Supporting Information

Effects of Protein Structure on Iron-Polypeptide Vibrational Dynamic Coupling in Cytochrome c

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Figure S1. Structure of the active site model of ferric \( Ht \) c-552 (PDB code: 1YNR) used to mimic the extent of heme ruffling in A7F (left), wt (middle), and M13V and M13V/K22M (right). The structure shows the Cys12-Met13-Ala14-Cys15-His16 pentapeptide loop on the proximal side of the heme and Met61 on the distal side. The yellow, purple, red, blue, and gray tubes represent sulfur, heme iron, oxygen, nitrogen, and carbon, respectively. The hydrogen atoms are omitted to emphasize the heme. The positions of the \( \alpha \)-and \( \beta \)-pyrrole carbons were adjusted to change the twisting along the Fe-N\(_\text{Pyr}\) bonds.
Figure S2. Resonance Raman spectrum of ferric wt *Ht* c-552 (black) and the variant M13V/K22M (red) in 50 mM Hepes buffer at 77 K. The spectrum was obtained by exciting the samples at 413 nm.

Figure S3. Left: Complete VDOS starting from 0 cm\(^{-1}\) for ferric wt *Ht* Cyt c and the variants M13V, M13V/K22M, and A7F (red traces). Gaussian fits of these data are included (black traces). Right: NRVS spectra of the same set of enzymes with error bars calculated from photon statistics.
**Figure S4.** Simulated NRVS spectra of ferric *Ht* c-552 as a function of modifying the $\delta$(C$_a$-S$_1$-C)$_{PyrI}$ Cys 12 bending force constant.

**Figure S5.** Simulated NRVS spectra of ferric *Ht* c-552 as a function of modifying the Pro25 C=O (formaldehyde in model) to His Hδ1 H-bonding force constant.
Figure S6. Average scan of raw data for wt Ht Cyt c and the variants M13V, M13V/K22M, and A7F before converting to their corresponding vibrational density of states.

QCC-NCA refit of the NRVS spectrum of Ht c-552 (full details).

The bands in the NRVS spectrum of $^{57}$Fe Ht c-552 representing vibrational modes that arise from the $\text{N}_{\text{His}}$-$\text{Fe}$-$\text{S}_{\text{Met}}$ axial unit, the heme and the polypeptide (especially the CXXCH segment) have recently been assigned by our group.$^1$ The assignment has been achieved by constructing a model of the Ht c-552 active site, comprised of the heme, the axial Met, and the CXXCH loop including the His ligand to heme. DFT was then used to calculate the force field for this model, which was subsequently refined using our Quantum Chemistry Assisted Normal Coordinate Analysis (QCC-NCA)$^2$ to reproduce the NRVS spectrum of wt and isotopically labeled proteins. In this way, the experimental vibrational energies, NRVS intensities, and isotope shifts were well reproduced (Figure 3). Deviations are observed in the lower energy region (150-325 cm$^{-1}$), which is dominated by heme and $\text{N}_{\text{His}}$-$\text{Fe}$-$\text{S}_{\text{Met}}$ axial ligand motions that are strongly coupled to vibrations of the polypeptide. At higher energy (325-400 cm$^{-1}$), the most intense NRVS features arise from in-plane (ip) iron-pyrrole stretching vibrations, $\nu$(Fe-$\text{N}_{\text{Pyr}}$), and out-of-plane (oop)
pyrrole tilting and swiveling modes. Beyond this region at 400-500 cm\(^{-1}\), weaker features are attributed to pure heme modes, and mixed heme and polypeptide motions. A complete assignment of the vibrational modes in the 150-450 cm\(^{-1}\) region from our previous study on \(Ht\ c\)-552 is available in ref. 1.

Despite the success in reproducing the experimental NRVS spectrum of \(Ht\ c\)-552 with our QCC-NCA simulation, discrepancies between the fit and the experimental data remained (see Figure 3). For example, the vibrational energies of the “three-band” feature in the 250-325 cm\(^{-1}\) region are shifted to lower energy by \(\sim 15\) cm\(^{-1}\) compared to experiment. In addition, a weak feature at 316 cm\(^{-1}\) in this region is noticeable in the simulated spectrum but is absent in the experimental NRVS data. Finally, the DFT-optimized structure of the CXXCH loop in the model used in ref. 1 shows deviations from the \(Ht\ c\)-552 crystal structure (see below). These inconsistencies prompted us to investigate whether a more accurate model of the active site would be able to reproduce the wt NRVS data with even better accuracy, which is important to explore how the NRVS data of the mutants M13V, M13V/K22M, and A7F investigated here can be reconstructed from the wt spectrum. Thus, a model of the \(Ht\ c\)-552 active site with a conformation of the CXXCH loop that better mimics the protein crystal structure was generated. In the previous model,\(^1\) the DFT optimization of the model (required to calculate the force field) led to a readjustment of the loop configuration such that the H-bonding distance between the Cys12 C=O group and the His16 N-H group became too long relative to the X-ray crystal structure.\(^3\) Since the H-bonds within the CXXCH loop could be critical for the geometry of the loop, it is imperative to accurately mimic this part of the loop structure. In our new model, the CXXCH loop region was allowed to optimize while the two key H-bonds within the loop were kept within a fixed distance. This generated a \(Ht\ c\)-552 active-site model with a CXXCH orientation that now resembles the protein crystal structure more closely than the previous model does (Figures 1 (right) and 4). The calculated force constants for the new model were then again refined against the experimental NRVS data on \(Ht\ c\)-552 including the isotopically \((^{13}C,\ ^{15}N)\) labeled derivatives using the QCC-NCA approach. This new fit, which delivers force constants very close to those of the previous model (see Table S3 for a comparison), resulted in a redistribution of spectral intensities in the 250-325 cm\(^{-1}\) region, leading to an improved fit that mimics the experimental data more accurately (see Figure 3). A condensed list of the force constants that played the most significant role in the new simulation of the NRVS data of \(Ht\ c\)-
552 is provided in Table 2.

