Magnetic Resonance Studies of Cyanide and Fluoride Binding to Galactose Oxidase Copper(II): Evidence for Two Exogenous Ligand Sites†

Beverly J. Marwedel, Daniel J. Kosman, Robert D. Bereman, and Robert J. Kurland*

Contribution from the Bioinorganic Graduate Research Group, Departments of Chemistry and Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214.
Received July 10, 1980

Abstract: Evidence for a second, inner-sphere coordination site at the copper(II) center in galactose oxidase (GOase) has been obtained from an analysis of cyanide–fluoride competition manifested in ^{19}F NMR relaxation rate changes. A quantitative description of the ^{19}F NMR relaxation rate deenhancement produced by added CN^- is provided by the following model. (1) At equatorial sites CN^- and F^- (both detected by ESR superhyperfine splitting effects in the GOase ESR spectra) bind strongly to the copper(II) atom. (2) In addition, fluoride (detected by ^{19}F NMR relaxation rate enhancement) binds more weakly to an axial coordination site. (3) The binding of axially coordinated F^- is weakened and/or the hyperfine coupling constant of this fluoride is diminished when equatorially coordinated F^- is displaced by CN^-. The effect of CN^- coordination on unpaired electron spin density near the copper(II) center is also shown by changes in ^{14}N ESR hyperfine splittings attributed to endogenous GOase ligands. The model proposed above is consistent with previously reported magnetic resonance data and results of kinetic competition studies.

The extracellular fungal enzyme galactose oxidase (D-galactose:oxygen oxidoreductase, EC 1.1.3.9, herein referred to as GOase) catalyzes the oxidation of many primary alcohols to the corresponding aldehydes.[1,2] As possibly the only protein which contains just a single type 2 copper atom and no other metal atoms or prosthetic groups,[3,4] GOase is of particular interest as a possible prototype for this type of copper site in other metalloenzymes. The enzymic Cu(II) is apparently coordinated to two protein imidazoles, a solvent H_2O (or OH^-), and a third, nonnitrogenous ligand (–S– or –O–).[5a] Although a number of spectral and kinetic studies of this enzyme have been reported,[5-13] coordination chemistry of exogenous ligands is still not completely elucidated.

Indeed, the binding properties of F^- deduced from ESR studies[11] are not consistent with those obtained from an analysis of ^{19}F NMR relaxation rate data.[13] According to the ESR results,[11] a large fraction of GOase is complexed to F^- at 0.22 M fluoride concentration; however the dissociation constant derived from the ^{19}F NMR results[13] is 1 order of magnitude too large to account for this degree of F^- complexation (see Table I). Moreover, the ^{19}F superhyperfine coupling constants obtained from the ESR and the ^{19}F NMR studies are not in agreement, as can be seen from the values reproduced in Table I; those from ESR are more characteristic of fluoride coordinated equatorially to Cu(II), as in $CuF^+(aq)$[14] (see Table I). Since the ESR studies indicated that CN^- at millimolar concentrations completely replaced the ESR detectable F^-, we undertook a similar study of the effect of added CN^- on the ^{19}F NMR relaxation rates, in order to clarify the apparent anomalies.

A similar CN^- competition study,[15] utilizing water proton relaxation rates to probe the type 2 Cu(II) sites of benzylamine oxidase, has shown that there are two types of water coordination to each of the Cu(II) atoms in this enzyme, one axial and one equatorial. The work reported here shows that two types of coordination sites are available for exogenous ligands binding to GOase Cu(II) also. One site corresponds to relatively strong equatorial coordination and the other to relatively weak axial coordination. Additional ESR data is presented to show that equatorially coordinated CN^- can alter the unpaired electron spin distribution at the GOase Cu(II) center.

Fluoride ion has been used in other studies as an NMR relaxation probe of the paramagnetic centers of metalloproteins.[16-19] The special utility of fluoride in such applications arises from the presence of unpaired electron spin density in p orbitals centered

Table I. Superhyperfine Coupling and Dissociation Constants of Fluoride Bound to Cu(II)

	$CuF^+(aq)$ (^{19}F NMR)[a]	GOase $Cu^{II}F^-$ (ESR)[b]	GOase $Cu^{II}F^-$ (^{19}F NMR)[c]
A_l,[d] Hz	1.0×10^9	4.1×10^8	2.6×10^8
A_a,[e] Hz	8.2×10^8	3.7×10^8	1.9×10^8
K_D,[f] M	1.0	≤ 0.2[g]	5×10

[a] Reference 14. [b] Reference 11. [c] Reference 13. [d] A_l is the isotropic coupling constant; see ref 13. [e] $A_a = |A_{\parallel} - A_{\perp}|$ is the anisotropic coupling constant; see ref 13. [f] K_D is the dissociation constant for the $Cu^{II}F^-$ complex. [g] Estimated from data in ref 11, from the assumption that at least 50% of the intensity of the observed ESR spectra ($T \approx 70$ K) is due to a GOase–F^- complex.

† A portion of this work has been taken from the dissertation of B. J. Marwedel, submitted in partial fulfillment of the requirements for the Ph.D. degree, State University of New York at Buffalo.

* To whom correspondence should be addresssed at the Department of Chemistry.

