EPR and Electron Nuclear Double Resonance (ENDOR) Studies Show Nitrite Binding to the Type 2 Copper Centers of the Dissimilatory Nitrite Reductase of *Alcaligenes xylosoxidans (NCIMB 11015)*

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ABSTRACT: EPR and ^{1}H, $^{14,15}N$ ENDOR spectra are described for the type 1 and type 2 Cu(II) centers of dissimilatory nitrite reductase (NiR) from *Alcaligenes xylosoxidans*. The study was carried out on preparations of NiR containing both type 1 and type 2 Cu sites, and also on preparations of lower activity which contained essentially only type 1 Cu centers. This has enabled ENDOR studies of type 1 and type 2 sites to be carried out largely independently of each other, by appropriate choice of the excitation field. Spectra were recorded both in the absence and presence of nitrite, allowing a clear determination of which of the two types of Cu center constitutes the substrate binding site. The EPR results show large changes in the type 2 site $g_{\parallel}$ (which decreases by 0.065) and $^{Cu}A_{\parallel}$ (which increases by 2.0 mT) while the type 1 site EPR is not affected. In addition, both ^{1}H and ^{14}N ENDOR of the type 2 Cu site undergo considerable changes on addition of nitrite whereas the type 1 Cu site ENDOR is unaffected. Our results clearly demonstrate that nitrite binds to the type 2 copper and that this process significantly perturbs the ligation of this copper by the protein histidine residues. No ^{15}N ENDOR resonances were observed from ^{15}N nitrite. The accessibility of the copper sites to solvent has been studied using $^{2}H_2O$. The results indicate that nitrite binds to the type 2 Cu by displacing a proton, probably on a water molecule bound to the copper atom.

Dissimilatory[1] nitrite reductase is the key enzyme in the anaerobic respiratory process by which denitrifying bacteria reduce nitrate to the gaseous products NO, N_2O, or N_2, since it is at this point in the denitrification pathway that significant losses of fixed nitrogen from the soil to the atmosphere occur. Dissimilatory nitrite reductases (NiR) have been purified from a number of organisms, and it is clear that there are two classes of enzyme which have different types of redox centers, one class containing diheme (heme *c,d1*) and the other copper as the redox active centers [see Hochstein and Tomlinson (1989) and Zumft (1991)].

The copper-containing NiRs are apparently a highly conserved group of enzymes since a 1.9-kb DNA probe carrying the structural gene of NiR (nir U) of *Pseudomonas* sp. G-179 hybridized to DNA of 15 out of 16 species with putative Cu-NiRs, whereas DNA of organisms with a heme-NiR mostly (7 out of 10) showed no hybridization (Ye et al., 1993). In addition, significant homology between Cu-NiRs has been revealed by immunoblot analysis (Michalski & Nicholas, 1988; Coyne et al., 1989).

The X-ray crystal structure of *Achromobacter cycloclastes* NiR at 2.3-Å resolution shows a trimeric structure with a type 1 Cu center buried in each of the subunits, and the type 2 Cu centers at the subunit interface and coordinated by ligands from two subunits (Godden et al., 1991). Comparison of the amino acid sequence of *A. cycloclastes* NiR with the derived sequence of NiR of *Pseudomonas* sp. G-179 shows 78% identity and that the ligands involved in coordination of both type 1 and type 2 Cu in *A. cycloclastes* are conserved (Ye et al., 1993). Consistent with the strong homology indicated above, the enzymes of *A. cycloclastes* (Iwasaki et al., 1975), *Bacillus halodenitrificans* (Denariaz et al., 1991), *Rhodobacter sphaeroidies* (Michalski & Nicholas 1985), and *Alcaligenes faecalis* Strain S-6 have been purified and shown to contain both type 1 and type 2 copper centers. These observations strongly suggest that the Cu-NiRs have similar structures and redox centers.

In agreement with this, and contrary to previous reports (Masuko et al., 1984), we have shown that NiR of *Alcaligenes* (formerly *Achromobacter*) *xylosoxidans* contains both type 1 and type 2 Cu centers (Abraham et al., 1993), and also, using the small angle X-ray scattering technique, that the enzyme is a trimer in solution (Grossman et al., 1993).

The presence of two types of Cu center in these enzymes raises the question as to which of them provides the substrate binding site? In general, for Cu proteins, type 1 Cu centers have been found to mediate electron transfer, and type 2 centers are the active sites where substrate binding and reduction occur (Spiro, 1981).

In the present paper, we report ^{1}H, ^{14}N and ^{15}N ENDOR studies on preparations of NiR of *A. xylosoxidans* containing both type 1 and type 2 Cu centers and also preparations of lower activity which contain essentially only type 1 Cu centers (type 2-deficient NiR). Data are presented which show clearly that NO_2^- binds to the type 2 Cu center to displace a proton, probably from bound water, from the Cu atom. The ligation of the type 2 Cu by nitrogen of protein histidine residues is also considerably perturbed by the binding of NO_2^-.

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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MATERIALS AND METHODS

Isotopes and Sample Preparation. $Na^{15}NO_2^-$ 98.8 gatom % was obtained from Prochem (BOC Ltd.) and was added either as aliquots of a 100 mM solution or as a solid to solutions of NiR as appropriate. ^2H_2O (99.9 gatom %) was obtained from Goss Scientific. Samples of NiR in deuterated buffer were prepared using a P6DG desalting column (0.8 × 8 cm) equilibrated with the deuterated buffer and were then concentrated by centrifugation at 3000*g* using Amicon Centricon microconcentrators at 5 °C.