As described above and shown in Figure 3, one remarkable improvement in the current fit is the excellent agreement of vibrational energies and NRVS intensities in the 250-325 cm\(^{-1}\) region with experiment. While the NRVS features in this spectral region are shifted to lower energy by ~15 cm\(^{-1}\) in the previous model, they are only shifted by ~6-8 cm\(^{-1}\) in the current fit. Importantly, the weak feature at 316 cm\(^{-1}\) that is present in the QCC-NCA simulations of the previous model now better resembles the experimental data. In the high-energy region (340-425 cm\(^{-1}\)), the energies and intensities of the NRVS spectral features are also well-reproduced, notably for the most intense band at 344 cm\(^{-1}\), and the peaks at 366, 385, and 418 cm\(^{-1}\). One spectral feature whose intensity is overestimated in the new QCC-NCA simulations is the band at 397 cm\(^{-1}\). This peak is particularly sensitive to the bending modes of the porphyrin core.\(^1\) The intensity of this band can theoretically be decreased at the expense of considerably changing one of the several in-plane bending pyrrole force constants. However, given the quasi-symmetric nature of the current model, this adjustment was avoided to retain comparable force constants among the related pyrrole in-plane bending internal coordinates (see Table S3). Overall, the new QCC-NCA NRVS simulation displays improved agreement with the experimental data, and forms the basis by which to analyze the NRVS data of the mutants.

Based on the agreement between the experiment and current QCC-NCA simulation, the NRVS spectrum of \(Ht\ c\)-552 can be assigned (see Tables S4 and S5). Notably, compared to the previous model,\(^1\) the vibrational assignments in the 340-425 cm\(^{-1}\) region are identical, but some changes are noted for the vibrational features in the 250-325 cm\(^{-1}\) region. In general, the low energy region is still dominated by polypeptide (pp) modes with contributions from vibrations of both the heme and the axial N\(_{\text{His}}\) - Fe - S\(_{\text{Met}}\) unit. Compared to the previous \(Ht\ c\)-552 model, the vibrational modes have been redistributed within this energy region and thus, the assignments show some alterations. First, the weakest feature at 222 cm\(^{-1}\) displays increased His bending and torsional contributions and less pyrrole motions in the new fit. The \(\delta\)(His) and \(\tau\)(His) modes extend to the subtle shoulder at 273 cm\(^{-1}\) with additional contributions from an oop methine motion of the heme, \(\gamma\)(C\(_{\alpha}\)C\(_{m}\)). Further, while the peak at 284 cm\(^{-1}\) showed dominant \(\delta\)(pp) and \(\tau\)(pp) contributions in the previous assignment, this feature now corresponds to a \(\nu\)(Fe-N\(_{\text{Pyr}}\)) stretch, mixed with torsions and bends involving the axial His and Met ligands. These vibrations are also accompanied by pp bending modes. The weak shoulder at 306 cm\(^{-1}\), which is only
discernible from a Gaussian fit of the data, has mixed contributions from pp and heme oop vibrations. Lastly, the band at 316 cm\(^{-1}\) has dominant contributions arising from \(\delta\)(His) and \(\tau\)(His) modes. The only spectral feature within the 250-325 cm\(^{-1}\) region whose assignment did not change to some degree from the previous simulation is the band at 297 cm\(^{-1}\), which displays an oop \(\gamma(C_\alpha C_m)\) motion mixed with contributions from \(N_{\text{His}} - Fe - S_{\text{Met}}\) axial vibrations.

Throughout the 150-400 cm\(^{-1}\) region, the relative amounts of mixing of the heme/\(N_{\text{His}} - Fe - S_{\text{Met}}\) unit vibrations with the pp modes are comparable to those of the previous model. However, one important difference is the \(\nu(Fe - S_{\text{Met}})\) stretching mode. In the new \(Ht\ c-552\) model, the \(\nu(Fe - S_{\text{Met}})\) mode is now more localized in the lower energy region. The QCC-NCA fits reveal 13\% \(\nu(Fe - S_{\text{Met}})\) contribution (mixed with \(\nu(Fe - N_{\text{Pyr}})\)) for the simulated band at 177 cm\(^{-1}\), which is in excellent agreement with the assignment of the experimentally observed band at 178 cm\(^{-1}\). On the other hand, the \(\nu(Fe - N_{\text{His}})\) mode is still distributed throughout the 200-325 cm\(^{-1}\) spectral region, similar to what has been observed for the original model. Because of this ‘delocalization’, this vibrational mode only shows small contributions to the vibrations occurring at 199, 222, 284, 297, and 306 cm\(^{-1}\). Experimentally, the \(\nu(Fe - N_{\text{His}})\) stretch has never been assigned with certainty for ferric Cyt \(c\). Our QCC-NCA simulations demonstrate why the \(\nu(Fe - N_{\text{His}})\) stretching mode has been elusive for ferric Cyt \(c\), which is due to the fact that there is no distinct band that can be associated with this feature.