(1) D. Amaral, F. Kelly-Falcoz, and B. Horecker, *Methods Enzymol.*, **9**, 87 (1965).
(2) G. A. Hamilton, J. deJersey, and P. K. Adolf, *Oxidases Relat. Redox Syst. Proc. Int. Symp.*, **1**, 103–124 (1973).
(3) R. D. Bereman, M. J. Ettinger, D. J. Kosman, and R. J. Kurland, *Adv. Chem. Ser.*, **No. 162**, 263 (1977).
(4) T. Vänngård in "Biological Applications of Electron Spin Resonance", H. M. Swartz, J. R. Bolten, and D. C. Borg, Wiley-Interscience, New York, 1972, pp 411–447.
(5) (a) B. J. Marwedel, L. Kwiatkowski, D. Melnyk, P. Tressel, R. D. Bereman, R. J. Kurland, M. J. Ettinger, and D. J. Kosman, *Oxidases Relat. Redox Syst. Proc. Int. Symp.*, in press. (b) R. S. Giordano, R. D. Bereman, D. J. Kosman, and M. J. Ettinger, *J. Am. Chem. Soc.*, **96**, 1023 (1974). (c) R. S. Giordano, Ph.D. Thesis, State University of New York at Buffalo, 1977.
(6) M. J. Ettinger and D. J. Kosman, *Biochemistry*, **13**, 1247 (1974).
(7) B. J. Marwedel, R. J. Kurland, D. J. Kosman, and M. J. Ettinger, *Biochem. Biophys. Res. Commun.*, **63**, 773 (1975).
(8) D. J. Kosman, M. J. Ettinger, R. D. Bereman, and R. S. Giordano, *Biochemistry*, **16**, 1597 (1977).
(9) R. E. Weiner, M. J. Ettinger, and D. J. Kosman, *Biochemistry*, **16**, 1602 (1977).
(10) L. D. Kwiatkowski, L. Siconolfi, R. E. Weiner, R. S. Giordano, R. D. Bereman, M. J. Ettinger, and D. J. Kosman, *Arch. Biochem. Biophys.*, **182**, 712 (1977).
(11) R. D. Bereman and D. J. Kosman, *J. Am. Chem. Soc.*, **99**, 7322 (1977).
(12) D. J. Kosman, J. Peisach, and W. B. Mims, *Biochemistry*, **19**, 1304 (1980).
(13) R. J. Kurland and B. J. Marwedel, *J. Phys. Chem.*, **83**, 1422 (1979).
(14) M. Eisenstadt and H. L. Friedman, *J. Chem. Phys.*, **48**, 4445 (1968).
(15) R. Barker, N. Boden, G. Cayley, S. C. Charlton, R. Henson, M. C. Holmes, I. D. Kelly, and P. F. Knowles, *Biochem. J.*, **177**, 289 (1979).
(16) G. Navon, R. G. Shulman, B. J. Wyluda, and T. Yamane, *J. Mol. Biol.*, **51**, 15 (1970).
(17) A. S. Mildvan, J. S. Leigh, and M. Cohn, *Biochemistry*, **6**, 1805 (1967).

0002-7863/81/1503-2842$01.25/0

Table II. Decrease of Fluoride Relaxation Rates Due to Added Cyanide Ion[a]

β, mole ratio added CN^-:GOase	$R_{1,obsd} = 1/T_{1,obsd}$[b] (% spin–lattice rate decrease)	$R_{2,obsd} = 1/T_{2,obsd}$[b] (% spin–spin rate decrease)	$R_{2,obsd}/R_{1,obsd}$	f_B[c]	$R_{1,obsd}$(calcd)[d]	$R_{2,obsd}$(calcd)[d]
0.0	9.79 (0)	27.1 (0)	2.77	1.00	(9.79)	(27.1)
1.0	5.74 (41.4)	17.1 (36.7)	2.98	0.645	6.67	19.6
2.0	4.54 (53.6)	14.5 (46.3)	3.20	0.448	4.94	15.4
3.0	3.99 (57.2)	13.2 (51.1)	3.32	0.334	3.94	13.0
4.0	3.59 (63.3)	12.2 (54.8)	3.41	0.264	3.32	11.6
5.0	3.24 (66.9)	11.2 (58.7)	3.45	0.218	2.92	10.6

[a] NMR samples contained 1.0 M F^-, (0.75 ± 0.04) × 10^{-5} M galactose oxidase in 0.5 M, pH 7.0 sodium phosphate buffer at 28 ± 1 °C. [b] Values were obtained by interpolation from data shown in Figure 1; small corrections (≲5%) were made to account for relaxation effects due to nonspecific binding of F^-, as determined from measurements on apoenzyme samples.[26] [c] Calculated from eq 4 with values K_{eC} = 4.4 × 10^{-5} M and K_{eF} = 1.0 M, obtained from kinetic competition studies;[27] $f_C = 1 - f_B$. [d] Calculated from eq 3a,b from values of f_B given in this table; $R^\circ_{iB} = R_{i,obsd}$ for β = 0 (no CN^- added); $R^\circ_{1C} = 1.0$, and $R^\circ_{2C} = 6.0$.

at the fluorine nucleus. The dipolar interaction between this ligand-centered unpaired spin density and the ^{19}F nucleus can be an appreciable or even dominant factor in the NMR relaxation mechanism; thus the ^{19}F relaxation rates can be used to monitor changes in metal–fluoride bonding upon changes in the binding of other ligands. Even though only a small fraction of unpaired spin density is present in the fluorine *p* orbital, the NMR relaxation effect can be much greater than that due to the greater unpaired spin density on the metal ion because the average distance of the unpaired spin to the ^{19}F nucleus is much shorter in the fluorine p orbital than in the metal centered orbitals (i.e. $\langle 1/r^6 \rangle_{2p}$ is much larger than $1/R^6$, R being the metal ion–fluoride bond length).[20] The major contribution of ligand p orbital spin density to the electron–nuclear dipolar interaction is manifest in ^{19}F anisotropic shift data for crystalline transition-metal fluorides[21] and in ^{19}F NMR relaxation studies of aqueous CuF^+.[14] However, previous fluoride NMR relaxation studies of paramagnetic metalloproteins have not extensively considered or utilized this aspect of the relaxation mechanism. The changes in fluoride-centered spin density deduced from ^{19}F relaxation rates can be expected to correlate qualitatively with changes in shfs of the galactose oxidase ESR spectrum when the ligands binding to the Cu(II) are changed, if these indicators are indeed sensitive to the nature of ligand–Cu(II) bonding.

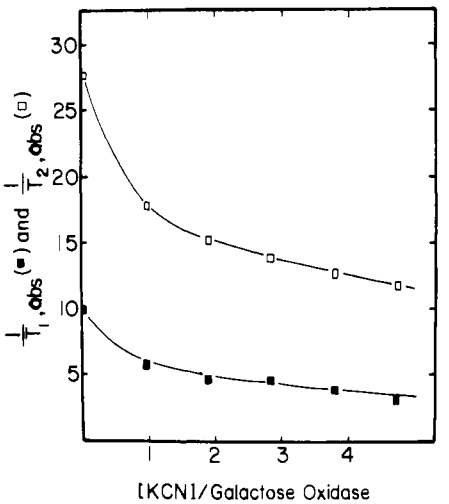

Figure 1. Spin–spin (upper trace, □) and spin–lattice (lower trace, ■) F^- ion relaxation rate enhancements observed for samples containing 1.00 ± 0.01 M F^- and varying amounts of CN^- in the presence of (0.75 ± 0.04) × 10^{-4} M galactose oxidase. Ambient temperature was 26.7–27.3 °C. Samples were prepared in 0.05 M, pH 7.0 phosphate buffer.

