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Comparison of Thioethers and Sulfoxides as Axial Ligands for *N*-Acetylmicroperoxidase-8: Implications for Oxidation of Methionine-80 in Cytochrome *c*

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Methionine-80 (Met-80) in mitochondrial cytochrome *c* (cyt *c*) can be oxidized to the corresponding sulfoxide by reactive oxygen species, a reaction of potential biological significance. As an approach to investigating how oxidation of Met-80 would influence its interactions with heme iron, we have examined binding of 2-(methylthio)ethanol (MTE) and dimethyl sulfoxide (DMSO), models for the side chains of Met and Met(SO), respectively, to ferrous and ferric *N*-acetylmicroperoxidase-8 (AcMP8). We find that DMSO coordinates 1.2 kcal/mol less strongly to Fe(III)-AcMP8 than does MTE, although both ligands form low-spin complexes. Comparison of spectroscopic data for the DMSO complex of Fe(III)-AcMP8 with published data for the Met(SO)-80 form of ferric cyt *c* allows us to conclude that Met(SO)-80 does not coordinate to iron in the latter. DMSO coordinates to Fe(II)-AcMP8 1.3 kcal/mol more strongly than does MTE, whereas Met-80 and Met(SO)-80 are reported to have approximately equal affinity for Fe(II) in cyt *c*. This result suggests that the steric environment near the heme iron in cyt *c* discriminates against coordination of Met(SO)-80. Vacuum quantum chemical density functional theory calculations confirm the greater affinity of the sulfoxide and show that coordination via oxygen is strongly favored. Resonance Raman spectroscopic data indicate that the preference for coordination via oxygen is maintained in solution. The computational data further indicate that the DMSO complex derives significant enthalpic stabilization from π back-bonding but that iron to sulfur π back-bonding does not make a significant contribution to bonding in the thioether complex.

Exposure of proteins to reactive oxygen species (ROS)[1] generated during oxidative phosphorylation (respiration) and other processes can result in detrimental changes in stability and function.[2,3] Although physiological mechanisms have evolved to minimize protein oxidation by ROS, accumulation of oxidatively modified proteins occurs during aging and under some pathological conditions.[2,3] The thioether moiety of methionine (Met) is particularly susceptible to oxidation, with the reaction typically yielding methionine sulfoxide [Met(SO)].[4,5]

The Met-80 side chain serves as a ligand to heme iron in mitochondrial cytochrome *c* (cyt *c*),[6] which mediates transfer of electrons between ubiquinol cyt *c* reductase and cyt *c* oxidase in eukaryotic oxidative phosphorylation.[7] The side chain of histidine-18 (His-18) coordinates trans to Met-80, and the heme is attached to the protein via thioether linkages to two cysteine residues (Cys-14 and Cys-17). Because mitochondria represent a major source of ROS,[8] oxidation of Met-80 to Met(SO) in cyt *c* may occur even under normal physiological conditions, although evidence for such a transformation has not been presented.

Another source of ROS is the respiratory burst accompanying phagocytosis of microbes by neutrophils.[9] Hypochlorous acid (HOCl), the major bacteriocidal agent

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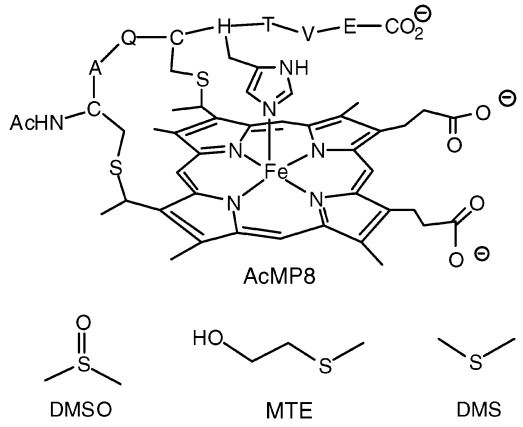
in neutrophils, is generated from a myeloperoxidase-catalyzed reaction between chloride ion and hydrogen peroxide.[10,11] HOCl activity has been associated with oxidation of cytochromes in the target microbes and resultant destruction of their respiratory chains.[12−15] Leakage of HOCl from neutrophils is believed to result in damage to host proteins as well.[16] It is noteworthy in this regard that HOCl has been shown to react with mitochondrial cyt *c* in vitro, converting Met-80 to Met(SO).[17]

Conversion of Met-80 in cyt *c* to Met(SO) might be expected to alter the heme-binding characteristics of the ligand and, hence, the properties of the protein. This idea was originally pursued in horse heart cyt *c* derivatives, wherein Met-80 had been converted to Met(SO).[18−24] NMR studies by Ivanetich et al.[19] and by Myer and co-workers[23] showed that Met(SO)-80 functions as a ligand to iron in the ferrous protein. However, the two groups reached different conclusions as to whether the sulfur[19] or oxygen atom[23] of the ambidentate sulfoxide moiety coordinates to the metal. Furthermore, Met(SO)-80 in the ferrous protein is displaced by CO,[22,25] while native ferrous cyt *c* is inert toward CO.[26] Recent photolysis studies by Larsen suggest that oxidizing Met-80 to Met(SO)-80 in cyt *c* does not cause a significant change in the ligand-binding equilibrium however.[25] Rather, he has shown that it dramatically slows both the ligand dissociation and rebinding rates, thereby allowing CO to compete for coordination.[25] The ferric sulfoxidized protein is significantly less stable toward thermal denaturation than is the native protein.[23] Studies by Myer and co-workers suggested that Met(SO)-80 coordinates to iron in the low-spin ferric protein above pH 7.5 but dissociates with p$K \approx$ 6.5 to generate a high-spin form of the protein.[23] In contrast, native cyt *c* exhibits low-spin His/Met coordination at neutral pH, converting to a high-spin form with cooperative dissociation of the His and Met axial ligands with p$K = 2.5$.[27] It has been demonstrated that ferrous Met(SO)-80 cyt *c* reacts with cyt *c* oxidase at nearly the same rate as the native protein but that ferric Met(SO)-80 cyt *c* reacts very slowly with cyt *c* reductase.[18,21,22] On the basis of these findings, one may conclude that physiological oxidation of Met-80 in cyt *c* to Met(SO) would disrupt the electron transport chain.