**Table S1.** Number of scans collected and adjustable parameters for the conversion of raw NRVS intensity to vibrational density of states (VDOS) using PHOENIX.

| Sample     | Number of Scans | Padd\(^a\) | Phox\(^a\) |
|------------|-----------------|-------------|-------------|
|            |                 | Temp | Asymmetry | Temp | Background | Asymmetry |
| \(Ht\ c-552\) | 18              | 140  | 1.07      | 150  | 0.5        | 1.08      |
| A7F        | 18              | 150  | 1.00      | 42   | 1.9        | 1.02      |
| M13V       | 15              | 150  | 1.00      | 97   | 2.6        | 1.07      |
| M13V/K22M  | 16              | 150  | 1.08      | 105  | 2.6        | 1.09      |

\(^{a}\)The conversion is performed in two stages, using the programs Padd and Phox.\(^{b}\)
Table S2. Contributions from different out-of-plane (oop) distortions in *Ht* c-552 models and Cyt c proteins (Å) using normal-coordinate structural decomposition (NSD) analysis.

| Model          | B2u  | B1u  | A2u  | E(\phi)(x) | E(\phi)(y) | A1u  |
|----------------|------|------|------|-------------|-------------|------|
| Less ruffled   | -0.36| -0.65| -0.04| -0.04       | 0.03        | -0.00|
| Current model  | -0.36| -0.78| -0.01| -0.06       | 0.05        | -0.01|
| More ruffled   | -0.27| -1.00| -0.01| -0.09       | 0.05        | -0.04|
| *Ht* c-552\textsuperscript{a} | -0.08| -0.62| -0.08| 0.06        | -0.07       | -0.03|
| *Pa* c-551\textsuperscript{b} | -0.10| -0.48| 0.00  | 0.02        | 0.01        | 0.00 |

\textsuperscript{a}Crystal structure taken from ref. 3. \textsuperscript{b}Crystal structure taken from ref. 7.

Table S3. Complete list of force constants of ferric wt *Ht* c-552 invoked in the fit of the NRVS data, based on the BP86/LanL2DZ\textsuperscript{*} DFT result.

| Force constant\textsuperscript{c} | BP86/LanL2DZ\textsuperscript{*} | QCC-NCA |
|-----------------------------------|----------------------------------|---------|
| Fe-S\textsubscript{Met} [1]       | 0.7905                           | 0.8404  |
| Fe-N\textsubscript{His} [17]      | 1.4835                           | 1.2493  |
| Fe-N\textsubscript{Pyr} [13-16]   | 1.6094, 1.6255, 1.6396, 1.6919   | 1.6981, 1.6771, 1.6961, 1.8058 |
| ν(pp)                             | 4.4079, 5.6041, 3.8388, 4.3474, 5.1805, 3.4718, 4.7723, 5.4258, 3.7457, 3.7530, 2.7487, 2.3613, 5.0847, 5.0881, 2.7990, 2.3680 | 5.2754, 6.7916, 3.6215, 5.2548, 6.9429, 3.9030, 5.0051, 5.2322, 4.1163, 3.8993, 2.9974, 2.7038, 5.8847, 5.8881, 2.8990, 2.8852 |
| ν(His)                            | 4.4768, 3.6772, 5.5075, 6.3193   | 4.8030, 3.7548, 5.4300, 6.8063 |
| pp H-bonds [154-155]              | 0.0915, 0.1915                   | 0.0750, 0.2005 |
| N\textsubscript{Pyr}-Fe-N\textsubscript{Pyr} [190-193] | 0.6531, 0.7224, 0.6534, 0.7212 | 0.7287, 0.7664, 0.8018, 0.8058 |
| N\textsubscript{His}-Fe-N\textsubscript{Pyr} [186-189] | 0.6484, 0.6607, 0.7528, 0.8163 | 0.7022, 0.9032, 0.9030, 0.9145 |
| [17]/[188]                        | 0.1187                           | 0.1139  |
| S\textsubscript{Met}-Fe-N\textsubscript{Pyr} [182-185] | 0.7725, 0.9375, 0.7187, 0.8310 | 0.8228, 0.9002, 0.7633, 0.8775 |
| δ(pp)                             | 0.7940, 0.8429, 0.7663, 0.4935, 0.7320, 0.6208, 0.5948, 0.6621, 0.5463, 0.8197, 0.6601, 0.5861, 0.5529, 0.7100, 0.7293, 0.6534 | 0.9057, 0.9968, 0.9963, 0.6531, 0.8320, 0.8208, 0.2548, 0.8921, 0.6463, 0.8197, 0.5601, 0.6001, 0.5529, 0.7100, 0.7893, 0.4034 |
| δ(His) [371, 372, 377, 378, 385, 391, 397] | 0.9225, 0.7040, 0.4785, 0.5857, 0.5743, 0.9837, 0.7051 | 0.8901, 0.8640, 0.4356, 0.5001, 0.7027, 1.0885, 0.8095 |
| δ(Met) [163, 181]                 | 0.6771, 1.1543                   | 0.8871, 0.9553 |
| Fe-S\textsubscript{Met}-C [161-162] | 0.9723, 0.9843                   | 1.2577, 1.1588 |
| δ(\textsubscript{pp})(porphyrin): pyrrole | 2.3760, 2.5627, 2.5611, 2.3767, 2.4540, 2.5016, 2.5153, 2.4329 | 2.2800, 2.2001, 2.4486, 2.3845, 2.3167, 2.4091, 2.2853, 2.3195 |
| δ(\textsubscript{pp})(porphyrin): methine | 0.9629, 0.9816, 0.9537, 0.9914, 0.7523, 0.8175, 0.8010, 0.7904, 0.7783, 0.8916, 0.7553, 0.8091, 0.8356 | 0.9223, 0.9010, 0.8880, 0.9107, 1.0900, 1.009, 1.2825, 1.3971, 1.2993, 0.8816, 0.8853, 1.1591, 0.9999 |
| δ(\textsubscript{oop})(porphyrin): pyrrole | 0.2992, 0.2749, 0.2709, 0.2468, 0.4464, 0.4399, 0.4425, 0.4495, 0.4418, 0.4635, 0.4409, 0.4221 | 0.1854, 0.1844, 0.1870, 0.1988, 0.4553, 0.4671, 0.4301, 0.4439, 0.4774, 0.4635, 0.4409, 0.4221 |
| \(\delta_{\text{oop}}\) (porphyrin): methine | 0.3952, 0.3442, 0.3807, 0.3643, 0.3647, 0.3748, 0.3454, 0.3675, 0.2393, 0.2479, 0.2393, 0.2475 | 0.4464, 0.4179, 0.4271, 0.4365, 0.4396, 0.4039, 0.4241, 0.3844, 0.2593, 0.2579, 0.2593, 0.2575 |
| \(\delta\) (propionates) | 0.7362, 0.8089, 0.6996, 0.6865, 0.4464, 0.4179, 0.4271, 0.4365, 0.4396, 0.4039, 0.4241, 0.3844, 0.2593, 0.2579, 0.2593, 0.2575 |
| \(\tau\) (Met) \[459-461\] | 0.0797, 0.0794, 0.1711 | 0.0681, 0.1190, 0.0720 |
| \(\tau\) (Fe-S<sub>Met</sub>), \(\tau\) (Fe-N<sub>His</sub>) \[458, 462\] | 0.1742, 0.7799 | 0.3879, 0.8214 |
| \(\tau\) (His) | 0.1931, 0.9253, 1.4613, 1.1402, 0.1358, 0.8600, 1.0601, 0.6909, 0.2102, 3.2671, 1.3095, 0.1846 |
| \(\tau\) (pp) | 0.6082, 0.2192, 0.2689, 0.7477, 0.3144, 0.3300, 0.4773, 0.2566, 0.1451, 0.1957, 0.3853, 0.1913, 0.2549, 0.3168, 0.2727 |
| \(\tau\) (porphyrin) | 0.3090, 0.0533, 0.4084, 0.0497, 0.3189, 0.0657, 0.3473, 0.0991, 0.0209, 0.0679, 0.2854, 0.0328, 0.0226, 0.5144, 0.3844, 0.0487, 0.2941, 0.2467, 0.2667, 0.2253, 0.3416, 0.2864, 0.3003, 0.2668, 0.2821, 0.2646, 0.2403, 0.2716, 0.2886, 0.3027, 0.2687, 0.2876, 0.2451, 0.2350, 0.2472, 0.2343, 0.2730, 0.2427, 0.2828, 0.2019, 0.3571, 0.2561, 0.2304, 0.3604, 0.2978, 0.2012, 0.2001, 0.3226, 0.2231, 0.2514, 0.2241, 0.2258, 0.3585, 0.3585, 0.3557, 0.3450 |
| \(\tau\) (propionate) | 0.2445, 0.2617, 0.0546, 0.0084, 0.6031, 0.6066, 0.2558, 0.1996 |

