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Ligation and Reactivity of Methionine-Oxidized Cytochrome *c*

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Abstract

Met80, one of the heme iron ligands in cytochrome *c* (cyt *c*) is readily oxidized to Met sulfoxide (Met-SO) by several biologically-relevant oxidants. The modification has been suggested to impact both the electron-transfer (ET) and apoptotic functions of this metalloprotein. The coordination of the heme iron in Met-oxidized cyt *c* (Met-SO cyt *c*) is critical for both of these functions but has remained poorly defined. We present electronic absorption, NMR, and EPR spectroscopic investigations as well kinetic studies and mutational analyses to identify the heme iron ligands in yeast *iso-1* Met-SO cyt *c*. Similar to the alkaline form of native cyt *c*, Lys73 and Lys79 ligate to the ferric heme iron in the Met80-oxidized protein but this coordination takes place at much lower pH. The ferrous heme iron is ligated by Met-SO implying the redox-linked ligand switch in the modified protein. Binding studies with a model peptide microperoxidase-8 provide a rationale for alterations in ligation and for the role of the polypeptide packing in native and Met-SO cyt *c*. Imidazole binding experiments have revealed that Lys dissociation from the ferric heme in K73A/K79G/M80K (M80K[#]) and Met-SO is more than three orders of magnitude slower than the opening of the heme pocket that limits Met80 replacement in native cyt *c*. The Lys-to-Met-SO ligand substitution gates ET of ferric Met-SO cyt *c* with Co(terpy)₂²⁺. Owing to the slow Lys dissociation step, ET reaction is slow but possible, which is not the case for non-switchable M80A and M80K[#]. Acidic conditions cause Lys replacement by a water ligand in Met-SO cyt *c* ($pK_a=6.3\pm0.1$) increasing intrinsic peroxidase activity of the protein. This pH-driven ligand switch may be a mechanism to boost peroxidase function of cyt *c* specifically in apoptotic cells.

Table of Contents Synopsis

Several biological oxidants cause oxidation of Met80, one of the ligands to the heme iron in cytochrome *c*, to Met sulfoxide. Spectroscopic studies, analysis of mutants, and model binding experiments identify and rationalize iron ligands in the modified protein. pH titrations, kinetics of imidazole binding, model electron-transfer reactions, and peroxidase activity assays probe the role of the switchable Lys-iron ligation in function of cytochrome *c*.

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Supporting Information Available. One table summarizing the ¹H chemical shifts of Met and Met-SO in ferrous WT* and WT-M80SO, respectively. Nine figures showing differences in Met64(65) location in yeast and horse heart cyt *c*; CD spectra of WT* and WT-M80SO; EPR “truth diagram” of various low-spin heme proteins; EPR spectra of Met-SO variants; WT-M80SO and structures of Met, *R* and *S* diastereomers of Met-SO and 2D ¹H NOESY of ferrous WT*; heme-ring current shifts of Met80 protons; results of pH titrations for M80A; imidazole binding data for WT*; and plots of results from spectroelectrochemistry titrations for WT-M80SO.

Introduction

Oxidation of protein methionine residues is a common modification related to oxidative stress. A number of biologically-relevant oxidants have been reported to cause oxidation of Met80, one of the ligands to the heme iron in cytochrome *c* (cyt *c*, Figure 1).^{1–4} The transformation is accompanied by the loss of the characteristic 695 nm charge transfer band in the electronic absorption spectrum of the ferric protein.⁵ The disruption of the native Met80-iron ligation is a key step in the transformation of cyt *c* from an electron-transfer (ET) carrier to a peroxidase.^{6–9} As a peroxidase, cyt *c* oxidizes the lipid cardiolipin (CL), which is a critical reaction in early stages of apoptosis.¹⁰ Previous studies of cyt *c* have found that methionine oxidation increases the protein's affinity for CL and its peroxidase activity, suggesting that this modification can amplify the pro-apoptotic response of cyt *c*.¹¹

Both ET and peroxidase functions of cyt *c* are dependent on the ligands that coordinate to the heme iron. Several studies have examined the changes in cyt *c* structure upon methionine oxidation;^{5,12–14} however, the ligands in the modified protein have not yet been clearly defined. Lys has been considered as a possible ligand to the ferric heme iron in early studies of methionine-oxidized cyt *c* (Met-SO cyt *c*) but this proposal was dismissed.⁵ Myer and coworkers have noted that the ¹H NMR spectrum of Met-SO cyt *c* is similar to that of alkaline cyt *c* but no definitive assignment of the ligand, apart from it not being Met80 sulfur, has been made.¹³ Analysis of the spectra of ferrous protein has suggested that Met80-SO is still located in the heme pocket that led to the conclusion of the Fe-O=S linkage in the modified protein.¹⁵ More recently Murgida *et al.* have considered Lys/His and hydroxide/His as possible ligands to the heme iron in the ferric protein.¹² The Lys/His and hydroxide/His heme species have very similar resonance Raman spectra but the hydroxide/His reference appeared to fit better the spectra of Met-SO cyt *c*. In addition, the pH dependence of the reduction potential of Met-SO cyt *c* was similar to that of M80A mutant of cyt *c*, the variant with hydroxide/His ligation at neutral pH.

The redox reactivity of Met-SO cyt *c* has also been a subject of controversy. While some assays of cyt *c* oxidase activity have found minimal differences between the native and Met-SO cyt *c*,^{5,16} others suggested that ET from cyt *c* to cyt *c* oxidase is impaired by the modification.³ Multiple studies have found that succinate-cyt *c* reductase activity is greatly reduced upon Met80 oxidation,^{5,16,17} but there have also been reports of no effects on this redox reaction.¹⁸ The reduction potentials and ET rates with a common reductant sodium ascorbate have also differed from one study to another.^{5,12,16,17} Interestingly, ascorbate experiments have revealed a kinetic phase independent of the reductant.¹⁹ These findings suggest that a population of Met-SO cyt *c* cannot be reduced by this weak reductant ($E = -60$ mV)²⁰ directly and requires an intramolecular process for its conversion to the reducible form.

Herein, we compare spectroscopic features and redox reactions of yeast *iso*-1 Met-SO cyt *c* to that of M80A and the recently described Lys-ligated variant K73A/K79G/M80K (M80K[#]).²¹ We find that the Met80 oxidation results in the formation of Lys-ligated species to the ferric heme iron already at neutral pH and rationalize the role of this seemingly unreactive species in the ET and peroxidase function of cyt *c*. Binding studies with the model heme peptide *N*-acetyl microperoxidase-8 (AcMP8) quantify interactions of Met-SO with the heme iron and explain the choice of the ligands in ferric and ferrous Met-SO cyt *c*.

Experimental Section

Chemicals were purchased from Fisher Scientific Inc., unless noted otherwise. Buffers were prepared using reagent-grade chemicals. Water was purified to a resistivity of 18.2 MΩ cm using Barnstead E-Pure Ultrapure Water Purification System. Data analyses were carried out using Origin 8 (OriginLab Corporation).