Experimental Section

NMR. ^{19}F-pulsed NMR relaxation rate measurements at 56.4 MHz were taken on a Bruker-321s variable-frequency spectrometer modified for external lock field-frequency control. Spin–lattice relaxation times T_1 were obtained by use of the Carr-Purcell 180°–τ–90° pulse sequence.[22] Spin–spin relaxation times T_2 were measured by use of the Carr-Purcell, Meiboom-Gill method[22,23] or from free-induction decay curves. Relaxation rates were calculated from a linear least-squares fit of the appropriate plots. The enzyme was prepared by use of modified literature procedures.[24] Successive additions of 5–25-μL volumes of KCN stock solutions were pipetted into F^-, enzyme samples contained in a 7-mm o.d. Kel-F NMR sample tube. Since it had been observed that the presence of micromolar amounts of paramagnetic impurities, especially cupric ion, results in a significant enhancement of NMR relaxation rates measured in fluoride solutions,[14] special care was taken to minimize free metal ion contamination. The buffer solutions used were made from deionized, glass-distilled water which contained less than 10^{-8} M cupric ion according to copper atomic absorption (limit of detection 10^{-8} M copper). Buffers used to prepare stock solutions were passed through a CHELEX-100 (BIO-RAD laboratories) column to remove cupric ion impurity present in the phosphate and NaOH components. The GOase used in the NMR experiments was exhaustively dialyzed against Cu(II)-free buffers to which 10^{-7} M ethylenediamminetetraacetic acid was added, to ensure the complexation of free cupric ion present due to enzyme decomposition. Stock solutions and NMR samples were maintained at pH 7.0 ± 0.10 by use of 0.05 M NaH_2PO_4 and NaOH. The ionic strength of all fluoride samples was maintained at 1.0 ± 0.1 M.

ESR. ESR spectra were obtained by using a Varian E-9 instrument operating at 9.1 GHz and 100 K. The enzyme used contained isotopically pure ^{63}Cu (Oak Ridge) and was prepared as described.[11] The magnetic field was calibrated at $g = 2$ by using diphenylpicrylhydrazyl; the microwave frequency was determined by using a Hewlett-Packard frequency meter. $Na^{13}CN$ was obtained from Merck.

Results

Cyanide Effects on Fluoride Ion Relaxation Rates. Figure 1 shows how the fluoride ion NMR relaxation rates, in the presence of galactose oxidase, are modified by successive additions of KCN. At a 1:1 mole ratio of CN^- to enzyme, the F^- relaxation rates decrease by 37% (spin–spin) and 41% (spin–lattice) from their value in the absence of CN^-, with only holoenzyme present. As the mole ratio of CN^- to enzyme increases from this value additional, but smaller, incremental reductions in the F^- relaxation rates are observed.

Unlike the results for the competition of Cl^- and CN^- for the metal ion site of bovine carbonic anhydrase,[25] where 1:1 CN^- to BCA yields essentially 100% loss of the quadrupolar Cl^- relaxation due to the presence of enzyme, the data presented here for galactose oxidase are consistent only with the presence of more than one type of site, at or near the enzymic Cu(II) center, which is accessible to exogenous ligands. If several such types of sites are

(18) J.-M. Guo, C. Chang, N. C. Li, and K. T. Douglas, *Biochemistry*, **17**, 432 (1979).
(19) P. Viglino, A. Rigo, R. Stevanato, G. A. Ranieri, G. Rotilio, and L. Calabrese, *J. Mag. Reson.*, **34**, 265 (1979).
(20) B. R. McGarvey and R. J. Kurland in "NMR of Paramagnetic Molecules", G. N. LaMar, W. DeW. Horrocks, and R. H. Holm, Eds., Academic Press, New York, 1973, pp 564–565.
(21) R. G. Shulman and K. Knox, *J. Chem. Phys.*, **42**, 813 (1965).
(22) H. Y. Carr and E. M. Purcell, *Phys. Rev.*, **94**, 630 (1954).
(23) S. Meiboom and D. Gill, *Rev. Sci. Instrum.*, **29**, 688 (1958).
(24) D. J. Kosman, M. J. Ettinger, R. E. Weiner, and E. J. Massaro, *Arch. Biochem. Biophys.*, **165**, 456 (1974).
(25) R. L. Ward, *Biochemistry*, **8**, 1879 (1969).

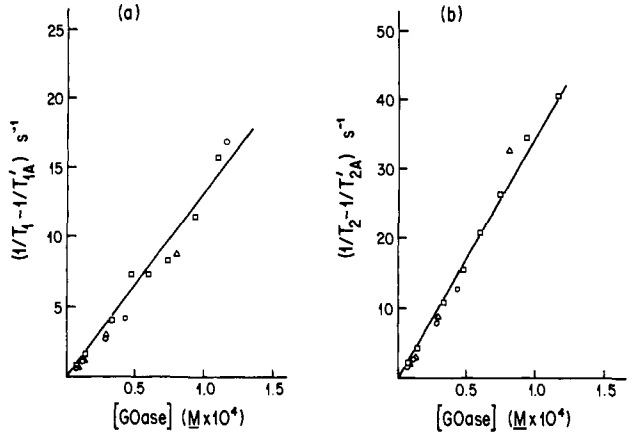

Figure 2. (a) Spin–lattice and (b) spin–spin relaxation rate enhancements observed for fluoride ion. Samples were prepared in 0.05 M, pH 7.0 phosphate buffer. Ambient rates were observed for the following: □, 1.015 M; △, 0.753 M; ○, 0.462 M F^-.