*The unit for the force constants of stretching modes is mdyn/Å; for bending modes and torsions it is mdyn-Å.*
Table S4. Condensed vibrational assignments for ferric wt *Ht* c-552 based on the QCC-NCA simulation of the NRVS data presented here. More detailed assignments are presented in Table S5.

| Experiment | v[cm⁻¹](Δ) | sym/int | polarization | assignment                      |
|------------|------------|---------|--------------|---------------------------------|
| 165        | /vw        | ip      | Pyr. rot /ν(Fe-Ne₄) |
| 177        | /vw        | ip/oop = 1:3 | ν(Fe-S₅) | δ(Fe-S₅-C) |
| 184        | /vw        | ip/oop = 1:2 | δ(C₆-C₆-S₅)₈₁₈₁ / δ(C₆-S₅-C)₈₁₈₁ |
| 199        | /vw        | ip      | δ(pp)/ν(Fe-Ne₄) |
| 222        | /vw        | ip      | τ(His)/ν(C₆-C₆)/δ(ω₁₈₁) |
| 273 (+4/+4/-1) | /w        | ip/oop = 1:14 | τ(His)/ν(C₆-C₆) |
| 284 (+4/+6/0) | /mw      | ip/oop = 1:2 | ν(Fe-Ne₄)/ν(Fe-S₅) |
| 297 (+2/+4/0) | /m       | ip/oop = 1:9 | δ(Fe-S₅-C) / τ(Met)/ν(Fe-S₅-C) |
| 306 (0/+5/na) | /w        | ip/oop = 1:2 | δ(pp)/ν(Fe-Ne₄) |
| 307        | /w        | ip/oop = 2.4:1 | δ(ω₁₈₁)/τ(His) |
| 334 (-7/na-12) | E₈        | ip      | ν(Fe-Ne₄)/Pyr tilt/ν(C₆-C₆) |
| 335 (-7/-1/-5) | E₈/vs    | ip      | ν(ω₅)/Pyr. tilt/δ(C₆-C₆) |
| 366 (-2/+3/-3) | A₂/s     | oop  | γ₆ |
| 375 (na/na-1) | /m        | ip/oop = 1:4 | ν(Fe-Ne₄)/Pyr. tilt/δ(C₆-C₆) |
| 385 (-7/-3/-1) | /ms      | ip      | ν(Fe-Ne₄)/H₂S₃ / Pyr. tilt/δ(C₆-S₅-C)₈₁₈₁ |
| 397 (-5/-5/-3) | /ms      | ip      | ν(Fe-Ne₄)/Pyr. tilt/δ(C₆-S₅-C)₈₁₈₁ |
| 418 (-12/+1/-1) | /w       | ip      | δ(C₆-C₆-C₆) / δ(C₆-S₅-C)₈₁₈₁ |
| 464        | /w        | ip      | Pyr. rot |
| 485        | /w        | ip      | Pyr. rot |

*(Δ) = shifts in the M13V, M13V/K22M, and A7F variants. *Based on the BP86/LanL2DZ* calculation. **Symmetry (sym, in D₅₀) and calculated intensity (int): vs = very strong, s = strong, ms = medium strong, m = medium, mw = medium weak, w = weak, vw = very weak. *Abbreviations: Met = methionine, His = histidine, Pyr = pyrrole, pp = polypeptide. v, stretch; δ, bend; τ, torsion; γ, oop wag. Specific heme vibrations: γ₇, oop meso carbon wag; γ₆, oop Pyr. tilt (A₂/s); ν₅₀, ν(Fe-Ne₄) (E₈); ν₅₂, δ(C₆-C₆)₈₁₈₁ (E₈). Classifications of heme vibrations are taken from ref. 8. See also ref. 9.
Table S5. Detailed vibrational assignments for ferric \textit{Ht} c-552 based on the QCC-NCA simulation, using the active site model shown in Figures 1 and 4.

