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Resonant inelastic X-ray scattering on Ferrous and Ferric bis-imidazole porphyrin and cytochrome c: Nature and Role of the Axial Methionine – Fe bond

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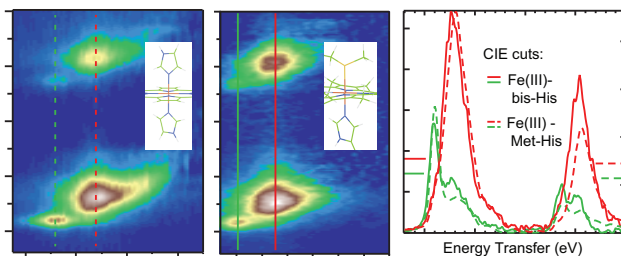
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Abstract

Axial Cu-S(Met) bonds in electron transfer (ET) active sites are generally found to lower their reduction potentials. An axial S(Met) bond is also present in cytochrome *c* (cyt *c*) and is generally thought to increase the reduction potential. The highly covalent nature of the porphyrin environment in heme proteins precludes using many spectroscopic approaches to directly study the Fe site to experimentally quantify this bond. Alternatively, L-edge X-ray absorption spectroscopy (XAS) enables one to directly focus on the 3d-orbitals in a highly covalent environment and has previously been successfully applied to porphyrin model complexes. However, this technique cannot be extended to metalloproteins in solution. Here, we use metal K-edge XAS to obtain L-edge like data through 1s2p resonance inelastic X-ray scattering. RIXS has been applied here to a bis-imidazole porphyrin model complex and cyt *c*. The RIXS data on the model complex are directly correlated to L-edge XAS data to develop the complementary nature of these two spectroscopic methods. Comparison between the bis-imidazole model complex and cyt *c* in ferrous and ferric oxidation states show quantitative differences that reflect differences in axial ligand covalency. The data reveal an increased covalency for the S(Met) relative to N(His) axial ligand and a higher degree of covalency for the ferric states relative to the ferrous states. These results are reproduced by DFT calculations, which are used to evaluate the thermodynamics of the Fe-S(Met) bond and its dependence on redox state. These results provide insight into a number of previous chemical and physical results on cyt *c*.

Introduction

A common structural motif across electron transfer (ET) metalloprotein active sites is the presence of an axial metal-thioether bond, where this ligand is provided by an endogenous methionine residue.¹ These are found in the mononuclear type one copper (T1 Cu), binuclear CuA, and cytochrome (cyt *c* and cyt *b*) containing ET metalloproteins. In T1 Cu and CuA active sites, the function of the Cu-S(Met) bond is now well understood:²⁻⁴ the presence of the axial S(Met) ligand lowers E^0 due to the stronger stabilization energy (i.e., larger axial ligand bond strength) in the oxidized than in the reduced state,⁵ while not greatly affecting the reorganization energy.²⁻⁴

Interestingly, in contrast to T1 Cu and CuA, the Fe-S(Met) bond in cyt *c* is generally thought to be stronger in the reduced rather than the oxidized state. This consideration for cyt *c* is based on the decrease in E^0 when the Fe-S(Met) bond is lost,^{6,7} ligand competition^{8,9} and binding,¹⁰⁻¹² protein folding studies,^{10,13-15} and the shorter Fe(II)-S(Met) bond (2.29 Å) relative to Fe(III)-S(Met) (2.33 Å) from extended X-ray absorption fine structure (EXAFS).¹⁶ Loss or rupture of the Fe-S(Met) bond in cyt *c* is also functionally relevant. Besides carrying out ET, cyt *c* is also involved in programmed cell death or apoptosis.¹⁷ During this process, cyt *c* switches function from an ET protein to a peroxidase enzyme. This functional switch is correlated to the loss of the axial Fe-S(Met) bond and the formation of an open coordination sphere for the activation of H_2O_2 in the peroxidation of cardiolipin.¹⁸⁻²⁰ In addition, the Fe(II)-S(Met) bond can be photo-dissociated, while the Fe(III)-S(Met) bond cannot.²¹⁻²⁴ Clearly, the difference in metal-ligand bonding, and thus bond strengths for the Fe(II) and Fe(III) states, is directly related to both functions of cyt *c*: ET and lipid peroxidation.

In order to understand these differences in bonding, it is necessary to employ a spectroscopic method that directly probes the electron structure of the Fe site; however, the highly covalent porphyrin obscures the spectral features associated with the Fe-S(Met) bond. S K-edge X-ray absorption spectroscopy (XAS) has proven to be a powerful spectroscopic tool

to quantitate the covalency of thiolate-metal bonds in proteins and models through the intensity of the pre-edge feature (i.e., the $1s \rightarrow 3d$ transition(s)).²⁵ However, the higher Z_{eff} of the thioether sulfur relative to a thiolate increases the energy of the pre-edge transition such that it is located within the envelope of the edge transitions (i.e., the $1s \rightarrow$ valence np).

The direct way to investigate the electronic structure of a heme protein, where the Fe is in the highly covalent porphyrin environment, would be to probe the unoccupied valence orbitals of the central Fe ion by Fe L-edge soft X-ray absorption spectroscopy (XAS). L-edge XAS is a powerful method to extract the differential orbital covalency (DOC, differences in the covalency of the different symmetry sets of d orbitals),²⁶⁻²⁹ that is particularly important for heme complexes and enzyme intermediates. However, the Fe L-edge excitation energy lies in the soft X-ray energy region (≈ 710 eV), which requires ultra-high vacuum conditions and generally involves the detection of electrons within 10 \AA of the surface of the sample due to the low electron escape depth. The escape depth can be enlarged to a few micrometers by detecting the fluorescence decay. However, this suffers from significant self-absorption effects, mainly on the L_3 -edge. These restrictions make it challenging to apply high-resolution metal L-edge XAS to metalloproteins and enzymes.

Metal K-edge XAS does not have these restrictions due to the high excitation energy in the hard X-ray region (the Fe K-pre-edge is at ≈ 7110 eV). However, due to the short lifetime of the $1s$ core-hole, the broadening of the $1s \rightarrow 3d$ transitions is significantly larger than for the $2p \rightarrow 3d$ transitions. Since the $1s \rightarrow 3d$ transitions are quadrupole allowed, they often gain intensity from small amounts of $4p$ mixing into the d orbitals in non-centrosymmetric ligand environments, while $2p \rightarrow 3d$ transitions are electric dipole allowed and their intensities are a direct probe of the metal d-character in valence orbitals involved in bonding. Thus, by going from soft to hard X-rays, one wins on experimental conditions, but loses resolution and insight into bonding.

The desired combination of accessible experimental conditions, higher resolution, and d-orbital covalency can be realized by applying $K\alpha$ resonant inelastic X-ray scattering ($1s2p$

RIXS). Here, a 1s electron is excited into an unoccupied 3d orbital (1s→3d) via a quadrupole transition to a $1s^1 2p^6 3d^{n+1}$ intermediate state and the subsequent electric dipole allowed decay of a 2p electron into the 1s hole (2p→1s) to the $1s^2 2p^5 3d^{n+1}$ final state, is detected by its photon emission. Thus, L- edge absorption and 1s2p RIXS reach the same final state configurations^{30,31} and allow a direct comparison but with complementary selection rules.³² Varying the incident X-ray energies and collecting the respective emission spectra, allows one to generate the RIXS plane. "L-edge-like" spectra are obtained as vertical cuts through the RIXS plane, providing the energies of the same final state configurations that could be reached through direct L-edge XAS, but via an intermediate state.

It has been recently shown that the valence bond configuration interaction (VBCI) model with the same parameters reproduces both L-edge XAS and RIXS results, but with selection rules³² involving the intermediate states in RIXS, as described by the Kramers-Heisenberg scattering equation^{33,34}

$$F(\Omega, \omega) \propto \sum_f \left| \sum_i \frac{\langle f | H_{1s2p} | i \rangle \langle i | H_{1s3d} | g \rangle}{E_g - E_f + \Omega - \frac{i\Gamma}{2}} \right| \times \delta(E_g - E_f + \Omega + \omega), \quad (1)$$

where Ω and ω are the incident and emission energy, respectively, with $\Omega - \omega$ being the energy transfer, that gives "L-edge-like" spectra.

To investigate the electronic structure of Fe in a covalent porphyrin environment, and in particular the Fe-S(Met) bonding properties of reduced and oxidized cyt *c*, we applied 1s2p RIXS to this protein, which cannot be reasonably studied by L-edge XAS. Parallel RIXS data were collected on tetraphenyl porphyrin (TPP) complexes with bisimidazole axial ligands (FeTPP(ImH)₂). These complexes have already been studied by L-edge XAS.²⁸ The two main differences in the structures of cyt *c* and FeTPP(ImH)₂, include replacement of one axial imidazole ligand with a thioether (Met-80), and the porphyrin ring is crosslinked to two cysteine derived thioether bonds (cys-14 and cys-17) (see Figure 1). Here, RIXS data

on $\text{FeTPP}(\text{ImH})_2$ are correlated to the L-edge XAS data and to 1s2p RIXS data on reduced and oxidized cyt *c*, and analyzed using the VBCI multiplet model. These results are then used to correlate to DFT calculations on large models to distinguish specific contributions to bonding. Truncated models, which reasonably represent the large models, are then used to explore the thermodynamic differences between the Fe-S(Met) and Fe-N(His) bond in the reduced and oxidized states. The results of these studies are then related to past experimental results on thioether Fe bonding.

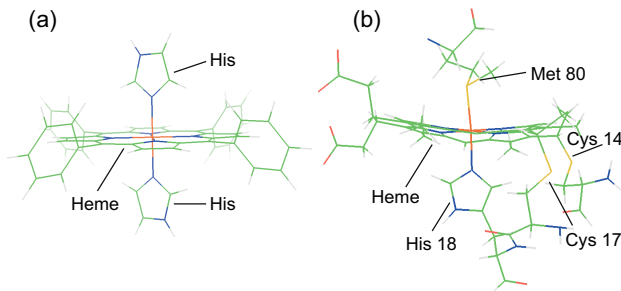


Figure 1: Molecular structures of (a) $\text{FeTPP}(\text{ImH})_2$, and (b) cyt *c*.

Experimental Section

Sample Preparation

Ferrous and ferric $\text{FeTPP}(\text{ImH})_2$ All chemicals were obtained from Sigma-Aldrich Co. as the highest purity available and were used without further purification unless otherwise indicated. Anaerobic preparations were done in a nitrogen glove box using anhydrous solvents that were degassed by 5 freeze-pump-thaw cycles under an argon atmosphere.

$[\text{Fe}^{\text{III}}(\text{tpp})(\text{ImH})_2]\text{Cl}$ (tpp = 5,10,15,20-tetraphenyl-21*H*,23*H*-porphine, ImH = imidazole) was synthesized according to published methods.³⁵ 500 mg (0.71 mmol) of $\text{Fe}(\text{tpp})\text{Cl}$ was mixed with 6 equivalents of imidazole in 60 mL of chloroform. A suitable product for analysis was obtained through the slow addition using hexane or by crystallization by hexane diffusion.