present, CN^- could either displace fluoride bound at or near the Cu(II) and/or bind at one or more sites not populated by exchangeable fluoride. The observed relaxation rates decrease most sharply with added CN^- at CN^-:enzyme mole ratios less than or equal to 1:1. However, even at CN^-:GOase ratios as high as 6:1, complete deenhancement (relaxation rates the same as those in the presence of apoenzyme) is not observed. Another striking feature of the reduction in relaxation rates produced by added CN^- is that the ratio of the F^- spin–spin to spin–lattice rates in the presence of CN^- are consistently larger than the ratio observed in the absence of CN^- (see Table II). If the effects of CN^- were due to a simple, single-site competition with fluoride bound to Cu(II), this ratio, $(1/T_{2,obsd})/(1/T_{1,obsd})$, would be independent of CN^- concentration. The relaxation rates observed at mole ratios of CN^- to galactose oxidase as high as 6:1 are too large to be attributed to F^- at nonspecific binding sites not near the Cu(II) ion; the apoenzyme F^- rates (i.e., those due to nonspecific binding effects) are only 3–5% of the rates observed in the presence of holoenzyme at the enzyme concentrations used in these experiments.

Two types of CN^-, F^- competitive binding schemes could be developed (without recourse to other data or information) that could account for the deenhancement effects of CN^- described above. (1) The total ^{19}F NMR relaxation rate could be taken as the sum of that due to F^- coordinated to GOase Cu(II) at two different types of sites; at both sites F^- exchanges rapidly (on the appropriate NMR time scale) with F^- in bulk solution. The deenhancement observed would then be due to the loss of the contribution from equatorially coordinated F^- as it is displaced by CN^-. The remaining, much smaller relaxation rates (with a large excess of CN^-) would be due to the contribution of axially coordinated F^-. (2) The total ^{19}F NMR relaxation rate is taken as that due only to axially coordinated F^-. In this latter model, exchange between equatorially coordinated F^- and bulk F^- is too slow to contribute to the observed relaxation. However, the replacement of equatorially coordinated F^- by CN^- alters the relaxation rate contribution of axially coordinated F^- (by change of binding constant and/or intrinsic relaxation rate of this axial ligand).

Although the first of the schemes above is simpler and possibly more appealing, it is not compatible with the dependence of the ^{19}F NMR relaxation rates on fluoride concentration. As can be seen from Figure 2, the ^{19}F NMR relaxation rates are essentially independent of F^- concentration over the range 0.467–1.0 M at 28 °C,[26] corresponding to a large dissociation constant for the NMR detectable F^-. From this apparent lack of concentration dependence, an approximate lower limit to the dissociation constant, $K_D(\text{NMR } F^-) \gtrsim 10$ M, can be set.[26] A slight concentration dependence at 50 °C and the temperature dependence of the ^{19}F relaxation rates yield the estimate for $K_D \approx 50$ M quoted in Table I.[13] Were the major contribution to the observed ^{19}F relaxation given by exchangeable F^- equatorially coordinated to GOase Cu(II) (as suggested in the competition scheme 1 above), the value of the dissociation constant for equatorial F^- (derived either from kinetic competition[27] or, implicitly from ESR results[11]—see Table I) and the deenhancement data (see Figure 1 and Table II) would predict a greater than 40% variation in ^{19}F relaxation rates over the concentration range employed, 0.462–1.0 M. Such a variation (well outside the precision of the relaxation rate measurements) was not observed.[26]

Therefore, scheme 2 above appears to be a more appropriate model. The analysis of the relaxation rate reduction due to CN^- addition proceeds as follows. We assume that only axially coordinated F^- contributes to the observed relaxation rates. Moreover we assume that there are three types of sites for axially coordinated F^-, which differ only in the nature of the equatorially coordinated exogenous ligand: H_2O (or OH^-), F^-, or CN^-. It is also reasonable to assume that the relaxation parameters and binding constants for axially coordinated F^- are essentially the same whether H_2O or F^- is equatorially coordinated, so that these two types of sites can be considered as one, designated B. This assumption is justified by the lack of F^- concentration dependence of the relaxation rates.[26] In any case, since the total F^- concentration was kept constant in the competition experiments reported here and since F^- was greatly in excess, the *ratio* of the concentration of GOase with equatorially coordinated F^- to that of enzyme with equatorially coordinated H_2O will remain constant, and the assumption of only one type of site (B) is operationally valid. The third type of axial site, associated with equatorial coordination of CN^-, will be designated as C. We assume also that F^- at sites B or C is in fast exchange with F^- in bulk solution, i.e., that slow or intermediate exchange effects do not contribute to the observed relaxation rates. The appropriate equilibria (eq 1a–e) for this competition scheme are given below. The observed

$$E{\cdot}F^-(eq) \overset{K_{eF}}{\rightleftarrows} E + F^- \quad (1a)$$

$$E{\cdot}CN^-(eq) \overset{K_{eC}}{\rightleftarrows} E + CN^- \quad (1b)$$

$$E{\cdot}F^-(ax) \overset{K_{aF}}{\rightleftarrows} E + F^- \quad (1c)$$

$$E{\cdot}F^-(eq){\cdot}F^-(ax) \overset{K_{aF}}{\rightleftarrows} E{\cdot}F^-(eq) + F^- \quad (1d)$$

$$E{\cdot}CN^-(eq){\cdot}F^-(ax) \overset{K'_{aF}}{\rightleftarrows} E{\cdot}CN^-(eq) + F^- \quad (1e)$$

relaxation rates (corrected for nonspecific and outersphere binding effects) are then given by eq 2. In eq 1a–e, K_{eF} and K_{eC} are the

$$1/T_{i,obsd} = R_{i,obsd} = f_B R^\circ_{iB} + f_C R^\circ_{iC} \ (i = 1 \text{ or } 2) \quad (2)$$

dissociation constants for equatorially coordinated F^- and CN^-, respectively, and K'_{aF} and K_{aF} are the respective dissociation constants for axial F^-, with and without equatorially coordinated CN^- present. In eq 2, f_B is the fraction of enzyme with type B sites (equatorially coordinated H_2O (OH^-) or F^-) and $f_C = 1 - f_B$ is the fraction with type C sites (equatorially coordinated CN^-). The relaxation rates R°_{iB} are those which are observed in the absence of CN^- (eq 3a), and R°_{iC} are the relaxation rates that

$$R^\circ_{iB} = \frac{C_E}{K_{aF} T_{iM,B}} \ (i = 1 \text{ or } 2) \quad (3a)$$

$$R^\circ_{iC} = \frac{C_E}{K'_{aF} T_{iM,C}} \quad (3b)$$

would be observed if all GOase Cu(II) had equatorially coordinated CN^- bound to it (eq 3b). In eq 3a,b it is assumed that the quantities C_E/K_{aF} and C_E/K'_{aF} are much less than unity, where

(26) B. J. Marwedel, Ph.D. Thesis, State University of New York at Buffalo, 1978.