Organism and Enzyme Purification. The organism used was *A.* (formerly *Achromobacter*) *xylosoxidans* subsp. *xylosoxidans* (NCIMB 11015). In some earlier biochemical work this organism was designated as *Pseudomonas denitrificans* [see Zumft (1991)]. Conditions for the growth, harvesting, and purification of NiR from crude extracts which had been activated by the addition of $CuSO_4$ were as previously described (Abraham et al., 1993). NiR activity was assayed by measuring NO_2^- utilization with the dithionite-reduced methyl viologen system as electron donor. The specific activity of the NiR used in this study was 243 μmol of NO_2^- reduced/(min·mg of protein) (Abraham et al., 1993). The type 2-deficient NiR used in these studies was purified using essentially the same procedures except that the activation step of adding $CuSO_4$ to crude extracts was omitted; the specific activity of this protein was 20.2 μmol of NO_2^- reduced/(min·mg of protein).

EPR and ENDOR Measurements. ENDOR spectra were recorded on an updated Bruker ER 200D-SRC spectrometer equiped with an ENDOR/TRIPLE accessory and a radiofrequency amplifier of 100 W nominal output power (ENI 3100LA). Low-temperature measurements were made using an Oxford Instruments ESR 900 cryostat modified to take sample tubes of up to 4-mm internal diameter. Recording conditions were as follows, unless specified otherwise: temperature, 15 K; microwave power, 5 mW; radiofrequency power, 0 dB attenuation; modulation depth, 50 kHz; scan rate, 0.21 MHz s^{-1}. All ENDOR spectra were accumulated over extended periods, of up to 6 h, to obtain adequate signal-to-noise ratios.

EPR spectra were recorded under the following conditions, unless specified otherwise: temperature, 120 K; microwave power, 10 mW; microwave frequency, 9.4 GHz; modulation amplitude, 0.5 mT. EPR spectral parameters were determined by simulation (Lowe, 1978). Hyperfine tensors were determined from ENDOR spectra by visual inspection following the concepts of Hoffman et al. (1984).

The electronic *g* tensor for Cu(II) ions has typically axial or nearly axial symmetry. The EPR spectra reported in this work for both the type 1 and type 2 Cu centers are consistent with a $d_{x^2-y^2}$ ground state and $g_\parallel > g_\perp > 2$. Type 1 Cu centers are characterised by an intense blue colour and $^{Cu}A_\parallel < 7$ mT. Type 2 Cu centers are generally representative of typical Cu(II) complexes with $^{Cu}A_\parallel > 13$ mT. The reader is referred to reviews (Solomon et al., 1992; Adman, 1991) for a full discussion of copper centers in proteins.

The ENDOR spectrum for a set of magnetically equivalent protons is a pair of lines centered on the proton Larmor frequency and split by the orientation dependent superhyperfine coupling constant *A*, with frequencies given by

$$\nu_\pm = \nu^H \pm A/2 \qquad (1)$$

A resonance can be clearly identified as resulting from protons by exploiting the proportional relationship between the proton Larmor frequency and the applied magnetic field, and therefore the microwave frequency. Tuning the spectrometer to a different microwave frequency mode shifts the magnetic field at the *g* value of interest, and consequently the Larmor frequency is shifted by a known amount.

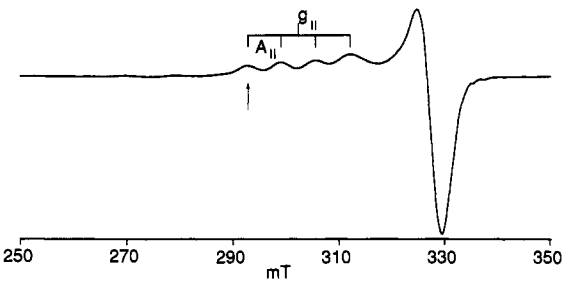

FIGURE 1: EPR spectrum of a sample of type 2-deficient nitrite reductase as isolated, comprising approximately 90% type 1 and 10% type 2 copper by integrated intensity. The protein concentration was 240 mg/mL in 50 mM Hepes buffer, pH 7.2. The stick diagram above the spectrum shows the positions of the four features resulting from the copper nuclear spin of $^3/_2$ for the type 1 site. The arrow shows the position of the type 1 copper $M_I = -^3/_2$ line where the ENDOR spectra of Figures 4 and 9 were measured.

The samples employed in the present study are frozen solutions and thus contain a random distribution of all protein orientations. However, it is well known that ENDOR spectra recorded with the magnetic field set at the extreme of the EPR spectrum give single-crystal-like patterns (Rist & Hyde, 1970). An ENDOR spectrum obtained at an intermediate *g* value does not result from a single orientation but from a well-defined subset of molecular orientations. A ^1H set that gives rise to only a pair of ENDOR peaks at the extrema of the EPR spectrum can display up to 12 ENDOR features at intermediate *g* values as a consequence of the multiple orientations present at such *g* values. Therefore, instead of only a pair of peaks at ν_+ and ν_-, one can expect an identical series of (up to six) peaks for ν_+ and ν_- (Hoffman et al., 1984). To simplify presentation, it is easier to consider only one of the series ν_+ or ν_-. The principal values of a hyperfine tensor and its orientation with respect to the *g*-tensor frame can be determined by obtaining a series of ENDOR spectra across the EPR spectrum (Hoffman et al., 1984; True et al., 1988). This information provides a means of placing the nucleus in question into the local structure around the metal center.

The ^{14}N ($I = 1$) ENDOR pattern consists of four peaks at frequencies given by (Atherton, 1973):

$$\nu_{\pm,m}(^{14}N) = \frac{A(^{14}N)}{2} \pm \nu(^{14}N) + \frac{3P(^{14}N)}{2}(2m-1) \qquad (2)$$

where *A* and *P* are orientation-dependent ^{14}N hyperfine and quadrupole coupling constants, respectively, the nuclear spin projection is $m = 1$ or 0, and $\nu(^{14}N)$ is the nitrogen Larmor frequency. When $A/2 > \nu > P$, eq 2 results in a four-peak pattern centered on $A/2$, split by 2ν, and further split by the quadrupole interaction.

