Electron paramagnetic resonance studies on *Pseudomonas* nitrosyl nitrite reductase

EVIDENCE FOR MULTIPLE SPECIES IN THE ELECTRON PARAMAGNETIC RESONANCE SPECTRA OF NITROSYL HAEMOPROTEINS

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The e.p.r. spectra of reduced ^{14}NO - and ^{15}NO -bound *Pseudomonas* nitrite reductase have been investigated at pH 5.8 and 8.0 in four buffer systems. At pH 8.0, absorption spectra indicated that only the haem d_1 was NO-bound, but, although quantification of the e.p.r. signals in all cases accounted for NO bound to the haem d_1 in both subunits of the enzyme, the precise form of the signals varied with buffer and temperature. A rhombic species, with $g_x = 2.07$, $g_z = 2.01$ and $g_y = 1.96$, represented in the low-temperature spectra seen in all the buffers was converted at high temperatures (approx. 200 K) into a form showing a reduced anisotropy. Hyperfine splitting on the g_z component of this rhombic signal indicated a nitrogen atom *trans* to NO and it is proposed that histidine provides the endogenous axial ligand for haem d_1 . At pH 5.8, absorption spectra indicated NO binding to both haems c and d_1 and e.p.r. quantifications accounted for NO-bound haems c and d_1 in both enzyme subunits. The e.p.r. spectra at pH 5.8 were generally similar to those at pH 8.0 with respect to g -values and hyperfine coupling constants, but were broader with less well defined hyperfine splittings. As at pH 8, rhombic signals present in spectra at low temperatures were converted to less anisotropic forms at high temperatures. The results are discussed in relation to work on model nitrosyl–protohaem complexes [Yoshimura, Ozaki, Shintani & Watanabe (1979) *Arch. Biochem. Biophys.* **193**, 301–313]. No e.p.r. signal was observed from oxidized NO-bound *Pseudomonas* nitrite reductase at pH 6.0, over the temperature range 6–100 K.

Pseudomonas nitrite reductase (ferrocytochrome c_{551} - O_2 oxidoreductase, EC 1.9.3.2) is a terminal respiratory enzyme of *Pseudomonas aeruginosa* (Horio *et al.*, 1958, 1961). By accepting electrons from either *Pseudomonas* ferrocytochrome c_{551} or *Pseudomonas* azurin, the enzyme can accomplish both the four-electron reduction of O_2 to water and the single-electron reduction of nitrite to NO; the latter process is considered to be the physiological function (Yamanaka *et al.*, 1963). The purified protein, of total mol.wt. 120 000, consists of two identical subunits each of which contains a haem

c and a haem d_1 (Kuronen & Ellfolk, 1972; Silvestrini *et al.*, 1979).

Spectrophotometric investigations (Shimada & Orii, 1975; Silvestrini *et al.*, 1979) have shown that NO will bind to the haem c and haem d_1 components in both oxidized and reduced states. In fact, the NO-binding behaviour is pH-dependent and, in the reduced state, the haem c moiety will only bind the ligand below pH 7.0. This has enabled us to examine species of the reduced enzyme that are either NO-bound at both haems c and d_1 , or bound solely at the haem d_1 . We have used the inherent ability of the protein to generate NO from nitrite in the presence of excess reductant to produce the appropriately bound species.

A study of the e.p.r. signals from the various NO-

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

bound forms of *Pseudomonas* nitrite reductase, as characterized by absorption spectrophotometry, has been performed with three principal aims: (a) to compare the e.p.r. signals of a reduced NO-bound haemoprotein of physiological importance with those from other reduced NO-bound haemoproteins such as haemoglobin, myoglobin and cytochrome *c* as well as with model haem complexes; (b) to quantify the e.p.r. signals so as to assess the percentage of total haem present that is NO-bound and hence confirm the conclusions arrived at by absorption spectrophotometry; (c) to utilize bound ^{14}NO and ^{15}NO as spin-label probes to characterize the endogenous axial ligands of haems *c* and d_1 . During these investigations it was observed that the forms of the e.p.r. spectra varied considerably with both temperature and with the buffer employed, and we have attempted to rationalize these phenomena in the light of work by Yoshimura *et al.* (1979) on model haem-NO complexes. The implications of our investigations bear important consequences for the interpretation of e.p.r. signals from nitrosyl haemoproteins in general.