The effects of Met-80 oxidation on the structure and function of cyt *c* have been demonstrated, and the potential physiological relevance of this transformation has been recognized. To what extent the local protein environment impacts interactions between Met(SO) and heme in ferrous and ferric cyt *c* has yet to be determined however. As an approach to investigating this issue, we have examined binding of 2-(methylthio)ethanol (MTE) and of dimethyl sulfoxide (DMSO), models for the side chains of Met and Met(SO), respectively, to ferrous and ferric *N*-acetylmicroperoxidase-8 (AcMP8). AcMP8 is a monomeric hemepeptide obtained by proteolysis of horse heart mitochondrial cyt *c*[28,29] followed by acetylation of the N-terminal amino group,[30,31] which retains the native His-18 axial ligand of the parent protein at neutral pH but lacks Met-80.



Experimental Section

Materials. All reagents were of commercial grade and were used without further purification. AcMP8 was prepared from horse heart cyt *c* (Sigma) using the method of Low et al.[32]

Ligand Binding Studies. Electronic absorption spectra were recorded on Kontron Uvikon 9410 and Varian Cary 100 Bio UV/visible spectrophotometers. The former contains a thermostated cell compartment whose internal temperature is controlled by a circulating water bath. The internal temperature was monitored using an Omega model HH200 thermometer with a T thermocouple (±0.2 °C). The latter spectrophotometer includes a Peltier thermostated

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cell holder, with temperature monitoring effected via a dedicated temperature probe accessory (±0.1 °C).

Ligand binding studies with Fe(II)-AcMP8 employed an anaerobic glass cuvette, while those for Fe(III)-AcMP8 were carried out in standard quartz cuvettes. The AcMP8 concentration was held constant at 3−5 μM, while the concentration of the ligand was varied. For analysis of MTE and DMSO binding by Fe(III)-AcMP8, all samples contained 10 vol % CH_3OH and were unbuffered (see text for details). For binding studies involving Fe(II)-AcMP8, the solutions were buffered to pH 8.0 with 100 mM potassium phosphate. In studies with Fe(II)-AcMP8, sample and ligand solutions were degassed by bubbling with N_2 for at least 30 min, and a positive N_2 pressure was maintained during the titrations. The N_2 was passed through a chromous chloride solution in order to remove adventitious oxygen. Reduction of Fe(III)-AcMP8 was accomplished by adding a small aliquot of a freshly prepared, degassed aqueous sodium dithionite solution. Aliquots of neat ligand were added using Hamilton gas-tight positive displacement syringes. Titration data were fit (Igor Pro, version 4.0; Wavemetrics, Inc.) to a standard equation describing a 1:1 binding isotherm (eq 1),

$$A_\lambda = A_{\lambda(\mathrm{AcMP8})} - \left[\left(\frac{[\mathrm{AcMP8}][\mathrm{L}]}{K_\mathrm{d}}\right)/\left(1+\frac{[\mathrm{L}]}{K_\mathrm{d}}\right)\right](\epsilon_{\lambda(\mathrm{AcMP8})} - \epsilon_{\lambda(\mathrm{L-AcMP8})}) \quad (1)$$

where A_λ is the absorbance at a given wavelength, $A_{\lambda(\mathrm{AcMP8})}$ is the corresponding absorbance in the absence of ligand, $\epsilon_{\lambda(\mathrm{AcMP8})}$ and $\epsilon_{\lambda(\mathrm{L-AcMP8})}$ are the extinction coefficients at that wavelength in the absence of added ligand and in the presence of a saturating concentration of the ligand, respectively, [L] is the concentration of free ligand, and K_d is the dissociation constant. The value of $\epsilon_{\lambda(\mathrm{L-AcMP8})}$ was allowed to vary to obtain the best fit.

Computations. Density functional theory based quantum chemical methods were employed in the prediction of molecular energetics, geometries, orbital structures, and charge distributions within analogues of DMSO and dimethyl sulfide (DMS) complexes of AcMP8. All calculations employed the Gaussian 98 suite of programs,[33] with 6-31G(d,f) split-valence polarized basis sets[34−36] and B3-LYP hybrid functionals,[37,38] using a default threshold for integral cutoffs and a self-consistency criterion of density matrix convergence to within 1×10^{-9} electronic units. At this level of theory, practical limitations on the model system size dictated judicious truncation of AcMP8. We thus chose to represent the system as an unsubstituted Fe(II) porphyrin, with the polarizing effects of His-18 simulated by the presence of an axial NH_3 group coordinated trans to the DMSO or DMS ligand.

Optimized geometries for DMS, DMSO, and the corresponding ligand−Fe(II) porphyrin−NH_3 complexes were obtained using the above method. Ligand and NH_3 degrees of freedom were relaxed, but the porphyrin was restricted to a planar structure (local symmetry of D_{4h}), with bond distances and angles corresponding to averaged values from the crystal structure by Sharma et al.[39] (ring substituents and ligands omitted). All optimization thresholds were left at default values.

Complexation enthalpies for each ligand−Fe(II) porphyrin−NH_3 complex were estimated via the supermolecular method, subtracting the total electronic plus nuclear energy of the optimized complex from the total energy for similar structures wherein the ligand was translated 15 Å (from its optimal coordinated position) along an axis normal to the porphyrin surface. Basis set superposition error was mitigated in all cases by Boys−Bernardi counterpoise corrections.[40]

Electronic structure analysis was performed for the theoretically optimized geometries of all ligand and ligand−Fe(II) porphyrin−NH_3 complexes. Orbital structures were analyzed for the ligands and their corresponding complexes by performing B3-LYP/6-31G-(d,f) calculations similar to those above but with the orbital localization scheme of Boys.[41]

Resonance Raman Spectroscopy. Resonance Raman spectra were recorded using a spectrometer comprising a 0.6 m spectrograph equipped with a 2400 groove/mm grating and a liquid-nitrogen-cooled CCD detector. Raman excitation was achieved with the 413.1 nm emission line from a Kr^+ laser, which was focused to a line on the sample. Scattered light was collected using an f1 lens, filtered through a holographic notch filter to remove Rayleigh scattered light, and passed through a polarization scrambler. Samples were contained in a 5 mm NMR tube, which was spun at ∼20 Hz during Raman acquisition. Spectra were calibrated against the known Raman frequencies of toluene and dimethylformamide bands.

