Nitrous oxide reductase from *Wolinella succinogenes*, an enzyme containing one heme c and four Cu atoms/subunit of $M_r = 88,000$, was studied by electron paramagnetic resonance (EPR) at 9.2 GHz from 6 to 80 K. In the oxidized state, low spin ferric cytochrome c was observed with $g_e = 3.10$ and an axial Cu resonance was observed with $g_l = 2.17$ and $g_t = 2.035$. No signals were detected at $g$ values greater than 3.10. For the Cu resonance, six hyperfine lines each were observed in the $g_l$ and $g_t$ regions with average separations of 45.2 and 26.2 gauss, respectively. The hyperfine components are attributed to Cu(I)-Cu(II) $S = \frac{1}{2}$ (half-met) centers. Reduction of the enzyme with dithionite caused signals attributable to heme c and Cu to disappear; exposure of that sample to $N_2O$ for a few min caused the reappearance of the $g = 3.10$ component and a new Cu signal with $g_l = 2.17$ and $g_t = 2.055$ that lacked the simple hyperfine components attributed to a single species of half-met center. The enzyme lost no activity as the result of this cycle of reduction and reoxidation. EPR provided no evidence for a Cu-heme interaction. The EPR detectable Cu in the oxidized and reoxidized forms of the enzyme comprised about 23 and 20% of the total Cu, respectively, or about one spin/subunit.

The enzyme offers the first example of a nitrous oxide reductase which can have two states of high activity that present very different EPR spectra of Cu. These two states may represent enzyme in two different stages of the catalytic cycle.

Two kinds of nitrous oxide reductase have been described. The one contains four Cu atoms/subunit of $M_r = 70,000$, is acidic, and is found in denitrifying and photodentifying bacteria (1–6). The other has been reported to contain three Cu atoms and one heme c/subunit of $M_r = 88,000$ and is basic (7). The Cu/Fe enzyme was purified from an electron-transport containing Gram-negative anaerobe, *Wolinella succinogenes*, originally isolated from bovine rumen (8, 9). Although not actually a denitrifying bacterium, *W. succinogenes* can exhibit considerable dithionite-$N_2O$ oxidoreductase activity (7). The cytochrome c domain of the Cu/Fe enzyme from *W. succinogenes* is linked by a protease-sensitive sequence, thus allowing the enzyme to be cleaved into an apparently inactive dimer with subunit $M_r = 70,000$ and a basic cytochrome c with $M_r = 13,000$ (7). This circumstance prompted Teraguchi and Hollocher (7) to suggest that the Cu/Fe nitrous oxide reductase may represent the product of a gene fusion between a basic, low $M_r$, cytochrome c and a peptide homologous with the Cu only type enzyme from denitrifying bacteria. One might expect on this basis that the Cu centers of nitrous oxide reductase of *W. succinogenes* would resemble those of the Cu only enzyme, at least to the extent that enzyme activity may depend on particular electronic and spin states of Cu.

The Cu only enzyme contains Cu probably coordinated with N and S ligands, as suggested by resonance Raman (12), magnetic circular dichroism (13), electron spin echo (14), and x-ray spectroscopy (13, 15). The “purple” oxidized form shows some or all of the seven EPR hyperfine lines expected from coupled Cu(I)-Cu(II) $S = \frac{1}{2}$ (half-met) centers (1, 16, 17).

We summarize herein the first EPR observations on nitrous oxide reductase from *W. succinogenes*.

**EXPERIMENTAL PROCEDURES**

Nitrous oxide reductase was purified from *W. succinogenes* to a peptide purity $\geq 99\%$ by the method of Teraguchi and Hollocher (7) and concentrated by pressure dialysis to a concentration of 3.64 mg × ml$^{-1}$ (41.3 μM with respect to the subunit of $M_r = 88,000$). The concentrated enzyme contained by analysis 36.6 μM of cytochrome c and 169 μM Cu. The Cu content of this and other recent preparations was unexpectedly great and indicated a Cu/subunit ratio of 3.7–4.0 instead of 3 as reported previously (7). The ratio of subunit to cytochrome c was found to be 1.92 before pressure dialysis and 1.13 after. The ratios are not considered to be significantly different from 1. The specific activity of the enzyme in the benzyl viologen-based assay (4, 18) was 142 and 146 μmol of $N_2O$ × min$^{-1}$ × mg$^{-1}$, respectively, before and after the EPR experiments. Dithionite solution (40 mM) was prepared anaerobically immediately before use in 40 mM potassium phosphate buffer, pH 7.2.