| Experiment | v[cm$^{-1}$(Δ)] | sym/int | polarization | assignment | QCC-NCA$^a$ | contribution$^d$ | Literature$^e$ |
|------------|----------------|---------|--------------|-------------|--------------|------------------|----------------|
| 165        | /vw           | ip      | Pyr. rot / v(Fe-N$_{Pyr}$) | 4% v(Fe-N$_{Pyr}$) + 2% v(Fe-S$_{Met}$) + Pyr. rot + τ(methyl) |
| 177        | /vw           | ip/oop = 1:3 | v(Fe-S$_{Met}$)/ δ(Fe-S$_{Met}$-C) | 13% v(Fe-S$_{Met}$) + 10% δ(Fe-S$_{Met}$-C) + 4% v(Fe-N$_{Pyr}$) + 3% τ(His) + 2% τ(Fe-N$_{His}$) |
| 184        | /vw           | ip/oop = 1:2 | δ(C$_{b}$-C$_{a}$-S$_{2}$)$_{pyr}$/ δ(C$_{a}$-S$_{1}$-C)$_{pyr}$ | 7% τ(His) + 5% v(Fe-N$_{Pyr}$) + 5% δ(C$_{b}$-C$_{a}$-S$_{2}$)$_{pyr}$ + 5% δ(C$_{a}$-C$_{1}$-S$_{2}$)$_{pyr}$ + 4% δ(C$_{b}$-C$_{a}$-S$_{1}$)$_{pyr}$ + 3% δ(C$_{b}$-C$_{a}$-S$_{1}$)$_{pyr}$ + 2% v(Fe-S$_{Met}$) + 2% δ(Fe-N$_{His}$-C) + δ(pp) |
| 199        | /vw           | ip      | δ(pp)/ v(Fe-N$_{His}$) | 11% δ(pp) + 5% v(Fe-N$_{His}$) + 5% v(Fe-N$_{Pyr}$) + 4% δ(C$_{b}$-C$_{a}$-S$_{2}$)$_{pyr}$ + 4% δ(C$_{b}$-C$_{a}$-S$_{2}$)$_{pyr}$ + 3% δ(Fe-N$_{His}$-C) + 2% τ(His) + v(pp) |
| 222        | /vw           | ip      | τ(His)/γ(C$_{a}$C$_{m}$)/ δ$_{oop}$(His) | 12% τ(His) + 7% τ(Fe-N$_{His}$) + 6% v(Fe-N$_{Pyr}$) + 4% δ$_{oop}$(His) + γ(C$_{a}$C$_{m}$) + 2% τ(Fe-S$_{Met}$) |
| 204        | /vw           | ip      | δ(His)/τ(His) | 13% δ(His) + 12% τ(His) + 4% δ(Met) + 2% v(Fe-N$_{His}$) + 2% v(Fe-N$_{Pyr}$) + 7% τ(pp) + δ(pp) |
| 207        | /w            | ip      | δ(His)/τ(His) | 13% δ(His) + 12% τ(His) + 4% δ(Met) + 2% v(Fe-N$_{His}$) + 2% v(Fe-N$_{Pyr}$) + 7% τ(pp) + δ(pp) |
| 211        | /vw           | ip/oop = 1:8 | δ(Met)/τ(Met)/ δ(Fe-S$_{Met}$-C) | 28% δ(Met) + 18% δ(Fe-S$_{Met}$-C) + 15% τ(Met) + τ(pp) + δ(pp) + τ(methyl) |
| 223        | /vw           | ip/oop = 13:1 | δ(pp)/τ(pp) | 32% δ(pp) + 14% τ(pp) + 2% τ(C$_{a}$-S$_{1}$)$_{pyr}$ + 2% τ(C$_{b}$-C$_{b}$)$_{pyr}$ + δ(C$_{b}$-C$_{a}$)$_{asym}$ + δ(Met) + Pyr. swivel |
| 228        | /vw           | ip/oop = 10.5:1 | τ(C$_{a}$-C$_{b}$)$_{pyr}$/ τ(C$_{a}$-C$_{b}$)$_{pyr}$/ τ(methyl)$_{pyr}$ | 28% τ(C$_{a}$-C$_{b}$)$_{pyr}$ + 20% τ(C$_{a}$-C$_{b}$)$_{pyr}$ + 11% τ(methyl)$_{pyr}$ + 6% τ(Met) + 3% δ(Fe-S$_{Met}$-C) + δ(pp) + δ(C$_{b}$-C)$_{asym}$ |
| 273 (+4/+4/-1 ) | /w       | ip/oop = 1:14 | τ(His)/δ$_{oop}$(His)/ γ(C$_{a}$C$_{m}$) | 19% τ(His) + 6% δ$_{oop}$(His) + 4% δ(pp) + 2% v(Fe-N$_{Pyr}$) + γ(C$_{a}$C$_{m}$) |
| 252        | /w            | ip/oop = 1:2 | τ(Met)/v$_{52}$ | 13% τ(Met) + 8% δ(C$_{b}$-C)$_{sym}$(v$_{52}$) + 6% τ(His) + 4% δ(Fe-S$_{Met}$-C) + 3% v(Fe-N$_{Pyr}$) + 3% δ(C$_{a}$-S$_{1}$-C)$_{pyr}$ + 4% δ(Met) + γ(C$_{a}$C$_{m}$) + δ(pp) + τ(pp) + τ(methyl) |
| 270        | E$_{d}$/w     | ip/oop = 1:2 | v(Fe-N$_{Pyr}$)/ δ(His)/τ(Met) | 5% v(Fe-N$_{Pyr}$) + 4% v(Fe-N$_{His}$) + 4% δ(Fe-S$_{Met}$-C) + 4% δ(His) + 4% τ(Met) + 2% τ(His) + δ(pp) + Pyr. swiv. + δ(C$_{b}$-C)$_{sym}$ + Pyr. tilt |
| 276        | E$_{d}$/w     | ip/oop = 1:5.5 | τ(Met)/v$_{52}$/ | 10% τ(Met) + δ(C$_{b}$-C)$_{sym}$(v$_{52}$) + 7% δ(Fe-S$_{Met}$-C) + 6% v(Fe-N$_{Pyr}$) |
| Sample | Dim. | oop | ip | Ex. 1 | Ex. 2 | Ex. 3 |
|--------|------|-----|----|-------|-------|-------|
| 284    | /mw  | ip/oop = 3:1 | δ(pp)/τ(pp)/δ(His)/δ(C<sub>p</sub>-C<sub>sym</sub>) | + 4% ν(Fe-N<sub>His</sub>) + 3% δ(Met) + 2% τ(His) |
| 286    | /m   | ip/oop = 1:6 | Pyr swiv./δ(C<sub>p</sub>-C<sub>sym</sub>) | Pyr. swivel + δ(C<sub>p</sub>-C<sub>sym</sub>) + 4% δ(His) + 2% ν(Fe-S<sub>Met</sub>) + δ(pp) |
| 297 (+2/+4/0) | 291 | /m | ip/oop = 1:9 | γ/δ(Fe-S<sub>Met</sub>-C)/τ(Met)/ν(Fe-S<sub>Met</sub>) | γ(C<sub>p</sub>-C<sub>met</sub>) (“pseudo”γ<sub>p</sub> + 11% δ(Fe-S<sub>Met</sub>-C) + 9% τ(Met) + 8% ν(Fe-S<sub>Met</sub>) + γ(C<sub>p</sub>-C<sub>met</sub>) + 5% δ(His) + 4% δ(Met) + 3% ν(Fe-N<sub>His</sub>) + 2% δ(S<sub>Met</sub>-Fe-N<sub>pyr</sub>) + δ(C<sub>p</sub>-C<sub>sym</sub>) |
