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Exploring second coordination sphere effects in nitric oxide synthase

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Abstract

Second coordination sphere (SCS) effects in proteins are modulated by active site residues and include hydrogen bonding, electrostatic/dipole interactions, steric interactions, and π -stacking of aromatic residues. In Cyt P450s, extended H-bonding networks are located around the proximal cysteinate ligand of the heme, referred to as the ‘Cys pocket’. These hydrogen bonding networks are generally believed to regulate the Fe–S interaction. Previous work identified the S(Cys) $\rightarrow$ Fe σ CT transition in the high-spin (hs) ferric form of Cyt P450cam and corresponding Cys pocket mutants by low-temperature (LT) MCD spectroscopy [Biochemistry 50:1053, 2011]. In this work, we have investigated the effect of the hydrogen bond from W409 to the axial Cys ligand of the heme in the hs ferric state (with H₄B and L-Arg bound) of rat neuronal nitric oxide synthase oxygenase construct (nNOSoxy) using MCD spectroscopy. For this purpose, wt enzyme and W409 mutants were investigated where the H-bonding network with the axial Cys ligand is perturbed. Overall, the results are similar to Cyt P450cam and show the intense S(Cys) $\rightarrow$ Fe σ CT band in the LT MCD spectrum at about 27,800 cm⁻¹, indicating that this feature is a hallmark of {heme-thiolate} active sites. The discovery of this MCD feature could constitute a new approach to classify {heme-thiolate} sites in hs ferric proteins. Finally, the W409 mutants show that the hydrogen bond from this group only has a small effect on the Fe–S(Cys) bond strength, at least in the hs ferric form of the protein studied here.

Graphical abstract

Low-temperature MCD spectroscopy is used to investigate the effect of the hydrogen bond from W409 to the axial Cys ligand of the heme in neuronal nitric oxide synthase. The intense S(Cys) $\rightarrow$ Fe σ -CT band is monitored to identify changes in the Fe–S(Cys) bond in wild-type protein and W409 mutants

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Keywords

Cytochrome P450; Nitric oxide synthase; Second coordination sphere effects; Magnetic circular dichroism; Electronic Spectra; Heme

Introduction

Cytochrome P450s (Cyt P450s) belong to a super-family of *b*-type heme-containing enzymes found in various forms of life [1–4]. The hallmark of these enzymes is the axial thiolate (cysteinate) coordination to the heme iron. Cyt P450s generally activate O₂ to catalyze diverse types of reactions, including hydroxylations and heteroatom oxidations of many natural or non-natural substrates. Closely related to the Cyt P450s with respect to active site properties and reactivity are nitric oxide synthases (NOSs) [1, 4–11] which are responsible for the biosynthesis of the diatomic gas nitric oxide (NO) in mammals via the two-step hydroxylation and oxidation of L-arginine. For the purpose of signaling, NO is produced by endothelial NOS (eNOS) and neuronal NOS (nNOS) for blood pressure control and nerve signal transduction, respectively [12]. In addition, NO is generated in macrophages by inducible NOS (iNOS) for the purpose of immune defense [13–17]. The fascinating and perplexing versatility of all of these enzymes with respect to their catalyzed reactions requires a subtle fine-tuning of their {heme-thiolate} active sites. This fine-tuning is generally facilitated by second coordination sphere (SCS) interactions within the heme pocket. Here, subtle effects of active site residues on the axial ligand properties (potentially including the substrate), the heme conformation, and the heme propionate side chains are thought to mediate the electronic structure, redox potential, and reactivity of the heme [18]. In this way, SCS effects allow principally similar active sites, like the {heme-thiolate} active sites in all members of the Cyt P450 and NOS families, to perform a surprisingly diverse range of functions. This requires adjustments in the electronic structures, redox potentials, and reactivities of the {heme-thiolate} catalytic units in the different enzymes. Despite this central importance of SCS effects for Cyt P450 and NOS functions [19], however, it has been very difficult to obtain *quantitative* insight into how exactly SCS effects moderate the electronic structures and properties of the heme protein active sites. In particular, it has frequently been observed that changes in the hydrogen bonding network of heme-axial ligands can have a distinctive effect on redox potential, but it is not clear whether this relates to changes in the heme-axial ligand bond strength, the ground state of the heme, and/or the heme conformation, etc., and whether the ferrous or ferric oxidation state (or both) is primarily affected by the change.