Site-Directed Mutagenesis, Protein Expression and Purification.

Point mutations in the Rbs (WT*) plasmid²² were introduced using a Quikchange kit (Agilent). The Rbs (WT*) plasmid contains genes CYC1 and CYC3 that encode the yeast *iso*-1 cyt *c* and yeast cyt *c* heme lyase, respectively. The parent pseudo-wild-type cyt *c* construct WT* had two additional mutations, K72A and C102S, to prevent Lys72 coordination to the heme and formation of Cys102-linked dimers, respectively. DNA was extracted and purified with a QIAprep Spin Miniprep Kit (Qiagen). Mutations were confirmed by sequencing, carried out at the Molecular Biology & Proteomics Core Facility (Dartmouth College). Expression and purification of the proteins were performed following the published procedures.²³ Protein conversion to ferric and ferrous forms has been accomplished by adding excess of potassium ferricyanide and sodium dithionite, respectively.

Preparation of Met-SO Derivatives.

Chloramine-T reaction was performed according to the published procedures with the following modifications.^{12,16} For both WT* and K79G, 0.5 mM protein was exchanged into a 60 mM Tris-HCl buffer at pH 8.4, then 2.5-fold molar excess of chloramine-T was added into the protein solution dropwise while stirring at room temperature. The excess of chloramine-T was removed by using a desalting column to stop the reaction after 3 h. The reaction mixture was loaded onto a HiTrap SP HP 5 mL column, which was pre-equilibrated with buffer A (10 mM sodium phosphate at pH 7.4), and eluted using a linear gradient of 0–100% buffer B (10 mM sodium phosphate containing 0.75 M NaCl at pH 7.4) for 120 min. The major peak was pooled and further purified using a Mono S 5/50 column. For the methionine oxidation of K73A/K79G variant, the reaction was kept at room temperature for 6 h. In order to separate the Met80-SO species from residual Met80 form, the buffer A and B for the purification step using Mono S 5/50 column were changed to 10 mM Tris-HCl at pH 8.4 and 10 mM Tris-HCl containing 0.5 M NaCl at pH 8.4, respectively.

Preparation of Microperoxidase-8.

AcMP8 was prepared by proteolytic degradation of horse heart cyt *c* (Sigma-Aldrich) and subsequent reaction of the heme-containing eight-residue peptide with acetic anhydride as previously described.²⁴

Spectroscopic Measurements.

Absorption spectra were acquired on an Agilent 8453 diode-array or a Shimadzu UV-1201 (Shimadzu Scientific) scanning spectrophotometers. CD spectra and CD temperature melting curves were recorded on a J815 CD spectropolarimeter equipped with a variable temperature Peltier cell device (JASCO).

Low temperature (10 K) EPR spectra were recorded on a Bruker EMX 300 X-band EPR spectrometer (Bruker Biosciences Corp.) Experimental parameters were set to 9.49 GHz microwave frequency, 3.21 mW microwave power, 100 kHz modulation frequency, 1.00 G modulation amplitude and a 20.48 ms time constant. Protein concentrations were 500 μ M and samples contained 30% v/v of glycerol, unless otherwise noted. ¹H NMR spectra were recorded on a 500 MHz Bruker NMR spectrometer (Bruker Biosciences). The ferric proteins were prepared in 100% D₂O, while the ferrous samples contained 10% (v/v) D₂O; 50 mM sodium phosphate was used to buffer these solutions. Protein concentrations were around 500 μ M and the buffers were exchanged by repeated ultrafiltration using 10 kDa centrifugal ultrafiltration devices (Millipore). Sodium dithionite (1 mM) was kept in the solution for NMR measurements of samples of ferrous proteins. With ferrous proteins, excitation sculpting with gradients was used to suppress water in 1D ¹H NMR or 2D ¹H NOESY experiments. With ferric proteins, 1D ¹H NMR spectra were collected using a superWEFT pulse sequence²⁵ with a whole recycle time of 220 ms for low-spin heme species of cyt *c* or 110 ms for high-spin heme species, which have been optimized for detection of both high-spin and low-spin signals.²¹ All NMR data were analyzed using Bruker TopSpin 3.2.

Protein extinction coefficients were determined by the pyridine hemochrome method.²⁶ All experiments with ferrous proteins were performed under anaerobic conditions in a nitrogen-filled glove box (COY Laboratory Products). Thermal denaturation, pH titrations, and studies of AcMet-SO binding to AcMP8 were performed according to the published procedures.²⁷

Calculation of Heme-Ring Current Shifts.

The heme-ring current shifts for protons of Met80 in cyt *c* were calculated using the Johnson-Bovey 8-loop model.^{28–30} The Johnson-Bovey model attributes the ring-current shift to two current loops above and below the plane of the aromatic ring with a distance (p) of 0.64 Å. The heme is considered as four pyrrole and four hexagon rings in the 8-loop model. The crystal structure of ferrous yeast *iso*-1 cyt *c* (1YCC.pdb)³¹ was used for initial coordinates and protons were added in Chimera (<http://www.cgl.ucsf.edu/chimera>).³² The cylindrical coordinates (ρ , z , ϕ) of protons relative to each ring were determined through translation and rotation in Chimera and VMD (<http://www.ks.uiuc.edu/Research/vmd/>).³³ The ring radius (a) for pyrrole and hexagon ring is 1.182 Å and 1.56 Å, respectively. The empirical ring-current intensity factor (i) was set as 0.374 and 1.992 for pyrrole and hexagon

ring, respectively. The ring-current shift (δ_R , shielding constant, in ppm) to each ring was calculated according to eq 1, where ρ and z are measured in units of a and e , m and c are standard constants (in esu).

$$\delta_R \times 10^{-6} = i \frac{3e^2}{6\pi m a c^2} \left[\frac{1}{[(1+\rho)^2 + (z-p)^2]^{1/2}} \left\{ K(k_-) + \frac{1-\rho^2 - (z-p)^2}{(1-\rho)^2 + (z-p)^2} E(k_-) \right\} + \frac{1}{[(1+\rho)^2 + (z+p)^2]^{1/2}} \left\{ K(k_+) + \frac{1-\rho^2 - (z+p)^2}{(1-\rho)^2 + (z+p)^2} E(k_+) \right\} \right] \quad (\text{eq 1})$$

K and E are the complete elliptic integrals of the first and second kind, respectively. The modulus, k , is given by eq 2. K and E were calculated in MATLAB 2011a (MathWorks) with the command $[K,E]=\text{ellipke}(k^2)$.

$$k_- = \left\{ \frac{4\rho}{(1+\rho)^2 + (z-p)^2} \right\}^{1/2} \quad (\text{eq 2})$$

$$k_+ = \left\{ \frac{4\rho}{(1+\rho)^2 + (z+p)^2} \right\}^{1/2}$$

The net shift was calculated as the sum of the shifts from the four pyrrole rings and four hexagon rings. For methyl protons, the Boltzmann weighted average shift for 12 equidistant values of θ in the interval 0 to 2π were used. To test the effects of ligand orientation relative to the heme normal, Met80 was tilted away from its original position by changing the bond angle of ND(heme)-Fe-SD(M80) and the heme-ring current shifts for protons of Met80 in this new orientation of the ligand were calculated.