| 293    | A<sub>2</sub>/m | ip/oop = 1:7 | γ/δ(Fe-S<sub>Met</sub>-C)/τ(Met)/ν(Fe-S<sub>Met</sub>) | γ(C<sub>p</sub>-C<sub>met</sub>) (“pseudo”γ<sub>p</sub> + 8% ν(Fe-S<sub>Met</sub>) + 6% δ(Fe-S<sub>Met</sub>-C) + 5% τ(His) + 5% ν(Fe-N<sub>His</sub>) + 4% τ(Met) + 3% ν(Fe-N<sub>pyr</sub>) + 2% δ(Met) + 2% δ(S<sub>Met</sub>-Fe-N<sub>pyr</sub>) + δ(C<sub>p</sub>-C<sub>sym</sub>) |
| 306 (-1/+5/na) | 304 | /w | ip/oop = 1:1 | δ(pp)/Pyr tilt/δ(C<sub>p</sub>-C<sub>sym</sub>) | 13% δ(pp) + 9% τ(His) + 7% ν(Fe-N<sub>pyr</sub>) + 5% τ(pp) + 4% δ(C<sub>p</sub>-C<sub>sym</sub>) + 3% τ(Met) + δ(S<sub>Met</sub>-Fe-N<sub>pyr</sub>) + δ(C<sub>p</sub>-C<sub>sym</sub>) + 3% ν(Fe-N<sub>His</sub>) + 3% δ(C<sub>p</sub>-C<sub>sym</sub>) + 2% δ(S<sub>Met</sub>-Fe-N<sub>pyr</sub>) + δ(C<sub>p</sub>-C<sub>sym</sub>) + 2% τ (Fe-N<sub>His</sub>) + 2% τ (Fe-N<sub>His</sub>) + 2% δ(S<sub>Met</sub>-Fe-N<sub>pyr</sub>) + δ(C<sub>p</sub>-C<sub>sym</sub>) |
| 307    | /w   | ip/oop = 2:4:1 | δ(pp)/Pyr. swiv./Pyr. tilt/δ(C<sub>p</sub>-C<sub>sym</sub>) | 26% δ(pp) + 7% τ(His) + 3% ν(Fe-N<sub>pyr</sub>) + Pyr. tilt + Pyr. swivel + δ(C<sub>p</sub>-C<sub>sym</sub>) + ν(pp) |
| 316    | /mw  | ip | τ(His)/δ(His) | 30% τ(His) + 27% δ(His) + 3% ν(Fe-N<sub>pyr</sub>) + ν(pp) |
| 324    | /w   | ip | δ(pp)/τ(His) | 38% δ(pp) + 22% τ(His) + 3% ν(Fe-N<sub>pyr</sub>) + ν(pp) + τ (pp) |
| 334 (na/na/-12) | 332 | E<sub>2</sub>/mw | ν<sub>S</sub>/His. rot/δ(C<sub>b</sub>-C<sub>as</sub>-S<sub>2</sub>)pyr<sub>¬</sub>/ν(Fe-N<sub>pyr</sub>) | δ(C<sub>p</sub>-C<sub>sym</sub>) (ν<sub>S</sub>2) + 5% ν(Fe-N<sub>pyr</sub>) + 5% δ(C<sub>b</sub>-C<sub>as</sub>-S<sub>2</sub>)pyr<sub>¬</sub> + 4% δ(Fe-N<sub>pyr</sub>) + 4% τ (His) + Pyr. tilt + 4% τ (Fe-N<sub>His</sub>) + δ(pp) + τ (pp) |
| 344 (-1/-2/-4) | 343 | E<sub>2</sub>/vs | ν<sub>50</sub> | 12% ν(Fe-N<sub>pyr</sub>) (ν<sub>50</sub>) + 9% τ(Fe-N<sub>His</sub>) + 5% τ(Fe-S<sub>Met</sub>) + τ (pp) + δ(pp) + τ (Prop) |
| 340    | /mw  | ip/oop = 1:1 | ν(Fe-N<sub>pyr</sub>)/Pyr. tilt/δ(C<sub>p</sub>-C<sub>sym</sub>) | 8% ν(Fe-N<sub>pyr</sub>) + Pyr. tilt + δ(C<sub>p</sub>-C<sub>sym</sub>) + 5% δ(C<sub>b</sub>-C<sub>as</sub>-S<sub>2</sub>)pyr<sub>¬</sub> + 5% δ(Prop) + 2% τ (His) |
| 347    | /s   | ip | Pyr. tilt/δ(C<sub>p</sub>-C<sub>sym</sub>) | Pyr. tilt + δ(C<sub>p</sub>-C<sub>sym</sub>) + 4% ν(Fe-N<sub>pyr</sub>) + 2% τ(Fe-N<sub>His</sub>) + 4% τ(His) |
| 349    | /ms  | ip | τ(pp)/γ(N-H)<sub>pp</sub> | 20% τ(pp) + 18% δ(pp) + 16% γ(N-H)<sub>pp</sub> + ν(pp) + δ(Met) + δ(C<sub>p</sub>-C<sub>sym</sub>) |
| 351    | /ms  | ip/oop = 3:1 | τ(pp)/δ(Met) | 33% τ(pp) + 13% δ(Met) + 10% δ(pp) + 11% γ(N-H)<sub>pp</sub> + ν(pp) |
| 355 (na/-1/-5) | 355 | E<sub>2</sub>/vs | ν<sub>50</sub>/Pyr. tilt/ | 12% ν(Fe-N<sub>pyr</sub>) (ν<sub>50</sub>) + 11% τ(Fe-N<sub>His</sub>) + 10% δ(C<sub>p</sub>-C<sub>sym</sub>) + 8% |
| 366 (−2/+3/-3) | 365 | A2/s | oop | δ(C=O)_{C_\text{sym}} | δ(Met) + 4% δ(Fe- S_{Met-C}) + 2% δ(C_{2-S}C_{3}PyrII) + τ (pp) + Pyr. tilt | γ_6 | Pyr. tilt (γ_6) + 5% v(Fe-N_{His}) + 5% v(Fe-S_{Met}) + 3% δ(pp) + τ (pp) | 369, E_{uu}, ν_{50} |
| 375 (na/na/-1) | 370 | /m | ip/oop = 1:4 | Pyr. swiv/δ(pp) | 16% δ(pp) + 8% v(C_{2-S}C_{3}PyrII + Pyr. swivel + Pyr. tilt + 7% δ(C_{3-C}=C_{4}S_{1}PyrII + 2% v(Fe-N_{Pyr}) + τ (pp) |
| 385 (−7/-3/-1) | 380 | /ms | ip | v(Fe-N_{Pyr})/His. rot/Pyr. tilt/δ(C_{2-S}C_{3}PyrII | 9% v(Fe-N_{Pyr}) + 6% His. rot. + Pyr. tilt + 4% τ(Fe-N_{His}) + 3% δ(C_{2-S}C_{3}PyrII + 3% δ(C_{3-C}=C_{4}S_{1}PyrII + 3% δ(pp) + 2% δ(Fe-S_{Met-C}) + 2% τ (His) + δ(C_{β-C}_{\text{sym}} |
| 397 (−5/0/-3) | 396 | /ms | ip/oop = 10.5:1 | Pyr. tilt/Pyr. swiv/v(Fe-N_{Pyr}) | Pyr. swivel + Pyr. tilt + 4% v(Fe-N_{Pyr}) + 2% τ (His) + 2% τ(Fe-N_{His}) + v(pp) + δ(pp) |
| 418 (−12/+1/+1) | 412 | /w | ip | δ(C_{β-C}=C_{3}b_{PyrII}/v(Fe-N_{Pyr})/δ(C_{β-C}_{\text{sym}} | 9% δ(C_{β-C}=C_{3}b_{PyrII} + 8% v(Fe-N_{Pyr}) + 8% δ(C_{β-C}_{\text{sym}} + 4% δ(S_{2-C}=C_{3}b_{PyrII} + Pyr. tilt |
| 419 | 412/418, δ(C_{β-C}=C_{3}b_{}) | /w | ip | v(Fe-N_{Pyr})/γ(N-H)_{pp}/Pyr. swiv | 5% v(Fe-N_{Pyr}) + δ(pp) + His. rot. + 8% γ(N-H)_{pp} + Pyr. swivel + δ(C_{β-C}_{\text{sym}} |
| 464 | 467 | /w | ip | Pyr. rot | Pyr. rot + v(pp) + δ(pp) |
| 485 | 494 | /w | ip | Pyr. rot | Pyr. rot |