[Fe^{II}(tpp)(ImH)₂] was prepared under an anaerobic atmosphere by direct reduction with excess sodium borohydride (NaBH₄). According to the procedure of Mink *et al.*³⁶ 100 mg (0.14 mmol) of Fe(tpp)Cl was dissolved in 50 ml of tetrahydrofuran (THF) and reduced using a 100 fold excess (14.2 mmol) of NaBH₄, which turned the solution from brown to red. The reaction was monitored by UV-vis absorption to ensure the complete reduction of Fe^{III} to Fe^{II}. After 48 hours, 12 equivalents (1.70 mmol) of imidazole were added and the reaction was stirred for 30 minutes, after which the solvent was removed under reduced pressure to yield a product suitable for XAS.

Reduced and oxidized cytochrome *c* Horse heart cyt *c* (type IV) was obtained from Sigma (>95% purity, SDS-page). The protein was further purified using cation exchange chromatography and an ÄKTApurifier UPC 100 manufactured by GE Healthcare Life Sciences. Samples were buffer exchanged into 100 mM phosphate buffer, pH 6.8, and concentrated to ~4 mM. Reduction of cyt *c* samples was performed by anaerobic addition of aliquots of sodium dithionite (Na₂S₂O₄). Protein samples were injected into Lucite cells with Kapton windows and immediately frozen and stored under liquid nitrogen.

Methods

L-edge X-ray absorption spectra were recorded at the Stanford Synchrotron Radiation Laboratory (SSRL) on the undulator beam line 10-1 under ring operating conditions of 350 mA and 3 GeV. The radiation was dispersed using a spherical grating monochromator set at 1000 lines/mm and 20 μ m entrance and exit slits (0.15 eV resolution). All measurements were done at around 240 K. Measurements were performed using the total electron yield mode, where the sample signal was collected using a Galileo 4716 channeltron electron multiplier aligned 45° relative to the sample surface normal, which was aligned parallel to the incident beam. The signal was flux normalized by the photocurrent of a gold-grid reference monitor. Data for all samples were recorded in a sample chamber maintained at

$\approx 3 \times 10^{-9}$ Torr. The photon energy was calibrated using the Fe L-edge XAS of powdered Fe_2O_3 (hematite) run at intervals between scans. The second feature in the L_3 -edge and the first feature in the L_2 -edge were calibrated to 708.5 and 720.1 eV, respectively. Samples were finely ground and spread across double-sided adhesive conductive graphite tape attached to a copper sample holder aligned at 45° to the incident beam. Data were taken over the range 670-830 eV to permit normalization, as described previously.^{26,28} No photo-damage was observed during one run in any of the samples described herein.

K-edge Ferrous and ferric $\text{FeTPP}(\text{ImH})_2$ sample were mixed with boron nitride and ground into a fine powder. The powder was loaded into a 1 mm thick Al spacer and sealed with $63.5 \mu\text{m}$ Kapton tape windows. Fe K-edges were measured in transition mode with N_2 -filled ionization chambers at SSRL beam line 7.3. Two or three scans were measured per sample to ensure reproducibility. Energies were calibrated against the first inflection point at 7111.2 eV of an internal foil standard.³⁷ A second-order polynomial was fit to the pre-edge and subtracted from the data. A two-segment spline of order 2 was fit to the EXAFS region, and all data were normalized to the edge jump at 7130 eV.

1s2p RIXS *Data collection:* 1s2p RIXS experiments were carried out at SSRL beam line 6-2 and APS beam line 9-ID. They are equipped with a liquid nitrogen cooled $\text{Si}(3,1,1)$ double-crystal monochromator, allowing for high resolution of the incoming radiation (0.2 eV) in the energy range of 7.100-7.300 keV. The (440) Bragg reflection of five (SSRL) and one (APS) $\text{Ge}(110)$ crystals arranged in a Rowland geometry were used to select the K_α emission energy, which were arranged in a Rowland geometry. A single element silicon drift detector (SSRL) and a line detector (APS) were used to measure the X-ray emission. The emitted beam path was enclosed by a He-filled bag to reduce the signal attenuation at SSRL. The total resolution was ≈ 0.6 eV, which is sufficient for a detailed analysis. To reduce photo-damage, a liquid He cooled cryostat for measurements at 10 K was used, as well as a sample stage that is equipped with motors to allow for horizontal and vertical movement for multiple

sampling positions. Energies were calibrated against the first inflection point at 7111.2 eV of an internal foil standard.³⁷ The RIXS plane has been recorded by scanning the incident energy in a step-wise mode at fixed emission energy.

Only oxidized cyt *c* was sensitive to photo reduction under X-ray irradiation. Photo-damage has been excluded by minimizing the count time and shortening the incident energy scan region, in addition to movement of the sample stage for each emission energy. To get a reasonable signal to noise, the 1s2p RIXS plane was measured four times and summed.

Rising-edge subtraction: The pre-edge in a 1s2p RIXS experiment suffers from the tail of the intense $1s \rightarrow 4p$ excitations at around 15 eV higher energy. This leads to an increase of the pre-edge background and further complicates a direct comparison of L-edge data with constant incident energy (CIE, i.e. vertical) cuts through the RIXS plane. Furthermore, it overlaps with intensity contributions due to π back-bonding. In order to estimate the intensity from the tail of the rising-edge, the RIXS data were fit with up to 15 Pearson VII functions depending on the complexity of the pre-edge and edge structure, as described in Ref.³⁰ (See Figure S1 in the Supporting Material for Fe(II)TPP(ImH)₂ as an example).

All data sets (L-edge, K-edge, 1s2p RIXS) have been measured a minimum of twice to ensure reproducible and reliable data.

Computational Details

Multiplet Calculations Charge transfer multiplet calculations were performed using the atomic theory developed by Cowan³⁸ and the crystal field interactions described by Butler,³⁹ including electronic Coulomb interactions and spin-orbit coupling for each sub-shell.⁴⁰⁻⁴² The Slater-Condon-Shortley parameters F_i and G_i were reduced from their Hartree-Fock calculated values to account for the over-estimation of electron-electron repulsion found in the calculations of the free ion.^{40,43,44} Covalent mixing of ligand character is modeled in the simulations using a valence bond configuration interaction (VBCI) model as implemented

by Thole.⁴⁵ A three configuration model is employed that includes a ligand-to-metal charge transfer (LMCT) configuration $d^{n+1}\underline{L}$ and a metal-to-ligand charge transfer (MLCT) configuration $d^{n-1}L^-$ mixed into the d^n configuration in the ground state. (\underline{L} =ligand with an e^- hole, L^- =ligand with an additional e^- electron) The corresponding charge transfer energies Δ (LMCT) and Δ^* (MLCT) are defined as

$$\begin{aligned}\Delta &= E(d^{n+1}\underline{L}) - E(d^n) \quad \text{and} \\ \Delta^* &= E(d^{n-1}L^-) - E(d^n).\end{aligned}\tag{2}$$

The configuration interaction is represented by the mixing terms $T_i = \langle d^n | h | d^{n+1}\underline{L} \rangle$ and $T_i^* = \langle d^n | h^* | d^{n-1}L^- \rangle$, where h and h^* are the LMCT and MLCT mixing operators, respectively. Thus T_i and T_i^* are proportional to the ligand-metal and metal-ligand overlap for each of the i^{th} symmetry blocks.

T_i and T_i^* as well as Δ and Δ^* were kept constant in the ground and final states, giving final state configuration energy separations of $E(\underline{c}d^{n+2}\underline{L}) - E(\underline{c}d^{n+1}) = \Delta + U_{dd} - Q$ and $E(\underline{c}d^n L^-) - E(\underline{c}d^{n+1}) = \Delta - U_{dd} + Q$, where U_{dd} is the 3d-3d and Q as the core-hole Coulomb interactions. The 1s2p RIXS includes an intermediate state with a 1s core-hole. In our simulations, it was assumed that $Q_{sd} = Q_{pd}$. However, the 1s3d and 2p3d Coulomb and exchange interactions are very different and these differences were incorporated in the simulations. All calculations have been performed in D_{4h} symmetry for all four complexes.

The covalency value for each of the symmetry blocks was generated via the projection method²⁶ leading to the differential orbital covalency (DOC).

The theoretical spectra have been broadened by a Gaussian to include the experimental broadening and a Lorentzian to account for the life-time broadening with values given by the various peak widths of the reference sample ferri-cyanide,^{27,32} which were measured before each run. For the L-edge spectra we used values of 0.4 and 0.4 (0.8) eV for Gaussian and L_3 (L_2) Lorentzian FWHM broadening, respectively. In the 1s2p RIXS simulations, all

transitions are broadened by a Gaussian for simplicity, with an intermediate energy life-time broadening of 1.2 eV and experimental broadening of 0.3 eV. Final state broadenings were 0.7 (L₂) and 0.3 eV (L₃) (all FWHM).

While the Kramers-Heisenberg scattering equation (Equation 1) allows for interference effects between intermediate states,³³ this is not included in the present simulations. Its significance to 1s2p RIXS is system dependent,⁴⁶ and simulations that did also include interference effects showed no improvement in the fit to the data.⁴⁷

DFT Calculations The starting structure for FeTPP(ImH)₂ was taken from the ferric complex crystal structure,³⁵ while the starting structure for cyt *c* optimization employed the horse heart oxidized cyt *c* structure.⁴⁸ Ground state DFT calculations and geometry optimizations were performed with Gaussian 09⁴⁹ using the unrestricted functional BP86,^{50,51} modified to include Hartree-Fock mixing of 10 and 20% with Lee, Yang, and Parr correlation,^{52,53} with a triple-zeta (6-311G*) basis set on Fe, N and S, and a double-zeta (6-31G*) basis set on all other atoms. For energy calculations, a high level basis set (6-311+G(d,p)) and a polarized continuum model ($\epsilon = 4.0$) were used in combination with a BP86+20% HF exchange-correlation functional. Full Mulliken populations and Mulliken fragment populations were analyzed using QMForge.⁵⁴ Unoccupied metal d-character has been determined by subtracting the sum of their contribution to all occupied orbitals from 100%. The ligand donor character has been obtained from the occupied fragment orbital character summed over the unoccupied orbitals.

Results and Analysis

Ferrous Spectroscopy

Ferrous TPP-bisIm: Figure 2 presents a collection of data sets comparing the 1s2p RIXS, L-edge and K-edge XAS data. Figure 2(a) shows the full 1s2p RIXS plane, while Figure 2(b)

gives the result with the rising edge subtracted as described in the Supporting Information. The features at an incident energy of ≈ 7112 eV and energy transfers of ≈ 706.5 and ≈ 720 eV reflect excitations into the K-pre-edge. The L_3 - and L_2 -edge-like features on the energy transfer axis are split by the spin-orbit coupling within the $2p$ core-hole in the final state. The intense feature starting at ≈ 7115 eV originates from the tail of the strong dipole allowed $1s$ - $4p$ transition at ≈ 7130 eV. Note that possible shake-up features due to σ + π -donation and π -back-bonding could be present at the onset of the main-edge tail that have been neglected in the background subtracted RIXS in Figure 2(b).