SCS effects in proteins and transition metal complexes correspond to interactions of the metal center and its primary ligands with groups that are not part of the intimate coordination environment of the metal [18]. In proteins, SCS effects are usually mediated by amino acid side chains in the substrate binding (active site) pocket of the protein. The most important types of interactions are hydrogen bonding, electrostatic (between charged groups) and dipole interactions, steric interactions, and π -stacking of aromatic side chains. These interactions are used by proteins for substrate recognition (binding and precise orientation in the active site), for fine-tuning of ligand donor strengths and redox potentials of transition metal centers, to enforce unnatural coordination geometries on transition metal complexes (the entatic state), for proton and electron transfer, etc. The most important SCS interaction is hydrogen bonding. A well-known example in this respect is the distal His in hemoglobin (Hb) and myoglobin (Mb), which forms a hydrogen bond with dioxygen bound to the heme iron, and in this way, stabilizes the oxy-Hb/oxy-Mb complex. In heme proteins, hydrogen bonds to the heme's proximal ligand(s), bound substrates, and the propionate side chains of the heme are most common, although it is unclear whether the latter interaction contributes to a fine-tuning of the heme properties itself [20].

Cyt P450s generally exhibit a hydrogen bonding network around the proximal Cys ligand (the 'Cys' pocket) as shown in Fig. 1 that is thought to regulate the Fe–S interaction. In fact, site-directed mutagenesis of Cys pocket residues in Cyt P450cam to alter the hydrogen bonding network of the axial Cys ligand leads to pronounced effects on the heme redox potential [21]. Compared to wild-type (wt) Cyt P450cam with an Fe(III)/Fe(II) redox potential of –134 mV, the variants L358P and Q360L (in which one amide hydrogen bond is removed) possess corresponding redox potentials of –170 and (one amide and one backbone hydrogen bond removed) the –180 mV, and for Q360P Fe(III)/Fe(II) redox potential drops to –205 mV [21]. The redox potential of the five-coordinate (5C), high-spin (hs) ferric heme is of key importance for the catalytic mechanism of Cyt P450s, as this state receives an electron from either another redox cofactor in the protein or an external electron source, leading to the reduction of heme, and hence, activation for O₂ binding as the next key step of catalysis [2, 3, 22]. Despite this central role for Cyt P450 catalysis, it is not known how exactly the hydrogen bonding network of the heme proximal pocket modulates the redox potential [23]. Possible explanations are that the hydrogen bonds primarily affect changes in Fe–S covalency, that the ground state of the heme is affected, or that the changes in the hydrogen bonding network alter the electrostatic environment of the heme. Work from the Lu group on blue copper proteins shows that electrostatics can in fact have a pronounced effect on redox potential [24]. Resonance Raman (rR) studies by Morishima and coworkers on Cyt P450cam show identical Fe–S stretching frequencies for high-spin ferric wt, L358P and Q360L proteins at 351 cm^{–1} and a small downshift of this mode by 4 cm^{–1} in Q360P [21]. This indicates that the amide hydrogen bonds from L358 and Q360 only play a minor role for the strength of the Fe–S bond in the high-spin ferric form of the enzyme. In addition, the observed shift in Q360P is actually opposite to the trend that would be expected if the removal of the hydrogen bonds would strengthen the Fe–S interaction as expected. This might be due to the fact that removal of two hydrogen bonds in Q360P could lead to a minor rearrangement of the Cys pocket conformation. Using magnetic circular dichroism (MCD) spectroscopy in combination with DFT calculations, we identified the S(Cys) $\rightarrow$ Fe σ