Results and Discussion

Thioethers as Ligands for Iron Porphyrins. Thioether sulfur atoms can be described as sp^3-hybridized, with two lone electron pairs and two alkyl groups occupying points of a distorted tetrahedron. The high polarizability (softness) of sulfur results in a low affinity of thioethers for hard acids (pK_a of $(CH_3)_2SH^+ \approx -7$)[42] but a good affinity for electron-rich (soft) metal ions. The ferric ion is categorized as a hard Lewis acid, while Fe(II) is intermediate between hard and soft.[43] Thioethers have long been known as good ligands for ferrous porphyrins, but they coordinate very weakly to ferric porphyrins.[44] These relative affinities are maintained in ferrous and ferric cyt *c*.[45,46]

N-Acetylmethionine (AcMet) has been examined as a ligand to AcMP8 and related hemepeptides by a number of

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researchers.[29,47,48] Solubility of AcMet in water requires that its carboxylic acid group be maintained in conjugate base form. One therefore does not observe saturation binding between Fe(III)-AcMP8 and AcMet in aqueous solution because the maximum achievable concentration of AcMet is about 2 M.[47] At this concentration, the binding sites are only about 80% saturated.[47] To circumvent this limit, we chose to examine MTE as a ligand in ligand binding studies with AcMP8, which required the inclusion of 10 vol % CH_3-OH in all solutions. By further omitting buffers from the solutions in titrations with Fe(III)-AcMP8, we achieved MTE concentrations in excess of 7 M, thereby approaching saturation binding. For consistency, methanol was also used as a cosolvent in the binding titration with Fe(II)-AcMP8, but the solution was buffered to pH 8.0 with potassium phosphate.

Sulfoxides as Ligands for Iron Porphyrins. Sulfoxides are pyramidal, with an oxygen atom replacing one of the lone pairs of electrons of the thioether. The sulfur−oxygen bond is described well by two resonance contributors [$(CH_3)_2S{=}O \leftrightarrow (CH_3)_2S^{(+)}{-}O^{(-)}$]. Sulfoxides are ambident ligands, capable of coordinating to metal ions via sulfur or oxygen,[49,50] typically coordinating to hard acids such as H^+ and Fe(III) via O but to soft metals via S.

Ivanetich et al.[18] and Myer et al.[23] examined binding of DMSO by the unacetylated precursor of AcMP8 (MP8) as part of their studies of Met(SO)-80 cyt *c*. While Fe(III)-AcMP8 is monomeric at concentrations below 30 μM in aqueous solution, Fe(III)-MP8 exists predominantly as a low-spin dimer in water.[51] As a result, only small changes in the spectrum of Fe(III)-MP8 were observed in the presence of up to 7 M DMSO.[18,23] Spectroscopic changes clearly indicating the formation of a low-spin complex were observed when DMSO was added to a solution of Fe(II)-MP8 however.[23] Free energies for DMSO binding were not determined in either case. Results reported below thus represent the first quantitative studies of sulfoxide binding by a cyt *c* heme-peptide. Titrations of Fe(III)- and Fe(II)-AcMP8 with DMSO were performed using conditions identical to those employed in the corresponding titrations with MTE, described above.

Binding of MTE and DMSO by Fe(III)-AcMP8. The electronic absorption spectrum of Fe(III)-AcMP8 is characteristic of high-spin ($S = {}^5/_2$) Fe(III) porphyrins, with the Soret band λ_{max} near 396 nm, a broad featureless band from 450 to 560 nm, and a ligand-to-metal charge-transfer band around 630 nm (Figure 1). Iron in Fe(III)-AcMP8 is hexacoordinated, with the His-18 side chain as one axial ligand and a water molecule likely occupying the other axial position.[31,52,53] Addition of MTE to a solution of Fe(III)-AcMP8 caused a gradual shift of the Soret band λ_{max} from 396 to 408 nm (see Table 1), consistent with the converson of Fe(III) from high spin to low spin ($S = {}^1/_2$). Changes in the Q-band region of the spectrum are also consistent with such a transition, including the disappearance of the π-iron charge-transfer band near 620 nm (the final spectrum from the titration is shown in Figure 1; see also Figure S1 in the Supporting Information). Several isosbestic points are present in this and all other titrations reported herein, and saturation behavior is observed. A weak ligand-to-metal charge-transfer band appears near 695 nm in the complex (Figure 1), indicative of S−Fe(III) coordination.[6] The final spectrum in the titration with MTE is similar to the spectrum of ferric horse heart cyt *c*. Nonlinear regression analysis of the titration data using eq 1 yields a dissociation constant (K_d) of 0.7 M (Table 1).

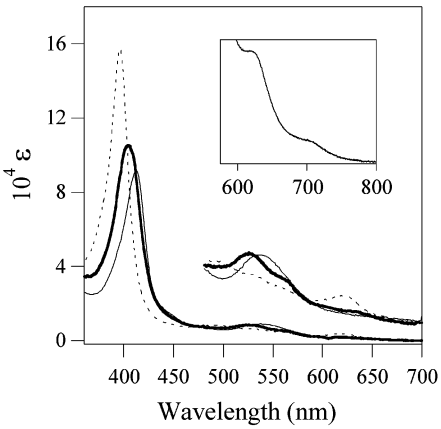

Figure 1. Electronic absorption spectra of Fe(III)-AcMP8 (5 μM) in 9:1 (v/v) H_2O/CH_3OH (dashed line), in the presence of 7 M MTE (bold line), and in the presence of 8 M DMSO (thin line). The concentration of the ligand in each case represents ≥95% saturation binding. The inset highlights the 695 nm band of the MTE complex.

In the presence of increasing DMSO concentration, the Soret band λ_{max} of Fe(III)-AcMP8 shifts from 396 to 412 nm, again consistent with a transition from high-spin to low-spin iron (Figures 1 and S2). Changes in the visible region of the spectrum support this conclusion. As with MTE, a very high concentration of DMSO is required in order to approach saturation binding. The extinction coefficient of the Soret band in the DMSO complex is lower than that in the MTE complex, and no charge-transfer band is observed near 695 nm. A dissociation constant of 2.5 M was determined from the binding data, demonstrating not only that DMSO is a weaker ligand than MTE by ∼1.2 kcal/mol but that binding of DMSO is actually endergonic (Table 1). This finding is consistent with the results of studies with cyt *c*, which suggested that oxidation of Met-80 to Met(SO) diminishes its affinity for ferric heme.[18,23]

Oxidizing Met-80 to Met(SO) in ferric cyt *c* causes a shift of the Soret band λ_{max} from 408 to 406 nm and an increase in the extinction coefficient (Table 1).[18,23] In contrast, the Soret band λ_{max} shifts from 408 to 412 nm upon replacement of MTE with DMSO as a ligand for Fe(III)-AcMP8 (Table 1). In addition, the extinction coefficient for the DMSO complex is lower than that for the MTE complex. These findings suggest that the Met(SO)-80 side chain does not

