Azide and Fluoride Binding to *E. Coli* Iron Superoxide Dismutase as Studied by Solvent Proton Magnetic Relaxation Dispersion

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Abstract: Nuclear magnetic relaxation dispersion (NMRD) is used to probe solvent exchange with the Fe^{III} site in *E. Coli* superoxide dismutase (FeSOD). We find that the water proton magnetic relaxation rates in aqueous solutions of the native enzyme are largely determined by innersphere interactions with Fe^{III} but that the outersphere contribution is not negligible. Azide binding dramatically *increases* the FeSOD relaxivity and alters the functional form of the NMRD profile. The enhanced relaxivity of the FeSOD–N_3^- complex is proposed to result from a rapidly exchanging water molecule that is hydrogen bonded to coordinated azide, with an Fe^{III}–^1H distance of ~3 Å. In marked contrast, fluoride binding to the Fe^{III} ions of FeSOD does not significantly alter the FeSOD relaxivity. Coordinated azide and fluoride must therefore interact with solvent very differently, suggesting that these two FeSOD–anion complexes have different structures.

Iron-containing superoxide dismutases (FeSODs) have been isolated from plants, protozoa, and both aerobic and anaerobic bacteria. *E. Coli* FeSOD is a dimer of two identical subunits with a dimeric molecular weight of 38 000 daltons.[2-4] Amino acid sequence analysis and X-ray crystallography of Fe- and MnSODs have established that these two enzymes are closely related and that both are completely different from the Cu,ZnSOD found in eukaryotic cells.[2-4] The structures of the Mn^{III} and Fe^{III} sites appear very similar: four amino acid ligands are evident in the *E. Coli* and *Pseudomonas ovalis* FeSOD crystal structures[3] and in the *Thermus thermophilus* MnSOD structure.[4] Comparison of the tertiary structures and available sequence data indicates that the metal ion ligands are three histidines and one aspartic acid.[4,5] H_2O may also be coordinated to Fe^{III}, producing a five-coordinate structure.[3] Extensive kinetics studies on the *E. Coli* FeSOD fully support a catalytic mechanism based on a redox cycle involving innersphere electron-transfer reactions between Fe^{III}/Fe^{II} and O_2^-.[6] Proton transfer from a general acid to a metal-bound intermediate is thought to be rate limiting,[6] and the existence of a labile metal–ion coordination site in both oxidation states is a key feature of the proposed mechanism. The Fe^{III} ligand-substitution chemistry of SOD is consistent with a single labile coordination position. Azide and fluoride bind to Fe^{III}, whereas cyanide and thiocyanate do not.[7,8] Azide binding produces characteristic absorption bands at 440 nm (ϵ = 1660 M^{-1} cm^{-1}) and 320 nm (ϵ = 3300 M^{-1} cm^{-1}) that may be assigned as $N_3^- \rightarrow Fe^{III}$ charge-transfer transitions.[7] Addition of fluoride partially bleaches the 350 nm absorption band of the resting enzyme; a similar effect, observed at high pH in the absence of fluoride, has been interpreted as ionization of a coordinated H_2O with $pK_a \simeq 9$.[6,7] The X-ray diffraction data show that a single N_3^- binds to Fe^{III}.[3] Results from studies of azide binding to simple Fe^{III} chelates suggest that H_2O is the leaving group in the ligand-substitution reaction of FeSOD with N_3^- as well.[9]

Extensive thermodynamic and kinetic studies of anion binding to FeSOD have been conducted, in part because small anions may serve as useful O_2^- analogues.[8,10] $\Delta H°$ dominates azide–iron(III) complex formation, but $\Delta S°$ controls fluoride binding ($\Delta H° \approx 0$ in this case).[10] Qualitatively, both ions bind rapidly; for example, the rate constant for N_3^- coordination to Fe^{III} is $k = 3.9 \times 10^4$ s^{-1} at pH 7.4.[8] Fluoride and azide are competitive inhibitors of FeSOD.[6] Several other anions, e.g., SCN^-, ClO_4^-, Cl^-, SO_4^{2-}, also competitively inhibit FeSOD but do not bind to the Fe^{III}.[6] These anions apparently bind to a specific anion-binding site on the protein.