RESULTS

EPR Studies

Two Forms of Enzyme. EPR spectra of type 2-deficient NiR, as isolated, and NiR (activated by the addition of Cu^{2+} to crude extracts; Abraham et al., 1993) are shown in Figures 1and 2,respectively. Integration of these EPR spectra indicates that the type 2-deficient enzyme contains less than 10% type 2 Cu centers whereas NiR contains approximately 50% type 1 and 50% type 2 Cu centers as reported previously by Abraham et al. (1993). Comparison of Figures 1 and 2 shows that the type 2-deficient sample displays essentially an EPR spectrum characteristic of type 1 Cu centers and very weak

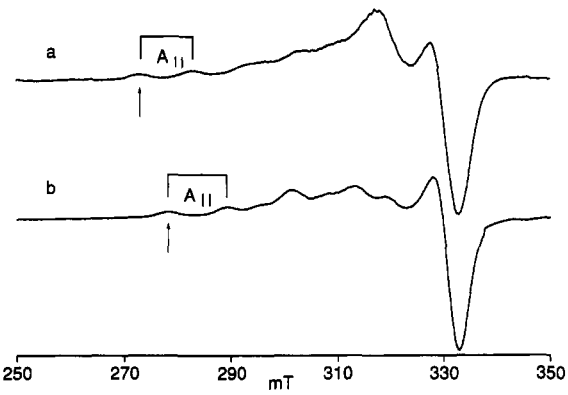

FIGURE 2: EPR spectra of nitrite reductase comprising approximately 50% type 1 and 50% type 2 copper by integrated intensity. The protein concentration was 110 mg/mL in 50 mM Hepes buffer, pH 7.4. The sample is (a) in the absence of nitrite and (b) in the presence of 6 mM nitrite. The stick diagrams above the spectra show the magnitude of the type 2 $^{Cu}A_{\|}$ value in each case. The arrows show the position of the type 2 copper $M_I = -3/2$ line where the ENDOR spectra of Figures 3, 5, and 7 were measured. Spectra were recorded at 10 K and 1 mW microwave power.

Table 1: EPR Parameters of Type 1 and Type 2 Cu Sites

	$g_{\|}$	$g_{\perp}$	$^{Cu}A_{\|}$ (mT)	$^{Cu}A_{\perp}$ (mT)[a]
type 1	2.212	2.036	6.3	0
type 2				
without NO_2^-	2.355	2.038	9.0	0
with NO_2^-	2.290	2.045	11.0	0

[a] The $^{Cu}A_{\perp}$ value is poorly defined within the EPR line shape and was set to zero in the simulations.

type 2 Cu features to low field [see Solomon et al. (1992) for a review of type 1 and type 2 Cu centers]. NiR gives an EPR spectrum which is the sum of type 1 and type 2 subspectra with the two lowest field features derived solely from type 2 Cu centers. The availability of these two types of sample allows us to make observations that distinguish the type 1 and type 2 Cu sites, largely independently of each other, by appropriate choice of the excitation field in the ENDOR study.

Effect of Substrate. Figure 2 also shows the effects on the EPR of the type 2 Cu centers caused by addition of 6 mM nitrite, a concentration twice that of the protein monomer and equivalent to one nitrite per copper binding site. The $g_{\|}$ of type 2 Cu decreases by 0.065 and the $^{Cu}A_{\|}$ increases by 2.0 mT. No further changes occur up to a nitrite concentration of at least 4 M. The EPR of the type 1 Cu center is unaffected by addition of nitrite up to at least 1 M. The EPR parameters of all the samples are listed in Table 1. Abraham et al. (1993) reported a type 2 $^{Cu}A_{\|}$ value for *A. xylosoxidans* NiR of 14.2 mT in their best defined spectrum, contrasting with our value of 9 mT, although a range of values was observed for the type 2 species shown in their spectra. However, in the earlier work, it was noted that these values were dependent on the preparation for reasons which remain unclear although they were not due to the nature of the sample buffer. The enzyme preparations used in the present study repeatedly gave values consistent with those shown in Table 1.

Type 2 Cu Center ENDOR

An ENDOR study of the type 2 Cu center by excitation at the $^{Cu}M_I = -3/2$ feature of the type 2 EPR, using NiR, showed pronounced changes in the ^1H and ^{14}N ENDOR patterns on addition of 6 mM nitrite (Figure 3) which were unaffected on addition of further nitrite up to at least 4 M.

^{14}N ENDOR. The $g_{\|}$ single-crystal-like spectrum of the enzyme in the absence of substrate (Figure 3a) shows three peaks at approximately 18, 20, and 22 MHz which are

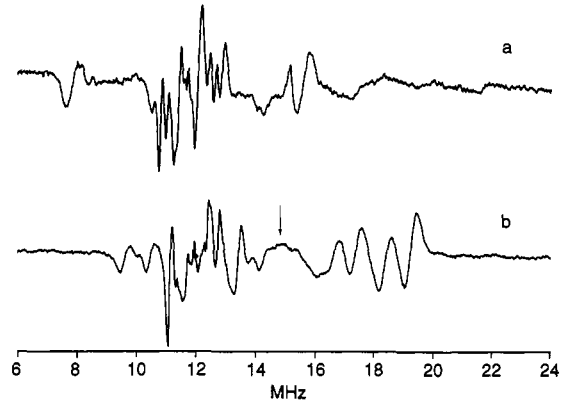

FIGURE 3: ENDOR spectra of nitrite reductase excited at the $^{Cu}M_I = -3/2$ feature of the type 2 EPR (a) in the absence of nitrite and (b) in the presence of 6 mM nitrite. The samples were those of Figure 2. The arrow indicates a peak at 14.8 MHz provisionally assigned to a protein ^{14}N nucleus. Spectra were recorded at 10 K and 6.3 mW microwave power.