(27) M. E. Winkler, R. J. Kurland, and R. D. Bereman, *J. Inorg. Biochem.*, in press.

C_E is the total enzyme concentration.

The mass action expressions corresponding to eq 1a–e can be solved for f_B in terms of the dissociation constants and concentrations

$$f_B = \frac{-u + (u^2 + 4\alpha\gamma)^{1/2}}{2\alpha} \quad (4)$$

where the parameters in eq 4 are given by $\alpha = (C_E/C_F)/(K_{eC}/K_{eF})$, $\gamma = 1 + (K_{eF}/C_F)$, $u = \gamma + \alpha(\beta - 1)$, β is the ratio of the total CN^- concentration to total GOase concentration, and C_F is the total concentration of F^-. If values for the dissociation constants K_{eC} and K_{eF} are taken from kinetic competition studies,[27] the values of f_B listed in Table II are obtained. Since the values of R°_{iB} are known from the measured relaxation rates in the absence of CN^-, the values of R°_{iC} can be determined via eq 2 from the calculated values of f_B and f_C and the observed relaxation rates at a given CN^- concentration. Thus the proposed competition scheme can be tested by a comparison of the calculated relaxation rates (i.e., the calculated contribution from both sites B and C) with the observed, as shown in Table II. We emphasize that this test is reasonably stringent: the only two freely adjustable parameters are R°_{1C} and R°_{2C}. Another important feature is that the values for the dissociation constants are taken from entirely independent experiments.[27] Since the value for K_{eC} taken from the kinetic competition studies[27] corresponds to an inhibition constant, rather than a true equilibrium dissociation constant, it should not be surprising that a decrease in K_{eC} to 1.8×10^{-5} M, with a concomitant change of R°_{1C} and R°_{2C} to 2.6 and 9.7 s^{-1}, respectively (K_{eF} remaining at 1.0 M), gives a better fit between calculated and observed relaxation rates. With this change the maximum deviation between observed and calculated relaxation rate values for the CN^-/GOase ratios shown in Table II is 5.1% and the average deviation is 3.5%.

The values of $R^\circ_{1C} = C_E/(K'_{aF}T_{1M,C}) = 1.2$–$2.6$ and $R^\circ_{2C} = C_E/(K'_{aF}T_{2M,C}) = 6.0$–$9.7$ derived from this analysis are much less than those for R°_{1B} and R°_{2B} (9.79 and 27.1, respectively). Thus the presence of equatorially coordinated CN^- evidently alters the intrinsic relaxation rates of F^- at the axial site (i.e., modifies the superhyperfine interaction) and/or increases the axial dissociation constant. The latter effect may be more significant; however, the large difference between the ratio $R^\circ_{2C}/R^\circ_{1C} = R_{2M,C}/R_{1M,C} \gtrsim 4$ and that for no CN^-, $R^\circ_{2B}/R^\circ_{1B} = R_{2M,B}/R_{1M,B} = 2.77$, strongly suggests that equatorial coordination of CN^- alters the superhyperfine coupling constants of axial F^-. One would expect that the electron distribution at the Cu(II) center is altered by CN^- ligation, and it is evident that ^{19}F NMR relaxation effects can provide a probe of these changes.

Cyanide Effects on Galactose Oxidase ESR Spectra. To assess these changes further, we compared the ESR spectra of galactose oxidase and its $^{12}CN^-$ and $^{13}CN^-$ complexes in order to establish the nature and effect of CN^- coordination.[28] These spectra are shown in Figure 3, and the spin Hamiltonian parameters are listed in Table III. The presence of 0.4 M F^- has no apparent effect on these spectra. Moreover, addition of 1:1 NaCN to the galactose oxidase–F^- complex converts the ESR spectrum of that complex[11] to that seen in Figure 3 (B or C). Changes in $g_\parallel$ and $A_\parallel$ upon CN^- coordination have been noted and discussed before.[5,11,12] Coupling between the unpaired electron spin and the cyanide ^{13}C nucleus is manifest in the broadening of the $M_I = +3/2$ and $+1/2$ transitions in the $g_\parallel$ region (see insert, Figure 3) and the splitting of the Kivelson line[29] centered at 3250 G. This latter "overshoot" line has been useful in previous analyses of galactose oxidase ESR spectra.[11] The splitting of this transition into two components in the $^{13}CN^-$ species (centered at 3221 and 3272 G, respectively) is evident. Also apparent is the shift of the highest field transition from 3276 to 3298 G; this transition is associated with the perpendicular Cu(II) signal and contains ^{14}N superhyperfine splittings attributed to endogenous ligands (protein imidazoles).[11,12] The other "half" of this transition is located at 3257 G. The ^{13}C and

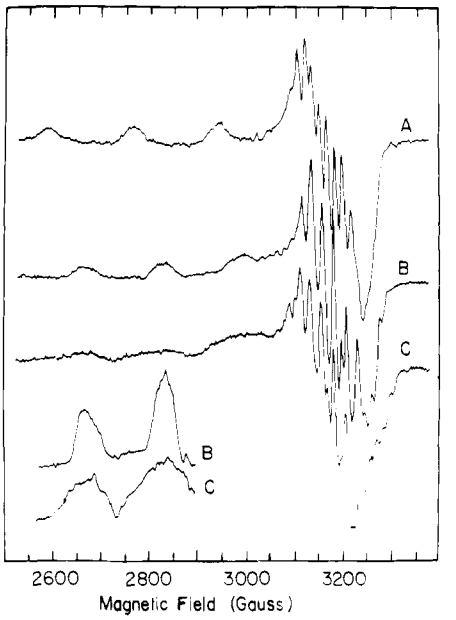

Figure 3. ESR spectra of ^{63}Cu galactose oxidase and its $^{12}CN^-$ and $^{13}CN^-$ complexes. Conditions: [enzyme] = 0.9 mM; [cyanide] = 1.0 mM in 0.1 M sodium phosphate buffer (pH 7.0). Sample temperature was 100 K at 9.115 GHz; microwave power is 20 mW with a modulation amplitude of 2 G. The following spectra were observed: A, enzyme alone; B, $+Na^{12}CN$; C, $+Na^{13}CN$. Insert shows low field regions of B and C at higher gain.