Water ^1H magnetic relaxation measurements have been previously carried out on FeSOD solutions.[11] The principal conclusion was that solvent water relaxation rates in the 6–48-MHz range are dominated by outersphere interactions with the Fe^{III}. Cyanide had no effect on the FeSOD-induced water relaxivity,[11] but this is a trivial result since CN^- does not coordinate to Fe^{III}. Numerous previous studies on a variety of metalloproteins have established that NMRD experiments, in which the solvent ^1H longitudinal proton relaxation rate $(T_1)^{-1}$ is measured over a wide range of magnetic field, can provide considerable insight into the metal–ion coordination chemistry of metalloproteins.[12] Further, it is now clear that metalloprotein–solvent interactions, and the associated relaxation effects, are more complex than originally anticipated.[12,13] Here we report measurements of the NMRD

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profiles of FeSOD solutions. The effects of N_3^- and F^- binding on the profiles have also been carefully examined in order to probe the Fe^{III} ligand-substitution chemistry. Unexpectedly, we found that FeSOD complexes with N_3^- and F^- interact with solvent very differently, implying that these complexes have different structures.

Experimental Section

FeSOD was purified from *E. Coli B* (Grain Products, Inc.) essentially as described by Slykhouse and Fee, using a hydroxylapatite column as the final step.[7] Our preparation was homogeneous, judged by SDS gel electrophoresis (protein staining), activity measurements, and spectral characteristics. SOD activity was measured with use of the cytochrome *c* reduction assay.[14] Samples used for relaxation studies were extensively dialyzed against chelexed 50 mM HEPES, 0.1 M NaCl buffer (pH 7.5), concentrated (Amicon YM 10 membrane) to 0.40 mM protein (0.8 mM Fe), and then passed through a Millipore 0.22 μm filter. NaN_3 and NaF solutions were also prepared in the same buffer immediately prior to the NMRD measurements. Initial relaxation rate measurements were performed at Amherst College on a JEOL FX-100 NMR spectrometer in HEPES buffered D_2O solutions, using the inversion recovery technique and a 180°–τ–90° pulse sequence. The relaxivities at 100 MHz followed the same pattern as the NMRD profiles presented herein: N_3^- significantly increased the FeSOD relaxivity whereas F^- had little effect. Complete NMRD profiles were obtained at IBM with use of instrumentation and analytical methods previously described.[15] All absorption spectra were obtained with a Varian 219 spectrophotometer interfaced to an Apple IIe computer.

Results

Background. It is well established that N_3^- and F^- bind to the Fe^{III} ions in FeSOD.[3,7,8,16] The azide complex appears five-coordinate at ~3 Å resolution, which suggests that H_2O, if it is a ligand in the native FeSOD, is the leaving group in the reaction with N_3^-. In addition to the putative coordinated water, another potential anion coordination position has been identified in the MnSOD structure and may also be present in FeSOD.[17] If so, anion binding may increase the metal–ion coordination number. Subject to certain constraints, NMRD measurements can distinguish the two cases.

The basic theory for solvent relaxation by paramagnetic ions has been extensively described in the literature.[13,18] Equations 1 and 2 summarize the contributions to the relaxivity that must be considered. The NMRD profile in solutions containing

$$(T_1)^{-1} = (T_1)_w^{-1} + (T_1)_{p\alpha}^{-1} + (T_1)_{p\beta}^{-1} + (T_1)_d^{-1} + (T_1)_{os}^{-1} \quad (1)$$

$$(T_1)_{p\alpha,\beta}^{-1} = \frac{[M]}{55.5}(T_{1M_{\alpha,\beta}} + \tau_{M_{\alpha,\beta}})^{-1},\ [M] \equiv [\text{paramagnetic ion}] \quad (2)$$