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Table 1. Spectroscopic and Ligand Binding Data for AcMP8 and Its Complexes with MTE and DMSO at 298 K

	ligand	Soret λ_{max}, nm (ϵ, mM^{-1} cm^{-1})	K_d, M	$\Delta G°$, kcal/mol	$\Delta H°$, kcal/mol	$-T\Delta S°$, kcal/mol
Fe(III)-AcMP8	none	396 (157)				
Fe(III)-AcMP8	MTE	408 (105)	0.7	−0.2	[a]	[a]
Fe(III)-AcMP8	DMSO	412 (92.0)	5.0	+1.0	[a]	[a]
Fe(III)-cyt *c*	Met	408 (104)[b]				
Fe(III)-cyt *c*	Met(SO)	406 (109)[b]				
Fe(II)-AcMP8	none	410 (96), 430 (sh)				
Fe(II)-AcMP8	MTE	414 (151)	4.8×10^{-3}	−3.2	−16.0	12.8
Fe(II)-AcMP8	DMSO	415 (210)	5.3×10^{-4}	−4.5	−18.7	14.2
Fe(II)-cyt *c*	Met	416 (138)[b]				
Fe(II)-cyt *c*	Met(SO)	418 (178)[b]				

[a] Not determined. [b] From ref 23.

function as a ligand to iron in the ferric Met(SO)-80 protein at pH 7.5, contrary to the conclusions of Myer et al.[23] and of Ivanetich and co-workers.[18] Myer et al. attributed a change from a low-spin species to a high-spin species with p$K \approx$ 6.5 in Met(SO)-80 cyt *c* to dissociation of the Met(SO) ligand.[23] A transition to a second high-spin form with p$K \approx$ 2.5 can be attributed to subsequent dissociation of the His ligand. In contrast, Met-80 and His-18 in native cyt *c* dissociate cooperatively with p$K \approx$ 2.5, concomitant with unfolding of the protein.[27] Gray and co-workers replaced Met-80 in yeast cyt *c* with alanine and reported that hydroxide was coordinated to iron.[54,55] Protonation of the low-spin hydroxide complex yields a high-spin aquo complex with p$K \approx$ 6.5. Similar pH-dependent spectroscopic characteristics have been observed for other cyt *c* variants in which Met-80 is replaced by an amino acid lacking a strong field ligand on its side chain.[24] The results of our binding studies, combined with data published for Met(SO)-80 cyt *c*[18,23] and the Ala-80 mutant of cyt *c*[54,55] suggest that the transition with p$K \approx$ 6.5 in Met(SO)-80 cyt *c* corresponds to protonation of a hydroxide ligand.

Binding of MTE and DMSO by Fe(II)-AcMP8. Reduction of Fe(III)-AcMP8 to Fe(II)-AcMP8 results in a shift of the Soret band from 396 to 410 nm, but a strong shoulder is present near 425 nm (Figure 2). A significant change also occurs in the Q-band region of the spectrum, with two signals near 513 and 545 nm appearing atop a much broader band. Others have noted that the electronic absorption spectrum of Fe(II)-AcMP8 is consistent with a mixture of pentacoordinated (high spin) and hexacoordinated (low spin) iron at equilibrium.[47,56] The low-spin component, with the Soret band λ_{max} at 410 nm and the signals at 513 and 545 nm, most likely arises from coordination of water (or methanol, in the present case) to a population of the molecules.

Spectra of Fe(II)-AcMP8 recorded in the presence of increasing MTE concentrations display enhanced features characteristic of low-spin iron and diminished signals arising from the high-spin component (Figures 2 and S3). The Soret band λ_{max} in the MTE complex is found at 414 nm, and the Q_o (α) and Q_v (β) bands are centered at 550 and 521 nm,

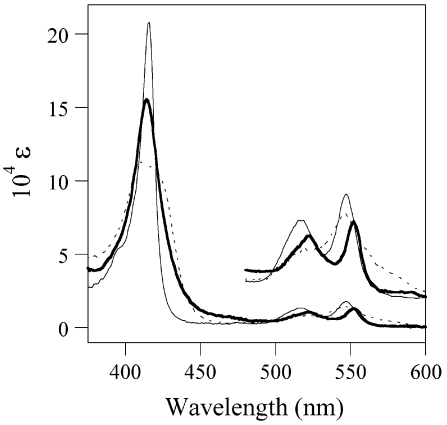

Figure 2. Electronic absorption spectra of Fe(II)-AcMP8 (5 μM) in 9:1 (v/v) H_2O/CH_3OH buffered to pH 8.0 with 100 mM potassium phosphate (dashed line), in the presence of 49 mM MTE (bold line), and in the presence of 51 mM DMSO (thin line). The concentration of the ligand in each case represents ≥95% saturation binding.

respectively. The final spectrum in the titration with MTE is nearly identical with the spectrum of ferrous horse heart cyt *c* (not shown). The complex between MTE and Fe(II)-AcMP8 is 3.0 kcal/mol more stable than the complex between MTE and Fe(III)-AcMP8, consistent with expectations from previous studies.[29,47,48]

Conversion of Fe(II)-AcMP8 to the low-spin form was also observed in the presence of DMSO (Figures 2 and S4). Nonlinear regression analysis of the binding data shows that DMSO coordinates to Fe(II)-AcMP8 about 10-fold (1.3 kcal/mol) more strongly than does MTE (Table 1). The Soret band in the DMSO complex is narrower than that in the MTE complex and exhibits a higher molar extinction coefficient. Similar changes following conversion of Met-80 to Met(SO) in ferrous cyt *c* were reported by Myer et al. (Table 1).[23] The DMSO−Fe(II)-AcMP8 complex is 5.5 kcal/mol more stable than the corresponding Fe(III) complex. Thus, reduction of Fe(III) to Fe(II) enhances DMSO binding free energy significantly more than it does for MTE.

Enthalpies and Entropies of Ligand Binding. Binding titrations with DMSO and MTE were performed over a range of temperatures (Figure 3 and Table 1) to determine the enthalpic and entropic partitioning of free energies of ligation to Fe(II)-AcMP8 (extremely weak binding precluded a similar analysis of the corresponding Fe(III)-AcMP8 complexes). The data reveal that both complexes are enthalpically very stable but that DMSO binding is 2.7 kcal/mol more

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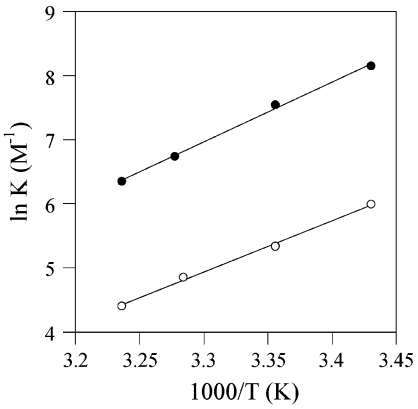

Figure 3. van't Hoff plot of ligand binding data for the MTE (open circles) and DMSO (filled circles) complexes of Fe(II)-AcMP8.

exothermic than MTE binding. The more favorable $\Delta H°$ in the case of DMSO complexation is partially counterbalanced by a more negative entropy of binding however.