\(^{a}\)(Δ) = shifts in the M13V, M13V/K22M, and A7F variants. \(^{b}\)Based on the BP86/LanL2DZ* calculation. \(^{c}\)Symmetry (sym, in D_{4h}) and calculated intensity (int): vs = very strong, s = strong, ms = medium strong, m = medium, mw = medium weak, w = weak, vw = very weak. \(^{d}\)Abbreviations: Prop = propionate groups, Met = methionine, His = histidine, Pyr = pyrrole, pp = polypeptide, v, stretch; δ, bend; τ, torsion; γ, ip orientation. Classification of porphyrin modes by Spiro and co-workers: γ_7, oop meso carbon wag; v_{50}, v(Fe-N_{Pyr}) (E_{a}); v_{52}, δ(C_{β-Y})_{sym} (E_{a}); γ_{6}, oop Pyr. tilting (A_{2a}); γ_{24}, γ(C_{4-C}_{m}) (E_{g}); v_{33}, Pyr. rotation (B_{2g}); ν_{9}, δ(C_{β-Y})_{sym} (A_{1g}); v_{8}, v(Fe-N_{Pyr}) (A_{1g}); v_{51}, δ(C_{β-Y})_{asym} (E_{a}). \(^{e}\)Refs. 10 and 4.
Table S6. Complete list of force constants of ferric *Ht* Cyt *c* variants invoked in the fit of the NRVS data, based on the BP86/LanL2DZ* result.