Figure 2(c) compares the K-edge XAS of the Fe(II)TPP(ImH)₂ model complex with the constant emission energy (CEE) cut through the RIXS plane as indicated by the diagonal red line in Figure 2(a). Both spectra show the same structure that is better resolved in the CEE spectrum. The peak at 7112.2 eV involves excitation to the $1s^1 3d_{t2g}^6 3d_{eg}^1$ final state, while the second weak peak at 7115.3 eV likely originates from π back-bonding. The existence of a small π back-bonding contribution (10%) in the ground state has already been observed in Ledge XAS and is present DFT calculations.²⁸

The x^2-y^2 and z^2 orbitals are separated in energy due to the difference in axial and equatorial bonding, associated with effective D_{4h} symmetry. Since the xy and xz/yz orbitals are occupied, the K-pre-edge is dominated by excitations into these empty x^2-y^2 and z^2 orbitals. To estimate the maximum D_{4h} final state splitting, the Fe(II)TPP(ImH)₂ result was compared to the $1s2p$ RIXS of Fe(II)-tacn, which has O_h local symmetry and thus only one possible K pre-edge peak (see Figure S2 in the Supporting Material).⁵⁵ This comparison limits the possible e_g peak splitting to ≤ 0.4 eV.

While no spectroscopic difference is observed between the K-edge XAS and CEE RIXS, this is not the case in comparing the L-edge XAS data with a CIE cut at $h\nu = 7112.2$ eV through the RIXS plane. (red vertical line in Figure 2(b)) Figure 2(d) presents the Fe(II)TPP(ImH)₂ L-edge XAS data together with the CIE cut through the main RIXS peak.

The CIE cut shows additional intensity at lower energies for both the L_3 - and L_2 -like

curves ($h\nu = 707.3$ and 719.6 eV) and a reduced relative intensity at the L-edge main peak at $h\nu = 708.2$ eV. The high energy L₃-edge shoulder in the CIE cut at 710.2 eV is also less pronounced compared to the corresponding L-edge feature. Similar observations hold true for the L₂-edge energy region, together with the observation of a smaller L₃-/L₂- peak intensity ratio in comparing the CIE RIXS cut to the direct L-edge XAS for Fe(II)TPP(ImH)₂.

In our earlier 1s2p RIXS experiments on O_h samples, additional intensity in the CIE RIXS cuts relative to L-edge XAS was found to originate from electric dipole forbidden excitations³² that are allowed in 1s2p RIXS (see Equation 1 and Figure 3(a)). In the Fe(II)TPP(ImH)₂ model complex, the Fe local symmetry is approximately D_{4h} with an A_{1g} ground state. In D_{4h} symmetry, the L-edge electric dipole transition operator has a_{2u} and e_u symmetry so that only A_{2u} and E_u final states are allowed (Figure 3(b)). In the 1s2p RIXS experiment, one excites via a 1s → 3d quadrupole transition, having a_{1g} and b_{1g} character, into intermediate states with A_{1g} (Fe d_{z²}) and B_{1g} (Fe d_{x²-y²}) symmetry, respectively. The 2p→1s decay from these final states again has electric dipole character, thus a_{2u} and e_u symmetry, which allows 1s2p RIXS intensity to final states with B_{2u}, A_{2u} and E_u symmetry (Figure 3(b)). Final states with E_u symmetry can be reached both from A_{1g} and B_{1g} intermediate states resulting in different final state energies. Thus the additional intensity found in the 1s2p RIXS cuts throughout the main intensity peaks originates from both final states that cannot be reached in L-edge XAS and also from the splitting of the x^2-y^2/z^2 orbitals in the D_{4h} symmetry of the porphyrin complex.

Ferrous cytochrome c: Figure 4 presents the 1s2p RIXS plane of reduced cyt *c*. Figure 4(a) shows the full RIXS plane as measured, while in (b) the rising edge has been subtracted. The pre-edge peak structure contains mainly one broad intensity region at the L₃- and L₂-like energies, with an intense tail to higher energies. This tail however is weaker in intensity compared to the ferrous model RIXS plane (Figure 2(b)). In (c) we compare the CEE cuts of reduced cyt *c* with that of the Fe(II)TPP(ImH)₂ complex. Both have very similar pre-edge

intensities and peak widths, as well as additional intensity at ≈ 7115.3 eV, likely due to π back-bonding. However, reduced cyt *c* shows a steeper rising edge, which overlays the π^* region. Both curves merge at higher energies, reflecting the common spin and valence state (see Figure S3 in the Supporting Material). A direct comparison of the CIE cuts for the Fe(II)TPP(ImH)₂ and reduced cyt *c*, is presented in (d). Two characteristic changes in spectral features are visible: Fe(II) cyt *c* has lower intensity in the shoulder at 710 eV, but higher intensity in the L₂ relative to the L₃-edge.

Ferrous multiplet simulations:

For Fe(II)TPP(ImH)₂ one can analyze both L-edge XAS and 1s2p RIXS, while only 1s2p RIXS is possible in cyt *c*. Comparing CEE and CIE cuts of both defines their quantitative difference. Thus charge-transfer multiplet calculations were performed within the VBCI framework. In a previous study, Fe(II)TPP(ImH)₂ has been simulated using the VBCI model to determine approximately 10% π back-bonding in the ground state wave function.²⁸ A similar result was determined for the closely related ferrous picket-fence porphyrin complex.⁵⁶ Here we follow these previous studies, but set the core-hole relaxation $U_{pd} - U_{dd}$ constant and treat both donation and back-bonding charge-transfer interactions as unchanged in ground and final states. The goal of these simulations is to first determine the parameter set that describes all the experimental results (K-edge, L-edge and 1s2p RIXS) for the reference Fe(II)TPP(ImH)₂ model complex, and then systematically evaluate parameter variations that reproduce the observed experimental changes in cyt *c*.

Figure 5, shows the best fit to Fe(II)TPP(ImH)₂: (a) K-edge and K-edge-like CEE cut, (b) the 1s2p RIXS plane, and (c) the L-edge and L-edge-like CIE cut. The corresponding parameter set is given in Table 1. The simulations reproduce the experimental results well for all three experimental methods. In line with the experiment, the simulated 1s2p RIXS cut is broader than the L-edge XAS simulation. The peak in the L-edge XAS simulation at 710 eV is reduced to a shoulder in the RIXS cut, and the ratio of the L₃/L₂-peak maxima is

lower in the 1s2p RIXS cut. The projection of the VBCI ground state gives 70.4% $d_{x^2-y^2}$, 77.2% d_{z^2} metal, and 8.1% $d\pi^*$ back-bonding character (Table 2).

From the group theory considerations in Figure 3(b), B_{2u} final states are forbidden in direct L-edge XAS. In addition, final states with E_u symmetry can be reached through the z^2 and x^2-y^2 intermediate states that are split in energy due to the tetragonal geometry. Thus the E_u final states are also split in energy by the crystal field splitting of the $d\sigma$ orbitals. To identify all contributions to the 1s2p RIXS plane, we separate the excitations into their individual pathways as shown in Figure 6(b)-(e). Since intermediate states of different symmetries do not interfere, the full RIXS plane is given by a summation of all four pathways, which are compared to the L-edge XAS spectral splittings in Figure 6(a).

The E_u final states contribute at energies above and below the main peak with the excitation through both the z^2 (a_{1g}) orbital being lower in energy than through the x^2-y^2 (b_{1g}) orbital, reflecting the ligand field energy splitting of these orbitals with the z^2 being lower in energy. The 1s2p RIXS final state splitting however is significantly larger than the ground state energy splitting (≈ 1.1 eV as compared to ≈ 0.4 eV,²⁸ respectively), which results from the strong 2p3d repulsive interaction. The E_u core-hole final state has mainly p_x, p_y character (p_z is mixed into the wave function through 2p spin-orbit coupling), which have a lower spacial overlap with the z^2 than with the x^2-y^2 orbital, leading to the increased energy splitting. This is a characteristic of 1s2p RIXS, which is inaccessible through L-edge XAS due to the large differences in the electric dipole matrix elements for absorption.⁵⁷ In direct L-edge XAS, the transition matrix element $p_{x,y} \rightarrow d_{x^2-y^2}$ is four times stronger than $p_{x,y} \rightarrow d_{z^2}$, leading to an intensity distribution mainly at higher energies (Figure 6(a)).

The other two final state symmetries are energetically located between the E_u states. Both the B_{2u} and A_{2u} core-hole final states (Figure 3(b)) have mainly p_z character, which now has stronger spacial overlap with the z^2 orbital. Thus the crystal field and 2p core-hole repulsion influences on the energy oppose each other with the core-hole being dominant, which inverts the excitation energy order relative to the E_u states.

Table 1: Collection of all parameter sets used to simulate all four complexes. Note that all LMCT mixing parameters are zero but T_{eg}^* , and $Q = Q_{sd} = Q_{pd}$.

complexes	crystal field			configuration energies			LMCT			MLCT	
	10Dq	Ds	Dt	Δ	Δ^*	Q-U	T_{b1g}	T_{a1g}	T_{eg}	T_{b2g}	T_{eg}^*
Fe(II)TPP(ImH) ₂	2.5	0.02	0.04	1.3	0.6	1.0	1.8	1.5	1.5	0.5	0.7
red. cytochrome c	2.5	0.02	0.04	1.3	0.6	1.0	1.8	1.8	1.5	0.5	0.7
Fe(III)TPP(ImH) ₂	3.0	-0.06	0.06	1.5	0.8	1.2	1.8	1.7	1.0	0.5	0.4
ox. cytochrome c	2.8	-0.06	0.06	1.5	0.8	1.2	1.8	1.9	1.2	0.5	0.4

Simulations of the reduced cyt *c* data were generated by starting from the simulation of the Fe(II)TPP(ImH)₂ model complex, and systematically varying the VBCI parameters to identify those that are responsible for the weak, but characteristic changes in the experimental spectral features between the cyt *c* and model complex spectra in Figure 4. The resulting K-edge, 1s2p RIXS plane, and CIE cut comparisons are shown in Figure 7(a)-(c), respectively. They reproduce all relative changes, however they are less pronounced than in the experiment. Both K-edge simulations have equal peak widths, in line with the experimental result. The CIE cuts along the red line in the 1s2p RIXS plane in Figure 7(c) show slightly higher intensity in the shoulder at 710 eV for the model complex, while the L₂ intensity is higher for the *red. cyt c* simulation. In order to reproduce these spectral differences, the covalent mixing of the d_{z²} orbital must be increased in cyt *c* (T_{a1g} goes from 1.5 eV in Fe(II)TPP(ImH)₂ to 1.8 eV in reduced cyt *c*).

This leads to a projection of the VBCI ground state of 73.4% $d_{x^2-y^2}$, 70.4% d_{z^2} metal, and 7.1% $d\pi^*$ back-bonding character (Table 2). Thus, in going from Fe(II)TPP(ImH)₂ to Fe(II) cyt *c*, the d_{z^2} metal character decreases with the increase of the T_{a1g} mixing parameter, while the $d_{x^2-y^2}$ increases and the π back-bonding character slightly decreases due to the normalized wave function.