Protein was estimated by the bicinchoninic acid method (19) (reagents from Pierce Chemical Co); cytochrome c was estimated on the basis of published extinction coefficients for heart cytochrome c, as described by Teraguchi and Hollocher (7); and Cu content of the enzyme was determined by atomic absorption spectrometry (Perkin-Elmer Cetus Instruments model 2380) after wet ashing of the sample with HNO$_3$.

First derivative EPR spectra and double integrations of these spectra were obtained with the use of a Bruker ESP-3000 EPR spectrometer and associated software. Samples were containing in 3 mm inner diameter quartz tubes fitted with standard-taper joints that allowed nailing of the tubes to a gas line for the purpose of
making anaerobic additions and withdrawals. Sample temperature was maintained by means of the rate of flow of cold He in an Air Products cryostat (20). Instrument settings which were common to all spectra presented in the figures were microwave frequency, 9.18 GHz; modulation amplitude, 4.8 gauss at 100 KHz; microwave power at the cavity, 1 milliwatt; amplifier gain, \(2 \times 10^7\); number of data channels, 2,048. A power level of 1 milliwatt was slightly saturating from 6 to 15 K for the Cu EPR signals but not saturating at 45 K at which temperature spectra for the purpose of double integration were obtained. First derivative spectra, for which the first integral failed to sum exactly to zero, were spline-corrected according to standard routines to assure a zero sum. The area under the first integral (the second integral) was taken to be proportional to the amount of EPR-visible Cu and provided a spin count when corrected for small differences in tube diameters and when referenced to data on the double integration of the spectrum from 100 \(\mu\)M Cu-EDTA. The EPR spectra used for integrations were all taken under the same instrument conditions and temperature (\(4.2\) K).

**RESULTS**

Scans from 100–8,100 gauss showed EPR signals attributed to nitrous oxide reductase only at \(g = 3.10\) (2,116 gauss) and the \(g = 2.9\) (3,280 gauss) region. In particular, no signals were observed in the \(g = 4\) (1,640 gauss) and 6 (1,093 gauss) regions, so it would seem that weakly coupled cupric dimers (\(g = 4\)), adventitious iron (\(g = 4.3\)) and high spin heme iron (\(g = 6\)) were not detected. Spectra covering a scan range of 2,000 gauss are presented in Fig. 1. The oxidized enzyme showed (as prepared) a component at \(g = 3.10\) (2,100 gauss) (Fig. 1, *spectra A*) which is attributed to the \(g_1\) component of the (ferric) cytochrome \(c\) domain (21). The \(g_1\) component, which was expected to be quite broad and centered near \(g = 1.29\) (5,084 gauss) (16), was not observed, probably because of insufficient signal to noise ratio at the temperatures and concentrations used. The oxidized enzyme also exhibited an axial Cu signal with \(g_1 = 2.17\) and \(g_2 = 2.035\). A very small feature at the low field side of this signal would be expected to arise from the weak \(g_2 \approx 2.23\) component of ferric cytochrome \(c\), but it is doubtful whether its presence can be inferred from the data. At higher resolution (Fig. 2, *spectral A*) the Cu signal can be seen to show six hyperfine lines in the \(g_1\) region with first-derivative intensity ratios of about 1:2:3:3:4:0.9:2:3:1, and separations of 43, 44, 45, 46 and 48 gauss from low field to high (45.2 gauss average separation) and six hyperfine lines in the \(g_2\) region with average separation of 26.2 gauss. The fourth line counted from the low field side was the most intense in the \(g_1\) set. Similarly, the fourth line counted from the high field side was the strongest of the \(g_2\) set. A seventh hyperfine line in the \(g_1\) set was expected but not unambiguously observed, either because it was broadened, split into two parts (see the sixth line), or overlapped with the first line of the \(g_2\) set. The \(g_1\) set of hyperfine lines was observed to broaden and show increasing separations from lower to higher field, and there is the suggestion that the sixth line may be partly resolved into two components. Similarly, a seventh hyperfine line was expected but not unambiguously observed in the \(g_2\) set, perhaps due to overlap with the \(g_1\) set. The \(g_1\) and \(g_2\) hyperfine lines weakened with increasing temperature and the \(g_2\) set had virtually disappeared at 80 K. The trend suggested that the \(g_1\) set might also disappear at 100–120 K.

