore the haem must first be reduced to the ferrous state, as concluded from spectrophotometric observations (Aparicio *et al.*, 1975; Hucklesby *et al.*, 1976; Vega & Kamin, 1977).

We would expect the iron–sulphur centre to be in the oxidized state initially, since its midpoint potential is extremely low. Therefore the initial one-electron reduction of the haem would probably occur directly by ferredoxin. After substrate is bound the iron–sulphur centre is probably more easily reduced, by analogy with the effects of cyanide and CO.

Step 2

In the second stage nitrite already bound to the ferrous haem is reduced to a haem–NO complex (oxidation state +2). The electron for this process might be provided either by the iron–sulphur centre or by ferredoxin. Some evidence that the sirohaem–NO species is a true intermediate in the reaction, rather than a 'dead-end' complex, is provided by the observation that it disappeared at least within a few seconds of the completion of the enzyme reaction. However, its true status may only be conclusively demonstrated by more rapid freezing techniques than those described here.

Further reduction

Four more electrons are now required to be added to the nitrogen ligand while it is bound to the haem of the enzyme. E.p.r. signals from the intermediates of this reaction have not been seen, but observations of reactions with different electron donors are relevant to this topic. With nitrite as substrate and $Na_2S_2O_4$ alone as reductant, the reaction proceeds slowly to NH_3, reaching an apparent equilibrium with a relatively small haem–NO signal. This indicates that most of the NO bound to haem has been reduced to a further stage, but not as far as NH_4^+. (Alternatively the NO might have dissociated. However, the iron–sulphur centre is fully reduced, suggesting that a ligand is still bound to the haem.) Assuming that the iron–sulphur centre still has a comparatively low midpoint potential under these conditions, why does it not become reoxidized? A possible explanation of the block in activity in the absence of Methyl Viologen or ferredoxin is that one of the later stages of reduction requires two electrons

to be added simultaneously, one by the iron–sulphur centre and the other by the electron donor directly. $Na_2S_2O_4$ either does not react sufficiently rapidly or cannot do so at two sites simultaneously, but may function slowly in the slightly dissociated form, $SO_2^{\cdot -}$, as a single electron donor.

An alternative possibility for the origin of NO–haem intermediate

In the above scheme, the NO–haem complex is formed by reduction of a nitrite–haem complex. However, an alternative hypothesis, which we cannot exclude at this stage, is that NO generated by reaction of NO_2^- at the non-haem centre subsequently reacts with the sirohaem. This possibility is suggested by studies of the reaction between nitrite and ferredoxin, which may be characteristic in this respect of iron–sulphur proteins in general. Thus, whereas $Na_2S_2O_4$ does not react appreciably with nitrite at pH values above 6, NO is produced in stoicheiometric amounts at pH 6–7 when $Na_2S_2O_4$ and nitrite are incubated with ferredoxin (Garrioch & Hewitt, 1976). At pH 7–8.5, NO is formed together with NH_3, which predominates at the higher pH. If a similar reaction occurs during enzymic nitrite reduction, the NO generated might then combine with the reduced sirohaem. Nitrosylation of reduced haem has previously been described (Paul & Kumta, 1975) and was used by Garrioch & Hewitt (1976) as a sensitive detector of NO produced from nitrite and ferredoxin. They also showed that myoglobin–NO was formed with $Na_2S_2O_4$-reduced myoglobin in the presence of nitrite and that the comparatively slow reduction of nitrosyl-myoglobin to NH_3 by $Na_2S_2O_4$ was accelerated about 25% by the presence of ferredoxin. Ferredoxin and myoglobin may be viewed as an interesting model system for the iron–sulphur and haem components of nitrite reductase, although functioning at only approx. 1% of the rate obtained by a ferredoxin nitrite reductase.

It appears that the nitrite reductase reaction involves concerted changes in the redox potentials of the electron-transfer components that react with the various nitrogenous intermediates. A better knowledge of these potentials would be an essential pre-requisite for a full understanding of the enzyme mechanism.

Note Added in Proof (Received 3 April 1978)

After this paper was submitted, a paper by Stoller *et al.* (1977) appeared in which measurements of the midpoint potentials of the spinach enzyme were reported. They point out the difficulties inherent in using dithionite as reducing agent, producing sulphite, which is a ligand to haem. They obtained midpoint potentials of −50mV at pH 7.8 (with reduced Methyl Viologen as reductant) for the free haem, and approx. −550mV at pH 9 for the iron–sulphur centre (with dithionite as reductant), results that do not disagree seriously with our own.

Surprisingly, cyanide was reported not to affect the potential of the iron–sulphur centre, but the authors concluded, as we do, that this potential probably shifts to a more positive value under physiological conditions.

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