Ferric Spectroscopy

Ferric TPP-bisIm: Figure 8 presents the experimental results of Fe(III)TPP(ImH)₂ plotted as for the ferrous complexes: Figure 8(a) shows the full 1s2p RIXS plane, while Fig-

ure 8(b) is the rising-edge subtracted plane. As a clear difference relative to the ferrous results, a well separated feature is visible at 7111.2 eV incident energy and 706 eV energy transfer in the L₃-edge-like region (7111.2/718.6 eV in the L₂-edge-like region). The high intensity feature around 7112.9/708.1 eV (7112.9/720.6 eV) is similar to the low-spin ferrous case. Figure 8(c) gives a comparison of the direct K-edge XAS to the CEE cut along the diagonal red line in Figure (a). In K-edge XAS, the low energy peak at 7111.2 eV appears as a weak shoulder, while two peaks are well resolved in the CEE cut. This is an intrinsic advantage of the two-dimensional plane as given by 1s2p RIXS and not due to experimental resolution, since the CIE integrated RIXS plane (which represents the K-edge) overlays with the direct K-edge spectrum (see Figure S4).

One of the main advantages of 1s2p RIXS is the ability to select a specific incident energy (i.e. a specific K-pre-edge transition) and record the corresponding L-edge-like spectrum associated with this pre-edge peak. When exciting with 7111.2 eV incident energy, the low lying $d\pi$ hole (in the ferric d^5 configuration) is probed, while exciting at an incident energy of 7112.7 probes $d\sigma$ character in the L-edge-like spectrum. Note that due to the weak 1s3d interaction, little mixing occurs and pre-edge peak interpretation in terms of specific d orbital excitations is reasonable,⁵⁵ which is not the case for 2p L-edge final states.²⁹

In Figure 8(d), the direct L-edge XAS data are compared to two CIE cuts taken through the two features in the rising-edge subtracted RIXS plane (Figure (b)) along the green and red lines at 7111.2 (π) and 7112.9 eV (σ character), respectively. While the low energy cut aligns well with the lowest energy L-edge XAS feature, the higher energy cut has its maximum at a lower transfer energy than the main peak of the L-edge XAS spectrum, similar to what was observed above for the ferrous TPP(ImH)₂ complex.

Ferric cytochrome *c*: Figure 9(a) and (b) presents the 1s2p RIXS results for oxidized cyt *c*, Figure 9(a) gives the full 1s2p RIXS plane, while Figure 9(b) is the rising-edge subtracted plane. Three main differences relative to the ferric TPP(ImH)₂ model complex RIXS

data (Figure 8) are observed: 1) The π feature at 7111.1 eV incident energy is weaker for the protein, 2) the strong σ feature around 7112.6 eV is shifted down in incident energy by 0.3 eV towards the π region, and 3) the L_2 -like σ region at 7112.6/720.3 eV is higher in intensity relative to the L_3 -like σ region.

The first two points are also visible in Figure 9(c) where the two CEE cuts of Fe(III) cyt *c* and TPP(ImH)₂ are compared. The low energy peak is slightly less intense and the main peak is shifted to lower incident energy.

In Figure 9(d), the L-edge-like cuts through the cyt *c* RIXS plane (solid lines) are compared to the corresponding cuts through the ferric Fe(III)TPP(ImH)₂ RIXS plane (dashed lines). Comparing the two CIE spectra associated with π excitation (cuts along the green lines in Figures 8 and 9(b)), three observations can be made: 1) The peak position of the low energy feature is the same at 706 eV, 2) the protein peak is slightly lower in intensity, and 3) the shoulder at 708.2 eV is higher in intensity. CIE cuts associated with σ excitation (cuts along the red lines in Figures 8 and 9(b)) reveal two distinct differences: 1) The Fe(III) cyt *c* CIE spectrum is shifted towards lower energies by 0.3 eV at both the L_3 - and L_2 -edge, 2) the L_3/L_2 peak intensity ratio is lower for Fe(III) cyt *c*. Note that the lower L_3/L_2 peak intensity ratio was also found for the Fe(II) complexes, while the shift to lower energy of the σ excitation decay spectrum is only observed in the ferric complex comparison.

Ferric multiplet simulations:

In parallel to the ferrous case, charge-transfer multiplet calculations within the VBCI framework were performed to simulate the above Fe(III) experimental data. Used parameters are given in Table 1. The Slater-Condon parameters were reduced to 70% of the Hartree-Fock derived values in the initial and to 60% in the final states due to covalency effects. The difference in ground and final states reduction originates from the anisotropic effect of the mixing parameters on the electron distribution in the VBCI model with and without a 2p core-hole that are thus corrected using the Slater-Condon parameters. Figure 10 gives

the best fits to Fe(III)TPP(ImH)₂ spectra: (a) The K-edge and K-edge-like CEE cut, (b) the 1s2p RIXS plane, and (c) the L-edge and L-edge-like CIE cut. The corresponding parameter set is given in Table 1. The simulations reproduce the experimental results (see Figure 8) for all three methods. The CEE cut along the diagonal red line in Figure 10(b) (see Figure 10(a)) shows a higher intensity for the $d\pi$ peak at 7111.2 eV compared to the direct K-edge simulation due to the higher intrinsic resolution of the two-dimensional RIXS plane. The $d\sigma$ peak at ≈ 7113.2 eV appears at 0.3 eV higher energy in the CEE cut than the direct K-edge simulation, in line with the experimental result in Figure 9.

The direct L-edge simulation shown in Figure 10(c) reproduces the experimental L-edge, but underestimates the intensity of the lower energy peak at 706 eV. The $d\pi$ and $d\sigma$ CIE cuts (green and red vertical lines in Figure 10(b), respectively) follow the experimental results, with the $d\pi$ intensity being slightly overestimated, while the peakwidth, shape, and energy splitting match the experimental results. The main CIE peak at 708.2 eV appears ~ 1 eV lower in energy than the L₃-edge peak similar to the behavior experimentally observed for Fe(II)TPP(ImH)₂ above and in other ferric complexes.³² A DOC quantitative analysis of the VBCI ground state reveals 69% $d_{x^2-y^2}$, 67% d_{z^2} , 75% $d\pi$ metal character, and 3% $d\pi^*$ back-bonding into the porphyrin.

To simulate the oxidized cyt *c* data, the results of the Fe(III)TPP(ImH)₂ simulations were taken as a starting point, from which parameters were varied in a systematic manner to reproduce all experimentally observed spectral changes. In Figure 11, best fit simulations of oxidized cyt *c* data are compared to the above simulation results for Fe(III)TPP(ImH)₂: (a) A comparison of the CEE cut of oxidized cyt *c* (along the diagonal red line in Figure 11(b)) with the CEE cut of Fe(III)TPP(ImH)₂ (along the diagonal red line in Figure 10(b)), (b) the 1s2p RIXS plane, and (c) a comparison of the CIE cuts at lowest energy through the $d\pi$ regions (along the green vertical lines in Figures 11 and 10(b)), and the cut at higher energies through the $d\sigma$ regions (along the red vertical lines in Figures 11 and 10(b)).

The CEE cuts of the simulated RIXS planes for both complexes reproduce the exper-

imental spectral changes well: The intensity of the $d\pi$ peak at 7111.2 eV is decreased for cyt c , while the energy difference between the $d\pi$ and the $d\sigma$ peak at ≈ 7113.0 eV is 0.2 eV smaller in the oxidized cyt c simulations, in line with the experimental results in Figure 9.

In the L-edge-like CIE cuts through the simulated RIXS planes of Fe(III)TPP(ImH)₂ and oxidized cyt c (Figure 11(c)), three main changes in the spectral shape are observed: 1) A slight decrease of the low energy Fe(III) cyt c peak intensity at 706 eV with an increase in intensity in the shoulder at 708.2 eV in the CIE cut after π excitation (green curves), 2) a shift by 0.3 eV toward lower energy for the CIE cuts after σ excitation for oxidized cyt c (red curves), and 3) an increase of the L₂-like σ intensity. All of these characteristic changes are also observed in the CIE cuts through the experimental RIXS planes (Figure 9(d)).

To simulate the oxidized cyt c 1s2p RIXS plane, characteristic variations in the spectra were found to be associated with specific changes in the parameter set relative to Fe(III)TPP(ImH)₂. Similar to the ferrous case, an increase of the z^2 mixing parameter ($T_{a_{1g}}$) leads to an increase of the L₂-like intensity associated with σ excitation. However, an increase of $T_{a_{1g}}$ also leads to a shift to *higher* energies of the L₃-like peak (from σ excitation). To reproduce the shift to *lower* energies accompanied by the decrease in intensity of the L₃-like peak associated with π excitation, the mixing parameters for $d_{x^2-y^2}$ ($T_{b_{1g}}$) and $d_{xz/yz}$ (T_{e_g}) were increased, together with a decrease of the crystal field splitting $10Dq$. The best fit parameters are tabulated in Table 1. A projection analysis of the VBCI ground state DOC gives 69% $d_{x^2-y^2}$, 64% d_{z^2} , 73% $d\pi$ metal, and 3% $d\pi^*$ back-bonding character in the porphyrin. Thus in going from Fe(III)TPP(ImH)₂ to Fe(III) cyt c , the d_{z^2} metal character decreases with the increase of the $T_{a_{1g}}$ mixing parameter, and the $d\pi$ character decreases due to the increase in the T_{e_g} mixing parameter. As a consequence, the $d_{x^2-y^2}$ character increases and the π back-bonding character slightly decreases due to the normalized wave function.

Table 2: Unoccupied metal d-character derived from DFT calculations using the BP86 functional with 0, 10, and 20% Hartree-Fock for the full molecule as well as for the small molecule (s.m.), and for the multiplet simulations. Note that the full molecule cyt *c* is optimized with the Fe-S(met) distance being constrained, while the small molecule is fully optimized.

	$d_{x^2-y^2}$ [%]		d_{z^2} [%]		π^* [%]			
	model	protein	model	protein	model	protein		
<i>ferrous:</i>								
BP86	71.3	71.6	71.8	70.4	9.3	9.2		
BP86 + 10%HF	75.0	74.8	75.8	73.6	7.2	7.0		
BP86 + 20%HF	77.3	77.3	78.5	76.5	5.2	4.8		
BP86 + 20%HF (s.m.)	77.7	77.6	78.5	77.9	5.2	5.2	$d(\pi)$ [%]	
VBCI simulation	70.4	73.4	77.2	70.4	8.1	7.1	model	protein
<i>ferric:</i>								
BP86	65.5	66.2	65.0	62.2	2.5	3.3	86.4	82.1
BP86 + 10%HF	67.3	68.0	66.5	63.7	1.6	1.9	90.9	87.7
BP86 + 20%HF	69.0	68.9	68.0	65.3	1.1	1.2	92.6	91.4
BP86 + 20%HF (s.m.)	69.1	68.9	67.8	65.7	1.1	1.2	93.4	91.4
VBCI simulation	68.8	71.5	66.7	62.3	2.8	2.5	75.0	71.0

Density Functional Calculations

Correlating Electronic Structure Calculations to RIXS Data As described above, VBCI multiplet calculations reproduce all observed spectroscopic changes in the 1s2p RIXS planes when going from both the ferrous and ferric FeTPP(ImH)₂ model complex to cyt *c*. Both the ferrous and ferric complexes require an increase in the T_{a_{1g}} mixing parameter for cyt *c* resulting in lower d_{z^2} character (i.e. increased covalency). In addition, the presence of a $d\pi$ hole in the ferric case gives an increase of T_{e_g} for Fe(III) cyt *c*, leading to a decrease in $d\pi$ character.