Materials and methods

Sodium ascorbate was obtained from Sigma and KNO_2 was from BDH. $\text{Na}^{15}\text{NO}_2$ (99.2 atom % ^{15}N) and O_2 -free N_2 were supplied by British Oxygen Co., London SW19 1YD, U.K. NO was acquired from Matheson Gas Products, East Rutherford, NJ, U.S.A. The N_2 gas was dispensed from the cylinder and stored in glass vessels over an alkaline solution of anthroquinone sulphonate before use. *Pseudomonas* nitrite reductase was purified from cells of *Pseudomonas aeruginosa* (N.C.T.C. 6750) using the procedure of Parr *et al.* (1976); the ratios A_{410}/A_{280} and A_{640}/A_{520} for the oxidized enzyme were 1.18–1.2 and 1.17–1.20 respectively.

Before use, aliquots of concentrated enzyme were passed through a Sephadex G-25 column equilibrated with the required buffer at the appropriate pH. The concentrations of the samples were then measured spectrophotometrically using an absorption coefficient expressed on the basis of an enzyme molecule containing two haem *c* moieties and two haem d_1 moieties of $A_{410}^{2\%} = (2.82 \pm 0.15) \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Silvestrini *et al.*, 1979). Portions (0.5 ml) of the Sephadex G-25-treated enzyme solution was placed in e.p.r. tubes modified with a cupped end so as to be sealable by vaccine cap. These were then degassed by several cycles of evacuation and flushing with N_2 before being finally placed under an atmosphere of the gas. The enzyme was then reduced by addition of sodium ascorbate solution to give a final ascorbate concentration of 20 mM and, after approx. 45 min,

a portion of NO_2^- solution was injected into the sample to give a final NO_2^- concentration of 1.8 mM.

Absorption spectra were recorded on a Cary 118C spectrophotometer at room temperature. E.p.r. spectra were measured on a Varian E-3, X-band e.p.r. spectrometer fitted with an Oxford Instruments E.S.R.-9 continuous flow cryostat and DTC 2 temperature controller enabling the sample temperature to be varied over the range 4.2–300 K to an accuracy of ± 0.1 K. Quantification of e.p.r. spectra by double integration was performed according to the method of Wyard (1965) and Aasa & Vänngård (1975) with 1 mM copper-EDTA as standard. It should be noted that, although the number of haem centres recovered by integration of the NO e.p.r. signals were constant, within experimental error, at temperatures above 50 K when using 1.25 mW power, the number decreased significantly at 30 and 10 K using the same power, indicating that the signal was saturating at these temperatures and powers. The small, sharp resonance at $g = 2.01$, which is visible in all the spectra presented below and is specifically indicated in Fig. 4(b), was observed at low temperatures in the absence of a sample and hence is attributed to a paramagnetic impurity in the quartz insert of the cryostat. Aside from this feature at $g = 2.01$, control experiments, over the range 30–230 K with all the buffer solutions under an atmosphere of NO gas and in the presence of ascorbate, failed to detect any other signals. Therefore none of the e.p.r. signals observed in this work arise from free NO or from NO bound to any extraneous ions in the buffers. The e.p.r. spectra have been calibrated with reference to Freymy's salt and the g values quoted here are accurate to ± 0.01 .

Williams-Smith *et al.* (1977) and Orie & Morita (1977) have drawn attention to the fact that freezing of buffer solutions can bring about apparent pH changes. Since such possible pH variations may have a bearing on our experiments we have adopted the practice of Williams-Smith *et al.* (1977) and used BDH Universal Indicator to examine our buffer systems. Samples (2 ml) of 0.1 M-Tris/HCl, Mops/NaOH, Hepes/NaOH and potassium phosphate buffers, at both pH 6 and pH 8, were placed in 10 mm diameter test tubes and three drops of dye indicator were added to each test solution. The solutions were frozen by immersion in a bath of liquid N_2 . Additionally, a parallel series of tests was carried out in which each buffer solution contained 3 mg of bovine serum albumin/ml. Williams-Smith *et al.* (1977) have found that the presence of protein can decrease the apparent pH change on freezing, but, since *Pseudomonas* nitrite reductase is coloured, we have used albumin, in a concentration approximating that of our enzyme

solutions, as a model. The pH of the frozen solutions was assessed by comparison with a range of unfrozen 'standard' solutions (2ml volume), prepared in steps of 0.5 pH unit and containing the same amount of dye indicator as in the test buffers.