Table III. Spin Hamiltonian Parameters for Cu(II)[a]

Galactose Oxidase[b]		
$g_\parallel = 2.277$	$A_\parallel = 175.0$	$A_N^\parallel = 14.5$
$g_\perp = 2.055$	$A_\perp$ = small	$A_N^\perp = 15.1$
		$A_N^0 = 14.8$
Galactose Oxidase–$^{12}CN^-$ Complex		
$g_\parallel = 2.233$	$A_\parallel = 159.0$	$A_N^\parallel$ = nr
$g_\perp = 2.050$	$A_\perp$ = small	$A_N^\perp = 22.5$
		$A_N^0 = 13.7$
Galactose Oxidase–$^{13}CN^-$ Complex		
$g_\parallel = 2.23$[c]	$A_\parallel = 160$[c]	$A_N^\parallel$ = nr $A_{^{13}C}^\parallel = 96$[d]
$g_\perp = 2.050$	$A_\perp$ = small	$A_N^\perp = 22.5$ $A_{^{13}C}^\perp = (45)$[e]
		$A_N^0 = 13.6$ $A_{^{13}C}^0 = 49$

[a] A values are given in gauss. The notation $\parallel$, $\perp$, and 0 indicates the superhyperfine couplings in each of the respective orientations; 0 represents the overshoot line. N refers to the endogenous nitrogen ligands; ^{13}C refers to $^{13}CN^-$. nr means not resolved. [b] From ref 11. [c] Line width precludes more precise measurement. [d] Estimated from line widths of $^{12}CN^-$ and $^{13}CN^-$ complexes. [e] Assumed to be a near multiple of $A_N^\perp$; see discussion in text.

^{14}N coupling constant values given in Table III are derived from this simple, but consistent analysis which is aided by the ^{13}C splitting of overlapping ^{14}N superhyperfine transitions.[28]

The $^{13}CN^-$ coupling constant for the $g_\perp$ region is inferred from the following two features: (1) the appearance of a new transition at the low field side of the $g_\perp$ envelope and (2) the apparent resolution of some transitions due, presumably, to different spectral overlaps. The "new" low field transition can be used to estimate the value for the ^{13}C coupling in the $g_\perp$ region. As is typical, this value (given in Table III) is smaller than this constant in the $g_\parallel$ or overshoot line regions; the coupling in the latter represents a weighted average over the three principal orientations.[4,29]

An additional result derived from this analysis is that the ^{14}N shfs due to protein imidazole ligands apparently changes on CN^- coordination. The ^{14}N coupling in the $g_\perp$ region clearly increased (from 15.1 to 22.5 G), while the ^{14}N splitting in the overshoot line decreases slightly.

(28) P. H. Haffner and J. E. Coleman, *J. Biol. Chem.*, **248**, 6629 (1973).
(29) R. Neiman and D. Kivelson, *J. Chem. Phys.*, **35**, 156 (1961).

Discussion

Eisenstadt and Friedman have pointed out[14] that the point dipole–dipole and Fermi contact interactions alone are not large enough to account for the observed spin–lattice relaxation rate of fluoride in aqueous CuF^+. From theory developed for the analysis of anisotropic ^{19}F NMR shifts of crystalline paramagnetic transition-metal fluoride,[21,30] they proposed that the constants

$$A_a = D + A_\sigma + A_\pi \quad (5a)$$

$$A_i = A_s + A'_s \quad (5b)$$

be used to describe the hyperfine interaction giving rise to the relaxation of F^- in aqueous CuF^+. The constant, D, represents the usual point dipole interaction between the ^{19}F nucleus and unpaired electron spin density centered at the Cu(II). A_σ and A_π give the dipolar interaction due to unpaired spin in p_σ and p_π orbitals, respectively, centered at the bound F^-; A_s represents the usual Fermi contact interaction constant while A'_s is the constant for isotropic interaction due to the polarization of ligand fluoride s electrons by spin density in fluoride p orbitals. The use of eq 5a,b is appropriate if the anisotropy of the **g** tensor can be neglected and only one Kramer's multiplet is thermally populated,[20,31] as will be the case for Cu(II).

The source of the large ^{19}F hyperfine constant for aqueous CuF^+ has been attributed to overlap between Cu(II)-centered and F^--centered orbitals for equatorially coordinated F^-.[14] The argument for equatorial coordination of the aqueous CuF^+ complex implied that such overlap, and the corresponding hyperfine contribution to the relaxation mechanism of Cu(II)-bound F^-, would be much smaller for axially coordinated fluoride. The values of the hyperfine constants A_i and A_a derived from the ^{19}F NMR relaxation measurements (see Table I) are consistent with this proposition, in that they are much less than those obtained for aqueous CuF^+ and less than those for the ESR detectable F^-, presumably equatorially coordinated.

An upper limit for the value of D, the point dipolar contribution to A_a, can be estimated as about 0.58×10^8 s^{-1} (with $r = 2.05$ Å, taken as the sum of the ionic radii of Cu(II) and F^-); thus this maximum value of D can provide only a 30% contribution to A_a (galactose oxidase $Cu^{II}F^-$). For greater Cu–F distance (~2.6 Å), corresponding to axial coordination, the value of D would be even smaller. The values of A_a and A_i (deduced from ^{19}F NMR data) for galactose oxidase $Cu^{II}F^-$ are both 4 times smaller that those values calculated for aqueous Cu(II)-bound fluoride (see Table I); however, the ratios A_i/A_a are identical for both fluoride complexes. The smaller value of A_a (galactose oxidase $Cu^{II}F^-$) compared to A_a ($Cu^{II}(aq)F^-$) must be due to a reduction in the unpaired electron spin density in the p_σ and p_π orbitals of enzyme Cu(II)-bound fluoride. Similarly, the smaller value of A_i (galactose oxidase $Cu^{II}F^-$) can be attributed to a reduction in the Fermi contact interaction and/or the isotropic interaction due to the polarization of the s electrons of the fluoride ion by unpaired electron spin density in the p orbitals.