Predicting the Mode of Ligand Coordination in the DMSO−AcMP8 Complexes. It has been demonstrated that sulfoxides are protonated exclusively on oxygen,[57,58] and a Cambridge structural database search revealed that all sulfoxide complexes of Fe(III) involve coordination via oxygen. Relevant to the present report are the bis(tetramethylene sulfoxide) complex of Fe(III) tetraphenylporphyrin (TPP)[59] and the bis-DMSO complex of Fe(III) octaethylporphyrin (OEP).[60] On the basis of these results and consideration of hard−soft acid−base theory, we expect that coordination of DMSO to Fe(III)-AcMP8 occurs exclusively via oxygen.

The mode for DMSO coordination to Fe(II)-AcMP8 is more difficult to predict. Two sulfoxide−metalloporphyrin X-ray crystal structures with sulfur-to-metal coordination have been reported. One is an Ir(II) complex of OEP in which DMSO is coordinated trans to a σ-bonded propyl group,[61] and the second is a Ru(II)-TPP complex containing two axial S-coordinated diethyl sulfoxide ligands.[62] The metal ion in both of these complexes is categorized as soft. Interestingly, all three sulfoxide complexes of Fe(II) for which X-ray crystal structures are available also exhibit sulfur coordination.[63−65] This small group includes the bis-DMSO complex of Fe(II) phthalocyanine,[63] a structure closely related to the iron porphyrin examined herein. No examples of ferrous porphyrins with O-coordinated sulfoxides appear in the Cambridge structural database.

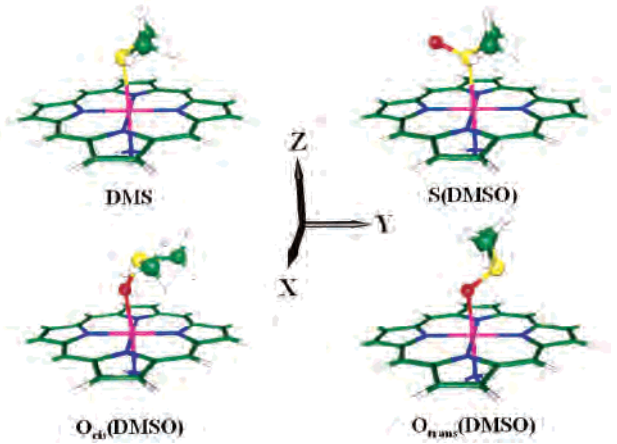

Figure 4. Theoretically optimized structures for the L-Fe(II) porphyrin-NH_3 complexes described in the paper. The color scheme is as follows: white (H); green (C); blue (N); red (O); yellow (S); magenta (Fe).

Density Functional Theory Calculations. To gain insight into the preferred mode of DMSO coordination to Fe(II)-AcMP8, as well as the factors responsible for DMSO being a stronger ligand than MTE, we turned our attention to density functional theory calculations. Model systems for the calculations entailed an unsubstituted iron porphyrin with either DMS or DMSO coordinated trans to an ammonia ligand. Geometry optimizations suggested one stable structure for the DMS complex and three stable structures for the DMSO complex, one with the ligand coordinated to iron via sulfur and two with coordination via oxygen. The structures are depicted in Figure 4, with supplemental structural and energetic information provided in Table 2.

Structures of the Calculated Complexes. Our predicted structure of the DMS complex is qualitatively similar to the one determined in other recent density functional theory calculations of Rovira et al.,[66] despite their use of a different model (imidazole as the trans ligand rather than ammonia) and level of theory (a supercell planewave method was used rather than a Gaussian orbital representation). The DMS ligand in each complex is oriented such that its C−S−C angle is approximately bisected by a line running through two opposing meso carbons of the porphyrin. The iron d_π orbitals are aligned with the Fe−$N_{porphyrin}$ bonds and likely have minimal overlap with d orbitals on sulfur in this orientation. Rotating the ligand by 45° maximizes the possibility for overlap but leads to a complex that is less stable by ~0.1 kcal/mol (data not shown).

The most significant difference between our lowest-energy DMS complex and that reported by Rovira et al. is a longer Fe(II)−S bond in the former (2.39 vs 2.26 Å). For comparison, the Fe(II)−S bond in the crystal structure of a ferrous porphyrin containing an intramolecular imidazole and an exogenous thioether as axial ligands is between these values (2.34 Å).[67] Shortening of the bond in our complex by 0.1 Å

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Table 2. Theoretical Geometric and Energetic Data for DMS and DMSO Ligands as a Function of Heme Binding Environment[a]

	R_{Fe-X} (Å)	R_{S-O} (Å)	R_{S-C} (Å)	R_{Fe-N} (Å)	∠Fe−X−Y (deg)	$\Delta H_{complex}$ (kcal/mol)
DMS (vacuo)			1.824			
DMSO (vacuo)		1.514	1.836			
DMS complex (X = S)	2.395		1.824	2.014		−8.1
S(DMSO) (X = S, Y = O)	2.285	1.504	1.823	2.021	118.4	−8.2
O_{cis}(DMSO) (X = O, Y = S)	2.086	1.530	1.830	2.003	142.5	−16.2
O_{trans}(DMSO) (X = O, Y = S)	2.041	1.540	1.824	2.010	123.0	−15.1

[a] All numbers have been derived via density functional theory (B3LYP/6-31G*) calculations; all energetic predictions have been subject to Boys−Bernardi counterpoise corrections. X refers to the ligand atom through which the ligand−heme binding occurs; Y refers to the nearest π-bonded neighbor to X within the ligand. The N for which R_{Fe-N} is reported belongs to the axial NH_3 ligand.

followed by energy minimization led to only a small increase in energy (approximately 1 kcal/mol), which is comparable to results reported by Rovira et al.[66]

In the lowest-energy S-coordinated DMSO complex, S(DMSO), the ligand is rotated by close to 45° relative to the DMS complex described above, with the C−S−C bond of DMSO bisected by one of the $Fe-N_{porphyrin}$ bonds. The Fe(II)−S bond length in this S(DMSO) complex is shorter than that in the DMS complex by ∼0.1 Å. Coordination of DMSO via sulfur has a small effect on the S−O bond, which is 0.01 Å shorter than that found in free DMSO (Table 2).