paramagnetic ions bound to macromolecules can be divided into five contributions: $(T_1)_w^{-1}$, the (solvent) background rate in the absence of paramagnetic ions and macromolecules; $(T_1)_{p\alpha}^{-1}$, the contribution from solvent exchange with the inner-coordination sphere of the paramagnetic ion; $(T_1)_{p\beta}^{-1}$, the contribution from exchange of weakly bound solvent beyond the first-coordination shell; $(T_1)_d^{-1}$, the diamagnetic contribution from the apoprotein itself;[19] $(T_1)_{os}^{-1}$, the outersphere contribution from the diffusion of solvent molecules into the vicinity of the paramagnetic ion. As indicated in eq 2, $(T_1)_{p\alpha,\beta}^{-1}$ depends on $T_{1M_{\alpha,\beta}}$, the longitudinal relaxation time of coordinated water protons, and $\tau_{M_{\alpha,\beta}}$, their residence lifetime. $(T_1)_{os}^{-1}$ is a function of the electron relaxation time, τ_s, and the correlation time for diffusion, $\tau_D = a^2/3D$, where a is the distance of closest approach between the solvent nuclei and the paramagnetic ion and D is the relative solvent–macromolecule diffusion constant.

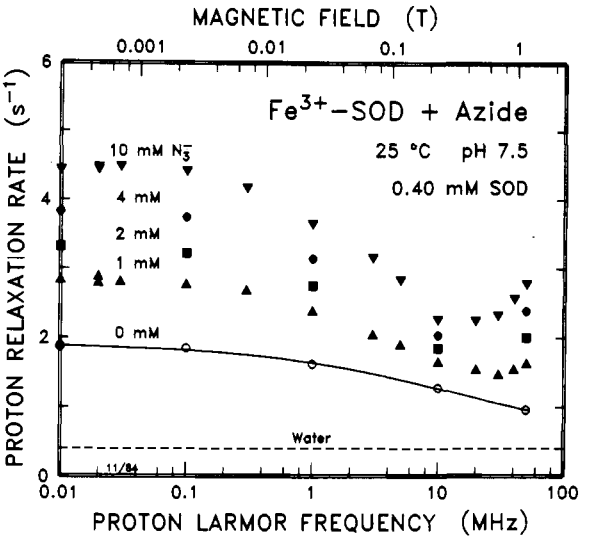

Figure 1. Longitudinal NMRD profiles for FeSOD in the absence and presence of azide in 50 mM HEPES, 0.1 M NaCl (pH 7.5) at 25 °C: (○) FeSOD; (▲) FeSOD plus 1.0 mM NaN_3; (■) FeSOD plus 2.0 mM NaN_3; (●) FeSOD plus 4.0 mM NaN_3; (▼) FeSOD plus 10.0 mM NaN_3. The dashed line indicates the relaxation rate of pure water; earlier data established that the diamagnetic contribution from the protein is appreciably smaller than this.

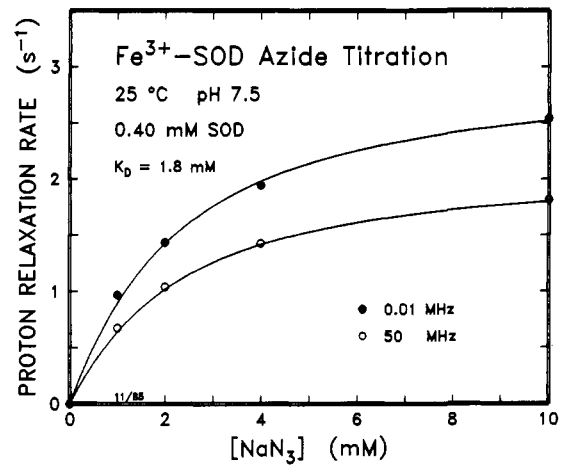

Figure 2. The increment in the longitudinal solvent proton relaxation rate in solutions containing FeSOD as a function of azide concentration: (●) rate at 0.01 MHz; (○) rate at 50 MHz. The data are from Figure 1 with the contribution from the azide-free sample subtracted.

Analysis. Figure 1 shows the NMRD profiles of FeSOD with successive additions of azide, at 25 °C. The background rate of the buffer ("water") is indicated. The diamagnetic contribution, $(T_1)_d^{-1}$, from the protein is negligible; from Figure 2, ref 19b, it is less than half the buffer contribution at low fields, and smaller at high fields. Thus in the absence of azide the NMRD profile can be regarded as arising entirely from paramagnetic effects. The low-field contribution from Fe^{III} is then about 1.4 s^{-1}, giving a relaxivity (per Fe^{III} ion) of 1.8 $(mM\ s)^{-1}$. This relaxivity is about 6-fold greater than that of methemoglobin and comparable to that displayed by the Fe^{III}–citrate complex,[20] which is known to arise from outersphere relaxation.