Kinetics of Imidazole Binding.

Equilibrium binding of imidazole was studied by examining changes in the Soret absorption band as previously described.³⁴ Changes in absorbance at 413 nm and 398 nm at increasing imidazole were analyzed and the apparent binding constants K_{app} were extracted by fitting these data to a one-site binding model. Kinetics of imidazole binding to WT* were measured with a BioLogic SFM-300 stopped-flow instrument monitoring the absorbance changes at 695 nm.²⁷ Kinetics of imidazole binding to M80K[#] and Met-SO derivative of WT* (WT-M80SO) were examined by performing manual mixing and monitoring the absorbance changes of the Soret band with an Agilent 8453 diode-array spectrophotometer. All kinetics experiments were performed with ferric protein, freshly oxidized and repurified before the measurements.

Spectroelectrochemical Measurements.

A spectroelectrochemistry kit (Pine Research Instrumentation) was used in a glove box (COY Laboratory Products) under a nitrogen atmosphere for reduction potential measurements. The path length of the optical cell was 1.7 mm. The working electrode, the

auxiliary electrode and the reference electrode were a honeycomb electrode, a platinum electrode and an Ag/AgCl gel (saturated KCl solution) electrode, respectively. The external potential was controlled by a WaveNow USB potentiostat and the electronic absorption spectra were measured using a Shimadzu UV-1201 spectrophotometer. Experiments and analyses were performed as previously described.²⁷

Co(terpy)₂²⁺ Reduction Experiments.

Bis(2,2':6,2''-terpyridine) cobalt (II) triflate Co(terpy)₂(CF₃SO₃)₂ was synthesized according to the published procedure.^{35,36} Proteins were freshly oxidized to yield ferric forms and repurified; kinetics of their reduction were examined by monitoring heme absorbance with an Agilent 8453 diode-array spectrophotometer; manual mixing was performed. All kinetics with WT-M80SO were monoexponential. The dependence of $k_{\text{obs}}^{\text{Red}}$ on the concentration of Co(terpy)₂²⁺ was fitted to eq 3³⁶ to extract the bimolecular ET constant k_{ET} and the ligand switching constants k_{f} (Lys-to-Met-SO) and k_{b} (Met-SO-to-Lys).

$$k_{\text{obs}}^{\text{Red}} = \frac{k_{\text{b}} \times k_{\text{ET}} [\text{Co(terpy)}_2^{2+}]}{k_{\text{ET}} [\text{Co(terpy)}_2^{2+}] + k_{\text{f}}} \quad (\text{eq 3})$$

Assays of Peroxidase Activity.

Guaiacol assay was performed as previously described.³⁷ The final concentrations of protein, guaiacol, and H₂O₂ were 3 μM, 5 mM, and 100 μM, respectively. Formation of tetraguaiacol ($\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$)³⁸ was observed every second for 1000 s by absorbance measurements. The initial rates were determined from the slopes of the linear phase of the reaction progress curves and the reaction stoichiometry (four guaiacol molecules consumed per one molecule of tetraguaiacol formed) has been accounted for in these calculations. Control experiments in the absence of guaiacol have confirmed that under these experimental conditions the degradation of the heme in the studied variants was minimal.

Results and Discussion

Yeast Cytochrome c.

All experiments in this study have been performed with yeast *iso-1* cyt *c*. The yeast protein with its high sequence and structural similarity to other mitochondrial cyt *c* proteins³⁹ is a useful model for understanding properties of this metalloprotein. The dynamics of the heme coordination loop have been thoroughly characterized for yeast *iso-1* cyt *c*.⁴⁰ Further, many of its previously characterized mutants, including M80K[#] from our recent work,²¹ facilitate analysis of the ligation to the heme iron.

Preparation of Yeast Met-SO Cyt c.

Addition of chloramine-T to ferric WT* causes gradual decrease of the 695 nm charge transfer band and a blue shift of the Soret band from 409 to 407 nm (Figure 2A) suggesting

changes in the Met80-iron coordination. The purification of the reaction mixture by ion-exchange chromatography has yielded one major product (Figure 2B). The MALDI spectra (Figure 2C) have identified the increase in molecular mass (+16 Da) of the protein upon oxidation, suggesting that only one Met is modified in WT-M80SO. In contrast, two major species have been reported in preparations of horse heart Met-SO cyt *c* and both Met64 and Met80 were oxidized.¹⁶ While Met65 in horse heart cyt *c* is surface-exposed,⁴¹ Met64 in the yeast protein is buried (Figure S1),⁴² providing an explanation for the differences between the two proteins. As previously reported for the horse heart protein, the modification does not introduce gross perturbations in the protein structure.¹³ Indeed, the far-UV and near-UV CD spectra of WT* and WT-M80SO are similar (Figure S2), suggesting that the secondary and tertiary structures of the protein remain largely intact.

Spectroscopic Properties and Ligands to the Heme Iron in Ferric Met-SO.

The Soret band at 407 nm in ferric Met-SO cyt *c* is indicative of the low-spin heme iron. Studies of cyt *c* mutants have revealed that His, Lys, and hydroxide ligands give rise to very similar positions of the Soret band, blue-shifted by a few nm from its position at 409 nm for the Met-ligated WT*.^{17,21,43–45} The far-UV and near-UV CD spectra of Met-SO and native cyt *c* (Figure S2) suggest only minor perturbations in the secondary and tertiary structure by the modification. Thus the ligation by His26, 33, or 39 residues, which are remote from the heme coordination loop, is unlikely. The modified protein does not have a split Q-band, a feature found in the spectrum of M80A.^{44,46} Thus hydroxide also does not seem to fit the spectral properties of the ligand to the heme iron in Met-SO, at least for the majority of the protein ensemble.

We next examined ¹H NMR and EPR spectra of WT-M80SO (Figure 3). The heme methyl resonances of WT-M80SO appear at the positions similar to those of Lys-ligated M80K[#]. The EPR spectra of WT-M80SO also resemble the spectrum of M80K[#], providing additional support for Lys coordination in this modified protein. Both ¹H NMR and EPR spectra are distinct from those of the hydroxide-ligated M80A.