provisionally assigned to ν_+ of three magnetically inequivalent ^{14}N nuclei directly coordinated to copper. The ν_- partners to lower frequency are too weak to be detected, and the absence of quadrupole splitting is consistent with previous observations for histidine nitrogens coordinated to copper (Werst et al., 1991). The pattern did not shift when the spectrometer was tuned to a higher frequency mode where larger Larmor frequencies result (data not shown). The shift in the nitrogen frequency would be barely perceptible whereas the proton frequency shift would be 0.6 MHz. The hyperfine couplings (~40 MHz) of these three features is also well within the range of ^{14}N nuclei directly coordinated to Cu(II) complexes (Iwaizumi et al., 1986). It is clear from Figure 3b that addition of $^{14}NO_2^-$ results in a completely new ^{14}N ENDOR pattern. The three features assigned to ^{14}N protein nuclei at 18, 20 and 22 MHz in the absence of NO_2^- appear to have been replaced by the four-peak ^{14}N pattern in the frequency range 16–20 MHz. The pattern once again did not shift on tuning to a higher microwave frequency mode and was invariant whether $^{15}NO_2^-$ or $^{14}NO_2^-$ was employed (data not shown). It is immediately apparent that the four-peak pattern cannot result from the substrate nitrogen as the different nuclear spin and magnetic moments of ^{15}N and ^{14}N would produce clearly observable changes. A degree of ambiguity can often exist in assigning ^{14}N resonances. This is because the observed ENDOR pattern depends critically on the magnitude of the quadrupole coupling. If the quadrupole splitting is well resolved, the expected four-peak pattern is observed. However, when this is not the case only a single Larmor split doublet centered on $A/2$ results. A four-peak pattern may therefore arise from a single type of nitrogen with resolved quadrupole splitting, from two (or even four) inequivalent nitrogens which do not exhibit resolved quadrupole splittings. In the present case, contrary to the previous observations (Werst et al., 1991) for histidine nitrogens coordinated to copper, the four-peak protein ^{14}N pattern at $g_{\|}$ can be assigned to a single type of nitrogen with resolved quadrupole coupling (A = 35.8 MHz, P = 0.3 MHz). Further ^{14}N ENDOR studies are in progress to define more clearly the nitrogen atoms which give rise to this four-peak pattern.

^1H ENDOR. In the absence of NO_2^- a number of weakly coupled sets of ^1H peaks ($A < 2.5$ MHz) and two strongly coupled sets of ^1H with A = 6.8 and 8.0 MHz are observed at the type 2 Cu center (Figure 3a). Following the addition of NO_2^-, the ^1H pattern is radically different (Figure 3b). In fact it is difficult to find any ^1H set that maintains its previous hyperfine coupling. Once again there are a number of ^1H sets

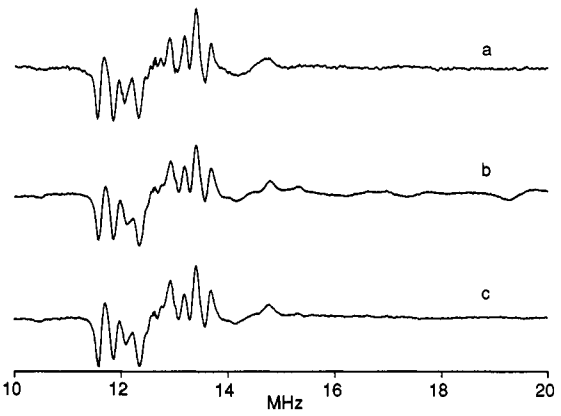

FIGURE 4: ENDOR spectra of type 2-deficient nitrite reductase excited at the $^{Cu}M_I = -3/2$ feature of the type 1 EPR (a) in the absence of nitrite, (b) in the presence of 1 M nitrite, and (c) panel b minus the proportion of a spectrum of (activated) nitrite reductase, recorded at the same g value, required to cancel out the ^{14}N features. The protein concentration was 240 mg/mL in 50 mM Hepes buffer, pH 7.2.

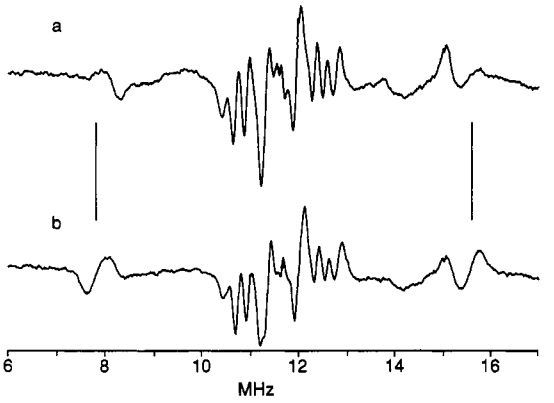

FIGURE 5: ENDOR spectra of nitrite reductase in the absence of nitrite excited at the $^{Cu}M_I = -3/2$ feature of the type 2 EPR (a) in ^2H_2O and (b) in ^1H_2O. The bars indicate the solvent exchangeable ^1H set discussed in the text. The protein concentrations were (a) 68 mg/mL in 50 mM Hepes buffer, pD 7.2, and (b) 269 mg/mL in 50 mM Hepes buffer, pH 7.2.

with couplings less than 2.5 MHz and two slightly more strongly coupled ^1H sets at $A = 3.6$ and 5.0 MHz. All but one of the features described above, for enzyme samples both with and without substrate, are unequivocally assigned to ^1H nuclei, as tuning to a higher frequency mode gives a shift in the ^1H patterns of the expected amount without perturbing the line shape. The exception is a peak at 14.8 MHz which we provisionally assign to protein ^{14}N. A number of ^1H sets for both samples shown in Figure 3 display a degree of asymmetry in their intensity that is the reverse of that normally observed in ENDOR spectra, with the low-frequency partner being the more intense. It is known that such effects can result from spin cross-relaxation (Poole & Farach, 1971) which has been proposed on a number of occasions (Gurbiel et al., 1989; Lubitz & Nyronen, 1980) to be active between proton and nitrogen nuclei. Such a mechanism is almost certainly the cause of the anomalous relative intensity effects observed in our ^1H ENDOR spectra.