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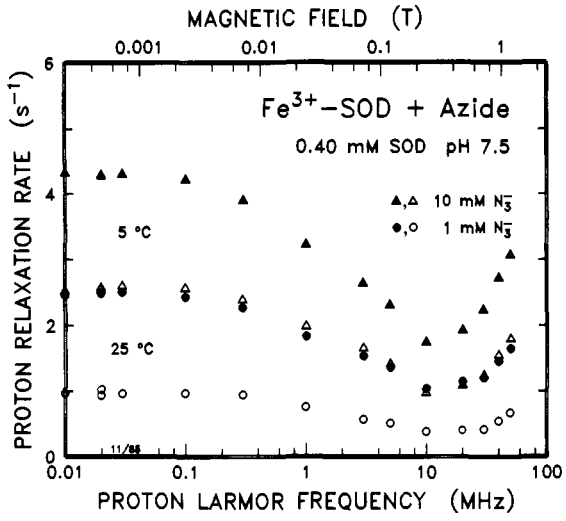

Figure 3. Temperature dependence of the FeSOD NMRD profiles. The relaxation rates of the native FeSOD (with no azide) have been subtracted from the profiles for FeSOD with 1.0 mM and 10.0 mM NaN_3: (○, △) NMRD profiles at 25 °C of FeSOD plus 1.0 and 10.0 mM NaN_3, respectively; (●, ▲) NMRD profiles at 5 °C of FeSOD plus 1.0 and 10.0 mM NaN_3, respectively.

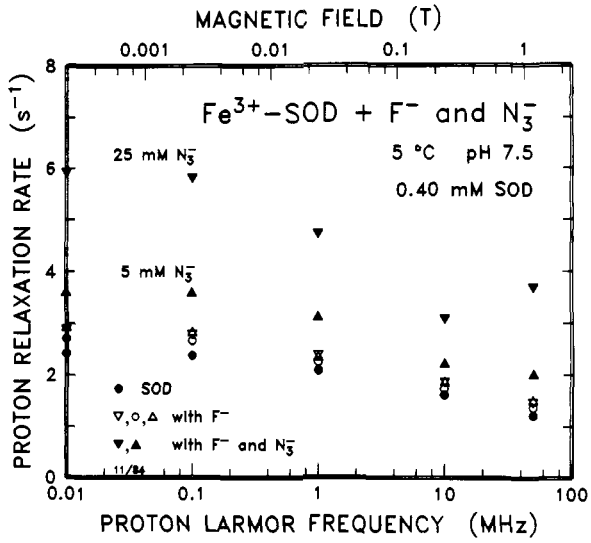

Figure 4. NMRD profiles for FeSOD and its anion complexes in 50 mM HEPES, 0.1 M NaCl (pH 7.5) at 5 °C: (●) native FeSOD; (○) FeSOD plus 5.0 mM NaF; (△) FeSOD plus 15 mM NaF; (▽) FeSOD plus 45 mM NaF; (▲) FeSOD plus 45 mM NaF and 5.0 mM NaN_3; (▼) FeSOD plus 45 mM NaF and 25 mM NaN_3.