At alkaline pH Lys73 and Lys79 residues within the heme coordination loop (residues 70–85) replace the Met80 ligand (Figure 1).⁴⁷ The Met-to-Lys ligand switch also takes place at near neutral pH in some mutants^{46,48–53} of cyt *c* and in the presence of denaturant urea.⁵⁴ Lysine ligation to the heme iron at near neutral pH has also been observed upon carboxymethylation⁵⁵ and tyrosine nitration of cyt *c*⁵⁶ and also upon binding to CL.⁵⁷ Since changes in the intraprotein hydrogen bonding and heme packing appear to facilitate the transition, we have hypothesized that structural perturbation associated with oxidation of Met80 could also favor Lys ligation to the ferric heme iron.

To further confirm Lys ligation and establish the identity of coordinating Lys residues, we have created two additional Met-SO variants, lacking Lys79 (K79G-M80SO) and both Lys79 and Lys73 (K73A/K79G-M80SO). The ¹H NMR spectrum of ferric K79G-M80SO shows fewer heme methyl resonances in the region between 13 and 25 ppm characteristic of the Lys-coordinated heme species than WT-M80SO and ferric K73A/K79G-M80SO has no detectable resonances from the Lys species in this region (Figure 4). These findings suggest that both Lys73 and Lys79 act as ligands to the ferric heme iron in Met-SO.

For K79G-M80SO and K73A/K79G-M80SO we have noted an additional set of heme methyl resonances in the ^1H NMR spectra, distinct from that of the Met- and Lys-ligated species. We have wondered if these resonances could arise from Met-SO ligated species. In the absence of the known spectra of ferric Met-SO/His-ligated heme proteins, we have explored the effects of the highly-soluble sulfoxide DMSO on the spectra of the model heme peptide AcMP8. The addition of 8.4 M DMSO causes a red shift of the electronic absorption spectra of AcMP8, consistent with prior observations.¹⁴ The ^1H NMR spectrum of AcMP8 in the presence of 8.4 M DMSO displays several new resonances at 20 ppm and between 30 and 40 ppm, at positions somewhat similar to those in K79G-M80SO and K73A/K79G-M80SO. There is also a set of signals in these regions for WT-M80SO but their intensity is much lower compared to the signals from the Lys-ligated species, suggesting that when Lys73 and Lys79 are available for coordination to the heme iron, the population of the Met-SO-ligated ferric species in Met-SO cyt *c* is low.

EPR spectra of cyt *c* variants (Figures 3B, S3, and S4) are consistent with our assignment of Lys being the major ligand to the ferric heme iron in WT-M80SO. The signals of the major species in WT-M80SO and K79G-M80SO resemble those of M80K[#] and alkaline cyt *c* and are distinct from those of the hydroxide-ligated M80A. The Blumberg and Peisach correlation, known as the “truth diagram”,^{58,59} has been widely used to predict the nature of the axial ligands in low-spin heme iron centers. The axial and rhombic ligand field parameters calculated from the experimental *g*-values for known Lys-ligated proteins yield a new distinct cluster (K) on this diagram (Figure S3). The Met80-oxidized derivatives WT-M80SO and K79G-M80SO also belong to this K cluster, supporting our assignment of Lys being the major ligand to the ferric heme iron in these variants. The high-spin species are present in the EPR spectrum of K73A/K79G-M80SO, but not in the spectrum of M80A (Figures 3B and S4). The lower Soret λ_{max} in the electronic absorption spectra of the K73A/K79G-M80SO variant is consistent with these EPR findings (Figure 5).

Spectroscopic Properties and Ligands to the Heme Iron in Ferrous Met-SO.

Electronic absorption spectra of ferrous Met-SO derivatives have revealed features of the low-spin six-coordinate heme iron.¹³ The ^1H NMR spectra (Figure 6A) suggest that this sixth ligand is Met-SO rather than Lys. In the upfield region of the ^1H NMR spectrum of ferrous Met-SO derivative, there are no proton resonances corresponding to the Lys ligand; ^{60,61} instead, there is a set resembling the resonances of the Met ligand in WT* but at a lower field. The most intense peak from the methyl protons of Met-SO is split into two. These results are consistent with the prior findings with the horse heart protein, which have been attributed to two *R* and *S* diastereoisomers,¹³ differing in the sulfur configuration of the sulfoxide ligand (Figure S5A). Two sets of cross-peaks from Met80-SO are also observed in the 2D ^1H NOESY spectrum (Figure S5B). One set has readily traceable correlations for protons of the entire Met-SO side chain and shares a similar cross-peaks pattern with Met in WT* (Figure S5C). The other shows only one clear cross-peak in the upfield ^1H region corresponding to the γ protons. Therefore, we have chosen to follow chemical shifts from the first well-defined set to avoid ambiguity in our subsequent analysis.

Both the sulfur and oxygen atoms have been previously suggested as the sites of the metal coordination in ferrous Met-SO cyt *c*.^{13–15} Comparison of the ¹H NMR spectra of AcMet and AcMet-SO (Figure 6B) has revealed that sulfur oxidation shifts to the lower field the neighboring ϵ -methyl (δ = 0.60 ppm), γ -methylene (δ = 0.36 ppm) and β -methylene (δ = 0.31 ppm) protons. In contrast, upon Met oxidation in cyt *c* protein, the direction and magnitude of the shifts differ: one of the γ -methylene protons encounters a relatively large magnitude of the downfield shift (δ = 1.01 ppm) and one of the β -methylene protons shifts upfield (δ = -0.25 ppm) (Figure 6A and Table S1). These findings suggest that the change in electron density upon Met oxidation is not the only factor responsible for the altered positions of these resonances in Met-SO cyt *c*.

In ferrous cyt *c*, the upfield position of the resonances of the ligands arises from the ring current interactions with the porphyrin. The reduction in the ring current effect implies that either the methyl and the methylene protons of Met-SO in WT-M80SO are placed further away from the heme plane than those of Met in WT* or the angle from the heme normal has changed. In principle, the sulfur oxidation could preserve the ligand coordination to the heme iron through sulfur but alter its orientation with respect to the heme and thus modify the ring current effects. To test this explanation, we have explored the effects of changes in the ligand orientation relative to the heme normal on the heme-ring current shifts of Met80 protons. The heme-ring current shifts have been calculated based on the original structure of ferrous WT*³¹ and those modeled with the tilt of the Fe-S bond for the Met80 ligand (Figure S6). The analysis suggests that even small changes in the orientation of the Met ligand relative to the heme plane could lead to significant changes in the ring current effects for the protons of the ligand. However, upon such reorientation, one of the γ -methylene protons would shift in the opposite direction compared to other protons, which is different from our experimental observations with Met-oxidized cyt *c* (Table S1). This result suggests not just a simple tilt but more substantial perturbations in the position of the ligand.

The coordination through oxygen would position protons of the Met-SO side chain further away from the heme, decreasing the ring current shifts, but then the side chain will likely also have to bend to meet steric requirements of this coordination within the heme pocket. Although Met80 in WT* and Met-SO in WT-M80SO have the same cross-peak pattern of resonances in the 2D ¹H NOESY NMR spectra (Figure S5B and S5C), there are differences in the magnitude of the separation for the two signals from β - and γ -methylene protons (Table S1), suggesting changes in the conformation of the Met side chain and the environment of these protons upon sulfur oxidation.