Type 1 Cu Center ENDOR

The type 1 Cu center can be studied without a large influence from the type 2 Cu center by using type 2-deficient NiR. However, due to the nonnegligible amount of type 2 Cu centers (<10%) present, some minor contribution to the ENDOR spectrum from type 2 Cu centers must be expected. Figure 4 shows the effects on the ENDOR at the magnetic field corresponding to the $M_I = -3/2$ line of the type 1 Cu centers in type 2-depleted NiR, caused by addition of NO_2^-.

In the absence of NO_2^- (Figure 4a) the ENDOR spectrum is characterized by a number of sets of protons with couplings less than 4.3 MHz. There is no evidence for ^{14}N couplings. On addition of substrate a series of new features appear in the spectrum (Figure 4b). The most prominent of these occur at 15.4, 17.6, and 19.5 MHz. These new features are shown to be due to the presence of a small amount of type 2 Cu centers by subtraction of an appropriate proportion of an ENDOR spectrum of the activated enzyme, recorded under identical conditions to those of Figure 4b. The removal of the contribution of the type 2 centers from Figure 4b leaves a spectrum (Figure 4c) which is essentially unchanged on addition of substrate. The small differences remaining in the relative intensities of the ^1H ENDOR, however, mean that we cannot completely exclude the possibility that there are minor changes in the local structure of the type 1 Cu site induced by the addition of nitrite.

It should be noted that there is no evidence from this ENDOR study for nitrogen coupling from NO_2^- to either Cu center. The effective low-frequency limit for detection of ENDOR peaks in the spectrometer used in the present work is 4 MHz, due to quite intense instrumental artifacts produced below this frequency at fractions of the proton Larmor frequency (ν^H/n). Any nitrogen nucleus with resonant frequencies in this range would have its ENDOR pattern centered on $A/2$, implying that the substrate nitrogen must have a hyperfine coupling $A \leq 8$ MHz. The X-ray structure defines an approximately tetrahedral structure for the Cu atom in the type 2 center (Godden et al., 1991). The weakness of this coupling leads us to speculate that the substrate is either coordinated at the apical position of the tetrahedron or through an oxygen in a planar position (Jiang et al., 1993). The latter possibility can be excluded (see below).

Solvent Exchangeable Protons

The data presented above demonstrate that NO_2^- binds to the type 2 Cu which the X-ray structure of *A. cycloclastes* NiR shows is ligated by three histidine residues and a water molecule. We have compared samples of NiR prepared in ^1H_2O and ^2H_2O to study the environment of this Cu center in *A. xylosoxidans* NiR.

Type 2 Center without NO_2^-. The ^1H ENDOR spectra of NiR in ^1H_2O and ^2H_2O in the absence of substrate, excited at the lowest field type 2 EPR feature (Figure 5), clearly demonstrate that there is at least one set of exchangeable protons ($A = 8.0$ MHz). There are small changes in relative intensity of other ^1H sets which may result from $^1H/^2H$ exchange. However, they could also be due to changes in relaxation pathways established by the ^2H nuclei. In the absence of ^2H ENDOR (as a consequence of the instrumental artifacts discussed above) we are reluctant to assign small changes of relative intensity as being the result of $^1H/^2H$ exchange.

The clearly exchangeable ^1H observed in Figure 5 has been monitored across the whole EPR envelope, and the results in Figure 6 show the positions of the peaks at the frequency extremes as a function of g value. The approximate hyperfine tensor determined from Figure 6 is reported in Table 2. The observation that the g and A tensors are coaxial, with $^H A_\parallel$ parallel to $g_\parallel$ since $\alpha = \beta = \gamma = 0$, allows a straightforward determination of the orientation of the Cu–^1H bond with respect to the local symmetry axes. In the present case with a $d_{x^2-y^2}$ Cu ground state and approximately tetrahedral symmetry, $g_\parallel$ is expected to be directed toward the apical

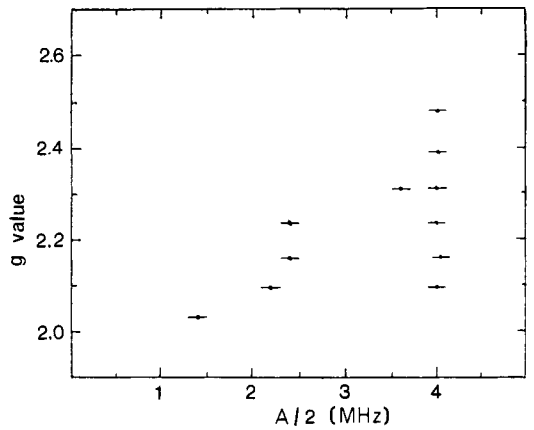

FIGURE 6: Plot of the peak postitions at the frequency extrema of the ν_+ exchangeable ^{1}H ENDOR pattern *versus* the observing g value for nitrite reductase in the absence of nitrite.