Adding azide *increases* the relaxivity of FeSOD (Figure 1) and changes the form of the NMRD profile. Increased relaxation rates following exogenous ligand binding to a metalloprotein are well documented and generally reflect $(T_1)_{p\beta}^{-1}$ mechanisms.[12,15] For example, fluoromethemoglobin has relaxivity values about an order of magnitude greater than those of methemoglobin, even though F^- unambiguously displaces the H_2O coordinated to the Fe^{III} centers in methemoglobin.[15] The methemoglobin relaxivity is low because the coordinated H_2O is in slow exchange and outersphere H_2O cannot closely approach the paramagnetic ion. On the other hand, water may hydrogen bond to F^- in fluoromethemoglobin, with a relatively short Fe^{III}–^1H distance, and simultaneously be in rapid (but not too rapid) exchange, thereby increasing the relaxivity. A similar mechanism can readily account for the increased relaxivity of the FeSOD–N_3^- complex, assuming that a rapidly exchanging water is hydrogen bonded to azide. The form of the NMRD profile indicates that the relaxivity of the FeSOD–N_3^- complex depends mostly on T_{1M}, which is a function of τ_s, τ_M, and τ_R (the rotational relaxation time of the protein). In fact, the upturn at high fields is related to the magnetic field dependence of τ_s. The "fluoromet" or $(T_1)_{p\beta}^{-1}$ mechanism has also been invoked to explain the relaxivity of Fe^{III}–transferrin,[12] the Cu^{II}- and VO^{2+}-substituted transferrin derivatives,[21] and nitroxide radical complexes with proteins;[22] it appears to be rather important for paramagnetic systems in which water exchange from the first-coordination sphere is either slow or absent. Azide binding to FeSOD could in principle increase the relaxivity by favorably altering τ_s. However, the increased EPR line width of the FeSOD–N_3^- complex,[7] compared to native FeSOD, shows the change to be in the wrong direction.

The increment in the FeSOD relaxation rates at two fields, as a function of the N_3^- concentration, is shown in Figure 2. The solid curves through both sets of data were derived by assuming that azide forms a binary complex with FeSOD, with a well-defined relaxivity at each field, and (in this case) a dissociation constant K_D equal to 1.8 mM at 25 °C. $K_D(N_3^-)$ determined by NMRD (Figure 2) is identical with that obtained by monitoring the $N_3^- \rightarrow Fe^{III}$ charge-transfer bands,[6] strongly suggesting that coordinated N_3^- mediates the enhanced relaxation.

Figure 3 shows the NMRD profile at 5 and 25 °C for the concentration extremes of the azide titration. It is apparent that the relaxivity increases with decreasing temperature, indicating that exchange is relatively rapid, and that $\tau_{M\beta}$ must be considerably smaller than $T_{1M\beta}$, eq 2. Further, exchange of H_2O molecules must be responsible for proton exchange as the experiments were conducted near neutral pH. From eq 2, and the data in Figure 3, one obtains $\tau_{M\beta} \leq 10^{-5}$ s. The most plausible explanation for the temperature dependence of the NMRD profiles is that the correlation time τ_c for the interaction of Fe^{III} ions with water protons is greater at 5 °C than at 25 °C. An estimate of the range of its value, made in the usual fashion from the results in Figure 3, is 10^{-9}–10^{-10} s. Since τ_R of the protein is $\sim 10^{-8}$ s, τ_c is determined by τ_s and τ_M. From the theory, an increase in either of these as temperature decreases would account for the observed temperature dependence of the FeSOD relaxation rates. The upturn in the relaxation rate at high field indicates that τ_s makes a substantial contribution to τ_c.

Figure 3 also shows that K_D is greater, i.e., binding is weaker, at 25 °C than at 5 °C by a factor of about 1.5 (at 5 °C, the data for 1 mM azide are more than halfway to saturation, whereas at 25 °C, they are much less than halfway). Thermodynamics measurements confirm this point as $\Delta H° = -8.3$ kcal/mol for FeSOD–N_3^- complex formation.[8]

Surprisingly, solutions of FeSOD and the FeSOD–F^- complex have nearly identical NMRD profiles (Figure 4). At $[F^-] \approx 45.5$ mM FeSOD is known to be more than 85% complexed, yet the relaxivity increases by, at most, ~20%. (Similar results were obtained for F^- binding to Mn^{II}–carboxypeptidase.[23]) Both spectroscopic results and X-ray diffraction data are consistent with F^- coordination to Fe^{III} in the FeSOD–F^- complex.[7,16] Since it is established that F^- coordinated to Fe^{III} can mediate enhanced relaxivity, as in methemoglobin, our data imply that bound F^- does not alter the solvent-exchange dynamics of the Fe^{III} sites. Accordingly, either fluoride binding is associated with a protein conformation change that prevents hydrogen bonding between water and coordinated fluoride or the fluoride and azide complexes have different coordination structures. As will be seen, the available data are more consistent with the latter possibility. If the α-carbon backbones of native FeSOD, FeSOD–N_3^-, and FeSOD–F^- are superimposed, examination of the difference electron-density maps (native vs. N_3^- complex, native vs. F^- complex, N_3^- complex vs. F^- complex) establishes that azide and fluoride bind in the same general site.[24] Moreover, N_3^- and F^-