charge-transfer (CT) transition around 28,000–29,000 cm^{-1} to higher energy of the Soret band in high-spin ferric Cyt P450cam and the above-mentioned mutants [23]. Given the small discrepancy in the energy of this sulfur-to-iron CT band between wt Cyt P450cam and the mutants L358P and Q360P, the effect of the hydrogen bonds from L358 and Q360 on the properties of the Fe–S bond is small in the ferric oxidation state [23, 25]. The main role of the “Cys” pocket therefore seems to be the stabilization (against diatomics and protonation) and pre-orientation (with respect to the iron center) of the thiolate group of Cys, and to a much lesser extent a fine-tuning of the Fe(III)–S(Cys) bond strength. Similar investigations have also been performed on Cyt P450 2B4 via rR spectroscopy, and again, the observed effect of Cys pocket mutants on the Fe–S stretching vibration is relatively small (about 6 cm^{-1}), indicating an overall small change in the Fe–S interaction [26]. Small effects were also reported for the Fe–CO stretch in the low-spin ferrous CO adducts, indicating that the hydrogen bonds to the axial Cys ligand might not have a strong effect on the Fe–S bond in the ferrous state either [26]. Similar observations were made for the CO adducts of Cyt P450cam and the above-mentioned mutants [21].

Structurally, mammalian *NOS isozyme* is a homodimer (α_2), where each monomer (α) contains an oxygenase domain (NOSoxy) and a reductase domain. Additionally, calmodulin (CaM) binding to a linker between the oxygenase and reductase domains activates NO synthesis in eNOS/nNOS [27–30]. The oxygenase domain, which catalyzes the conversion of L-arginine (L-Arg) to citrulline and NO, contains the {heme-thiolate} active site. A key feature of the NOS active site not observed in Cyt P450s is the presence of one strong, dominating hydrogen bond between the heme cysteinate ligand and a nearby tryptophan (Fig. 2) that is believed to control the Fe–S bond strength and regulate NOS activity [31–34]. Additionally, one of the heme propionate side chains is hydrogen-bonded to both the tetrahydrobiopterin (H_4B) cofactor and the substrate L-Arg [35]. Site-directed mutagenesis studies on mammalian and bacterial NOSs to remove or alter the Trp hydrogen bond to the axial thiolate ligand have been performed. Couture and coworkers studied the W to F, Y, and H mutants of bacterial NOS from *Staphylococcus aureus* (where the F and Y mutants eliminate the hydrogen bond to Cys (see “Results and Discussion”), whereas for the H mutant, the hydrogen bond is presumably stronger [36]), and found that although these mutants alter the redox potential of the heme by over 100 mV, the effect on the Fe–CO stretching mode in the low-spin ferrous form of the enzyme is very small ($\pm 2\text{--}3\text{ cm}^{-1}$ relative to wt) [37]. A similar set of mutants was investigated for bacterial NOS [38] from *Bacillus subtilis*, again leading to the observation of very similar Fe–CO stretching frequencies with $\nu(\text{Fe-CO}) = 501\text{ cm}^{-1}$ in wt enzyme, and $\nu(\text{Fe-CO}) = 500, 501.5$ and 504.5 cm^{-1} in W66F, W66Y and W66H, respectively [39]. Similar studies on the ferric NO complexes of wt rat nNOSoxy and the W to F and Y mutants have also been performed [33].

The present study investigates the effect of the hydrogen bond from W409 to the axial Cys ligand of the heme (see Fig. 2) in the high-spin ferric state (with H_4B and L-Arg bound) of rat nNOSoxy using low-temperature (LT) MCD spectroscopy. As mentioned above, we have previously shown for Cyt P450cam that the S(Cys) $\rightarrow$ Fe(III) σ CT transition in the high-spin ferric state gives rise to an intense, negative MCD feature to higher energy of the Soret band [23]. Although this is similar to the Cl $\rightarrow$ Fe(III) CT band in a model complex [40], there is currently only one example for this assignment. In order to determine whether this is

a general hallmark of {heme-thiolate} sites in the high-spin ferric state, we have now extended our studies to nNOS. In addition, using the mutants W409F and W409Y (hydrogen bond eliminated) and W409H (hydrogen bond strengthened [36]), we further evaluated whether the presence of the W409–S(Cys) hydrogen bond influences the properties of the Fe(III)–S(Cys) bond in nNOS. Note that Dawson and coworkers reported the MCD spectra of wt, W409F, and W409Y nNOSox at room temperature (RT) [41, 42]. However, RT MCD data lack the important *C*-term signals (since their intensity is inversely proportional to temperature) and, in particular, do not show the intense S(Cys) → Fe(III) CT transition, which is a pure *C*-term feature that can only be observed at cryogenic temperatures.