Table 2: Superhyperfine[a] Coupling Constants (MHz) of Type 2 Exchangeable Protons from ENDOR Spectroscopy

	$^HA_\parallel$	$^HA_\perp$	α	β	γ	Cu–^{1}H distance (Å)[b]
without NO_2^-	8.0	2.8	0	0	0	2.9
with NO_2^- [c]	8.6	5.2				3.5

[a] The hyperfine tensors were determined by visual inspection of the ENDOR spectra (see Materials and Methods), not simulation, and are thus approximate. [b] Values are approximate. See the text for a discussion. [c] Euler angles α, β, and γ have not been determined, but from the experimental behavior one or more are clearly nonzero.

position of the tetrahedron and $g_\perp$ lies within a plane which can be considered approximately equatorial containing the half-occupied d orbital. The largest principal value of the A tensor ($^HA_\parallel$) defines the metal–^{1}H direction. This is approximately coincident with $g_\parallel$ (Table 2) and thus supports the conclusion of the X-ray analysis that the apical position of the local Cu site structure is occupied by a water molecule or hydroxyl ion.

A note of caution must be raised at this point. The field-dependent behavior displayed by the exchangeable ^{1}H shown in Figure 6 could also be consistent with $^HA_\parallel$ perpendicular to $g_\parallel$ [see Hoffman et al. (1984) for an analysis of the behavior expected in both cases]. The doubt is based on the observation of ^{1}H features exhibiting apparent isotropic behavior across the EPR envelope with A = 8.0 MHz, coincident with a principal A value of the exchangeable ^{1}H under consideration. When the hyperfine interactions are small, if $^HA_\parallel$ is parallel to $g_\parallel$ one expects only a series of ENDOR peaks running smoothly from $^HA_\parallel$ to $^HA_\perp$ across the EPR envelope. However, if there is a hyperfine interaction present large enough to perturb the distribution of molecular orientations, as exemplified in the present work by ^{Cu}A, a similar trend is expected but the detailed behavior of the peak positions will differ. An interpretation in which $^HA_\parallel$ is parallel to $g_\parallel$ is favored for the following reasons: (1) the data of Figure 6 result from a sample of pH 7.4; however, very similar data result from a sample of pH 5.2 (data not shown). The relevance of the pH 5.2 data lies in the fact that the X-ray structure of NiR was determined on a sample at pH 5.2. This showed evidence for a coordinated water molecule in a direction approximately parallel to $g_\parallel$ but no ligands in the equatorial plane (perpendicular to $g_\parallel$). (2) If $^HA_\parallel$ is assumed perpendicular to $g_\parallel$, the data of Figure 6 require that $^HA_\perp > {}^HA_\parallel$, which is impossible. In the most general case, the ligand superhyperfine tensor is fully asymmetric, and it is impossible to find a coordinate system where the hyperfine tensor is diagonal. However, when the g anisotropy is small compared with the average g value, as is the case in the present work, association of a unique

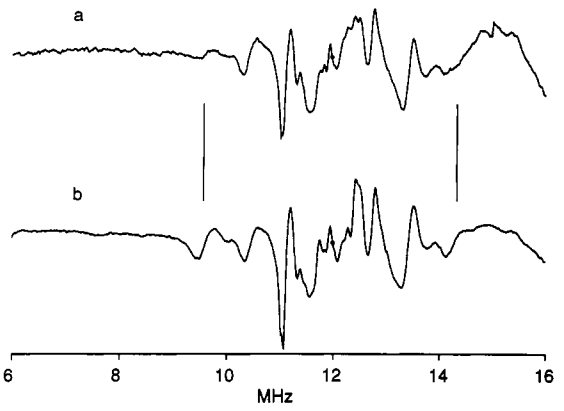

FIGURE 7: ENDOR spectra of nitrite reductase in the presence of nitrite excited at the $^{Cu}M_I = -^3/_2$ feature of the type 2 EPR (a) in ^2H_2O and (b) in ^1H_2O. The bars indicate the solvent exchangeable ^{1}H set discussed in the text. The protein concentrations were (a) 58 mg/mL in 50 mM Hepes buffer, pD 7.4, plus 6 mM nitrite and (b) 110 mg/mL in 50 mM Hepes buffer, pH 7.4, plus 6 mM nitrite. Spectra were recorded at 10 K and 6.3 mW microwave power.

tensor coordinate system with a ligand becomes a valid concept. In this case, the maximum hyperfine component occurs along the metal–nucleus vector ($A_\parallel$ axis) and the minimum component when the field is in the $A_\perp$ plane. The adoption of an interpretation, with $^HA_\parallel$ parallel to $g_\parallel$, implies that the peaks with an isotropic ^HA = 8 MHz belong to another exchangeable ^{1}H set. It is considered impossible that the peaks with ^HA of 8.0 MHz have intensity at field positions extending so far across the EPR envelope due to orientational admixture resulting from the large $^{Cu}A_\parallel$ value.

The point–dipole model together with the dipolar component of the ^{1}H hyperfine tensor determined from Table 2 enables the Cu–^{1}H distance to be estimated (Howes et al., 1991). The calculation relies on a knowledge of the effective electron spin density at the Cu nucleus. A value of 0.65 was found for the spin density remaining on the Cu ion which places the exchangeable ^{1}H distant 2.9 Å. The spin density value was determined from a comparison of the expressions for the total hyperfine coupling constants (Kivelson & Neimar, 1961; Solomon et al., 1983) and the experimentally obtained Cu hyperfine tensor reported in Table 1. A typical value of the Cu spin density found for Cu complexes representative of type 2 Cu sites is 0.8 [Solomon et al. (1983)—note a misprint p 6, α should be α^2]. The value of 0.65 is indicative of either a higher degree of covalency associated with the metal–ligand bonds than is normally the case for type 2 sites or mixing of the $4p_z$ orbital with the ground state 3d orbital. The mixing of states allowed by ligand field theory depends on the site symmetry. A tetrahedral distortion from tetragonal symmetry can mix a $4p_z$ orbital with a $3d_{x^2-y^2}$ orbital; these two orbitals contribute dipolar terms of opposite sign to $^{Cu}A_\parallel$ (Solomon et al., 1992), causing an apparent reduction in the estimated spin density on the Cu. An important point which would follow from greater electron delocalization is that the point–dipole approximation describing the copper electron charge density is less reliable. This has an obvious effect on the accuracy of distances calculated using the point–dipole model, which we estimate to be ca. 0.3 Å. Therefore, whichever mechanism is responsible for the reduced Cu spin density, the distance estimates of Table 2 must be taken only as indicative values.