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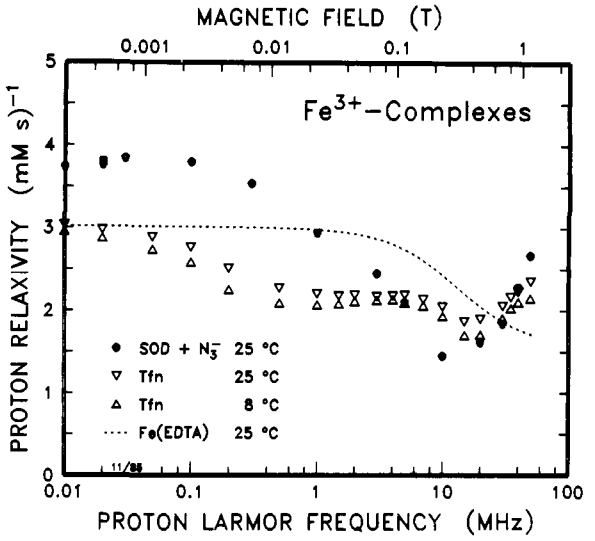

Figure 5. Paramagnetic components of the NMRD profiles for Fe^{III}–SOD, Fe^{III}–transferrin, and $[Fe(EDTA)(H_2O)]^-$. Data are expressed in relaxivity units. The diamagnetic contribution to the total FeSOD relaxivity was estimated from data in ref 19b: (●) FeSOD plus 10.0 mM NaN_3 at 25 °C; (▽) transferrin at 25 °C from ref 21; (△) transferrin at 8 °C; (---) $[Fe(EDTA)(H_2O)]^-$ at 25 °C from ref 20.

coordination to Fe^{III} induces identical shifts in the electron densities corresponding to the other Fe^{III} ligands.[24] Addition of azide to the FeSOD–F^- complex produces an increase in relaxivity, and changes in the NMRD profile, which also imply mutually exclusive binding of F^- and N_3^-. Comparison of Figures 4 and 3 demonstrates quite clearly that the increment in the profiles for the sample with 25 mM N_3^- (Figure 4) is close to that for the 10 mM N_3^- sample at 5 °C (Figure 3) and that both are close to saturation. Taken together, the data indicate that the bound anions in the FeSOD–N_3^-, F^- complexes interact with solvent H_2O very differently though both anions coordinate to the same metal ion site. Assuming that different contributions from $(T_1)_{p\alpha,\beta}^{-1}$ and $(T_1)_{os}^{-1}$ (see eq 1 and 2) do not fortuitously yield very similar NMRD profiles for FeSOD and its F^- complex, the simplest interpretation of the data is that F^- does not displace the coordinated H_2O, i.e., the F^- complex is six-coordinate. Previous work has demonstrated that the similar relaxivities of Mn^{II}–carboxypeptidase and its complex with F^- could be accounted for in this way.[23] In contrast, the NMRD results and steric considerations suggest a five-coordinate structure for the FeSOD–N_3^- complex.