While VBCI simulations are able to reproduce the experimental results due to the full multiplet treatment including the 1s and 2p core-holes, they are unable to distinguish between the two axial ligands and the specific contributions from the porphyrin, as all contribute to the T_{a_{1g}} mixing.

Therefore, DFT calculations were performed to determine which reproduce the experiment and can thus be used to obtain a more detailed description of the ground state. The

Fe-S(Met) distance in cyt *c* was constrained to the crystal structure values of 2.29 Å (Fe(II) and 2.33 Å (Fe(III)) since a fully optimized structure leads to an overestimated Fe-S(Met) bond length by more than 0.1 Å. The resulting DFT derived charge densities (orbital differentiated metal d-characters) are equivalent to the VBCI derived DOC values and are used to couple the experimental results to the DFT calculations.

The results for the ferrous calculations are shown in Table 2 (top) for three functionals: BP86, BP86+10% Hartree-Fock (HF), and BP86+20% HF (in the following labeled as B(XXHF)P86). All three functionals find unchanged $d_{x^2-y^2}$ metal d-character in cyt *c* compared to Fe(II)TPP(ImH)₂ and a small, reproducible decrease in d_{z^2} character. The π^* bonding character does not change. These results parallel the VBCI simulations of the data, where an increase in $T_{a_{1g}}$ leads to a decrease in d_{z^2} character. The associated change in $d_{x^2-y^2}$ and π^* back-bonding in the VBCI simulations are due to the normalized nature of the wave function.

A collection of the various ligand characters for d_{z^2} binding as derived from a fragment analysis of the DFT results is given in Table 3 (top). All three functionals give the same behavior. The decrease in d_{z^2} character is accompanied by a decrease in porphyrin character and an increase in axial ligand character. This is mainly due to the exchange of an imidazole by a thioether, resulting in the ruffling of the porphyrin and a decrease in the Fe-N(His) bond length.

The results of the DFT calculations for the ferric complexes for all three functionals are given in Table 2 (bottom). While the $d_{x^2-y^2}$ orbital shows unchanged metal d-character and π^* back-bonding between Fe(III)TPP(ImH)₂ and oxidized cyt *c*, a decrease in d_{z^2} metal character is found, similar to the ferrous case. The $d\pi$ hole that is present in the ferric complexes also shows a decrease in metal character for all functionals as found in the VBCI simulations of the 1s2p RIXS data.

The fragment analysis (Table 3 (bottom)) qualitatively gives the same ligand bond behavior for the d_{z^2} orbital in the ferric as in the ferrous case: A decrease in d_{z^2} and porphyrin

Table 3: Collection of metal and ligand character from a fragment analysis for three different functionals for the full molecules as well as for the small molecule models. Note that the full molecule *cyt c* is optimized with the Fe-S(Met) distance being constrained, while the small molecule is fully optimized.

	BP86		BP86+10% HF		BP86+20% HF		small molecule	
	model	protein	model	protein	model	protein	model	protein
<i>ferrous:</i>								
d_{z^2}	71.8	70.4	75.8	73.6	78.5	76.5	78.5	77.9
por	9.7	6.5	8.4	6.0	7.3	4.1	7.4	5.0
imh ₂ / met+imh	16.7	20.6	14.3	18.3	11.6	13.8	11.8	16.3
imh	–	10.8	–	9.6	–	7.7	–	8.4
met	–	9.8	–	8.7	–	6.1	–	7.9
<i>ferric: d_{z^2}</i>								
d_{z^2}	65.0	62.2	66.5	63.7	68.0	65.3	67.8	65.7
por	10.3	8.9	10.4	8.7	9.9	8.1	11.1	7.9
imh ₂ / met+imh	19.7	24.8	19.2	23.9	18.4	24.1	18.7	22.9
imh	–	12.5	–	12.0	–	11.6	–	11.2
met	–	12.3	–	12.0	–	12.5	–	11.7
<i>ferric: $d\pi$ hole</i>								
$d\pi$	86.4	82.1	90.9	87.7	92.6	91.4	93.4	91.4
por	13.3	18.7	9.1	12.8	6.7	10.7	6.9	9.8
imh ₂ / met+imh	2.9	3.1	2.7	1.8	2.2	1.4	2.4	0.0
imh	–	3.1	–	1.8	–	1.4	–	0.0
met	–	0.0	–	0.0	–	0.0	–	0.0

ligand character is compensated by an increase in axial ligand character due to the exchange of an imidazole with the more covalent thioether ligand. The $d\pi$ bonding is dominating between the $d\pi$ hole and the filled porphyrin $3e_g$ orbital, where the decrease in metal $d\pi$ character reflects an increase in $3e_g$ that is caused by the ruffling of the porphyrin ring upon the imidazole/thioether exchange. The imidazole bonding character in the $d\pi^*$ hole is small and unchanged between the Fe(III)TPP(ImH)₂ and Fe(III) *cyt c*, while there is no Met contribution present in the π^* hole in oxidized *cyt c*.

Truncated models with only the Fe-porphyrin ring and two axial ligands (bis-His and His-Met) were geometry optimized and compared to the results of the larger calculations. The last column in Table 3 gives the results on the truncated models for 20% HF. These quantitatively agree with the results for the large models with the same functional and basis

set, and are used below to quantitatively evaluate the thermodynamics of axial ligand metal bonding.

Axial Ligand Bond Strengths In this Section, axial ligand bond strengths are calculated for both N(His) and S(Met) axial ligands in both Fe(II) and Fe(III) oxidation states. The bond strength is taken as the difference in energy between the ligand on and off forms. Thus, in order to accurately calculate the axial ligand bond strengths, a DFT protocol was needed that reproduces the experimentally observed spin-states for both ligand on and off forms. Several different exchange-correlation functionals were tested; the results are given in Supporting Information Tables S1-S3 in the Supporting Material (for BP86 with 0, 10, and 20 % HF admixtures (B(XXHF)P86), OPBE, OLYP, TPSSh, and B3LYP). Experimentally, the ligand off state for Fe(II) in porphyrins and cyt *c* is high-spin, $S = 2.26$. This is reproduced with the B(20HF)P86, OPBE, OLYP, and B3LYP functionals (Table S1). Thus, the B(00HF)P86, B(10HF)P86, and TPSSh functionals were eliminated. The ligand off state of Fe(III) in porphyrins and cyt *c* is typically intermediate-spin ($S = 3/2$) or a quantum admixture of intermediate- and high-spin ($S=5/2$) states.⁵⁸⁻⁶¹ The $S = 3/2$ ground state is reproduced by all functionals, but only the B(20HF)P86, OPBE, OLYP, and B3LYP functionals have the $S = 5/2$ state near enough in energy for quantum mixing (< 5 kcal/mol) (Table S2). Lastly, the ligand on cground state for Fe(III)-S(Met) is low-spin, $S = 1/2$.⁵⁸ The OPBE, OLYP, and B3LYP functionals predict the intermediate-spin $S = 3/2$ state to be lower in energy than the $S=1/2$ state (Table S3), while the B(20HF)P86 functional has the $S = 1/2$ and $S = 3/2$ states at similar energy, with the electronic energy (ΔE) and enthalpy (ΔH) giving the $S=1/2$ state lowest (Table S3). Thus, the B(20HF)P86 functional gives the best agreement between theory and experiment for both redox states and is used below. (Note that all functionals predict similar trends in axial ligand bond strengths (Table S4). This functional is also consistent with the 1s2p RIXS data and simulations presented above and the DFT calculations with the large models with this functional (see Supporting

Information).

Table 4: Thermodynamics of Ligand Loss from Fe^{II/III}. All energies are in kcal/mol.

B(20HF)P86 ^a	Fe ^{II} - S(Met)	Fe ^{III} - S(Met)	$\Delta\Delta^b$ S(Met)	Fe ^{II} - N(His)	Fe ^{III} - N(His)	$\Delta\Delta$ N(His)	Fe ^{II} - H ₂ O	Fe ^{III} - H ₂ O	$\Delta\Delta$ H ₂ O
ΔE	5.0	7.1	2.1	9.9	16.9	7.0	-0.5	6.7	7.2
ΔH	2.6	5.5	5.5	7.2	14.7	7.5	-2.0	5.3	7.3
ΔG	-13.9	-8.8	5.1	-9.1	-0.4	8.8	-12.3	-4.2	8.1

^a 6-311+G(d,p) basis set for all atoms and PCM corrections ($\epsilon = 4.0$).

^b $\Delta\Delta$ is the difference in Fe^{III} and Fe^{II} axial ligand bond strengths.

The calculated axial ligand bond strengths for S(Met) and N(His) in both Fe(II) and Fe(III) oxidation states are given in Table 4. Note that values are reported for ligand loss. The axial ligand Fe(II)-S(Met) bond strength is calculated to be 5.0 kcal/mol (ΔE), while the Fe(II)-N(His) bond strength is 9.9 kcal/mol; the axial ligand Fe(III)-S(Met) bond strength is 7.1 kcal/mol, while the Fe(III)-N(His) bond strength is 16.9 kcal/mol. Thus, the Fe-S(Met) bond is weaker than the Fe-N(His) in both Fe oxidation states. Interestingly, from the above 1s2p RIXS data and the differential orbital covalency, the Fe-S(Met) bond is more covalent than the Fe-N(His) bond. This apparent discrepancy between covalencies and bond strengths is addressed in the Discussion. Furthermore, the Fe(III)-S(Met) bond is stronger than the Fe(II)-S(Met) bond ($\Delta E = 7.1$ vs. 5.0 kcal/mol, respectively). This is consistent with the higher S(Met) character for Fe(III) cyt *c* from the RIXS data/simulations and DFT calculations presented above. However, this observation is not consistent with the literature considerations summarized in the Introduction. Lastly, the difference in Fe(III) and Fe(II) bond strengths is larger for a N(His) axial ligand than for S(Met) (Table 4, $\Delta\Delta E = 7.0$ vs. 2.1 kcal/mol, respectively; note the $\Delta\Delta G$ for H₂O is also given in Table 4 and is referred to in the Discussion). The stronger bonds to the Fe(III) states are generally consistent with this higher degree of covalency than in the corresponding Fe(II) states.