Type 2 Site with NO_2^-. Figure 7 shows ^{1}H ENDOR spectra of activated enzyme in ^1H_2O and ^2H_2O in the presence of substrate. The spectra clearly demonstrate an exchangeable ^{1}H, most apparent in the low-fequency component, with a hyperfine coupling A = 5.0 MHz and some minor changes in

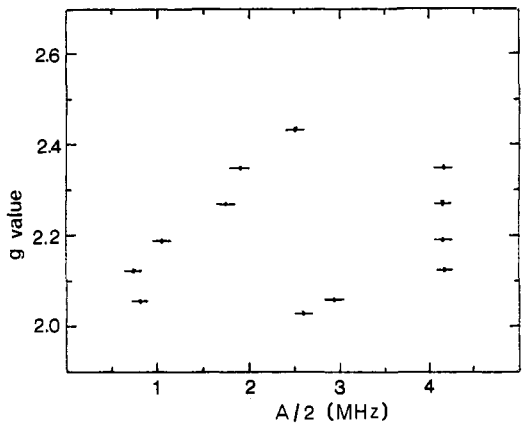

FIGURE 8: Plot of the peak postitions at the frequency extrema of the ν_+ exchangeable ^1H ENDOR pattern versus the observing g value for nitrite reductase in the presence of nitrite.

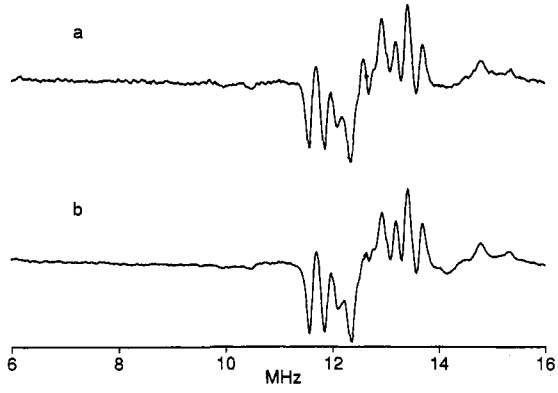

FIGURE 9: ENDOR spectra of type 2-deficient nitrite reductase excited at the $^{Cu}M_I = -3/2$ feature of the type 1 EPR (a) in ^2H_2O and (b) in ^1H_2O. The protein concentrations were (a) 85 mg/mL in 50 mM Hepes buffer, pD 7.2, and (b) 240 mg/mL in 50 mM Hepes buffer, pH 7.2.

relative intensity of other ^1H sets. The hyperfine tensor of this ^1H was determined in the same manner as described above by recording a set of ENDOR spectra at intervals across the EPR spectrum (Figure 8). The approximate A tensor is shown in Table 2. No angles are given for the relative orientation of the g and A tensors, but the complex behavior of the ^1H with field strongly indicates that the axes of these tensors are not parallel, so that the ^1H does not occupy the apical position of the tetrahedron nor a planar position, but some intermediate orientation. The Cu–^1H distance in this case is estimated to be 3.5 Å. These results thus indicate the absence of a water molecule in the apical position of the type 2 Cu structure in the presence of nitrite, suggesting that it has been replaced by a substrate molecule.

Type 1 Site. Study of the type 1 Cu center using type 2-depleted NiR enzyme shows no solvent $^1H/^2H$ isotope effects since the ^1H ENDOR spectra with the magnetic field set to the $^{Cu}M_I = -3/2$ feature of the type 1 EPR are the same in ^1H_2O and ^2H_2O. This is so whether or not substrate is present in the sample. Representative spectra are shown in Figure 9. There is a small change at the proton frequency (12.6 MHz), which is not unexpected as this is representative of distant ENDOR effects. The complete absence of exchangeable protons implies that the type 1 Cu center is isolated from the solvent. This result is consistent with the X-ray analysis which showed the type 1 center to be buried in each subunit of the trimer (Godden et al., 1991).

DISCUSSION

The existence of two types of copper center in nitrite reductase has caused some uncertainty about whether the type 1 or type 2 Cu provides the active site. It has recently been demonstrated that, for *A. cycloclastes* NiR, the loss of type 2 Cu resulted in a loss of activity and reconstitution of these sites generated activity in proportion to the type 2 Cu content (Libby & Averill, 1992). Clearly, the type 2 Cu center is required for activity, and it has been suggested that it constitutes the active site of NiR although no evidence has been presented. The X-ray analysis of *A. cycloclastes* gave different electron density maps between crystals of the enzyme as isolated and isomorphous crystals in the presence of nitrite, which show changes at both type 1 and type 2 copper centers. The changes at the type 1 centers are interpretable as a partial loss of type 1 Cu and those at the type 2 center as NO_2^- displacing a water ligand from the Cu atom (Godden et al., 1991). Small changes in the $g_\parallel$ region of the EPR arising from the type 2 Cu center of this enzyme in the presence of excess NO_2^- have been reported (but no data presented), suggesting that NO_2^- binds to this center (Libby & Averill, 1992).