Discussion

The NMRD results agree with the previous, limited (6–48 MHz) measurements on FeSOD.[11] The low paramagnetic relaxivity of the native enzyme is consistent with contributions from outersphere relaxation mechanisms. Transferrin is the most comparable Fe^{III}-containing protein that has been investigated by NMRD relaxometry. An $Fe^{II} \rightleftharpoons Fe^{III}$ redox cycle is involved in the function of both transferrin and FeSOD. The Fe^{III} sites in these two proteins are somewhat similar; at least two imidazoles, one or two anionic oxygen donors (carboxylate or tyrosinate), and a water molecule are ligands.[25] Both proteins display a rhombic high-spin Fe^{III} EPR spectrum with a g value of ~9.9 and a three-component feature centered at $g \sim 4.3$. Azide binding perturbs but does not drastically alter the FeSOD EPR spectrum;[7] the rhombic, high-spin character ($g \sim 4$ and 10) is retained. Thus the Fe^{III} electronic environment in FeSOD–N_3^- resembles that in Fe^{III}–transferrin. Figure 5 compares the Fe^{III}–transferrin NMRD profiles[26] at 8 and 25 °C to the FeSOD–N_3^- NMRD profile at 25 °C. We can see that the profiles are rather similar in important aspects: the absolute magnitudes of the relaxivities are very close, and the increase at high fields due to a field-dependent correlation time is evident in both proteins. Hydroxide is a probable metal ion ligand in Fe^{III}–transferrin at the pH employed in the experiments summarized in Figure 5.[25] Hence coordinated azide (in FeSOD–N_3^-) and hydroxide (in Fe^{III}–transferrin) may well mediate the paramagnetic contribution to the relaxivity in analogous ways. The detailed shapes of the profiles will depend on the exact form of the relevant spin Hamiltonian[27] and might not be expected to be identical. Also, the temperature dependences differ (cf. Figure 3), indicating a different mixture of contributions from τ_s and τ_M to τ_c for the two proteins. Nevertheless, the data suggest that the rapidly exchanging water molecule is approximately the same distance from the Fe^{III} ion in the two proteins, with a proton about 3 Å from the Fe^{III} ions. This should be regarded as only a rough estimate, but it is relatively insensitive to the source of τ_c.

It is also interesting to compare our results to NMRD measurements[9,20,28] on $[Fe(EDTA)(H_2O)]^-$ in order to illustrate some differences in the dynamics of Fe^{III}–solvent interactions in macromolecules vs. small chelates. $[Fe(EDTA)(H_2O)]^-$ has proved to be an especially useful FeSOD analogue that catalyzes superoxide dismutation. The NMRD profile of $[Fe(EDTA)(H_2O)]^-$ is shown in Figure 5. This complex is seven-coordinate in aqueous solutions, containing a single innersphere water that can be displaced by azide.[9,28] Solvent relaxation in $[Fe(EDTA)(H_2O)]^-$ solutions has been analyzed in detail.[20,28] A substantial fraction of the observed relaxivity has been attributed to innersphere H_2O exchange with the bulk solvent, i.e., to the $(T_1)_{p\alpha}^{-1}$ term (eq 1);[28] outersphere contributions were estimated[28,29] to be ~10–50% of the total $(T_1)^{-1}$. Azide binding *decreases* the observed $[Fe(EDTA)(H_2O)]^-$ relaxivity, consistent with displacement of a coordinated water molecule that contributed to solvent relaxation via exchange.[9] Similar effects have been observed for the ligand-substitution reactions of metalloproteins, excepting those containing Fe^{III}, where water is the leaving group.[12,30] Such behavior might have been expected for FeSOD if the $(T_1)_{p\beta}^{-1}$ contributions did not dominate the relaxivity of the FeSOD–N_3^- complex, since then the effects of ligand substitution on the exchange and magnetic relaxation dynamics of FeSOD and $[Fe(EDTA)(H_2O)]^-$ would likely be similar. The markedly different contributions from the $(T_1)_{p\alpha,\beta}^{-1}$ terms to the relaxivities of $[Fe(EDTA)N_3]^{2-}$ and FeSOD-N_3^- may be understood in terms of the following points: (1) since $\tau_R \sim 10^{-11}$ s for the EDTA complex, it will tend to dominate τ_c; (2) the contribution to the $[Fe(EDTA)N_3]^{2-}$ relaxivity from $(T)_{os}^{-1}$ is possibly much greater (vide infra), thereby decreasing the effects of the $(T_1)_p^{-1}$ terms. In addition, the protein moiety may also contribute to a favorable $\tau_{M\beta}$ for second-shell H_2O in the FeSOD–N_3^- complex. Clearly, the coordination of the single azide would not have dramatically altered the FeSOD NMRD profile if H_2O in the first or second coordination shell of the Fe^{III} ions already had a favorable $\tau_{M\alpha,\beta}$. The current interpretation of the X-ray map indicates that a channel connects the buried metal ion to the protein surface; the proposed anion binding site faces this channel.[17] Further analysis should await the refinement of the X-ray data. In fact, FeSOD may be an excellent system to test models[12,13,15,21] for solvent

(24) These results were obtained by using the unrefined, 2.8-Å electron-density maps of the *Pseudomonas ovalis* FeSOD and its complexes with fluoride and azide. At this time nothing can be concluded about the presence or absence of coordinated H_2O in the anion complexes of this FeSOD. Ringe, D.; Petsko, G., personal communication.