Results and discussion

Absorption spectra

Fig. 1 shows a comparison of the absorption spectra of the reduced NO-bound forms of *Pseudomonas* nitrite reductase at pH 8.0 and pH 5.8, together with a spectrum of the unbound reduced protein, which is similar at both pH values. These observations are in agreement with our previous work (Silvestrini *et al.*, 1979) and were not affected by the use of phosphate, Hepes, Tris or Mops buffers. The changes in the spectra around 460 nm and 650 nm can be attributed to NO binding to the haem d_1 component, whereas changes in the regions around 420 nm and 500–600 nm can be attributed to NO binding to the haem c component. It is apparent that, whereas NO binds to the haem d_1 at both alkaline and acidic pH values, the haem c only binds NO at the acid pH. Silvestrini

et al. (1979) have examined the NO complexes of reduced *Pseudomonas* nitrite reductase in the pH range 4–9 and found that whereas the haem d_1 component reacts with NO over the entire range, the haem c moiety only reacts readily with NO below pH 7 and not at all at alkaline pH values. These workers also established that the affinity of the haem d_1 for NO is much greater than that of the haem c , and ordinarily one would always expect binding of NO to the haem d_1 to take preference over binding to the haem c . It therefore seems unlikely that our e.p.r. experiments at pH 8 would be confused by a distribution of NO amongst the haems c and d_1 .

Effect of freezing on pH

The colorimetric comparison of our clear 'standard' solutions with the frozen buffer solutions presented some difficulty and we only felt able to assess the pH of the solutions to within a range of approx. 0.5 pH unit. In the presence of bovine serum albumin the situation was further complicated by the fact that the dye apparently interacted with the protein. Thus, the pH 8 albumin-containing solutions looked more acidic (approx. pH 7) and the

Fig. 1. Absorption spectra of reduced *Pseudomonas* nitrite reductase and its NO complexes at pH 8.0 and 5.8. The absorption spectra of reduced *Pseudomonas* nitrite reductase at pH 8.0 (—) and the NO-bound forms at pH 8.0 (---) and pH 5.8 (— · —). The spectra were run in 0.1 M-Hepes buffer in a 1 cm pathlength Thunberg cuvette in the presence of 4 mM-ascorbate and, in the case of the NO-bound samples, 400 μ M- KNO_2 under 1 atmosphere of N_2 at 20°C. The protein concentration was 5 μ M.

Table 1. Apparent pH changes on freezing buffer solutions

Apparent pH value at liquid-N₂ temperature

Buffer	pH value at room temperature	Apparent pH value at liquid-N ₂ temperature		pH change when frozen + albumin
		-Albumin	+ Albumin	
Tris/HCl	8.0	8.0 ± 0.5	8.0	Alkaline
	6.0	7.0–7.5	4.5–5.0	Acid
Potassium phosphate	8.0	6.5	8.0	Alkaline
	6.0	5.5–6.0	5.5–6.0	Acid
Mops/NaOH	8.0	8.0–8.5	7.5–8.0	Alkaline
	6.0	6.0–6.5	6.5	None
Hepes/NaOH	8.0	7.5–8.0	7.5–8.0	Alkaline
	6.0	6.0 ± 0.5	6.0	Acid

pH 6 albumin-containing solutions looked more basic (approx. pH 6.5) in spite of the fact that measurements on a pH meter gave the anticipated readings. Therefore, in the case of each albumin solution, the effect of freezing has been assessed both as a change relative to an otherwise identical but unfrozen control, as well as against the range of non-protein-containing 'standards'. The results of the freezing experiments are summarized in Table 1 and it is clear that the presence of protein has had a substantial effect on the behaviour of some buffer systems.

With regard to the pH variation in the ability of the haem *c* moiety of *Pseudomonas* nitrite reductase to bind NO, any changes in pH on freezing that cross pH 7 give cause for concern. In the absence of albumin, two of the buffers traverse this region; the Tris/HCl sample at pH 6, which had an apparent pH of 7–7.5 after freezing, and the potassium phosphate sample at pH 8, which underwent an apparent acidification to pH 6.5. However, examination of the results in Table 1 that pertain to the albumin solutions, and are therefore considered most likely to reflect the conditions of the e.p.r. experiments, indicates that in all cases the buffers at pH 8 appeared more alkaline on freezing, whereas those at pH 6 either appeared more acidic or did not appear changed. Such pH variations do not give grounds to suspect that any differences between the e.p.r. spectra of samples prepared at the same room temperature pH, but in different buffers, arise as a result of NO binding, or release, from the haem *c* component. Any alkaline changes in the pH 8 samples would still not allow binding except at the haem *d*₁, whereas any acidic changes in the pH 6 samples would still allow binding at both the haems *c* and *d*₁.