Experimental procedures

Expression and purification of nNOS and corresponding mutants

The rat nNOSox mutants were prepared by site-directed mutagenesis on a pCWori⁺ vector containing cDNA of the wt nNOSox construct. The forward primers for the mutations are (mutated bases are underlined): GCGCCAAGCATGCCTATCGGAACGCCTCTCGATGTG (W409Y), GCGCCAAGCATGCCTTTTCGGAACGCCTCTCGATGTG (W409F), and GCGCCAAGCATGCCCAACCGGAACGCCTCTCG (W409H). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Agilent Technologies-Stratagene, La Jolla, CA). The mutated plasmids were confirmed by DNA sequencing at the University of New Mexico DNA Research Services. The expression and purification of wt nNOSox and the corresponding mutants, W409F, W409Y, and W409H, were carried out as reported earlier [43, 44].

UV–Vis spectroscopy

Electronic absorption spectra were measured on an Analytical Jena Specord S600 instrument at room temperature. The UV–Vis and MCD spectra of the nNOS proteins were taken in a pH 7.2 buffer (100 mM bis–tris propane, 200 mM NaCl, 40 μM H₄B, 1 mM DTT, and ~50 % ethylene glycol). Ethylene glycol is added to the protein samples to make an optically transparent glass for MCD measurements (glycerol cannot be used as it causes instability of the protein) [45, 46]. The protein concentration for the MCD samples was typically 25–50 μM, and 1–5 mM L-arginine was added to create the high-spin ferric form of the proteins. Hence, for the low-spin ferric complexes, the (+H₄B, -L-Arg) forms were used, whereas all measurements on the high-spin forms of the proteins were conducted on the (+H₄B, +L-Arg) forms.

MCD spectroscopy

The MCD setup employs an Oxford SM4000 cryostat and a JASCO J-815 CD spectrometer. The SM4000 cryostat consists of a liquid helium-cooled superconducting magnet providing horizontal magnetic fields of 0–7 T. The J-815 spectrometer uses a gaseous nitrogen-cooled xenon lamp and a detector system consisting of two interchangeable photomultiplier tubes in the UV–Vis and NIR range. The samples were loaded into a 1.5–300 K variable-temperature insert (VTI), which offers optical access to the sample via four optical windows made from Suprasil B quartz. The MCD spectra were measured in $[\theta] = \text{mdeg}$ and converted into MCD extinction coefficients, ϵ [$\text{M}^{-1}\text{cm}^{-1}\text{T}^{-1}$], using the conversion factor $\epsilon = \theta'$

($32980 \cdot \epsilon \cdot d \cdot B$), where c is the concentration, B is the magnetic field, and d is the path length [40]. The product $\epsilon \cdot d$ can be substituted by $A_{\text{MCD}}/\epsilon_{\text{UV-Vis}}$, where A is the absorbance of the sample measured by the CD spectrometer, and $\epsilon_{\text{UV-Vis}}$ is the extinction coefficient from UV-Vis. The conversion is based on the Soret band of the heme. The spectra were recorded at different temperatures (2, 4, 8, 12, 20, and 50 K) and magnetic fields (0–7 T). Experiments were conducted by varying the field at the respective temperatures.

Gaussian deconvolution of the spectra

Gaussian fitting of the UV-Vis and MCD spectra was carried out using the program PeakFit (version 4.12). The smallest number of Gaussian functions necessary to fit the data was used for the analysis, and the quality of the fits was accessed by χ^2 .