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interaction with partially accessible paramagnetic ions in macromolecules once a high resolution crystal structure is available.

Given the available theory for NMRD produced by anisotropic, high-spin Fe^{III}, only an estimate of τ_M for a coordinated solvent molecule is possible from the data. $(T_1)_{os}^{-1}$ for FeSOD can be estimated from the theory[18b] to be $\leq 0.5\ mM^{-1}\ s^{-1}$. Equation 2 then gives $\tau_M \leq 2 \times 10^{-5}$ s, implying a solvent exchange rate that is faster than turnover ($25\,000\ s^{-1}$ in comparable conditions).[6] Thus the data reported here are consistent with innersphere electron-transfer steps in the catalytic mechanism of FeSOD. The on-rate for azide binding is $40\,000\ s^{-1}$ and also permits anion binding steps to be involved in turnover.[6,8]

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An Inclusion Complex Containing the Dioxygen Molecule and an Organic Guest Molecule Cohabiting within a Vaulted Cobalt(II) Cyclidene Host—A Rare Kind of Ternary Complex

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Abstract: A credible model of the ternary complex of cytochrome P450 has been demonstrated with use of NMR spin lattice relaxation determinations on guest protons in the presence of the dioxygen adduct of a vaulted cyclidene–cobalt(II) host. Hydrophobic forces produce regiospecific binding of the 1-butanol guest within the cavity of the vaulted cyclidene cobalt complex while the metal center is simultaneously coordinated to dioxygen. The ternary complex is formulated as [{vaulted cyclidene cobalt(II)}–dioxygen–guest]. The spin density localized on the coordinated dioxygen acts as the paramagnetic center, and results obtained at two spectrometer frequencies yield the same calculated distances. ESR and visible spectral measurements establish the stability and reversibility of the dioxygen adduct under the conditions used in the NMR studies. Distance determinations made with the deoxygenated form of the cobalt(II) complex are the same, within experimental error, as those found with the analogous copper(II) complexes of the ligands.

Given the enormous strides made recently in elaborating its active site chemistry, the static relationships and dynamic consequences of the ternary complex of cytochromes P450 should provide the basis for understanding the elegant oxidation chemistry orchestrated by that enzyme.[1] We present here the first credible model for this ternary complex. In the natural system, the binding of dioxygen to the heme iron atom is possible only after a substrate molecule is in place, a design feature that prevents activation of the enzyme in the absence of substrate.[2] The extreme reactivity of the activated site toward organic substances indicates that selectivity is achieved largely through the details of the substrate binding process. The crystal structure of the cytochrome-P450-cam from *pseudomonas putida* has confirmed this.[3] A hydrophobic region surrounds the coordination site at the heme group where the activation of dioxygen takes place. This enables the complex to bind organic substrates hydrophobically while simultaneously providing a site for the activation of dioxygen.

The elucidation of the interrelationships and possible synergisms that operate within such a ternary complex requires good models, and the family of cyclidene complexes developed in these laboratories[4-6] have now been applied to this purpose. The cyclidene ligand is especially favorable to the formation of dioxygen complexes by both iron(II)[7-9] and cobalt(II)[10,11] while the ease of synthesis and diversity of superstructures that can be appended to the cyclidene facilitate the study of inclusion complex formation with potential organic substrates.[12-15] Simultaneous substrate binding and dioxygen binding in a single synthetic complex are demonstrated for the first time here (Figure 1). Further, the spin density on the dioxygen species,[16,17] that is bound to cobalt(II), is exploited to disclose the regiospecific binding of the substrate.

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