E.p.r. spectra at pH 8.0

Multiple species. The e.p.r. spectra of the ¹⁴NO–haem *d*₁ derivative of the enzyme at 30 K in Tris/HCl, Mops, phosphate and Hepes buffers are shown in Fig. 2. The spectra are buffer-dependent, varying from a form in Tris/HCl buffer which appears due

Fig. 2. Effect of different buffers on the e.p.r. spectrum of NO-bound reduced *Pseudomonas* nitrite reductase at pH 8.0

The spectra were recorded at 30 K; the microwave power was 1.25 mW, microwave frequency 9.24 GHz, field modulation amplitude 0.63 mT and receiver gain 1.5×10^5 . All samples were at pH 8.0 in 0.1 M-buffer solution in the presence of 20 mM-ascorbate and 1.8 mM-KNO₂. Buffer and enzyme concentrations were as follows; (a) Tris/HCl, 29 μM; (b) Mops, 25 μM; (c) potassium phosphate, 29 μM; (d) Hepes, 32 μM.

to a pure rhombic species, to be called I, with $g_1 = 2.07$, $g_2 = 2.01$ and $g_3 = 1.96$, to that in Hepes buffer which is the most complex. The spectrum taken in Hepes buffer appears to consist of the rhombic species I but contains additional components at $g = 2.11$, $g = 2.05$ and $g = 1.98$. The hyperfine structure is less well resolved, presumably because of underlying signals from species other than I. Thus we conclude that there are multiple species in Hepes buffer.

The spectra of samples in Mops and phosphate are intermediate in character between those in Tris/HCl and Hepes. The hyperfine structure of species I becomes well resolved and new features can be seen at $g = 2.05$ and $g = 1.98$ (there is also a slight suggestion of a signal at $g = 2.11$ in phosphate buffer). It therefore appears that at least one other species, in addition to I, is also seen in Mops and phosphate buffers. Consideration of Figs. 2(b), 2(c) and 2(d) suggests that the signals at $g = 2.05$ and $g = 1.98$ need not necessarily arise from the same source as the feature at $g = 2.11$. Such a marked dependence of e.p.r. signals of nitrosyl haemoproteins upon the nature of the buffer does not appear to have been observed previously.

The low-temperature e.p.r. spectra of a wide range of nitrosyl ferrous haemoproteins are reported in the literature [e.g. cytochrome *c* oxidase (Blokzijl-Homan & Van Gelder, 1971; Stevens *et al.*, 1979), cytochrome *P*-450 (O'Keefe *et al.*, 1978), catalase, horseradish peroxidase and cytochrome *c* peroxidase (Yonetani *et al.*, 1972), cytochrome *c* (Kon & Kataoka, 1969; Yonetani *et al.*, 1972; Stevens *et al.*, 1979), myoglobin (Dickinson & Chien, 1971) and haemoglobin (Kon, 1968; Kon & Kataoka, 1969;

Shiga *et al.*, 1969; Chien, 1969; Yonetani *et al.*, 1972)] and all exhibit an e.p.r. absorption centred around $g = 2$, very similar to that of species I. Single-crystal studies on myoglobin (Dickinson & Chien, 1971) and haemoglobin (Chien, 1969) indicate g_x , g_z and g_y to be the low-, central- and high-field resonances respectively, where z is in the direction perpendicular to the haem plane. In the e.p.r. spectra of some of these nitrosyl haemoproteins additional signals have been observed. For example, Yonetani *et al.* (1972) reported an extra e.p.r. signal, labelled g_z , with a maximum between $g = 2.05$ and $g = 2.03$ in the nitrosyl derivatives of cytochrome *c* oxidase, catalase, cytochrome *c*, myoglobin and haemoglobin at 77 K in both K- and X-band studies. But no additional e.p.r. signals could be found in the spectra of the nitrosyl derivatives of horseradish peroxidase, cytochrome *c* peroxidase or lactoperoxidase under the same conditions.