Results and discussion

Substrate-free ferric nNOS

In the wild-type (wt) ferric resting form of rat nNOSoxy and the corresponding mutants (Trp409Phe, Trp409Tyr, and Trp409His), the heme is primarily five-coordinate (5C) and high-spin (hs) with a sextet ground state ($S = 5/2$) at RT [47]. This is different from Cyt P450cam where an exogenous water molecule is bound to the iron center in the absence of the substrate, causing the heme to be six-coordinate (6C) and low-spin (ls) with a doublet ground state ($S = 1/2$). The resting ferric heme complex of nNOSoxy has been extensively characterized by various spectroscopic methods, including UV-Vis, EPR, and rR spectroscopy, which all confirm the hs state of the heme (~90 % based on EPR spin quantification) [33, 42, 47]. In the wt and variant proteins, the UV-Vis spectra (Fig. S1) measured at room temperature in bis-tris propane buffer containing ~50 % (v/v) ethylene glycol (used as a glassing agent for MCD measurements) are consistent with these literature reports [41, 42]. However, at cryogenic temperatures the Soret band shifts from 395 to 416 nm (see Fig. S2) in wt protein and the mutants in the absence of substrate, which is characteristic for the ls ferric heme complex of {heme-thiolate} active sites. We speculate that entropy-driven binding of a water or ethylene glycol molecule to the heme at low temperature causes the observed spin state change of the heme in nNOSoxy in the absence of L-Arg (analogous observations have been made for Cyt P450s; see Ref. [21] as an example). Figure 3 shows the low-temperature (LT) MCD spectra of wt, W409F, W409Y, and W409H nNOSoxy. The most intense features in the spectra are observed in the Soret band region. Here, the Soret transition gives rise to two main C -term bands at ~23,500 and ~24,500 cm^{-1} with a pattern of $- \epsilon / + \epsilon$ intensities (from lower to higher energy). These features are extremely intense with the negative component of the wt protein showing a ϵ of $-5.50 \text{ mM}^{-1}\text{cm}^{-1}$ at 2 K/7 T, which corresponds to a magnetic extinction coefficient of about $0.8 \text{ mM}^{-1}\text{cm}^{-1}\text{T}^{-1}$ (at 5 K). This is indicative of a low-spin ferric heme with the $[d_{xy}]^2[d_{xz}, d_{yz}]^3$ ground state, due to the very effective spin-orbit coupling between the d_{xz} and d_{yz} orbitals in z direction (the heme normal), giving rise to strong C -term intensity. This is discussed in detail in Ref. [40]. These findings are in agreement with previous reports on the LT MCD spectra of ls ferric Cyt P450cam [23]. Notably, the feature at ~23,500 cm^{-1} in W409H shows a much lower magnetic extinction coefficient compared to wt and the other two mutants. The generally low signal intensity for the W409H mutant suggests that this is

an artifact caused by glass strain in the sample, leading to depolarization of the circular polarized light. More noticeable differences between the MCD-spectral features of these proteins are observed in the Q band region as shown in the right panel of Fig. 3. Finally, there are no distinct bands observed to higher energy of the Soret band, where the intense, negative *C*-term band of the S(Cys) → Fe(III) CT transition is observed in high-spin ferric Cyt P450cam [23].

In summary, the spectra of ls ferric nNOSoxy and of the mutants closely resemble the spectra of ls ferric Cyt P450cam, and are not distinct from LT MCD spectra of other ls ferric heme proteins [48–50]. No features that can be specifically attributed to S(Cys) → Fe(III) transitions are discernible. Further analysis of the variable-temperature variable-field (VTVH) saturation isotherms is hampered by the small amount of magnetic anisotropy for $S = \frac{1}{2}$ ground states. Hence, analysis of the VTVH data to determine the polarizations of the corresponding electronic transitions is not possible in this case [25]. This stresses the importance of using the hs form of the protein to investigate the effect of changes in the hydrogen bonding network of the axially bound Cys residue on the properties of the Fe(III)–S(Cys) bond. Nevertheless, this is the first report of the LT MCD spectra of ls ferric nNOSoxy wt and variant proteins.

High-spin ferric nNOS with L-arginine bound in the distal pocket

The addition of the substrate, L-arginine, results in a five-coordinate, high-spin ferric {heme-thiolate} complex in the active site of nNOSoxy at both RT and LT. Prior to the MCD measurements, the nNOSoxy variants with H₄B and L-arginine bound (the (+H₄B, L-Arg) form, in bis-tris propane buffer containing ~50 % (v/v) ethylene glycol) were tested with UV–Vis spectroscopy at room temperature, resulting in spectra identical to those reported previously, which confirms the ferric hs state at room temperature [41]. Previous characterization of these heme proteins by rR spectroscopy also shows that the L-arginine bound ferric form of rat nNOS is predominantly hs at RT, both for the wt protein and Trp409 mutants [33]. Cooling the (+H₄B, +L-Arg) forms to cryogenic temperatures preserves the hs state, as shown by EPR studies [47].