Discussion

The highly covalent environment in Fe porphyrin complexes do not allow for most methods to quantitatively determine the local electronic structure. Fe L-edge XAS using soft X-rays is a powerful tool to determine the metal d-characters for all orbitals (i.e. the DOC)²⁶ including the ability to separately determine the σ and π donation and back-bonding.^{27,28,56} The soft X-ray nature of Fe L-edge XAS leads to a low life-time broadening and a feature rich spectrum, but requires ultra-high vacuum conditions and generally involves the detection of electrons within a few Å of the surface of the sample. The sampling depth can be enlarged to a few micrometers by detecting the fluorescence decay; however, this can lead to significant self-absorption effects. Hard X-rays do not have these restrictions, but give lower resolution spectra due to the short life-time of the 1s core-hole as observed in Fe K-edges.⁵⁵

1s2p RIXS combines the advantages of both methods, leading to two-dimensional high resolution spectra for samples in a variety of environments. The same information regarding the DOC can be extracted from 1s2p RIXS with the advantage of accessing L-edge XAS forbidden final states.³² In complexes with tetragonal symmetry like FeTPP(ImH)₂, where both L-edge XAS and 1s2p RIXS have been obtained, the broadening of the L-edge-like (CIE) RIXS cut associated with σ excitation is further enlarged due to the contribution from the $d_{x^2-y^2} - d_{z^2}$ orbital energy splitting, while this equatorial vs axial splitting is not accessible in direct L-edge XAS due to the dipole selection rule and strong multiplet effects.

To access the electronic structure of the heme center in cyt *c* in its reduced and oxidized form, only 1s2p RIXS experiments could be performed; these were compared to the results from corresponding ferrous and ferric FeTPP(ImH)₂ model complexes. In the ferrous case, two characteristic spectral changes were observed for reduced cyt *c* relative to the ferrous bis-imidazole model: A decrease in intensity of the high energy shoulder of the L₃-like peak and an increase of relative intensity of the L₂-like peak in the CIE cut associated with σ excitation (Figure 4). These changes require an increase of the z^2 covalency. Note that these spectral changes can only be quantified by including the whole L-edge-like energy

range; often, only the L_3 -like energy region is taken into account, which would not have been sufficient for a unique identification of this increase in z^2 covalency. In the ferric complexes, two CIE cuts are required and their comparison between the ferric bis-imidazole model and oxidized cyt *c* defines four characteristic spectral variations: (i) The L_2 -like relative intensity increases for oxidized cyt *c* in the CIE RIXS cut associated with σ excitation, similar to the ferrous case; (ii) the energy positions of the L_3 - and L_2 -like peaks in the σ cut are shifted towards lower energies, which is different from the ferrous result; (iii) the main peaks in the CIE cut associated with π excitation appear at the same energy, but with weaker intensity for oxidized cyt *c*; and (iv) the intensity of the shoulder of the main peak in the π cut increases (Figure 9). An increase of z^2 covalency reproduces the increase in L_2 -like intensity, parallel to the ferrous case. The shift towards lower energy of the σ CIE cut is due to a decrease of the crystal field strength, $10Dq$, together with an increase of π covalency, reproducing the other three observations. From DFT calculations correlated to these data, the increase of z^2 covalency is due to the axial ligand change from imidazole to methionine, as methionine has a stronger σ donation than imidazole. The increase of π covalency is due to the porphyrin ring, which is ruffled due to this ligand exchange, rather than a π donation interaction with the methionine ligand. Finally, from the quantitative DOC's obtained from the VBCI fit to the 1s2p RIXS data, the axial methionine covalent interaction is found to be larger in oxidized than in reduced cyt *c*.

The experimental RIXS data and simulations have indicated that the Fe-S(Met) bond is more covalent than the Fe-N(His) bond. However, the calculated axial ligand bond strengths in Table 4 indicated that the Fe-S(Met) bond is weaker than the Fe-N(His) bond. This difference can be understood in terms of basic bonding concepts. Within the framework of perturbation theory, the bond energy (BE) is proportional to $(H_{M-L})^2/\Delta$, where H_{M-L} is the resonance integral between the metal and the ligand orbitals and Δ is the difference in energy between these interacting orbitals before bonding. The covalency (i.e., the coefficient squared of ligand character in the metal d-orbital, α^2) is proportional to $((H_{M-L})/\Delta)^2$. Thus, the

BE can be estimated from the covalency scaled by Δ (i.e., $BE = \alpha^2\Delta$). Thus, the stronger Fe-N(His) bonds relative to Fe-S(Met), despite their lower covalencies, can be understood in terms of the difference in their valence donor orbital energies, where the ImH σ donor bond is 1.1 eV lower in energy than the Met b_1 valence donor orbital. This results in a significantly larger Δ for N(His) and thus a stronger M-L bond.

It was found above that the Fe(III)-S(Met) bond is stronger than the Fe(II)-S(Met) bond (7.1 vs. 5.0 kcal/mol, respectively, Table 4). The energy of the axial ligand bond was calculated as the difference in energy between the ligand on ($S=1/2$ and $S=0$ for Fe(III) and Fe(II), respectively) and off ground states ($S=3/2$ and $S=2$ for Fe(III) and Fe(II), respectively). Thus, there can be additional contributions to the calculated bond strengths due to the different spin states involved in ligand loss. These can be taken into account using the potential energy surfaces (PESs) of ligand binding to the Fe(III) and Fe(II) states. Figure 12 gives the PESs for Met ligand loss for the ferrous state in the gas phase leading to an $S = 0$ to $S = 2$ surface crossing. The gas-phase axial S(Met) ligand bond strength is 5.6 kcal/mol. Note that energies for the corresponding two-box calculations (i.e., the porphyrin and axial ligand are calculated separately) are given in parentheses (e.g., 5.8 kcal/mol for the Fe(II)-S(Met) bond strength). The energy of ligand loss on the $S = 0$ surface is calculated to be 12.3 kcal/mol (Figure 12, right). The difference in energy for these two processes of ligand loss is the exchange stabilization energy corresponding to a change in Fe(II) spin-state (i.e., $S = 0 \rightarrow S = 2$) and is calculated to be 6.2 kcal/mol (Figure 12). (Note that the exchange stabilization energy is taken to be the difference in energy between the $S = 0$ and $S = 2$ spin states without an additional axial ligand in order to directly compare to Fe(III) case below.) Thus, this exchange stabilization lowers the calculated value for the axial ligand bond strength. Analogous calculations for axial ligand loss from the Fe(III)-porphyrin site give gas-phase axial ligand bond strengths of 8.1 kcal/mol (for the $S = 1/2 \rightarrow S = 3/2$ conversion) and 17.8 kcal/mol (for ligand loss along the $S = 1/2$ surface) and an exchange stabilization energy of 9.7 kcal/mol. Thus, even after correcting the Fe(III) and Fe(II) axial

ligand bond strengths for the different exchange stabilization energies (9.7 vs. 6.2 kcal/mol, respectively), the axial ligand bond in the Fe(III) site is still stronger than that for the Fe(II) (8.1 vs. 5.6 kcal/mol, respectively). Without exchange stabilization, the difference between the Fe(III) and Fe(II) bond strengths is even larger (17.8 vs. 12.3 kcal/mol). The fact that the Fe(III)-S(Met) bond is stronger than Fe(II)-S(Met) is consistent with the higher degree of covalency, as determined above via the 1s2p RIXS data and simulations.

As outlined in the Introduction, past considerations have concluded that, in cyt *c*, the Fe(II)-S(Met) bond is stronger than the Fe(III)-S(Met) bond. One argument for this is the shorter Fe(II)-S(Met) bond (2.29 Å) relative to Fe(III)-S(Met) (2.33 Å) bond.¹⁶ However, the bond lengths are not necessarily related to bond strengths, as these are affected differently by the ionic and covalent contributions to bonding.^{29,62} Furthermore, for considerations related to the decrease in E^0 upon Fe-S(Met) bond loss,^{6,7} ligand competition^{8,9} and binding,¹⁰⁻¹² and protein folding studies,^{10,13-15} it is essential to define and refer to the reference state (i.e. S(Met) bound vs. either N(His), N(Lys), or H₂O axial ligands). Importantly, from Table 4, the $\Delta\Delta G$ for axial ligand binding between Fe(III) and Fe(II) states for S(Met), N(His), and H₂O axial ligands is 5.1, 8.8, and 8.1 kcal/mol, respectively, and is thus larger for the N(His) and H₂O axial ligands. This difference results in a decrease in E^0 upon loss of the S(Met) ligand and replacement with either a nitrogen or oxygen based ligand (estimated to be 160 mV from the $\Delta\Delta G$ for S(Met) vs. N(His), Table 4). This difference in $\Delta\Delta G$ also leads to an apparent increase in S(Met) binding affinity to Fe(II) relative to Fe(III),¹⁰⁻¹² as binding to the Fe(III) (but not Fe(II)) state also involves displacement of an axial H₂O ligand. This difference in the $\Delta\Delta G$ for S(Met) vs. N(His) or H₂O ligands can be understood through hard/soft acid/base concepts, where the harder nitrogen- and oxygen-based ligands bind more strongly to the Fe(III) than Fe(II) oxidation state and while the softer sulfur-based ligand still binds more strongly to Fe(III), it has a higher relative (to N and O based ligands) affinity for Fe(II).

Previous studies have defined the function of the axial S(Met) ligand in T1 Cu and

CuA.^{2,4,5} The presence of the axial S(Met) bond lowers E^0 due to the stronger Cu(II)-S(Met) bond relative to Cu(I). This difference in E^0 is relative to the absence of axial ligand binding upon S(Met) ligand loss. A similar function is found here for cyt *c*, as the Fe(III)-S(Met) bond is indeed stronger than Fe(II); however, decreases in E^0 are observed upon ligand loss in cyt *c* due to its replacement by an endogenous (N(His) or N(Lys)) or exogenous ligand (H₂O). These ligands have larger ΔG s for Fe(III) vs. Fe(II) binding and thus effectively lower E^0 relative to the S(Met). Furthermore, the T1 Cu-S(Met) bond has been measured experimentally to be weak.⁵ Thus, the S(Met) bond would be subject to ligand loss due to entropic contributions at physiological temperature.^{4,5} This has provided insight into the 'entatic/rack'⁶³⁻⁶⁵ nature of T1 Cu and CuA ET active sites. Similar behavior is found here for cyt *c*, as the axial ligand bond strengths for both Fe(II) and Fe(III) are weak (i.e., ~ 5 kcal/mol) and on the order of the T ΔS free energy of bond loss. Thus, analogous to T1 Cu, the protein matrix of cyt *c* provides the opposing free energy necessary to keep the axial S(Met) bond intact. The axial Fe-S(Met) bond in cyt *c* can therefore be considered under entatic control. The weaker Fe(II)-S(Met) bond as compared to the Fe(III)-S(Met) bond is also consistent with the observed axial ligand photolysis for Fe(II) but not Fe(III) cyt *c*. Differentiating bond strength contributions to the photochemistry and photophysics in cyt *c* is an aim of current studies.