Resonance Raman studies of DMSO binding to ferrous AcMP8 have revealed the evidence for iron $d\pi$ to DMSO backbonding in this complex.¹⁴ In particular, the ν_4 band, which is a good π -electron density marker, occurs 5 cm⁻¹ higher in frequency in the DMSO complex than the ν_4 band in the complex with a model thioether 2-(methylthio)ethanol. This finding, together with results of computational analysis, has led to the conclusion that DMSO coordinates to the ferrous heme iron through its oxygen atom. Similar to these model studies, the resonance Raman spectra of Met-SO cyt *c* also show the ν_4 band at a higher frequency (1366 cm⁻¹) than that of WT (1363 cm⁻¹)¹³ suggesting an increase in $d\pi$ back bonding in the case of Met-SO cyt *c* as well. These findings, together with our NMR

analyses of chemical shifts, make us conclude that the oxygen atom of Met-SO is the coordination site to the heme iron in ferrous Met-SO.

Binding Affinity of Met-SO for the Heme Iron.

Our spectroscopic findings establish that in WT-M80SO, Lys and Met-SO coordinate to the ferric and ferrous heme iron, respectively, implying redox-dependent preferences for the coordination of these biological ligands. The heme microperoxidase peptides are excellent models to evaluate the intrinsic binding affinity of ligands to the iron center in the absence of the protein scaffold.^{14,27,61–63} Acetylation of the *N*-terminus of MP8 diminishes formation of the peptide dimers. Using AcMP8, we have previously quantified the binding constants to the ferric²¹ and ferrous⁶¹ heme iron for Lys. To enable the comparison of Lys and Met-SO, we now wanted to extend these measurements to Met-SO.

Addition of 2.5 M AcMet-SO to ferric AcMP8 did not affect the position of the Soret band, suggesting weak affinity of this ligand for the ferric heme iron (Figure 7A). Because of the low solubility of AcMet-SO, further increase in the concentration of this compound was not possible but the existing data still allowed us to set the lower limit for the dissociation constant $K_D > 2.5$ M. The switch to a more soluble sulfoxide ligand DMSO has captured the shift in the Soret band from 396 nm to 412 nm but again required very high concentration of the ligand, reiterating the conclusion that the binding affinity of the sulfoxide for the ferric heme is low (Figure 7B).

Importantly, the binding interactions between Met-SO and ferric AcMP8 are even weaker than those between Met and ferric AcMP8,⁶³ providing a rationale for the ligand displacement in ferric cyt *c* upon Met80 oxidation. The protein scaffold is a key determinant of Met80 coordination to the ferric heme iron in cyt *c*.^{21,46,63} The soft base Met is a poor match for the hard ferric iron yet it is brought by the protein fold to be the ligand to the heme iron in the native protein. The hard base Lys, on the other hand, binds strongly to the ferric heme iron, but requires deprotonation. Removal of the constraints of the surrounding protein that favor Met80 ligation, together with the presence of protein groups that can assist Lys deprotonation, shift the delicate balance of these forces to yield Lys-ligated cyt *c* conformers already at neutral pH. Oxidation of the Met sidechain not only reduces the intrinsic affinity of the ligand for the ferric heme but may also perturb the heme pocket to enable one of the nearby Lys residues to take its place at the metal center. Structural perturbations at the heme pocket appear to be essential for ligand switch as Lys coordination to the iron does not occur in M80A within the pH range for the alkaline transition in yeast WT* or horse heart WT cyt *c*.^{46,55}

Titration of the solution of ferrous AcMP8 with AcMet-SO have caused changes in the electronic absorption spectra (Figure 7C and 7D) yielding dissociation constants in Table 1. The red shift and the increase in intensity of the Soret absorption band are similar to the changes observed upon binding of other neutral ligands to AcMP8.⁶³ The binding affinity of Met-SO for ferrous AcMP8 is comparable to that of Met. Again, Lys binds stronger to the ferrous heme than either Met-SO or Met but requires deprotonation. The difference in the binding affinities, however, is not as large as in the case of the ferric heme iron and

presumably the structural perturbations caused by the oxidation of the Met residue are not sufficient to favor Lys coordination already at neutral pH.

pH-Dependent Transitions in Ferric Met-SO Cyt *c*.

Our experiments have revealed that Lys is the ligand to the ferric heme iron in yeast Met-SO cyt *c* at pH 7.4. Studies of other Lys-ligated systems suggest that at lower pH other ligands may replace this residue at the heme iron since the high pK_a of the free Lys (10.5 ± 0.1) favors its protonation and thus dissociation from the heme iron.^{21,46} We have monitored changes in the electronic absorption spectra of our three Met-SO cyt *c* variants as a function of pH (Figure 5B). As the pH of the solution is lowered, the Soret band increases in intensity and blue shifts and the charge-transfer band at 623 nm, indicative of the formation of the H₂O-ligated species, also becomes apparent. The changes in the pH range between 1.6 and 10.0 can be fit to a three-state transition with parameters in Table 2.

The lower pH transition ($pK_a \sim 3$) is associated with acid unfolding of cyt *c*. This transition is not affected by Met80 oxidation and Lys mutations performed in our studies here. Protonation of His26, with the breakup of the contact between residues 26 and 44, is an important step in the mechanism of acid unfolding of cyt *c*.⁶⁴ Because our modifications involve the heme coordination loop, away from these residues, they do not appear to influence the global acid unfolding process.

Spectroscopic changes from the low-spin to the high-spin species as well as distinct markers of the Lys-ligated heme iron at pH 7.4 in WT-M80SO and K79G-M80SO (Figures 3, 4, S3, and S4) are consistent with the higher pH transition in these variants being the Lys-to-H₂O ligand replacement. The similarity of the pK_a values (Table 2) for these two variants suggests that Lys73- and Lys79-ligated conformers in the Met-SO protein have similar stability.

The heme iron in K73A/K79G-M80SO is already H₂O-ligated (Figure 4) at pH 7.4 and for this variant the transition from the high-spin to the low-spin species at higher pH (Figure 5B) is related to deprotonation of the heme iron H₂O ligand. The pK_a of the H₂O-to-hydroxide transition in AcMP8 ($pK_a = 9.6$)⁶² is higher than that in M80A ($pK_a = 6.7 \pm 0.1$, Figure S7 and Table 2) and the differences in the values are related to the fact that in AcMP8 there is no protein scaffold to further tune the acid-base properties of the water ligand. Our findings of $pK_a = 8.2 \pm 0.2$ (Table 2) for deprotonation of the water ligand in K73A/K79G-M80SO suggest that in this variant the two Lys mutations and Met oxidation collectively result in a more open heme pocket than that in M80A.