ESR data, on the other hand, suggest that a fluoride ion coordinates equatorially to galactose oxidase Cu(II).[11] The most plausible interpretation of the fluoride relaxation data and these ESR data is that two sites are available for F^- at the enzyme Cu(II): at one site, F^- is equatorially coordinated and relatively strongly bound such that exchange with free F^- in solution is slow (on the appropriate NMR time scale);[32] at the other, F^- is weakly axially coordinated and exchanges rapidly with free F^-. The equatorially bound F^- is that detected in the ESR experiments, while the axially bound fluoride is that observed in the ^{19}F relaxation measurements.

Also consistent with this interpretation are values for the dissociation constants. The value for K_{aF} for the assumed axial F^-, as derived from the ^{19}F relaxation results,[13] is about 50 times greater than the dissociation constant for $CuF^+(aq)$ (see Table I); in the latter complex for the F^- is coordinated equatorially.[14] Moreover, the value for the fluoride inhibition constant, K_I, derived from kinetic measurements of galactose oxidase activity in the presence of F^-,[27] is equal to that for the dissociation constant for $CuF^+(aq)$,[14] a result which also argues for two-site F^- binding.

The superhyperfine coupling of $^{13}CN^-$, as detected by ESR, clearly shows that cyanide binds via carbon coordination to Cu(II) *at an equatorial site*, as has been observed for this ligand's interaction with superoxide dismutase and Cu(II) carbonic anhydrase.[28] Thus the ESR results establish that the effects of CN^- observed in the ^{19}F relaxation experiments (Figure 1) are due to equatorially coordinated CN^-. Moreover, the good agreement between the value derived in this study for K_{eC} and that for the CN^- inhibition constant[27] provides a strong argument that the kinetic effects of CN^- are due to an equatorially coordinated ion. Since CN^- has been shown to act as a "noncompetitive" inhibitor of GOase, i.e., substrate does not compete with CN^- for the same GOase binding site,[27] one can conclude that substrate does *not* bind at an equatorial site, but rather at an outersphere or axial position. Fluoride also acts as a noncompetitive inhibitor against alcohol substrate;[27] in order to reconcile this result with the observation that added alcohol substrate quantitatively deenhances fluoride ^{19}F NMR relaxation in the presence of GOase,[33] one must again invoke two types of F^- coordination at the GOase Cu(II).

A result of equal importance is the effect the CN^- coordination has on the electron spin distribution in the enzymic copper complex, as indicated by ESR data, and how this effect correlates with the ^{19}F relaxation data. In addition to changes in ^{14}N superhyperfine splittings manifested directly in the ESR spectrum, CN^- binding to GOase Cu(II) also changes the ESR spin–echo modulation envelopes in such a way as to suggest that there is less spin density at the distal, noncoordinated nitrogens of the protein imidazole groups in the CN^- complex than in the native ezyme.[11,12] These ESR results are in accord with the back-bonding properties of a strong π-bonding ligand such as CN^-. Since the Cu(II) orbitals involved in such CN^- bonding are, in part, the same as those which would be involved in a F^-(axial)–Cu(II) p_π–d_π bond, a decrease in the F^- (axial) intrinsic relaxation rate and/or an increase in the dissociation constant upon CN^- coordination is consistent with ESR and ESR spin–echo results.

The CN^- competition results are also consistent with our analysis of the F^- relaxation parameters,[13] in that both indicate that the NMR detectable F^- (in fast exchange with free F^-) is axially coordinated, while CN^- displaces equatorially coordinted (ESR detectable) F^- which exchanges too slowly with bulk fluoride to be detected by the NMR relaxation experiments.[32] The approximate magnitude of the F^- dissociation constant derived from the analysis of the relaxation rate changes on CN^- addition are consistent also with ESR competition results. At 1:1 ratio of added CN^- to GOase, the fraction of enzyme with equatorially coordinated F^- or CN^- can be estimated via eq 4 for the concentrations which obtained in the ESR or NMR experiments. In the ESR experiments,[11] where enzyme concentration was 9.0×10^{-4} M and F^- concentration 0.22 M, the estimated fractions of GOase with equatorially coordinated CN^- and F^- are about 80 and 4%, respectively. In the ^{19}F relaxation experiments, where GOase and F^- concentrations were 7.5×10^{-5} and 1.0 M, respectively, the estimated fractions of GOase with equatorially coordinated CN^-

(30) B. Bleaney, *Phys. Rev.*, **104**, 1190 (1956).
(31) R. J. Kurland and B. R. McGarvey, *J. Mag. Reson.*, **2**, 286 (1970).
(32) The assumption that equatorially coordinated F^- is in slow exchange with bulk F^- can be justified as follows. The values of the superhyperfine and dissociation constants appropriate for equatorial F^- would yield (see ref 13) a value for the correlation time, τ_c, operative in the relaxation mechanism of about 6×10^{-12} s, a value approximately 2 orders of magnitude smaller than any reasonable estimate. Moreover, one would expect that if exchange between axial and equatorial F^- lay in the fast or intermediate region (on the appropriate NMR time scale), the effect would be manifested in a significant concentration dependence of the observed relaxation rates, which was not observed.

(33) B. J. Marwedel and R. J. Kurland, *Biochim. Biophys. Acta.*, **657**, 495 (1981). The ^{19}F NMR relaxation deenhancement caused by added substrate can be rationalized by either of the following explanations: (1) bound substrate displaces axially coordinated F^-; (2) bound substrate prevents GOase-bound F^- from exchanging rapidly with F^- free in solution. The latter explanation might also apply to equatorially coordinated F^-.

and F^- are about 60 and 30%, respectively. These estimates are consistent with the changes observed in both the ESR and ^{19}F NMR experiments.