In an attempt to discover the origin of the multiple e.p.r. signals of nitrosyl derivatives of *Pseudomonas* nitrite reductase the temperature dependences of the e.p.r. signals have been investigated. The results are shown in Fig. 3. The first spectrum, Fig. 3(a), shows the temperature dependence of the e.p.r. signals in Tris/HCl buffer at pH 8.0. The spectrum collapses from the pure rhombic form of species I at 70 K towards a spectrum at 230 K with a much decreased anisotropy and peaks at $g = 2.05$ and $g = 1.98$. On the other hand, the e.p.r. spectrum in Hepes buffer, Fig. 3(b), which contains at least two species at 30 K changes steadily until at 190 K the features of species I are lost and a broad spectrum results. The high-temperature spectrum in Fig. 3(b) can be interpreted in a number of ways.

Fig. 3. Effect of temperature on the e.p.r. spectrum of NO-bound reduced *Pseudomonas* nitrite reductase. The e.p.r. spectra of NO-bound *Pseudomonas* nitrite reductase in (a) 0.1 M-Tris/HCl, pH 8.0; (b) 0.1 M-Hepes, pH 5.8 and (c) 0.1 M-Tris/HCl, pH 5.8, were recorded at the temperatures indicated. In all cases the spectra were run after the addition of ascorbate and KNO_2 to concentrations of 20 and 1.8 mM, respectively and in the case of (b) the conditions were as for Fig. 2(d). For (a) and (c) the protein concentrations were 29 and 23 μM , respectively, with the same spectrometer settings as described for Fig. 2 except that the receiver gain was 2.5×10^5 for (a) and 2×10^5 for (c).

One possibility is that it represents a single rhombic species with g factors of 2.10, 2.02 and 1.98. However, since the spectrum of species I in Tris/HCl has been shown to collapse to a form showing decreased anisotropy at high temperature and, since the spectrum in Hepes at 30K clearly contains a large proportion of species I, it seems more probable that the high-temperature spectrum of Fig. 3(b) also consists of the overlapping spectra from at least two species. Presumably one species, the high-temperature form of I, dominates the central region and the other species is apparent only at $g = 2.1$, the remaining portions underlying the bands in the centre of the spectrum. Attempts to deconvolute and simulate the high temperature spectrum do not help to decide between these possibilities because of the large uncertainties in the line-widths of the different species.

Integrations of each of the spectra in Figs. 3(a) and 3(b) recovered a constant number of spins at all temperatures and similar results were also obtained

in Mops and phosphate buffers. This implies that variations in the form of the spectra do not arise as a result of a difference in the temperature dependences of the bandwidths of two distinct species not in equilibrium, but rather as a result of the interconversion of one species into another. Given this background of interconversion it does not seem unreasonable to compare those signals seen at $g = 2.05$ and $g = 1.98$ at high temperatures in Tris buffer with the features observed in the 30K spectra in Mops and phosphate buffers not assignable to species I.

In view of the fact that Williams-Smith *et al.* (1977) have indicated the possibility that freezing different buffer solutions can give rise to different apparent pH changes it is conceivable that the variation in the spectra of Fig. 2 could arise as a result of temperature-induced pH changes. However, on the basis of the observations presented in Table 1 for the four buffer systems at pH 8.0 in the presence of albumin, Mops and Hepes would be expected to

Fig. 4. Expanded e.p.r. spectra of ^{14}NO and ^{15}NO -bound reduced *Pseudomonas nitrite reductase* at pH 8.0 (a) Shows the expanded e.p.r. spectrum of ^{14}NO -bound reduced *Pseudomonas nitrite reductase*. The protein concentration was $22\ \mu\text{M}$ in the presence of $1.8\ \text{mM-KNO}_2$. (b) Shows a similar spectrum of the ^{15}NO -bound reduced enzyme in which the protein concentration was $29\ \mu\text{M}$ in the presence of $1.8\ \text{mM-Na}^{15}\text{NO}_2$ (99.2 atom % ^{15}N). The e.p.r. spectra were recorded at 30K. The microwave power was 1.25 mW, microwave frequency 9.24 GHz, field modulation amplitude 0.2 mT and receiver gain 4×10^5 . Both samples were in 0.1 M-Tris/HCl buffer, pH 8.0, in the presence of 20 mM-ascorbate. X indicates a paramagnetic impurity in the cryostat insert.

behave in a similar way to, but to go slightly less alkaline than, Tris and phosphate. Therefore, the observed differences between the spectra in Fig. 2 do not correlate in a consistent manner since, for example, the spectrum in Mops resembles more closely the spectrum in Tris than that in Hepes.