The two lowest-energy O-coordinated DMSO complexes differ from one another by a formal 180° rotation about the S−O bond of the ligand. As in the S(DMSO) complex, the C−S−C bond of the ligand in each complex is bisected by an $Fe-N_{porphyrin}$ bond. We refer to these two complexes as O_{cis}(DMSO) and O_{trans}(DMSO) to indicate whether Fe(II) and the DMSO methyl groups are on the same side of the S−O bond in the complex or on opposite sides, respectively. These are analogous to the cis,cis and trans,trans notations used to describe other O-coordinated sulfoxide complexes.[50] Our calculations indicate elongation (weakening) of the S═O bond by 0.016 Å in O_{cis}(DMSO) and by 0.026 Å in O_{trans}-(DMSO) relative to free DMSO (Table 2).

Ligand Binding Enthalpies. Computed enthalpy changes for DMS and DMSO binding to the Fe(II) porphyrin−NH_3 complex are included in Table 2. Perhaps the most surprising result of the calculations is that coordination of DMSO to Fe(II) via sulfur is approximately as favorable enthalpically as coordination of DMS, suggesting that coordination of DMSO to Fe(II)-AcMP8 via sulfur is a distinct possibility. However, coordination of DMSO to Fe(II) via oxygen yields a bond nearly twice as strong. The results show that O_{cis}-(DMSO) is favored over O_{trans}(DMSO) by approximately 1 kcal/mol. In complexes between DMSO and metal ions and in protonated DMSO, the mode of coordination analogous to O_{trans}(DMSO) is strongly favored.[50] The reversed preference in the porphyrin complexes reported herein likely reflects favorable nonbonding interactions between the ligand methyl groups and the porphyrin ring in O_{cis}(DMSO).

Molecular Orbital Analysis. The low molecular symmetry (at best C_s and generally C_1) in the systems under consideration here and the dense energetic clustering of orbitals near the Fermi level complicate orbital analysis because of smearing of bonding features across multiple orbitals. Fortunately, it is still possible to identify a number of orbitals that contribute to bonding in the DMS and DMSO complexes. Isodensity plots generated for some of these orbitals, and simplified synopses of their atomic orbital composition, are included in the Supporting Information.

The DMS Complex. In the DMS complex, only one orbital exhibiting substantial ligand-to-metal σ donation was identified (Figure S5A), and it can be described as involving lone-pair electrons on sulfur interacting with the iron d_{z^2} orbital. A molecular orbital with electron density shared between a metal d_π orbital and a ligand orbital with s and p components was also found (Figure S5B). Similar orbitals were reported by Rovira et al. for the DMS−Fe(II) porphyrin−imidazole complex.[66]

It has been suggested that complexes between ferrous porphyrins and thioethers can involve overlap between a filled iron d_π orbital and an empty sulfur 3d orbital (π back-bonding).[6,68] As noted above, DMS is oriented such that the chance of achieving the necessary orbital overlap is minimal in our lowest energy complex as well as in the complex calculated by Rovira et al.[66] Consistent with this result, neither we nor Rovira et al. obtained direct orbital evidence of such bonding. In addition, no evidence for π back-bonding was found in the complex wherein DMS had been rotated by 45° in order to maximize the possibility for the necessary orbital overlap.

The S(DMSO) Complex. The main bonding feature in the S(DMSO) complex is the profusion of partial S−Fe σ bonds (Figure S6A). Ligand-to-metal σ-bonding appears to be somewhat more important in the S(DMSO) complex than in the DMS complex, as evidenced by the greater number of orbitals containing such interactions, as well as by the shorter Fe(II)−S bond. The presence of a plausible σ^* orbital (Figure S6B) can also be noted.

π back-bonding is theoretically possible in S-bonded sulfoxide complexes of Fe(II) porphyrins. An increased S═O bond length relative to free DMSO provides evidence for electron density donation from metal d_π orbitals into ligand orbitals exhibiting S−O antibonding (π^*) character.[50] While metal−DMSO complexes displaying this phenomenon have been observed,[50,69] it is generally accepted that S-coordinated sulfoxides are weak π-accepting ligands.[50] While the orientation of DMSO in the lowest-energy S(DMSO) complex maximizes the possibility for the necessary orbital overlap, we have not identified any orbitals with obvious π-back-bonding character. A marginal shortening of the S−O

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bond in the complex by 0.01 Å relative to in vacuo DMSO actually suggests the possibility of greater double-bond character. Rack and Gray suggest that shortened S−O bonds in S-coordinated sulfoxides represent a synergism in which greater S−O π-bond character increases sulfur's σ-donating ability.[69] It is noteworthy that the Fe−S and S−O bond lengths in the bis-DMSO complex of Fe(II) phthalocyanine are consistent with minimal π back-bonding.[63] Likewise, IR studies of the bis-sulfoxide complex of Ru(II) TPP suggest that the S−O bond is strengthened relative to the free ligand, suggesting that π back-bonding does not make a significant contribution.[70]

O-Coordinated DMSO Complexes. Complexes O_{cis}-(DMSO) and O_{trans}(DMSO) exhibit evidence of σ bonding (Figures S7A and S8A, respectively) but also have orbitals with iron-to-ligand π-bonding character. The two orbitals in question (Figures S7B and S8B, respectively) strongly suggest significant S═O π^* orbital population, in a fairly clear case of π back-bonding. Our calculations indicate elongation (weakening) of the S═O bond by 0.016 Å in O_{cis}-(DMSO) and by 0.026 Å in O_{trans}(DMSO) relative to free DMSO. Comparable S═O bond lengthening is found in crystal structures of O-bonded transition-metal sulfoxide complexes.[50] Protonation of sulfoxides also induces substantial S═O bond lengthening, a phenomenon attributable solely to interaction between the proton and S═O π electrons, consistent with the resonance description of DMSO noted above.[50,58] For the O-coordinated DMSO complexes predicted herein, bond lengthening likely arises from donation of electron density from the DMSO π bond to the iron d_{z^2} orbital coupled with metal-to-ligand π back-bonding.