| Force constant | wt | M13V | M13V/K22M | A7F |
|----------------|----|------|-----------|-----|
| Fe-S<sub>Met</sub> [1] | 0.8404 | 0.8100 | 0.8254 | 0.8404 |
| Fe-N<sub>His</sub> | 1.2493 | 1.3165 | 1.3203 | 1.2293 |
| Fe-N<sub>Pyr</sub> [13-16] | 1.6981, 1.6771, 1.6961, 1.8058 | 1.6981, 1.6771, 1.6961, 1.8260 | 1.6981, 1.6971, 1.6961, 1.7558 | 1.6881, 1.6771, 1.6861, 1.8058 |
| ν(pp) | 5.2754, 6.7916, 3.6215, 5.0548, 5.2754, 6.7916, 3.6215, 5.0548, 6.9429, 3.9030, 5.0051, 5.2322, 4.1163, 3.8993, 2.9974, 2.6538, 5.8847, 5.8881, 2.8990, 2.5852 | 5.2754, 6.7916, 3.6215, 5.0548, 6.9429, 3.9030, 5.0051, 5.2322, 4.1163, 3.8993, 2.9974, 2.6538, 5.8847, 5.8881, 2.8990, 2.8852 |
| ν(His) | 5.2754, 6.7916, 3.6215, 5.0548, 6.9429, 3.9030, 5.0051, 5.2322, 4.1163, 3.8993, 2.9974, 2.6538, 5.8847, 5.8881, 2.8990, 2.8852 | 5.2754, 6.7916, 3.6215, 5.0548, 6.9429, 3.9030, 5.0051, 5.2322, 4.1163, 3.8993, 2.9974, 2.6538, 5.8847, 5.8881, 2.8990, 2.8852 |
| pp H-bonds [154-155] | 0.0750, 0.2005 | 0.1050, 0.2205 | 0.1595, 0.2315 | 0.0750, 0.2005 |
| N<sub>His</sub>-Fe-N<sub>Pyr</sub> [186-189] | 0.7022, 0.9032, 0.9030, 0.9145 | 0.7122, 0.9193, 0.9030, 0.9345 | 0.7222, 0.9233, 0.9230, 0.9396 | 0.7022, 0.8933, 0.8930, 0.9145 |
| δ(pp) | 0.9057, 0.9968, 0.9963, 0.6531, 0.8320, 0.8208, 0.2548, 0.8921, 0.6463, 0.8197, 0.5601, 0.6001, 0.5529, 0.7100, 0.7893, 0.4034 | 1.2005, 0.9998, 0.9993, 0.6631, 0.8320, 0.8208, 0.2548, 0.9021, 0.6463, 0.8197, 0.5601, 0.7001, 0.5529, 0.7400, 0.8593, 0.4034 | 1.1199, 0.9868, 0.8006, 0.6731, 0.8320, 0.8208, 0.3548, 0.9621, 0.6463, 0.8197, 0.5601, 0.6501, 0.5529, 0.7100, 0.8793, 0.4034 | 0.9057, 0.9568, 0.9463, 0.6531, 0.8320, 0.8208, 0.2548, 0.8921, 0.6463, 0.8197, 0.5601, 0.6001, 0.5529, 0.7100, 0.7893, 0.4034 |
| δ(His) [371, 372, 377, 378, 385, 391, 397] | 0.8901, 0.8640, 0.4356, 0.5001, 0.7027, 1.0884, 0.8095 | 0.8800, 0.8201, 0.4555, 0.4302, 0.5027, 1.0000, 0.8595 | 0.9000, 0.8200, 0.4256, 0.4602, 0.7227, 1.0000, 0.8595 | 0.9000, 0.8740, 0.4356, 0.4902, 0.6827, 1.0185, 0.7895 |
| τ(Fe-S<sub>Met</sub>), τ(Fe-N<sub>His</sub>) [458, 462] | 0.3879, 0.8214 | 0.3879, 0.7700 | 0.3879, 0.7654 | 0.3879, 0.8114 |
| τ(His) | 0.1358, 0.8600, 1.0601, 0.6909, 0.2142, 3.2152, 1.1596, 0.1846 | 0.2158, 0.8400, 1.070, 0.6009, 0.2542, 3.2452, 1.0596, 0.2846 | 0.1458, 0.8800, 1.0900, 0.6909, 0.2442, 3.2952, 1.0500, 0.1846 | 0.1358, 0.8600, 0.9810, 0.6409, 0.2042, 3.1952, 1.1696, 0.1946 |
| τ(pp) | 0.5540, 0.3329, 0.2918, 0.2998, 0.1451, 0.1957, 0.3853, 0.1913, 0.2549, 0.3168, 0.2727, 0.1061 | 0.5540, 0.3289, 0.3218, 0.2998, 0.1451, 0.2957, 0.4253, 0.2113, 0.2849, 0.3568, 0.3227, 0.1061 | 0.5540, 0.2829, 0.3218, 0.2998, 0.2451, 0.3057, 0.4353, 0.2213, 0.2949, 0.3668, 0.3327, 0.1161 | 0.5540, 0.3329, 0.2918, 0.2998, 0.1451, 0.1957, 0.3853, 0.1913, 0.2549, 0.3168, 0.2727, 0.1061 |

*a*The unit for the force constants of stretching modes is mdyn/Å; for bending modes and torsions it is mdyn·Å.
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