Figure 4 shows the UV–Vis and LT MCD spectra of wt ferric nNOSoxy, together with a correlated Gaussian fit of these data. From the fit, 12 electronic transitions are identified. The parameters of the individual optical bands are listed in Table 1. The MCD data are remarkably similar to those of hs ferric Q360P Cyt P450cam, a variant that remains 100 % hs at cryogenic temperatures [23]. This indicates, as pointed out above, that wt ferric nNOSoxy in the (+H₄B, +L-Arg) form remains predominantly hs at cryogenic temperatures, and only contains a negligible amount of the ls form, if any at all. The optical data for wt ferric nNOSoxy can therefore be assigned based on the previous studies on Cyt P450cam. In the Q band region, five low-intensity features are identified in the MCD data of wt ferric nNOSoxy at 15,477, 16,553, 17,389, 18,135 and 20,058 cm^{–1} (bands 1–5; Fig. 4). The prominent bands 4 and 5 are assigned to the two components of the Q band, which show orthogonal polarizations (in the *x,y* plane; with the *z* axis being the heme normal and pointing roughly along the Fe–S(Cys) vector) and spin–orbit coupling between the two excited states, leading to the observed “pseudo *A*-term” signal with a– *ε*/+ *ε* intensity

pattern (from lower to higher energy) [51]. These features correspond to the broad Q-band centered around $19,000\text{ cm}^{-1}$ in the absorption spectrum. In Q360P Cyt P450cam, the corresponding Q-band features are observed at almost identical energies [23]. To higher energy, the MCD data of wt ferric nNOSoxy exhibit two positive bands at 21,157 and 22,228 cm^{-1} that slightly tail into the Soret region of the spectrum (bands 6 and 7 in Fig. 4 and Table 1). These are assigned to $\pi \rightarrow \pi^*$ transitions from lower-lying porphyrin(π) orbitals into the e_g -symmetric LUMO (in ideal D_{4h} symmetry) of π^* character of the porphyrin macrocycle [23]. Notably, the intense Soret band of the heme usually gives rise to 2–3 very intense MCD features in the LT data [25, 50]. This is also the case for ls ferric {heme-thiolate} proteins as discussed above. However, the hs ferric case of {heme-thiolate} active sites represents a notable exception to this rule, as first described for Cyt P450cam [23]. In this case, the Soret band at 391 nm in the absorption spectrum corresponds to three comparatively weak bands in the MCD spectrum (compared to ls and other hs ferric hemes) with a $- \epsilon/+ \epsilon/- \epsilon$ intensity pattern (from lower to higher energy). The data for hs ferric nNOSoxy are consistent with these previous findings, and provide further support for the idea that this is indeed a hallmark of hs ferric {heme-thiolate} complexes. In the nNOSoxy case, the Soret band at 396 nm can be deconvoluted into three MCD features at 23,512, 24,211, and 25,481 cm^{-1} (bands 8–10 in Fig. 4 and Table 1) that again show the alternating pattern of $- \epsilon/+ \epsilon/- \epsilon$ intensities. Here, band 10 appears as a shoulder on the more intense band 11. Note that the ls form does not have any negative features in this spectral region, so band 10 does indeed originate from the hs form. In theory, the Soret band should only give rise to a pseudo- A term signal with one positive and one negative signal, just like the Q band discussed above. However, our previous work on Cyt P450cam shows that the three-band pattern arises from a splitting of the negative component of the Soret transition into two features via selective mixing with a number of porphyrin $\rightarrow$ Fe(III) CT transitions [23]. Our new results on nNOSoxy confirm this previous finding. Interestingly, a similar three-band pattern of the Soret band is also observed for the hs ferric model complex [Fe(TPP)(Cl)] [40]. Finally, the very intense, negative band at 27,841 cm^{-1} (band 11) to higher energy of the Soret band is assigned to the S(Cys) $\rightarrow$ Fe(III) σ CT transition, again based on the appearance of an analogous feature in hs ferric Cyt P450cam [23]. In the latter case, this assignment was achieved by analyzing the MCD VTVH saturation curves for this transition, which show a large degree of z polarization for this feature [23]. Figure S3 shows a direct comparison of the VTVH data for this band for Cyt P450cam and nNOSoxy, illustrating the similar polarizations for this transition in the two enzymes, which further supports this assignment. Note in this regard that the ZFS parameters for the hs ferric forms of Cyt P450cam and nNOSoxy are quite similar [47]. On the other hand, the UV-Vis feature that corresponds to the S(Cys) $\rightarrow$ Fe σ CT transition is buried under the intense Soret band (see fit in Fig. 4). This demonstrates the utility of LT MCD spectroscopy to deconvolute overlapping optical features that are otherwise hard to identify by UV-Vis spectroscopy. With the LT MCD spectrum of wt ferric nNOSoxy analyzed, we can now use band 11 to examine whether changes in the hydrogen bonding network of the axial thiolate (Cys) ligand affect the Fe(III)-S(Cys) bond, via changes in the energy of this S(Cys) $\rightarrow$ Fe(III) CT feature. For this purpose, we have investigated the nNOS Trp409 variants W409F, W409Y and W409H.