The EPR parameters (Table 1) obtained from our study of *A. xylosoxidans* reveal a dramatic change at the type 2 Cu center on addition of NO_2^-. A decrease of 0.065 is observed for $g_\parallel$ and an increase of 2 mT for $^{Cu}A_\parallel$. This contrasts with no change in $g_\parallel$ or $^{Cu}A_\parallel$ for the type 1 center on addition of substrate. The clear implication is that the presence of NO_2^- causes a dramatic rearrangement of the ligand environment of the type 2 Cu atom, consistent with NO_2^- binding to the type 2, and not type 1, Cu atom.

It has been recognized for some time (Peisach & Blumberg, 1974; Addison, 1983) that the EPR parameters $g_\parallel$ and $A_\parallel$ for Cu(II) compounds are related to equatorial metal ligand composition and overall charge. (Axial ligand effects on the EPR parameters are much smaller and are neglected in this treatment.) It was therefore possible to describe metal ligand structures in Cu(II) proteins. Maps of $A_\parallel$ against $g_\parallel$ for Cu(II) model compounds and proteins are used in this method. In many cases this approach has proven successful in providing information on copper environments in proteins. Unfortunately, it is of relatively little use in cases where the copper site symmetry deviates significantly from square planar. The X-ray crystal structure of NiR in the absence of NO_2^- demonstrated that the type 2 center has an unusual near-tetrahedral site symmetry. Consequently, it is impossible to follow the treatment outlined above to correlate changes in EPR parameters on addition of substrate with specific structural changes. However, as pointed out by Addison (1983), the ratio $g_\parallel/A_\parallel$ can be used as an approximate guide to the degree of distortion from square planar symmetry. Thus one can qualitatively interpret the changes in the NiR type 2 Cu center EPR parameters on addition of NO_2^-, a reduction in the $g_\parallel/A_\parallel$ ratio, as being indicative of a smaller distortion from tetragonal when NO_2^- binds.

The ^1H and ^{14}N ENDOR spectra of the type 2 Cu center recorded at $g_\parallel$ (Figure 3) are completely changed by addition of NO_2^- to the enzyme. Equivalent ENDOR spectra of the type 1 Cu center (Figure 4) display little effect on addition of substrate. Clearly the ENDOR data strongly suggest that NO_2^- binds to the type 2 Cu atom resulting in a marked change in ligand nature or geometry. One possibility, resulting from an assignment of the four-peak ^{14}N pattern, found in the presence of NO_2^-, to a single type of nitrogen, is that the three histidine residues which the X-ray structure shows to be directly coordinated to type 2 Cu (Godden et al., 1991) move from a slightly inequivalent disposition about the copper in the absence of NO_2^- to occupy a more strictly equivalent planar disposition on binding NO_2^-. However, given the ambiguity discussed

above, associated with assigning a four-peak ^{14}N ENDOR pattern to one, two, or even four types of nitrogen, we have insufficient data at present to make a positive identification. Indeed, a fourth histidine residue shown by the X-ray structure analysis to be near the type 2 Cu site may move in closer on NO_2^- binding. At this stage we cannot discount completely the possibility that the four-peak pattern results from four inequivalent nitrogens. A detailed ^{14}N ENDOR study is in progress to clarify the nature of the histidine coordination changes at the type 2 center induced by NO_2^- binding.

Two solvent exchangeable ^1H are observed at the type 2 center in the absence of NO_2^- and one in its presence, whereas no exchangeable protons are observed at the type 1 center. This is quite consistent with the X-ray structure of *A. cycloclastes* NiR which showed the type 2 centers to lie in a deep solvent channel formed by two monomers and the type 1 centers to be buried in each monomer. The favored hyperfine tensor (see Results) of the anisotropic exchangeable ^1H in the absence of NO_2^- (Table 2) indicates that it is located in a direction approximately parallel to $g_\parallel$, at 2.9 Å from the Cu atom. This is long compared with the Cu–^1H distance deduced from the X-ray data of ~2.4 Å, but comparison with the X-ray analysis reveals that it is almost certainly a water molecule in the apical position of the local Cu site structure. The hyperfine tensor of the exchangeable ^1H in the presence of NO_2^- (Table 2) is quite different. An intermediate orientation is indicated at an approximate Cu–^1H distance of 3.5 Å. This implies that the coordinated water molecule has been lost and suggests it has been replaced by the NO_2^- ion. One can only speculate at present about the origin of the exchangeable ^1H in the presence of NO_2^-. One obvious possibility, however, is that the ^1H of the remote nitrogen from one (or more) histidinyl ligands becomes observable due to a change in local structure induced by substrate. A ^1H on the imidazole ring has a dipole interaction contribution from spin density delocalized onto the histidine ring in addition to the Cu–^1H dipole interaction. This is difficult to evaluate but can be significant (Hüttermann et al., 1988) and further obscures the Cu–^1H distance estimate in this case. The assignment of the second exchangeable ^1H observed in the absence of nitrite, with an apparent isotropic hyperfine tensor and coupling of 8 MHz, must be equally speculative. The isotropic character of the hyperfine tensor implies that the proton must be farther than 6 Å from the type 2 copper, otherwise significant hyperfine anisotropy would be expected. Presumably, therefore, it is located on a nearby amino acid residue.

The inaccuracies associated with the distances quoted above due to significant electron delocalization and consequent breakdown of the point–dipole model must be borne in mind. Nevertheless, the estimates do serve a useful purpose providing a guide to ^1H distances, and they do not affect the main conclusions of this work.

In summary, the EPR and ENDOR data reported herein demonstrate that the type 2 Cu centers constitute the NO_2^- binding sites of NiR. Furthermore, the effects of substrate binding are much more extensive, in terms of perturbation of the ligand coordination, than simply the substitution of an apical water molecule by NO_2^-. Nitrogen coupling from the substrate has not been observed, consistent with an apical position and consequent weak coupling to copper.

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