Resonance Raman Spectroscopy. Our calculations indicate that coordination of DMSO to Fe(II)-AcMP8 via oxygen is enthalpically favored over sulfur coordination. As discussed below, however, strong hydrogen bonding between water and DMSO oxygen is likely to diminish the binding enthalpy in aqueous solution. Whether this is likely to induce sulfur coordination is difficult to predict. Calculations further indicate that coordination of DMSO to Fe(II)-AcMP8 via oxygen will involve π-back-bonding interactions, whereas coordination via sulfur will not. The sensitivity of resonance Raman spectra to changes in electron density on iron[71] can potentially allow us to distinguish between these possibilities.

The resonance Raman spectrum of Fe(II)-AcMP8, with Soret band excitation at 406.7 nm, is shown in Figure 5A. The oxidation state marker band in the spectrum (ν_4) is positioned at 1357 cm^{-1}. Two ν_3 bands (spin state marker bands) are present in the spectrum, one centered at 1468 cm^{-1} and the other at 1492 cm^{-1}. These bands arise from five-coordinated high-spin Fe(II) and low-spin hexacoordinated Fe(II), respectively. We also observe a prominent peak at 1591 cm^{-1}, which we assign as ν_2, by analogy with the spectrum of ferrous cyt *c*, wherein ν_2 is centered at 1588 cm^{-1}. The spectrum of Fe(II)-AcMP8 differs in several respects from spectra reported by Othman et al.[56] and by

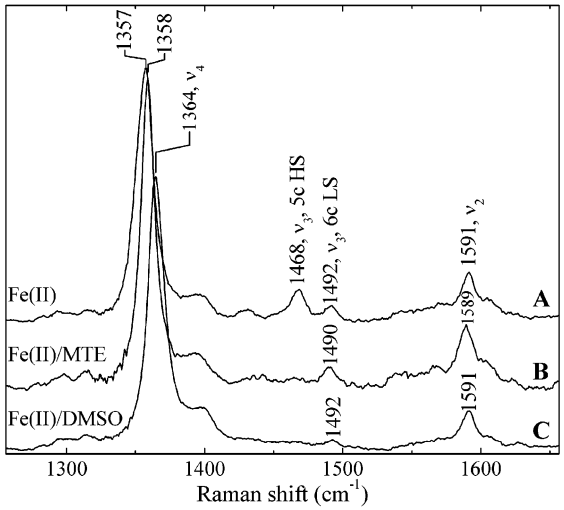

Figure 5. Resonance Raman spectra of AcMP8 complexes recorded with 406.7 nm excitation. All samples were 50 mM in phosphate, 50% MeOH by volume, and pH 7.0: (A) 39 μM Fe(II)-AcMP8; (B) 39 μM Fe(II)-AcMP8 + 108 mM MTE; (C) 14 μM Fe(II)-AcMP8 + 20 mM DMSO.

Tezcan et al.[47] (both with Soret band excitation at 413.1 nm). The ν_4 band in those spectra is centered at 1352−1353 cm^{-1}, corresponding to the five-coordinated high-spin form of the molecule. The 1357 cm^{-1} ν_4 band shown in Figure 5A is closer to that reported for ferrous cyt *c* (1358 cm^{-1})[47] but broad, suggesting that it arises from both high-spin penta-coordinated and low-spin hexacoordinated Fe(II). Furthermore, the ν_2 and low-spin ν_3 bands in our spectrum are more prominent than their counterparts in the previously reported spectra.[47,56] These differences are likely due to the fact that we excited our molecules at 406.7 nm rather than at 413.1 nm. Because the Soret band of the low-spin complex (∼410 nm) is blue-shifted relative to that of the high-spin complex (∼420 nm), our relative resonance enhancement of the low-spin component of the mixture is higher than that in the previously reported spectra.

The ν_3 band corresponding to five-coordinated Fe(II)-AcMP8 is significantly diminished in the spectra of solutions containing saturating concentrations of MTE and DMSO (Figure 5B,C), consistent with nearly complete formation of low-spin complexes in the presence of these ligands. The ν_2 bands corresponding to low-spin hexacoordinated Fe(II) are present in the spectra of these complexes as well. The most notable difference between the spectra of the MTE and DMSO complexes is the position of the oxidation state, or π-electron density marker band, ν_4. In the DMSO complex, ν_4 is shifted 6 cm^{-1} to higher frequency relative to ν_4 in the spectrum of the MTE complex. It is known that ν_4 is a sensitive indicator of π^* electron density in the porphyrin ring. For example, ν_4 occurs at 1370 cm^{-1} in the resonance Raman spectrum of ferric cyt *c*, whereas in ferrous cyt *c*, it is observed at 1358 cm^{-1}.[47,71,72] This oxidation-state sensitivity is largely attributed to greater iron d_π to porphyrin back-bonding in low-spin Fe(II), which increases the porphyrin LUMO (e_g*) population with a resultant decrease in the vibrational frequency. Back-bonding axial ligands such as

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CO that compete for d_π electron density shift this band to higher frequencies.[73] For example, the ν_4 band in the bis-imidazole complex of Fe(II) protoporphyrin IX is located at 1356 cm^{-1}.[73] For Fe(II) mesoporphyrin IX with pyridine as one axial ligand and the strongly π-acidic CO ligand as the other, ν_4 is substantially higher at 1371 cm^{-1}.[73] An analogous comparison can be made between the MTE and DMSO adducts of ferrous AcMP8. The hemes in both complexes are predominantly six-coordinated and low-spin, as evidenced by their ~1490 cm^{-1} ν_3 bands. However, the ν_4 band of the DMSO complex occurs 5 cm^{-1} higher in frequency than ν_4 of the MTE complex. This is consistent with DMSO being a more π-acidic ligand than MTE but a weaker one than CO, corroborating conclusions reached by Larsen in studies of the DMSO−CO and bis-DMSO complexes of Fe(II) protoporphyrin IX.[74] The resonance Raman evidence for iron d_π to DMSO back-bonding, in conjunction with the results of our computational studies, leads us to conclude that DMSO coordinates to Fe(II)-AcMP8 via its oxygen atom. Because the electronic absorption spectrum of ferrous Met(SO)-80 cyt *c*[23] is nearly identical with the spectrum of the DMSO complex of Fe(II)-AcMP8, it is reasonable to hypothesize that Met(SO)-80 coordinates to iron via oxygen as well. The fact that the ν_4 band in the low-spin form of Fe(II)-AcMP8 (~1357 cm^{-1}) is similar to that in the MTE complex (1359 cm^{-1}) also supports the conclusion reached in our computational studies, and those of Rovira et al.,[66] that the iron d_π orbitals do not donate significant electron density to the sulfur 3d orbitals.