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Supporting Information Available

Further information are given in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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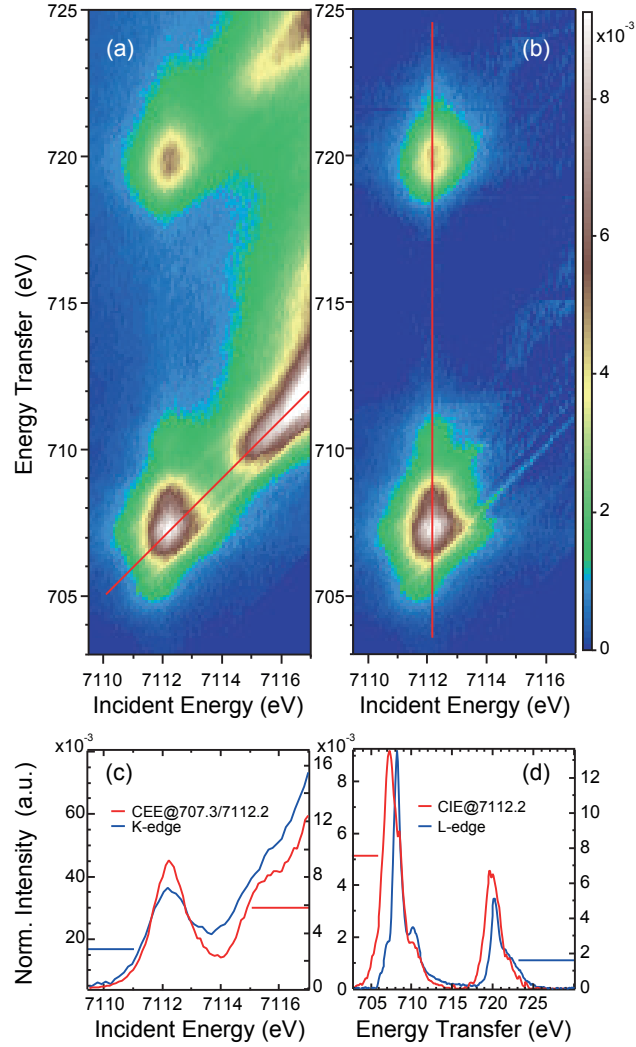


Figure 2: Fe(II)TPP(ImH)₂: (a) Full 1s2p RIXS plane, (b) rising-edge subtracted 1s2p RIXS plane, (c) comparison of the CEE cut through the uncorrected plane (red line in (a)) with the direct K-edge, (d) comparison of the CIE cut along the red line in (b) with the direct L-edge experimental data.

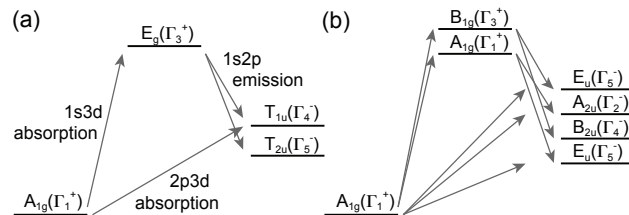


Figure 3: Possible excitation pathways for an L-edge and 1s2p RIXS experiment of a ferrous low-spin system for (a) O_h and (b) D_{4h} symmetry. The pathways through B_{2g} and E_g intermediate states in (b) have been omitted for clarity.

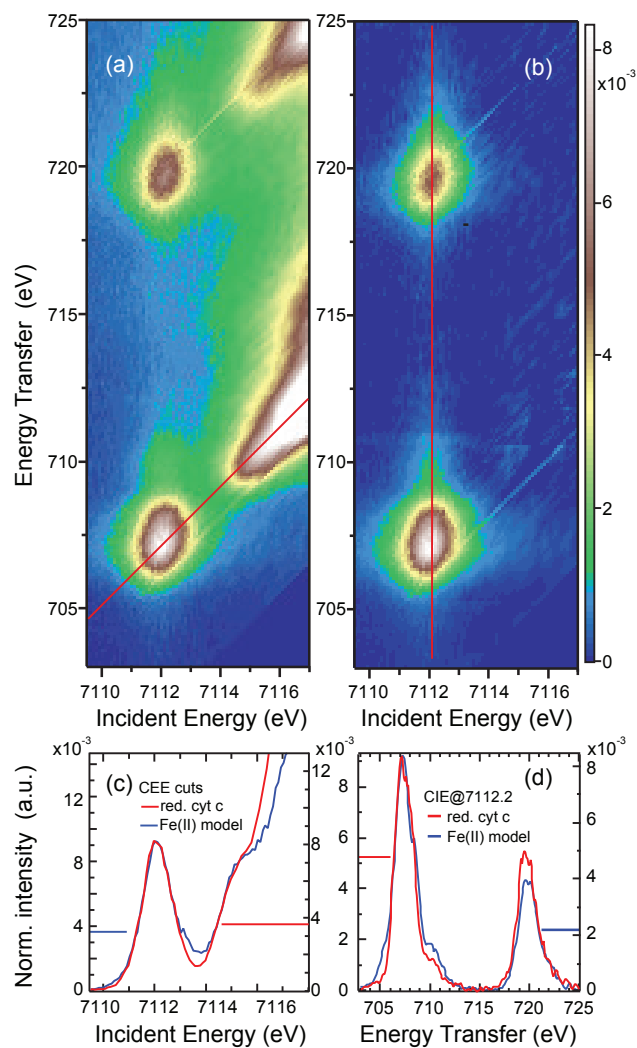


Figure 4: Reduced cyt *c*: (a) Full 1s2p RIXS plane, (b) rising-edge subtracted 1s2p RIXS plane, (c) comparison of the CEE cut through the uncorrected plane (red line in (a)) with the CEE cut of Fe(II)TPP(ImH)₂, (d) comparison of the CIE cut along the red line in (b) with the CIE cut of Fe(II)TPP(ImH)₂.

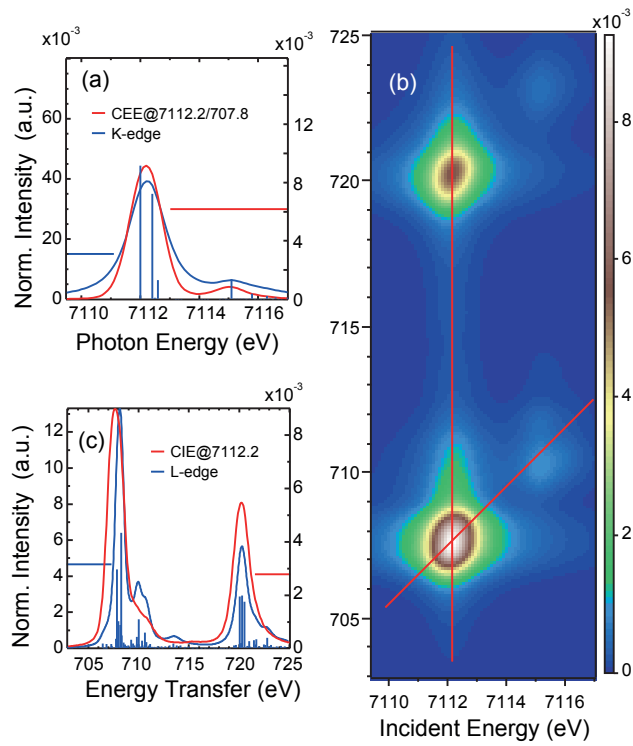


Figure 5: Fe(III)TPP(ImH)₂ simulations: (a) comparison of the CEE cut along the red line in (b) with the simulated K-edge, (b) 1s2p RIXS plane simulation, (c) comparison of the CIE cut along the vertical red line in (b) with the direct L-edge simulation.

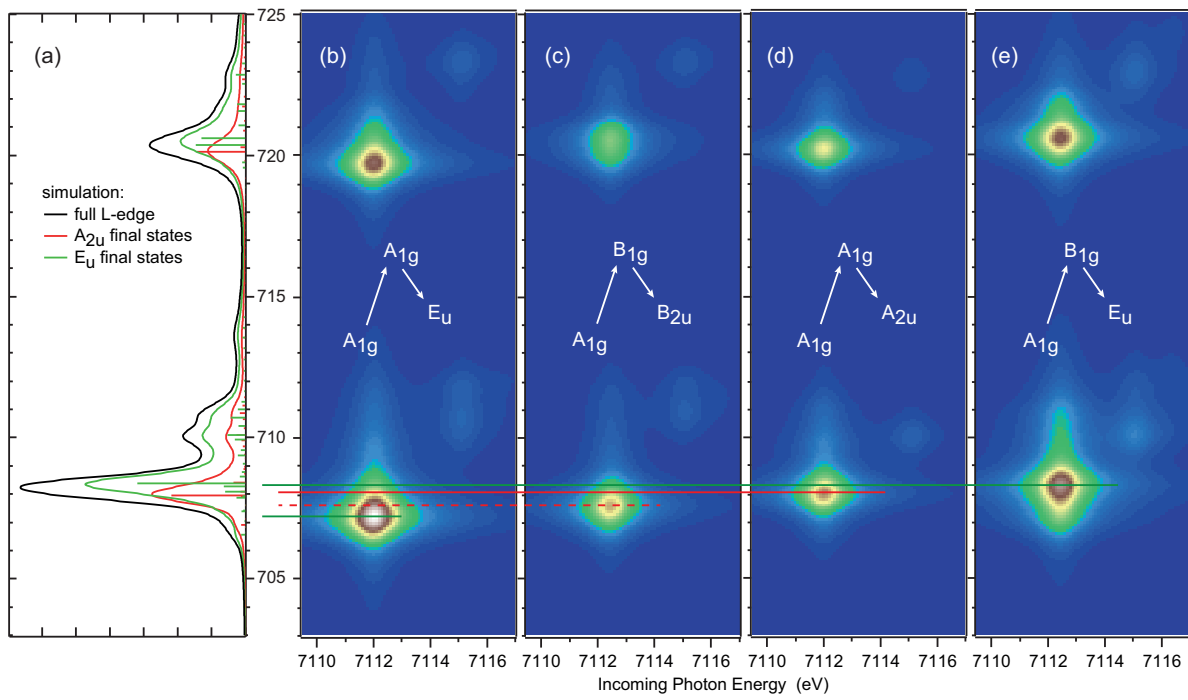


Figure 6: Comparison of the final state L-edge XAS simulation (a), and the four $1s2p$ RIXS pathways carrying most intensity (b)-(e). Pathways through the intermediate state with B_{2g} and E_g being omitted for clarity.