Quantification of e.p.r. signals. Quantification of the e.p.r. signals at pH 8.0 in phosphate, Hepes, Tris or Mops buffers for both ^{14}NO - and ^{15}NO -bound reduced *Pseudomonas* nitrite reductase, at various temperatures between 50 and 230 K and 1.25 mW microwave power, indicated in all instances that $45 \pm 5\%$ of the haems present were NO-bound. This result, together with the absorption data above, demonstrates that the haem d_1 moieties in both subunits of the enzyme are NO-bound at this pH. This is completely at variance with the spectrophotometric study of the same enzyme by Shimada & Oori (1978), who concluded that 'in the pH region higher than neutral the reduced haems remained unattacked [by NO] except at extreme alkaline pH'.

Hyperfine structure on the rhombic signal, I. The e.p.r. spectrum of the NO-bound haem d_1 derivative in Tris/HCl buffer at 30 K contained the smallest contribution from additional species and hence the hyperfine structure on the rhombic signal was more pronounced than in any of the other buffers used. Fig. 4 shows expanded e.p.r. spectra using a 0.2 mT modulation amplitude for both the ^{14}NO - and ^{15}NO -bound haem d_1 derivative at 30 K in Tris/HCl buffer. The hyperfine splitting on the central resonance of the ^{14}NO -bound haem d_1 spectrum is interpreted in terms of three sets of three lines arising from two non-equivalent ^{14}N atoms interacting with the unpaired electron. The origins of the hyperfine splittings were unambiguously assigned by analogous studies on ^{15}NO -bound haem d_1 , which has g values identical with those of the ^{14}NO -bound species, with a hyperfine pattern of a doublet of triplets. The transition from a triplet of triplets to a doublet of triplets, with a concomitant change in the larger hyperfine coupling constant from 2.4 to 3.3 mT on replacement of ^{14}NO with ^{15}NO , is consistent with the nuclear spin (I) and magnetogyric ratio (γ_N) of ^{14}N ($I = 1$, $\gamma_N = 1.934$) and ^{15}N ($I = \frac{1}{2}$, $\gamma_N = -2.712$). Thus, the larger hyperfine splitting results from the N nucleus of the bound NO. The magnitude of the smaller triplet hyperfine splitting, 0.65 mT, is unchanged, irrespective of whether ^{14}NO or ^{15}NO is bound and hence must be assigned to another axially bound, *trans* ^{14}N nucleus. Therefore, the sixth ligand on haem d_1 is shown to be N. These observations support the assignment of the central resonance to g_z and suggest that the unpaired electron from NO has undergone extensive delocalization over the Fe orbitals. The low- and high-field signals can be identified as the x and y absorptions in the plane of the

porphyrin ring and their broadness and apparent triplet hyperfine structure, of magnitude approx. 0.8 mT, irrespective of ^{15}NO or ^{14}NO binding, is probably best interpreted in terms of overlapping hyperfine structure from the four approximately equivalent ^{14}N atoms of the pyrrole groups in the porphyrin ring. Interactions with the pyrrole nitrogens would be maximal at these orientations if the unpaired electron is localized in the (d_{z^2}) orbital of the Fe (Kon & Kataoka, 1969).

The endogenous axial nitrogen ligand on haem d_1 could be contributed from a number of amino acids, such as histidine, arginine and lysine, or the peptide N atom. However, the magnitude of the hyperfine coupling constants for the *trans* axial nitrogen ligands in the reduced NO-bound derivatives of haemoglobin and cytochrome c (0.64 and 0.68 mT respectively) (Kon & Kataoka, 1969; Yonetani *et al.*, 1972) are very similar to those observed in this work. Imidazole from a histidyl residue is known to provide the axial ligand for both haemoglobin (Perutz, 1970) and cytochrome c (Dickerson *et al.*, 1971). Therefore, it is proposed that the endogenous axial nitrogen ligand for haem d_1 is supplied by the imidazole group of a histidyl residue.