Previous studies of Met-SO cyt *c* excluded the possibility of Lys ligation to the ferric heme iron based on the pH profile of this derivative.⁵ Similar to our findings, these researchers have observed two separate processes during acid unfolding of Met-SO, but argued that the iron ligand cannot be Lys because only one process is apparent during acid unfolding of Lys-ligated carboxymethyl cyt *c*. Our studies of M80K[#] have illustrated that there are also two acid transitions in this mutant and Lys80 is displaced from the heme iron before the acid-unfolding transition of the water-ligated conformer.²¹ In contrast, only one pH transition ($pK_a = 5.6 \pm 0.1$) is readily apparent in the largely unstructured Y67R/M80A,⁴⁶ at lower pH,

this protein and its heme rapidly degrade resulting in erratic changes in the absorbance spectra. The carboxymethyl cyt *c* might have experienced a similar fate of degradation in the prior work yielding only one transition. Regardless of what has been happening with carboxymethyl cyt *c*, it is clear from two earlier investigations^{5,13} and our study here that there are two distinct transitions in acidic titrations of Met-SO cyt *c*. Importantly, the similarities of pH profiles for M80K[#] and WT-M80SO further strengthen our assignment of Lys being the ligand to the ferric heme iron in the Met-oxidized protein.

Binding of Imidazole.

Addition of imidazole to M80K[#] and WT-M80SO cyt *c* modifies the electronic absorption spectra of these proteins (Figure 8A), suggesting changes in the heme environment. The increase in the intensity and red shift of the λ_{max} from 405 nm to 407 nm for the Soret band are similar to the changes previously reported for two other Lys-ligated cyt *c* variants.⁴⁶ The apparent binding constant (K_{app}) for imidazole of M80K[#] is similar to the value for WT* but is about ten-fold greater for WT-M80SO (Figure 8B and Table 3). Because dissociation of the endogenous heme ligand X (Met80 in WT*) and binding of imidazole involve rearrangements of the heme coordination loop (Ω -loop D) in cyt *c*,^{65,66} imidazole binding experiments provide insight into the properties of the heme pocket. The results of equilibrium binding experiments suggest that, compared to WT* and the reference M80K[#] mutant, WT-M80SO possesses a more open heme pocket favoring both the entrance of imidazole and dissociation of the Lys ligand.

Previous studies of ligand binding to cyt *c* have derived an expression describing the dependence of the observed rate constant k_{obs} on the concentration of the exogenous ligand, in our case imidazole, Im, (eq 5), where rate constants k_1 , k_{-1} , k_2 , and k_{-2} are defined according to Scheme 1). At high concentration of imidazole $\alpha \times k_{\text{obs}}^{\text{Im}} \approx k_1$, where $\alpha = K_{\text{app}} \times [\text{Im}] / (1 + K_{\text{app}} \times [\text{Im}])$. Equation 5 can be further rearranged to eq 6 and eq 7, provided that $k_{-1} \gg k_1 + k_2$.⁶⁵ While WT* shows the hyperbolic dependence of $\alpha \times k_{\text{obs}}^{\text{Im}}$ on the concentration of imidazole (Figure S8), the observed rate constants $k_{\text{obs}}^{\text{Im}}$ for the two other variants are independent of the concentration of imidazole in the investigated range (Figure 8C). Furthermore, the magnitudes of $k_{\text{obs}}^{\text{Im}}$ for Met-ligated WT* and the two Lys-ligated variants M80K[#] and WT-M80SO also differ.

$$K_{\text{app}} = \frac{k_1 \times k_2}{k_{-1} \times k_{-2}} \quad (\text{eq 4})$$

$$k_{\text{obs}}^{\text{Im}} = \frac{k_1 \times k_2 \times [\text{Im}] + k_{-1} \times k_{-2}}{k_1 + k_{-1} + k_2 \times [\text{Im}] + k_{-2}} \quad (\text{eq 5})$$

$$\alpha \times k_{\text{obs}}^{\text{Im}} = \frac{k_1 \times k_{-2} \times K_{\text{app}} \times [\text{Im}]}{k_{-2} \times K_{\text{app}} \times [\text{Im}] + k_1} \quad (\text{eq 6})$$

$$\frac{1}{\alpha \times k_{\text{obs}}^{\text{Im}}} = \frac{1}{k_1} + \frac{1}{k_{-2} \times K_{\text{app}} \times [\text{Im}]} \quad (\text{eq 7})$$

Studies of imidazole binding to horse heart cyt *c* have identified the limiting process in this Met-ligated protein to be opening of the heme pocket with the rate constant of about 60 s⁻¹.⁶⁵ We find a very similar k_1 value for yeast *iso*-1 cyt *c* WT* suggesting that, despite several alterations in the sequence of the heme coordination loop and differences in protein stability,^{23,40} the dynamics of the heme pocket opening of the two proteins are largely the same. In contrast, recent studies of azide binding to human WT cyt *c* have revealed a slower Met80 dissociation process with $k_1 = 5.77 \pm 1.5 \text{ s}^{-1}$,⁶⁷ which is accelerated by mutations in the Ω -loop C (residues 40–57) of the protein.⁶⁸

For Lys-ligated M80K[#] and WT-M80SO variants, $k_{\text{obs}} \approx k_1$ at all imidazole concentrations employed in our experiments. These rate constants are at least three orders of magnitude lower than k_1 of WT* (Table 3), implicating a change in the rate-controlling step. Evidently, when Lys is the ligand to the ferric heme iron, the exogenous ligand binding is no longer limited by the dynamics of the heme pocket but instead depends on the dissociation of the Lys ligand from the ferric heme iron. The larger k_1 value for WT-M80SO suggests that the heme pocket in this variant is more open than that in M80K[#]. Both Lys73 and Lys79 ligate to the heme iron in WT-M80SO (Figures 3 and 4). For the Lys73-ligated conformer, the heme pocket may be particularly open compared to that of Lys79- and Lys-80 ligated species, since this coordination involves the residue more distant from the axial ligand in the native protein.

Reduction Potential and Kinetics of Co(terpy)₂²⁺ reduction.

Spectroelectrochemistry experiments with WT-M80SO have revealed reduction potentials of 157±3 and 158±3 mV in the reductive and oxidative direction, respectively (Figure S9). These values fit within the range between 100 and 205 mV reported for the horse heart Met-SO derivative.^{5,12,16,17} The drop in the potential compared to that of WT* (274±4 mV)²¹ is consistent with the change in ligation and a more solvent-accessible heme pocket. The differences in the potentials of WT-M80SO and Lys-ligated M80K[#] (−94±2 mV)²¹ are in accord with Met-SO, rather than Lys, being the sixth ligand in the ferrous protein. Some of the heme proteins that switch ligands upon the change in the heme iron oxidation state exhibit a hysteresis in spectroelectrochemistry experiments, with distinct potential values from titrations in the reductive and oxidative direction.^{21,69} The origin of this effect is not always clear, but slow kinetics of ligand-substitution reactions provides a possible explanation. The redox-linked substitution reactions in WT-M80SO appear to be relatively uncomplicated, with clean conversion between the two differently-ligated forms.