After the anaerobic addition of 800 \(\mu\)M dithionite to the enzyme (41 \(\mu\)M in subunits; about 200 \(\mu\)M in Cu plus Fe), both the cytochrome \(c\) \(g_1 = 3.10\) and Cu \(g_2 = 2\) signals disappeared (Fig. 1, *spectra A*) and were replaced with a spectrum showing two, narrow, weak lines at \(g = 2.00\) and 1.97. Comparisons made with a second enzyme sample and a different EPR tube, indicate that these lines were largely or entirely a property of the tube and not the enzyme. Addition of 800 \(\mu\)M of dithionite, which previously had been destroyed by autoxidation and converted chiefly into bisulphite, to a separate sample of enzyme had no effect on the EPR spectrum. Exposure of the dithionite-reduced sample to \(N_2O\) for a few min to destroy the excess dithionite and reoxidize the enzyme brought about a return of the \(g_1 = 3.10\) signal, which is a clear indicator of the quantitative destruction of dithionite, and an axial Cu resonance (Fig. 1, *spectra C*; Fig. 2, *spectra B*) having \(g_1 = 2.17\) and \(g_2 = 2.035\). The latter resonance was quite different in detail from the original one and lacked or largely lacked the original sets of \(g_1\) and \(g_2\) hyperfine lines. The small feature at \(g = 1.27\) (3,292 gauss) (Fig. 2, *spectra B*) largely represents the weak, narrow line attributable to the sample tube; compare Fig. 1, *spectra C*, with Fig. 2,

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**FIG. 1.** EPR spectra of nitrous oxide reductase from *W. succinogenes* over a field range of 2,000 gauss. *A*, enzyme as prepared (oxidized) in tube 1: 7.2 K, 20 ms time constant, 81 ms/channel, 166 s sweep time, relative plotter gain = 2; *B*, enzyme in tube 2 after having been reduced with 800 \(\mu\)M dithionite: 9.2 K, 40 ms time constant, 163 ms/channel, 334 s sweep time, relative plotter gain = 17; *C*, enzyme in tube 1 after the oxidative destruction of excess dithionite by \(N_2O\): 8.7 K, 20 ms time constant, 81 ms/channel, 166 s sweep time, relative plotter gain = 1. The center field was 2,700 gauss.

**FIG. 2.** EPR spectra of nitrous oxide reductase from *W. succinogenes* over a field range of 1,000 gauss. *A*, enzyme as prepared (oxidized) in tube 1: 15.1 K, 5 superimposed scans; *B*, enzyme in tube 2 after the oxidative destruction of excess dithionite by \(N_2O\): 14.7 K. Other instrument conditions were as follows: 40 ms time constant, 163 ms/channel, 334 s sweep time, relative plotter gain = 2.
spectrum B, for which different EPR tubes were used. The cycle of reduction and reoxidation carried out evidently did not allow the Cu of the enzyme to return exactly to its original state.

The initial specific activity of the enzyme was 142 µmol of N₂O × min⁻¹ × mg⁻¹, and after the cycle of reduction and reoxidation (including three thaws) it was 146 µmol of N₂O × min⁻¹ × mg⁻¹. The EPR spectra of the oxidized and reoxidized enzyme and 100 µM Cu EDTA were obtained at 45 K under the same instrument conditions and were carried through double integration. On this basis, the EPR-detectable Cu was estimated to be 23 ± 5% and 20 ± 5%, of the total Cu for the oxidized and reoxidized enzyme, respectively. This would correspond to about one Cu spin/subunit.

**DISCUSSION**

It can be inferred from the EPR spectrum of the oxidized form of nitrous oxide reductase from *W. succinogenes* that its cytochrome c is largely or entirely in the low spin state with a normal *g*₁ value (21) and that its Cu centers resemble those reported previously for the Cu centers of the purple (oxidized) state of the Cu only enzyme from *P. stutzeri* (1, 16, 17), *P. aeruginosa* (15), and *P. denitrificans* (4). In particular, the hyperfine patterns observed support the idea that Cu(I)-Cu(II) exchange coupled or half-metal centers exist in the *W. succinogenes* oxidized enzyme. These *S* = ½ binuclear centers are expected in the simplest case to present seven hyperfine lines with intensity ratios of 1:3:4:3:2:1. The progressive increase in the separation and line width of the *g* set of hyperfine lines actually observed with the enzyme from *W. succinogenes* may be related to nuclear quadrupole interactions, an anisotropic component of the hyperfine interaction or the differences in nuclear magnetic moment between °Cu and °Cu (25), or, alternatively, to the composite effect of several species of enzyme each with slightly different *A* and/or *g* values (1, 14).