E.p.r. spectra at pH 5.8

Multiple species. Fig. 5 shows e.p.r. spectra at 30 K for ^{14}NO -bound derivatives of the enzyme at pH 5.8 in Tris/HCl, Mops, phosphate and Hepes buffers. The spectra are generally similar in form to those at pH 8.0; however, the signals are noticeably broader with less well defined hyperfine structure than at pH 8.0, as would be expected for a superposition of both ^{14}NO -bound c and d_1 haems. All the spectra show evidence for the presence of multiple species and, as at pH 8.0, a variation in the form of the spectra with the buffer is evident. The spectrum recorded in Hepes still shows a signal at $g = 2.11$, although the spectrum in phosphate now contains no evidence of this feature. A detailed interpretation of the spectra is complicated because we now expect contributions from both NO-bound haems c and d_1 which may have differing dependencies. It is, however, interesting to note that there is no obvious low field, g_x , resonance in addition to that which has been assigned, from pH 8.0 data, to the haem d_1 . The origin of the additional signal at $g = 2.05$, seen to different extents in the different buffers, is uncertain because of the fact that both haems are now in the NO-bound state. The temperature dependence of the enzyme at pH 5.8 in Tris buffer, shown in Fig. 3(c), demonstrates behaviour analogous to that seen at pH 8.0, and the results from integrations again indicated inter-conversion of two spectral types. Compared with the spectrum at pH 8.0 (Fig. 3a) the decrease in

Fig. 5. Effect of different buffers on the e.p.r. spectrum of NO-bound reduced *Pseudomonas nitrite reductase* at pH 5.8

The spectra were recorded at 30 K; the microwave power was 1.25 mW, microwave frequency 9.24 GHz and field modulation amplitude 0.63 mT. All samples were at pH 5.8 in 0.1 M-buffer solution in the presence of 20 mM-ascorbate and 1.8 mM- KNO_2 . Buffer, enzyme concentrations and receiver gains were as follows; (a) Tris/HCl, 23 μM , 1.25×10^5 ; (b) Mops, 20 μM , 1.25×10^5 ; (c) potassium phosphate, 25 μM , 0.8×10^5 and (d) Hepes, 22 μM , 0.8×10^5 .

anisotropy appears even more marked, the spectrum at 230 K appearing close to isotropic. Our data are not inconsistent with the temperature dependence of the haem c -NO complex being similar to that of the haem d_1 -NO complex, but this matter also remains uncertain because of the lack of a signal that is clearly identifiable with haem c -NO.

The facts that require explanation are that, at both pH 8.0 and pH 5.8, multiple species are observed to different extents in different buffers at different temperatures. Recent e.p.r. studies (Yoshimura *et al.*, 1979) on nitrosyl protohaem dimethyl ester

complexes with imidazole derivatives in chloroform and toluene show the existence of two molecular species in frozen solutions that give rise to axial and rhombic e.p.r. spectra, respectively. On raising the temperature, the relative proportions of these two signals varies, with the axial signal predominating at 190 K. The g values of the low-temperature rhombic species from the model system with imidazole as the base and chloroform as the solvent are $g_1 = 2.072$, $g_2 = 2.004$ and $g_3 = 1.971$, which are remarkably similar to the values obtained for species I at pH 8.0 in the present work. The axial species, having g values of 2.03 and 1.987, could well be comparable with the corresponding features seen in the spectra in Mops and phosphate buffers in Fig. 2. The similarity of the model systems to our protein data also extends to the temperature dependence. As the temperature is raised to approx. 200 K the relative proportion of the axial and rhombic signals change in the e.p.r. spectrum of the 1-methyl-imidazole derivative of nitrosyl protohaem dimethyl ester, so that above 190 K the spectra are very similar to those at high temperature in Figs. 3(a), 3(b) and 3(c), except that the broad weak feature at $g = 2.10$ in Fig. 3(b) is never seen. Thus the work of Yoshimura *et al.* (1979) strongly suggests that rhombic and axial species are present in the nitrosyl nitrite reductase of *Pseudomonas aeruginosa*. Yoshimura *et al.* (1979) have proposed that an increase in the Fe-N-O bond angle is responsible for the axial species they observed, although it is also possible that an axial signal might arise as a result of increased rotation of a bent Fe-N-O system at high temperatures. Whatever the reason behind the observed spectral interconversions, the nitrosyl protohaem model systems do not explain the signal at $g = 2.11$ seen in Hepes buffer. We are unable to account for this signal, although we note that g_x values around 2.1 are not unknown for nitrosyl haemoproteins [e.g. cytochrome c oxidase ($g = 2.09$)]. It is possible that there are in fact two types of rhombic species formed on haem-NO binding in Hepes.