Effect of the Solvent on Ligand Coordination. Our calculations indicate that O coordination of DMSO to an Fe(II) porphyrin, trans to a strong field nitrogenous ligand, is approximately 8 kcal/mol more favorable enthalpically than coordination of a thioether. The resonance Raman studies described above support the conclusion that the preference for O coordination is maintained in solution. However, ligand binding studies show that the enthalpy change accompanying coordination of DMSO to Fe(II)-AcMP8 is only 2.7 kcal/mol greater than that accompanying coordination of MTE. The most significant factor contributing to this smaller difference in solution ligand binding enthalpies is likely to be differences in solvent−ligand hydrogen-bonding interactions. To a first approximation, the formation of each complex will involve dissociation of one hydrogen-bonded water molecule, followed by its replacement with a coordinative bond between the ligand and iron (eqs 2−4; L = DMSO or MTE).

$$L + H_2O \rightarrow L{\cdot}H_2O \quad (\Delta H^\circ_{L,H_2O}) \tag{2}$$

$$L + Fe(II) \rightarrow Fe(II)\text{-}L \quad (\Delta H^\circ_{L,Fe(II)}) \tag{3}$$

$$L{\cdot}H_2O + Fe(II) \rightarrow Fe(II)\text{-}L + H_2O \quad (\Delta H^\circ_L = \Delta H^\circ_{L,Fe(II)} - \Delta H^\circ_{L,H_2O}) \tag{4}$$

Density functional theory calculations have shown that the enthalpy change associated with the formation of a hydrogen bond between H_2O and the DMSO oxygen atom is quite large ($\Delta H^\circ_{DMSO,H_2O} \sim -7.7$ kcal/mol) in comparison to the hydrogen bond formed between H_2O and DMS ($\Delta H^\circ_{MTE,H_2O} \sim -4.1$ kcal/mol).[75] Formation of an O-bonded DMSO−Fe(II)-AcMP8 complex would thus involve a larger enthalpic cost for ligand desolvation ($\Delta H^\circ_{DMSO,H_2O} - \Delta H^\circ_{MTE,H_2O} = \Delta\Delta H^\circ_{L,H_2O} \sim -3.6$ kcal/mol). We have shown experimentally that $\Delta H^\circ_{DMSO,Fe(II)} - \Delta H^\circ_{MTE,Fe(II)} = \Delta\Delta H^\circ_L = -2.7$ kcal/mol. Hence, we estimate that the intrinsic difference in DMSO and MTE binding enthalpies is closer to −6.3 kcal/mol, similar to the value obtained computationally (−8.1 kcal/mol).

The greater enthalpic cost for desolvating DMSO versus MTE will also diminish the difference in enthalpies of binding to Fe(III)-AcMP8 in solution relative to the intrinsic values. Recall that DMSO forms a weaker complex with Fe(III)-AcMP8 in solution than does MTE ($\Delta\Delta G^\circ \sim 1.2$ kcal/mol). Assuming that $\Delta\Delta H^\circ$ for coordination of DMSO and of MTE to Fe(III)-AcMP8 does not differ dramatically from $\Delta\Delta G^\circ$, it is possible to conclude that DMSO is an intrinsically stronger ligand than is MTE. This conclusion is consistent with expectations based on hard−soft acid−base considerations. However, the difference in intrinsic enthalpies for binding of DMSO and of MTE to Fe(III)-AcMP8 will almost certainly be smaller than those for binding to Fe(II)-AcMP8. We attribute this to the fact that only σ bonding is likely to be involved in DMSO and MTE complexes of Fe(III)-AcMP8 because the electron deficiency of Fe(III) is likely to make it a poor π electron donor.

Relevance of the Results to cyt *c*. Previously reported experiments have demonstrated that Met(SO) functions as an axial ligand in ferrous horse heart Met(SO)-80 cyt *c*,[19,23] but it was not unambiguously established whether the sulfoxide moiety coordinates to iron via sulfur[19] or via oxygen.[23] While our evidence that DMSO coordinates to Fe(II)-AcMP8 via oxygen is compelling, coordination via sulfur is energetically feasible. Similarity between the electronic absorption spectra of ferrous Met(SO)-80 cyt *c* and the DMSO complex of Fe(II)-AcMP8 supports the hypothesis that Met(SO)-80 coordinates to the heme iron of cyt *c* via oxygen.

Studies with ferrous Met(SO)-80 cyt *c* have shown that oxidation of the Met side chain exerts little, if any, effect on the thermodynamic stability of the ligand to iron bond; only the rate at which the side chain binds and dissociates is affected.[25] Our ligand binding studies have shown that oxidation of Met-80 to Met(SO) in cyt *c* should increase the intrinsic affinity of the side chain for Fe(II) by approximately 1.3 kcal/mol. We therefore conclude that the local environment experienced by Met(SO)-80 in the modified protein discriminates against its coordination by approximately this amount. This discrimination may be attributable to the size difference between Met(SO) and Met, with the larger sulfoxide being accommodated less favorably in the space

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around the heme iron atom. However, as noted by Ivanetich and co-workers,[18] the greater polarity of the Met(SO) side chain in comparison to the Met side chain may also play a role.

The nature of the coordination sphere in ferric Met(SO)-80 cyt *c* was not firmly established in the previously reported studies, although it was suggested that Met(SO)-80 is the ligand at neutral pH and above.[18,19,23] Comparisons of data from DMSO and MTE complexes of Fe(III)-AcMP8 with those published for ferric Met(SO)-80 cyt *c*[18,23] and the Ala-80 mutant of cyt *c*[54] have allowed us to conclude that Met-(SO)-80 is not coordinated to iron in the ferric protein but is likely replaced by hydroxide. The results of the ligand binding titrations with Fe(III)-AcMP8 indicate that oxidizing Met-80 to its sulfoxide should diminish the intrinsic affinity of the side chain for Fe(III) in ferric cyt *c* by ∼1.2 kcal/mol. As noted above, the unfavorable steric environment near iron should discriminate against coordination by an additional 1.3 kcal/mol. The intrinsically weaker affinity of Met(SO)-80 for Fe(III) relative to Met, coupled with the unfavorable steric environment, would thus be expected to result in dissociation of the axial ligand, as our spectroscopic data suggest. It is interesting to speculate that evolution has fine-tuned the steric environment in the vicinity of Met-80 in cyt *c* to disfavor coordination of the ligand were it to become oxidized to Met(SO) in vivo. Dissociation of the modified ligand would be expected to facilitate its repair by Met(SO) reductases.[76,77] We are investigating this possibility in studies with Met(SO)-80 cyt *c*.

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Supporting Information Available: Data from binding titrations and isodensity plots from density functional theory calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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