It is generally the case at X-band frequencies that only five or six of the expected seven-line set is observed in the *g*₁ region with the purple form of the enzyme from *P. stutzeri*, *P. aeruginosa*, and *P. denitrificans*. Reduction by dithionite revealed a fundamental difference between the Cu/Fe and Cu only enzymes. Resonances attributable to Cu (and Fe) were abolished in the former enzyme but the Cu resonances were not entirely abolished in the latter. It would appear therefore that all EPR detectable cupric centers are reducible in the enzyme from *W. succinogenes*. The dithionite-reduced Cu only enzyme exhibits, at or above a reducible ratio of 1 electron/Cu atom, a substantial signal attributable to 4–25% of the Cu with *g*₁ = 2.17 and *g*₂ = 2.05–2.06 (shifted from 2.03 to 2.04) (1, 4, 6, 14–17). The same EPR signal was obtained when photochemically generated methyl viologen radical cation replaced dithionite as reductant for the enzyme from *P. denitrificans* (4). Thus, the modified EPR parameters of Cu in the reduced enzyme cannot be easily attributed to something like the acquisition of sulfite ligands produced by the oxidation of dithionite.

The EPR spectrum of the reoxidized Cu/Fe enzyme from *W. succinogenes* resembled that of the purple form of the Cu only enzyme from denitrifiers following its reduction by dithionite (1, 4, 15–17). Upon reoxidation of the Cu/Fe enzyme, the *g* value was increased by 0.02, the hyperfine pattern attributed to half-metal centers was largely or entirely lost, and the up-field half of the resonance was broadened by some 35 gauss (44%) relative to the oxidized (as prepared) form of the enzyme. These are the same changes seen on reduction of the purple form of the Cu only enzyme. A point of difference between the reoxidized Cu/Fe and the reduced Cu only enzyme is the small feature that appeared at *g* = 2.15 (3,045 gauss) (Fig. 2, spectrum B), the cause of which is unknown but probably related to superposition of sets of unresolved Cu hyperfine lines. The EPR spectrum of the reoxidized Cu/Fe enzyme also resembles that of the highly active oxidized state of the pink, monomeric, Cu-only enzyme from *Achromobacter cycloclastes* (6), which has a *g* value of 2.05 and lacks resolvable hyperfine components, and the oxidized, activated state of the enzyme from *P. aeruginosa* (15) which at pH 7 also lacks the hyperfine sets attributable to single half-metal centers. The latter enzyme also exhibits the dip at *g* = 2.15. It may be relevant regarding EPR spectral similarities that the Cu/Fe enzyme, the enzyme from *A. cycloclastes* and the activated enzyme from *P. aeruginosa* all were either purified under aerobic conditions or exposed to air during activation.

The spin counts correspond to about one spin/subunit or one spin/four Cu atoms for both the oxidized and reoxidized forms of the enzyme. The spin density on the Cu only enzyme, which shows evidence for half-metal, the data are consistent with the presence of one Cu(I)-Cu(II) S = ½ half-metal center and a Cu(I)-Cu(II) S = 0 antiferromagnetically coupled cupric center. The involvement of a Cu(I) pair cannot be ruled out, however. For the reoxidized enzyme, which failed to show resolved hyperfine lines diagnostic for half-metal centers, the data may be rationalized by either of two models. The first model is exactly like the one above, except that there are at least two different species of half-metal centers present in similar proportion. These species are characterized by different hyperfine splitting constants and perhaps different *g* values and present EPR spectra which superimpose to give a complex, poorly resolved resultant spectrum. The second model proposes a coupled tetranuclear Cu(Ii)-Cu(I) S = ½ state. It may be expected that delocalization of the odd electron on one instead of two Cu atoms would result in smaller average hyperfine splitting constants, a substantially larger number of hyperfine lines, and an EPR spectrum largely lacking resolved hyperfine structure. This model suggests a possible analogy between tetranuclear nuclear spin coupling in the Cu-containing active site of nitrous oxide reductase and the Fe-containing clusters of non-heme iron proteins.

Irrespective of model, the spin counts indicate that the oxidized forms of the Cu/Fe enzyme contain substantial amounts of EPR silent Cu, just as does the Cu only enzyme from denitrifiers. The latter enzyme has been reported to contain 50–75% EPR silent Cu in oxidized states, depending on species and purification method (1, 4, 6, 14–17).

It was interesting to observe that two different EPR forms of the oxidized enzyme from *W. succinogenes* had the same extremely high specific activity. Thus, the hyperfine pattern indicative of a single type of half-metal center need not necessarily be evident in the EPR spectrum in order to have good activity. It is possible that the two EPR states of identical activity may represent enzyme captured in two different stages of its catalytic cycle.

The present study provides no evidence for a magnetic interaction or coupling between heme c and the Cu atoms of the Cu/Fe enzyme from *W. succinogenes*.

**REFERENCES**

EPR of N₂O Reductase