As was the case at pH 8.0, an argument can be raised as to whether different pH changes on freezing the different buffers are responsible for the various forms of spectrum in Fig. 5. Table 1 suggests that, on freezing the albumin-containing buffers at pH 6, Tris would be expected to be the most acidic, followed by phosphate, Hepes and Mops. Such a series does not rationalize the appearance of a signal at $g = 2.11$ in Hepes alone, nor does it, for example, parallel the relative prominence of the signal at $g = 2.05$. This being so, it seems more likely that it is a buffer-protein interaction that is the cause of the spectral variation.

Quantification of e.p.r. signals. Quantification of the e.p.r. signals at pH 5.8 at temperatures in the

range 50–230 K on numerous occasions consistently accounted for $85 \pm 10\%$ of the haems present being NO-bound, irrespective of the buffer used. This corroborates the indications of the spectrophotometric studies; namely, that NO is bound to the *c* and *d*₁ haems in both subunits of the enzyme.

Hyperfine structure on the rhombic signal, I. Fig. 6 shows expanded e.p.r. spectra at 30 K using a 0.2 mT modulation amplitude for both the ¹⁴NO- and ¹⁵NO-bound derivatives of the enzyme in Tris/HCl buffer, pH 5.8. A comparison with the spectra in Fig. 4, where only haem *d*₁ was NO-bound, indicates that the hyperfine splitting is less pronounced in this instance, but with identical hyperfine coupling constants. Whether this results from the NO-bound haem *c* having hyperfine splitting almost identical with that of NO-bound haem *d*₁, or from haem *c* having no hyperfine structure on *g*_z, cannot

be determined at the present time. Thus little can be stated concerning the nature of the axial ligand on haem *c* as a result of this investigation.

Finally, it is of interest to compare the results of this work with those of studies on the nitrite reductase from *Thiobacillus denitrificans*, which is an enzyme with *c* and *d*₁ haems and absorption spectral characteristics identical with the nitrite reductase from *Pseudomonas aeruginosa* in both the oxidized and reduced states (LeGall *et al.*, 1979). However, the low-spin haem *c* signal observed in the e.p.r. spectrum at 10 K of oxidized *Pseudomonas* nitrite reductase (Gudat *et al.*, 1973; Walsh *et al.*, 1979) was not observed for the oxidized nitrite reductase from *Thiobacillus denitrificans*. An NO-bearing derivative of the reduced enzyme at pH 7.6 (formed in a manner analogous to that in the present work) was also reported and the e.p.r. spectrum at 26 K in

Fig. 6. Expanded e.p.r. spectra of ¹⁴NO- and ¹⁵NO-bound reduced *Pseudomonas* nitrite reductase at pH 5.8 (a) Shows the expanded e.p.r. spectrum of ¹⁴NO-bound reduced *Pseudomonas* nitrite reductase, the protein concentration was 23 μ M in the presence of 1.8 mM-KNO₂ and receiver gain was 4×10^5 . (b) Shows a similar e.p.r. spectrum of ¹⁵NO-bound reduced enzyme in which the protein concentration was 25 μ M in the presence of 1.8 mM-Na¹⁵NO₂ (99.2 atom % ¹⁵N) and receiver gain was 3.2×10^5 . The spectra were recorded at 30 K and e.p.r. spectrometer settings (apart from receiver gains) were as for Fig. 4. Both samples were in 0.1 M-Tris/HCl buffer, pH 5.8, in the presence of 20 mM-ascorbate.

phosphate buffer was qualitatively similar to that observed in the present work, except that no hyperfine splitting due to a *trans* axial ligand was resolved. Quantification of this e.p.r. spectrum was reported to account for NO-binding to both *c* and *d*₁ haems, although the conditions under which such quantifications were performed are not stated (LeGall *et al.*, 1979). It should be noted that, in the present work with *Pseudomonas* nitrite reductase, quantifications of reduced NO-bound haem signals gave low results for spectra obtained at less than 50 K and 1.25 mW microwave power because of signal saturation effects.

NO-bound, oxidized Pseudomonas nitrite reductase at pH 6.0

Spectrophotometric studies indicate that oxidized *Pseudomonas* nitrite reductase binds NO to both haem *c* and *d*₁ at pH 6.0, when placed under an atmosphere of the gas (Silvestrini *et al.*, 1979). However, no e.p.r. signals were observed for this derivative over the temperature range 6–100 K, which suggests that the unpaired electron of the bound NO is spin-paired with the unpaired d electron on both low-spin haems *c* and *d*₁.

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