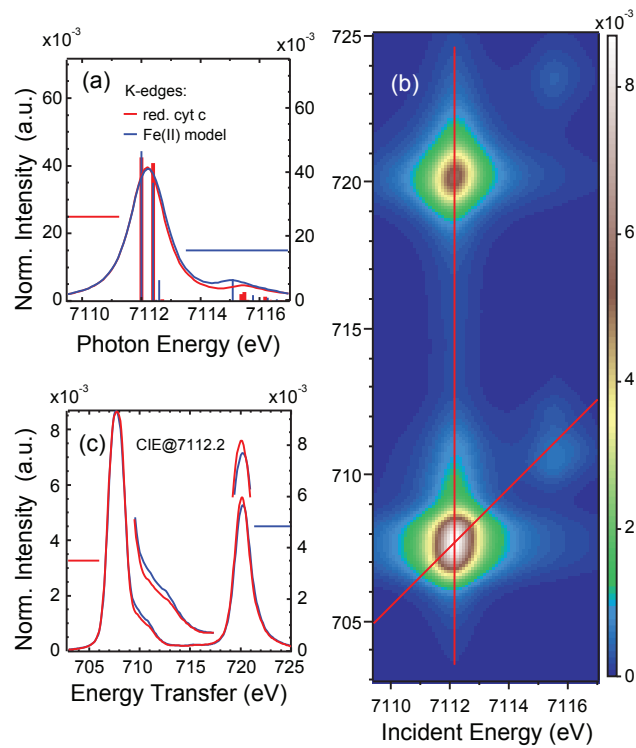


Figure 7: reduced cyt *c* simulations: (a) comparison of the CEE cut along the red line in (b) with the CEE cut of the model complex simulation, (b) 1s2p RIXS plane simulation, (c) comparison of the CIE cut along the vertical red line in (b) with the CIE cut of the model complex simulation.

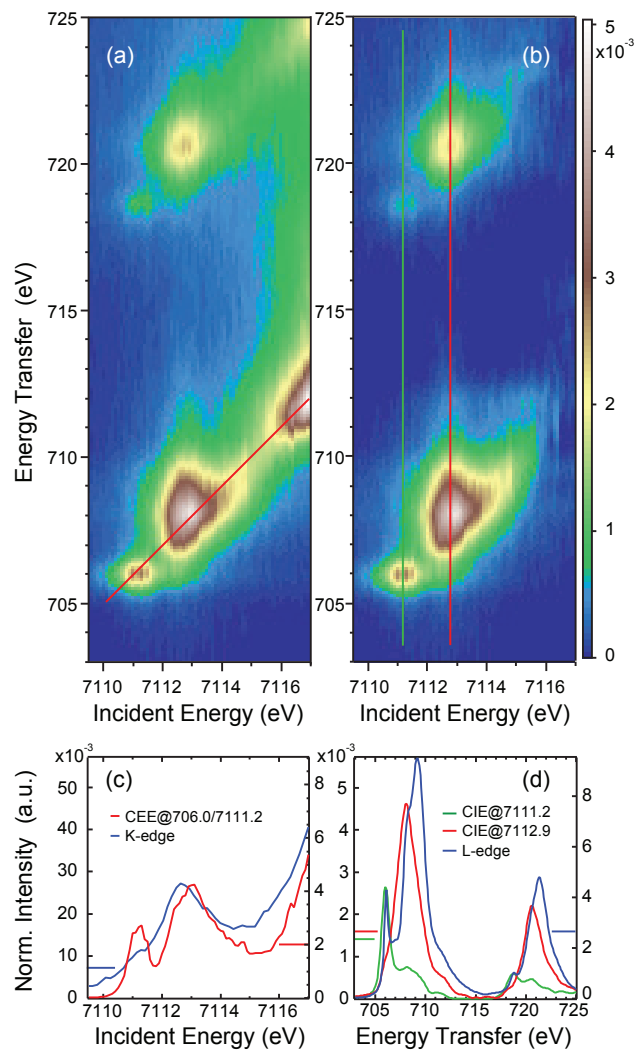


Figure 8: Fe(III)TPP(ImH)₂: (a) Full 1s2p RIXS plane, (b) rising-edge subtracted 1s2p RIXS plane, (c) comparison of the CEE cut through the uncorrected plane (red line in (a)) with the direct K-edge, (d) comparison of the CIE cuts along the green and red lines in (b) with the direct L-edge experimental data.

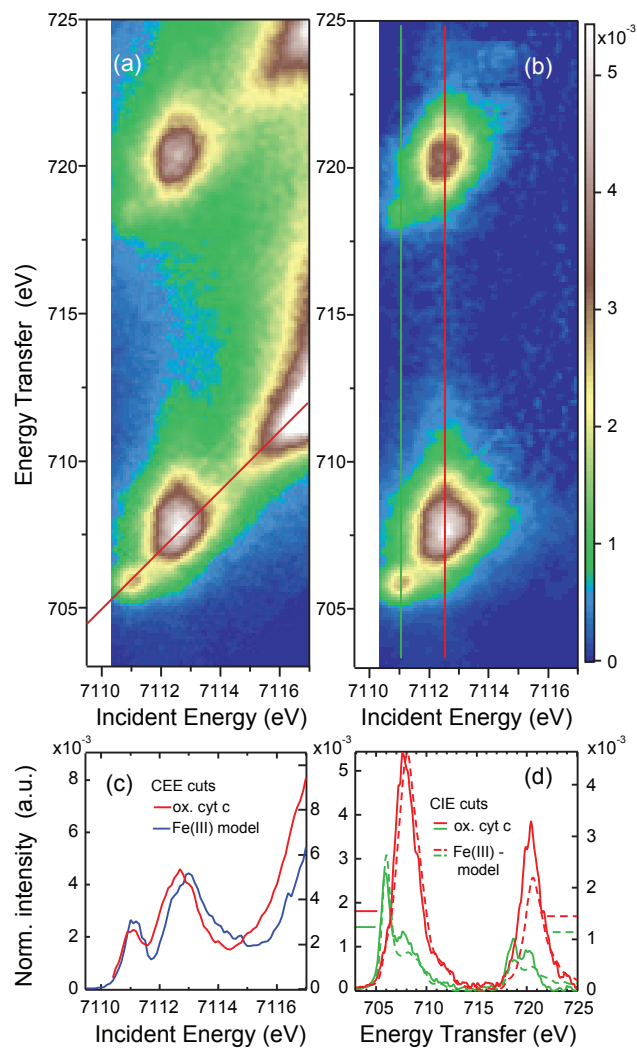


Figure 9: Oxidized *cytochrome c*: (a) Full 1s2p RIXS plane, (b) rising-edge subtracted 1s2p RIXS plane, (c) comparison of the CEE cut through the uncorrected plane (red line in (a)) with the CEE cut of Fe(III)TPP(ImH)₂, (d) comparison of the CIE cuts along the green and red lines in (b) with the corresponding CIE cuts of the ferric model complex.

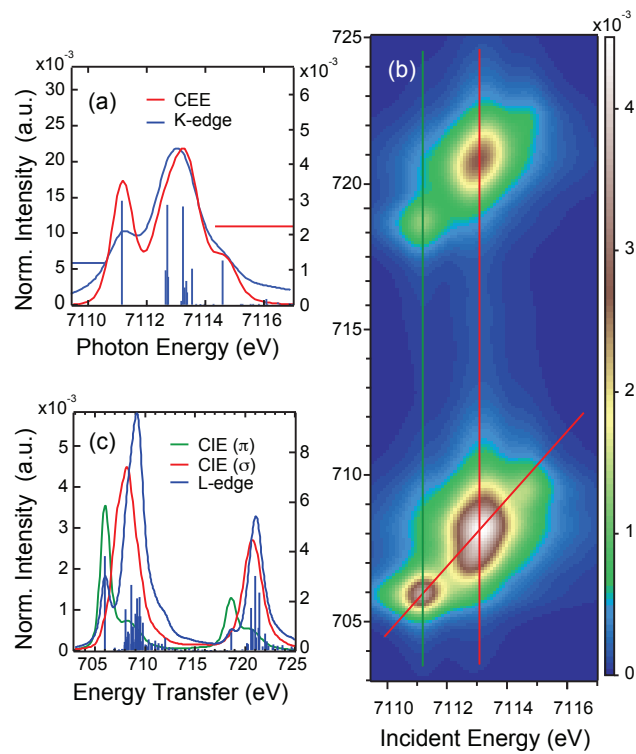


Figure 10: Fe(III)TPP(ImH)₂ simulations: (a) Comparison of the CEE cut along the red diagonal line in (b) with the simulated K-edge, (b) 1s2p RIXS plane simulation, (c) comparison of the CIE cuts along the green and red vertical lines in (b) with the direct L-edge simulation.

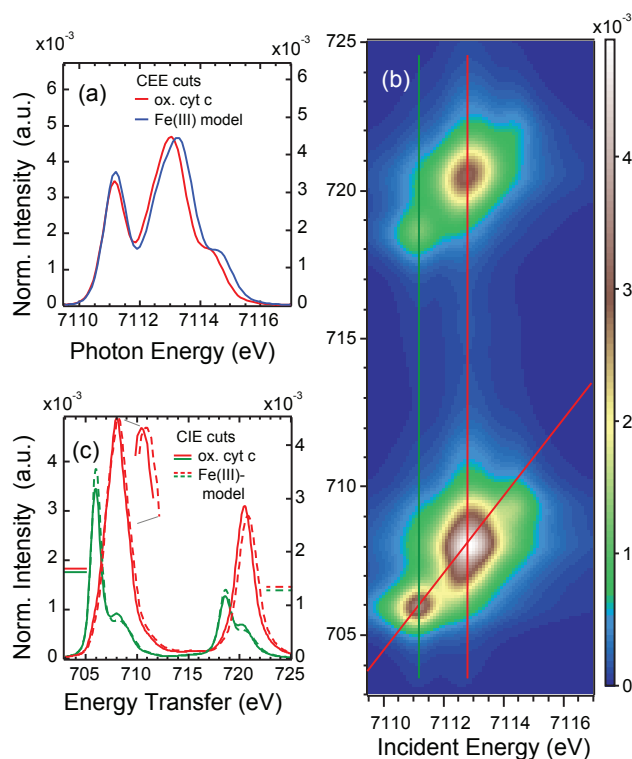


Figure 11: Oxidized *cytochrome c* simulations: (a) Comparison of the CEE cut along the red diagonal line in (b) with the CEE cut of the model complex simulation, (b) 1s2p RIXS plane simulation, (c) comparison of the CIE cuts along the green and red vertical lines in (b) with the CIE cuts of the model complex simulation.

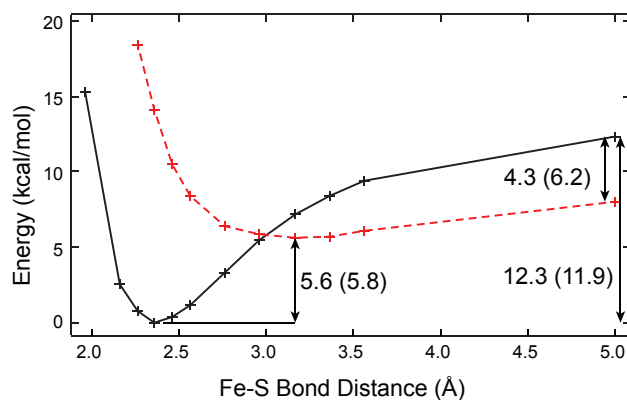


Figure 12: DFT calculated gas-phase potential energy surfaces of S(Met) binding to both Fe(II) $S = 0$ (black line and crosses) and $S = 2$ (red dashed line and crosses) spin-states. Values for one-box calculations are given and corresponding two-box values are given in parentheses. Values for Fe(III) $S = 1/2$ and $S = 3/2$ are given in the text