The model proposed here—one equatorial and one axial binding site for exogenous ligands—is compatible with the known five-coordinate chemistry of copper(II) centers.[34] If it is assumed that the GOase Cu(II) site consists of three equatorially coordinated endogenous ligands from the protein matrix, one labile equatorially coordinated ligand (in the native enzyme, H_2O or, possibly, OH^-) and a quite labile, weakly bound axially coordinated ligand (again in the native enzyme, H_2O), a square-pyramidal five-coordinate geometry would be present. In such Cu(II) complexes the axial bond distances are always significantly longer than equatorial ones. Thus ligands which would bind potentially via π bonds would coordinate preferentially at an equatorial site and would tend to stabilize four coordinate compared to five-coordinate geometries. This latter dictum can be interpreted in terms of competition for the same Cu(II) orbitals required for π bonds by both axial and equatorial ligands. Thus poor π-bonding ligands (e.g., F^-, H_2O) do not compete effectively with CN^- for an equatorial binding site. On the other hand, if CN^- is already equatorially coordinated, a second CN^- would be less likely to coordinate axially; in the case at hand, F^- (a "hard" ligand that binds predominantly through σ bonds) would compete effectively against excess CN^- for an axial site. The result that axially coordinated F^- binds more weakly than does equatorially coordinated F^- can be rationalized in terms of the longer bond distance for the former, a general feature of Cu(II) square-pyramidal five-coordinate geometry. The same argument can be used to justify the inference that axially coordinated F^- exchanges relatively rapidly with bulk F^- compared to equatorially coordinated F^-. Thus, for a five-coordinate Cu(II) square-pyramidal complex in which one fluoride was coordinated at an equatorial site and one at an axial position, one would expect CN^- to displace preferentially the equatorial F^- and also to find that the bonding of the axial F^- is weaker in the CN^- complex. Such effects are just those which have been inferred from the ^{19}F NMR relaxation measurements.

In summary, we conclude that the presence of two Cu(II) coordination sites in GOase for exogenous ligands is established by these results. The data presented in support of a high affinity, equatorial ligand site augment ESR results reported previously.[5b,c,11] That anions (and water) bind also, albeit weakly, to an axial site is a suggestion that may be relevant to the coordination of hydroxyl groups of substrate ligands.[5a] Moreover, the electronic interaction between Cu(II) and axially coordinated ligand can be modified, as shown in this work, if the labile equatorial ligand (H_2O or OH^-) ordinarily present is replaced by a strong π-bonding ligand such as CN^-. Thus this modulation may be related to the properties of these exogenous ligands as noncompetitive inhibitors[27] (i.e., inhibitors which do not seriously effect GOase substrate binding but do block enzyme catalysis). Kinetic and magnetic resonance studies are being carried out to elucidate the relationship between the bonding properties of an equatorially coordinated exogenous ligand and its effect on catalysis.

Acknowledgment. B.J.M. gratefully acknowledges fellowships from the Allied Chemical Corp. and from the Graduate School, SUNY/Buffalo. We thank the National Science Foundation for support for this research (Grant No. BMS73-01248-A01) and for some of the NMR instrumentation used (Grant No. CHE7506183-A02 to the Department of Chemistry, SUNY/Buffalo). We also thank our colleagues in the Bioinorganic Graduate Research Group for stimulating and helpful discussions.

(34) B. J. Hathaway and D. E. Billing, *Coord. Chem. Rev.*, **5**, 143 (1970), and references therein.

Electron Paramagnetic Resonance Study of Nitrosylhemoglobin and Its Chemistry in Single Crystals

D. C. Doetschman* and S. G. Utterback[1]

Contribution from the Department of Chemistry, State University of New York at Binghamton, Binghamton, New York. *Received July 21, 1980*

Abstract: The electron paramagnetic resonance (EPR) of nitrosylhemoglobin (HbNO) is measured in single crystals of converted human oxyhemoglobin (HbO_2). Several chemical and physical processes in the crystals are observable by means of the EPR. At 9 °C HbO_2 a subunits exchange O_2 for NO faster than b subunits. The subunit spectra display both ^{14}NO and [^{14}N]histidine hyperfine structure. Power saturation between 1.6 and 4.2 K and temperature dependence above 80 K point to efficient spin–spin and spin–lattice relaxation processes that involve heme–heme magnetic dipolar interactions between both kinds of subunits. With a Hg–Xe lamp, HbNO photolysis quickly occurs at 4.2 K, resulting in the disappearance of the EPR spectrum. The HbNO spectrum returns when the sample is warmed from 4.2 to 85 K.

Nitrosylhemoglobin (HbNO) resembles the physiologically important oxyhemoglobin (HbO_2) in several ways, presumably because of similar electronic structures.[2–5] Moreover, HbNO has the advantage of spin $S = {}^1/_2$ in the highest occupied molecular orbital, making electron paramagnetic resonance (EPR) possible, as first shown by Gordy and Rexrod.[6] The heme structures in HbNO and a free model oxyheme are very similar:[7–8] the FeNO and FeOO bond angles are less than 180° and in both structures more than one type of diatomic molecule projection on the heme

(1) From dissertation research in partial fulfillment of Ph.D. requirements.
(2) Kon, H. *J. Biol. Chem.* **1968**, *243*, 4350.
(3) Kon, H. *Biochemistry* **1969**, *8*, 4757.
(4) Doetschman, D. C. *Chem. Phys.* **1980**, *48*, 307.
(5) Doetschman, D. C.; Schwartz, S. A.; Utterback, S. G. *Chem. Phys.* **1980**, *49*, 1.
(6) Gordy, W.; Rexrod, H. N. In "Free Radicals in Biological Systems", Blois, M. S., Ed., Academic Press: New York; 1961.
(7) Deatherage, J. F.; Moffat, K. *J. Mol. Biol.* **1979**, *134*, 401.
(8) Collman, J. P.; Gagney, R. R.; Reed, C. A.; Halberty, T. R.; Lang, G.; Robinson, W. T. *J. Am. Chem. Soc.* **1975**, *97*, 1427.

0002-7863/81/1503-2847$01.25/0