For the Lys-ligated ferric WT-M80SO, reduction may proceed through the Lys-ligated species or, upon ligand substitution, through Met-SO species, to yield Met-SO-ligated ferrous protein (Scheme 2). Because the reductant Co(terpy)_2^{2+} ($E=270$ mV)⁷⁰ cannot reduce the low-potential metal centers directly, it has been employed to stimulate and probe ligand-substitution reactions in ferric cytochromes.³⁶ The WT* protein is readily reducible by Co(terpy)_2^{2+} with $k_{\text{ET}} = 2.61 \pm 0.06 \text{ mM}^{-1}\text{s}^{-1}$ under our experimental conditions (Figure 9A). In contrast, both Lys-ligated M80K[#] and hydroxide-ligated M80A cannot be reduced by Co(terpy)_2^{2+} (Figure 9B). The WT-M80SO protein, however, is reducible because of the ligand switch to the Met-SO-ligated form (Figure 9C). Analysis of the reduction kinetics has yielded the rate constant for the Lys-to-Met-SO ligand substitution k_b of $0.014 \pm 0.011 \text{ s}^{-1}$. This value agrees with the k_1 value from imidazole binding experiments, suggesting that, as in these latter experiments, Lys dissociation controls the rate of ligand substitution.

The Co(terpy)_2^{2+} experiments imply that the reduction potential of Met-SO-ligated ferric species is higher or comparable to that of this reductant and of Met-ligated cyt *c*. These results are in accord with our findings of comparable binding affinities of AcMet and AcMet-SO to the ferrous heme iron. Further, these results suggest that Met80 oxidation does not halt the electron flow in the biological ET chain but may slow down the process kinetics.

Peroxidase Activity.

The intrinsic peroxidase activities of M80A, M80K[#], and M80SO at pH 7.4 are 2–4 fold greater than those of WT* but this increase is minimal compared to that of AcMP8 (Figure 10). The Lys/His ligation in M80K[#] and M80SO provides an effective barrier to the activation of peroxidase function as it requires dissociation of the endogenous ligand to open up the sixth coordination site. This dissociation process is slow in the Lys-ligated variants, more so than in the Met-ligated WT* (Table 3). Therefore, the activity enhancement is unlikely due to the Lys-ligated species themselves but rather reflects the propensity of the protein to form the nonnative conformers, among which there might be some pentacoordinate species. The population of such species in the protein ensemble is likely very small, since spectroscopic characterization has only picked up features of the six-coordinate heme iron. As had been discussed in studies of cyt *c* folding,⁸ the assay of peroxidase activity is a more sensitive reporter for the presence of the nonnative conformers than many traditional methods of spectroscopic characterization.

The decrease in pH increases the activity of M80A, M80K[#], and WT-M80SO (Figure 10) but not of WT*⁷¹ cyt *c*. At this pH, the $\text{H}_2\text{O}/\text{His}$ heme iron species are readily apparent in the spectra of these proteins, explaining this finding. The activity of these proteins, however, is much less than that of AcMP8, suggesting that the polypeptide surrounding the heme inhibits the access of H_2O_2 and guaiacol to the heme.

The pH-driven increase in peroxidase activity of Met-SO cyt *c* may be physiologically significant. Oxidative stress is a common precursor of apoptosis and reactive oxygen species do readily oxidize Met residues, including Met80 in cyt *c*. Under normal cellular conditions, the pH of intermembrane space, where cyt *c* is located, is about 7.2.⁷² At neutral pH, peroxidase activity of the Lys-ligated Met-SO derivative is only slightly greater than that of the native Met-ligated cyt *c*. In apoptotic cells, however, the pH of intermembrane space

drops down to 5.8.⁷² Under these conditions the loss of Lys ligation ($pK_a=6.3\pm0.1$) increases intrinsic peroxidase activity of the Met-SO-modified protein cyt *c* suggesting that Met80 oxidation may add a switchable control to enhance peroxidase function of cyt *c* specifically in apoptotic cells. When coupled to the protein conformational changes upon CL binding, this activity is further amplified.¹¹

Conclusions

Our NMR and EPR findings, together with analyses of Met-SO derivatives of K79G and K73A/K79G mutants, have revealed that, similar to the alkaline form of cyt *c*, Lys73 and Lys79 coordinate to the ferric heme iron in Met80-oxidized cyt *c*. In contrast to the alkaline form, this coordination takes place at near neutral pH, assisted by perturbations of the polypeptide packing upon oxidation of Met80. The binding affinity of Met-SO for the ferric heme iron is much weaker than that of the deprotonated Lys explaining the formation of the Lys-ligated conformers. The ferrous heme iron in the modified protein is ligated by Met80-SO and our analyses suggest that this ambidentate side chain coordinates through the oxygen atom.

Lys dissociation from the ferric heme iron is a slow process compared to the opening of the heme pocket in native cyt *c*. Changes in the heme iron coordination upon Met80 oxidation decrease the reduction potential of the metal site. The Lys-to-Met-SO ligand substitution enables cyt *c* reduction by high-potential reductants such as Co(terpy)_2^{2+} to proceed but at a slow rate suggesting that Met80 oxidation may impede but not halt the function of the mitochondrial ET chain. When Lys ligation is lost at low pH, peroxidase activity of Met80-oxidized protein increases. The pH-driven Lys-to- H_2O ligand switch may be a mechanism to boost peroxidase function of cyt *c* specifically in apoptotic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structure of yeast *iso-1* cyt *c* (2YCC.pdb) showing positions of axial ligands His18 and Met80 as well as two Lys residues implicated in coordination to the heme iron at alkaline pH. The sequence of the Ω -loop D (residues 70–85) is listed on the top and the residues are highlighted in *forest green* in the structure. Structures of Met80 and ligands replacing it in the Met-SO cyt *c* are shown.

Figure 2.

(A) Electronic absorption spectra of ferric WT* (*black*) and WT-M80SO (*red*). (B) An FPLC chromatogram of the chloramine T-treated WT* solution during purification on a HiTrap SP HP cation-exchange column. Buffer A was 10 mM sodium phosphate at pH 7.4 and buffer B was 10 mM sodium phosphate at pH 7.4 containing, 0.75 M NaCl. (C) MALDI-TOF mass spectra of WT* (*black*) and purified WT-M80SO (*red*). The theoretical molecular mass of iso-1 WT* is 12594 Da, the m/z value of 12610 ± 2 for WT-M80SO reflects the addition of one oxygen atom.

Figure 3.

(A) ^1H NMR at 25 °C and (B) EPR at 10 K spectra of ferric WT*, WT-M80SO, WT* at high pH (WT*_alkaline), K73A/K79G/M80K (M80K[#]) and M80A. Samples for NMR measurements were prepared in a 50 mM sodium phosphate buffer in 100% D₂O at pD 7.4 except for WT*_alkaline which was made in a 50 mM sodium borate buffer in 100% D₂O at pD 9.2. EPR samples were prepared in a 50 mM sodium phosphate buffer at pH 7.4 except for WT*_alkaline which was made in a 50 mM sodium borate buffer at pH 10.5.

Figure 4.

The downfield region of ^1H NMR spectra of ferric WT*, horse heart Met-myoglobin, M80SO variants and DMSO-bound AcMP8 showing both high-spin and low-spin signals. WT*, myoglobin and M80SO variants are in a 50 mM sodium phosphate buffer in 100% D_2O at pD 7.4. DMSO-bound AcMP8 was made by dissolving lyophilized powder of AcMP8 in a solution consisting of 10% Methanol- d_4 , 60% DMSO- d_6 , and 30% sodium borate buffer (150 mM sodium borate in 100% D_2O at pD 9.5). The spectrum of myoglobin matching the literature reports⁷³ illustrates that our experiments are optimized for detection of both high-spin and low-spin signals.

Figure 5.

(A) Electronic absorption spectra in a 50 mM sodium phosphate buffer at pH 7.4 and (B) pH titration curves of ferric WT-M80SO (*black*), K79G-M80SO (*red*) and K73A/K79G-M80SO (*blue*).

Figure 6.

Oxidation of methionine (Met) into methionine sulfoxide (Met-SO) downshifts the NMR resonances of protons next to the sulfur atom. The shifts are indicated by arrows. (A) Structures of Met and Met-SO and the upfield region of ^1H NMR spectra for ferrous WT* and WT-M80SO. (B) Structures of acetyl-methionine (AcMet) and acetyl-methionine sulfoxide (AcMet-SO) and their ^1H NMR spectra.

Figure 7.

Electronic absorption spectra of ferric AcMP8 (A) in a 50 mM sodium phosphate buffer at pH 7.4 (*black*) and in the same buffer with 2.5 M acetyl-methionine sulfoxide (AcMet-SO, *red*), (B) in 9:1 (v/v) H₂O/CH₃OH (*black*) and 3:1:6 (v/v/v) H₂O/CH₃OH /DMSO (~8 M DMSO, *red*) buffered to pH 9.5 with 100 mM sodium borate. (C) Spectral changes and (D) a corresponding binding curve of ferrous AcMP8 upon gradual addition of AcMet-SO.

Figure 8.

(A) Electronic absorption spectra of WT-M80SO and corresponding difference spectra (*inset*) from imidazole binding experiments performed in a 50 mM sodium phosphate buffer at pH 7.4, (B) difference in absorbance values at 413 nm and 398 nm, and (C) plots of $\alpha \times k_{\text{obs}}^{\text{Im}}$ against imidazole concentrations for WT-M80SO and K73A/K79G/M80K (denoted as M80K#).

Figure 9.

(A) Dependence of the observed rate constant ($k_{\text{obs}}^{\text{Red}}$) of WT* on Co(terpy)₂²⁺ concentration. Linear fit of this dependence has yielded $k_{\text{ET}} = 2.61 \text{ mM}^{-1}\text{s}^{-1}$. (B) Representative traces for reduction process of WT-M80SO (*black*), M80K[#] (*red*) and M80A (*blue*) by 1 mM Co(terpy)₂²⁺ monitored by absorbance at 550 nm. The protein concentration was 5 μM . (C) Dependence of $k_{\text{obs}}^{\text{Red}}$ of WT-M80SO on Co(terpy)₂²⁺ concentration. Fit of this dependence to eq 3 has yielded the following parameters: $k_{\text{b}} = 0.014 \text{ s}^{-1}$, $k_{\text{f}} = 1.29 \text{ s}^{-1}$, and $k_{\text{ET}} = 0.29 \text{ mM}^{-1}\text{s}^{-1}$. The reduction yield was around 40%.

Figure 10.

Representative traces for guaiacol oxidation by H₂O₂ catalyzed by variants of cyt *c* at (A) pH 7.4 and (B) pH 4.5 as well as (C) rate constants from repeated measurements at these two pH conditions. The concentrations of protein, guaiacol, and H₂O₂ were 3 μ M, 5 mM, and 100 μ M, respectively. The slow rates relatively to those from assays with AcMP8 suggest that the heme pocket in all the studied cyt *c* variants is well protected at both pH values.

Scheme 1.

Scheme 2.

Table 1.Dissociation Constants (K_D , M) for Binding Amino Acids to Ferric or Ferrous AcMP8

Amino acid	ferric	ferrous
AcMet	0.38 ^a	2.4×10^{-3} ^a
AcMet-SO	5.0 ^b	1.4×10^{-3}
AcLys	1.6×10^{-4} ^c	1.2×10^{-4} ^d

^aFrom Ref. 63.^bFrom Ref. 14.^cFor the deprotonated AcLys, from Ref. 21.^dFor the deprotonated AcLys, from Ref. 61.

Table 2.

pK_a Values from Spectrophotometric pH Titrations and Proposed Ligand-Switching Processes for Ferric Variants of *Iso-1* Cytochrome *c*

Variant	pK_a	Ligand Switch
WT* ^a	8.8 ± 0.1	Lys→Met80
	3.0 ± 0.1	Met80→H ₂ O; His18/H ₂ O→H ₂ O/H ₂ O?
M80A	6.7 ± 0.1	OH ⁻ →H ₂ O
	4.4 ± 0.1	Remains H ₂ O (protonation of unknown group)
	2.9 ± 0.1	His18/H ₂ O→H ₂ O/H ₂ O?
M80K ^{#a}	4.9 ± 0.2	Lys80→H ₂ O
	3.3 ± 0.3	His18/H ₂ O→H ₂ O/H ₂ O?
WT-M80SO	6.3 ± 0.1	Lys73/79→H ₂ O
	3.1 ± 0.1	His18/H ₂ O→H ₂ O/H ₂ O?
K79G-M80SO	6.5 ± 0.1	Lys73→H ₂ O
	3.0 ± 0.1	His18/H ₂ O→H ₂ O/H ₂ O?
K73A/K79G-M80SO	8.2 ± 0.2	OH ⁻ →H ₂ O
	3.3 ± 0.3	His18/H ₂ O→H ₂ O/H ₂ O?

^aFrom Ref. 21.

Table 3.

Apparent Binding Constants (K_{app} , mM^{-1}) and Dissociation Rate Constants (k_1 , s^{-1}) for the Endogenous Ligand X from Imidazole Binding Experiments to Ferric Variants of *Iso*-1 Yeast Cytochrome *c*

Variant	X	K_{app} (mM^{-1})	k_1 (s^{-1})
WT*	Met80	0.36 ± 0.04	39 ± 13
M80K [#]	Lys80	0.43 ± 0.04	0.007 ± 0.001
WT-M80SO	Lys73, Lys79	4.3 ± 0.4	0.012 ± 0.001