Figures 5 and 6 show the UV–Vis and LT MCD data for the W409F and W409Y variants where the Trp hydrogen bond to the axial Cys415 ligand of the heme has been eliminated (see Fig. 2). Note that it has been proposed that the tyrosine OH group in W409Y is not correctly positioned to form a hydrogen bond with Cys415 [33]. Hence, one might predict that in these cases the increase in charge density on the Cys sulfur would lead to an overall strengthening of the Fe–S bond. The UV–Vis and MCD data of ferric W409F and W409Y nNOSoxy are overall very similar to those of wt enzyme, showing analogous spectral features and band positions. Some intensity differences are observed, for example with respect to the relative intensities of the three MCD components of the Soret band. In particular, W409F (see Fig. 5) does not show a clear MCD feature associated with the third (highest energy) component of the Soret band. However, since all other proteins studied here have this feature, the fit of the spectrum of W409F does include the third component of the Soret band (band 10), which in this case is not well defined from the fit. In both W409F and W409Y, the S(Cys) $\rightarrow$ Fe(III) CT transition is identified as a very prominent, negative feature to higher energy of the Soret band (band 11), with transition energies of 27,740 and 27,651 cm^{-1} , respectively, for W409F and W409Y (see Tables S1 and S2). As in the case of wt enzyme, the amount of ls ferric protein present in the MCD samples is negligible.

Finally, we also investigated the variant W409H, where the newly introduced His has been proposed (in the isozyme iNOS [36]) to make a stronger hydrogen bond to the axial Cys ligand of the heme. This conclusion is based on structural data and the fact that the imidazole ring has a lower pK_a than the indole N–H group, which makes the former a better H-bond donor [39]. This stronger H-bond in W409H should lead to a reduction of the Fe(III)–S(Cys) bond strength, but the magnitude of this effect is not clear [23]. The UV–Vis and LT MCD data of the hs ferric form of W409H nNOSoxy are shown in Fig. 7. These data are again very similar to the nNOSoxy wt and mutant data, indicating that no significant changes have occurred to the active site heme, and that, again, only small amounts of the ls form are present (if any) in the MCD sample. In W409H, the S(Cys) $\rightarrow$ Fe(III) CT transition (band 11) is observed at 27,608 cm^{-1} (Table S3).

Summary and implications

LT and VTVH MCD-spectroscopic investigations on hs ferric nNOSoxy wt protein and W409 mutants were conducted in this work. These data are surprisingly similar to those previously obtained for hs ferric Cyt P450cam and its corresponding Cys pocket mutants. Importantly, this indicates that the obtained spectral features are typical for hs ferric {heme-thiolate} complexes. This includes:

- the appearance of the Q band around 19,000 cm^{-1} as the expected pseudo-*A* MCD signal,
- the observation of the Soret band as a weak MCD signal with a characteristic $- \epsilon/+ \epsilon/- \epsilon$ three-band pattern around 24,000–25,000 cm^{-1} ,
- the presence of a broad, negative and intense feature around 27,000–28,000 cm^{-1} (see Table 2) to higher energy of the Soret band that is strongly *z*-polarized and that corresponds to the S(Cys) $\rightarrow$ Fe(III) σ CT transition.

To examine the potential modulation of the Fe(III)–S(Cys) bond strength in hs ferric nNOSoxy, we can now compare the energies of the S(Cys) → Fe(III) σ CT feature, easily identified in LT MCD, in the spectra of wt, W409F, W409Y, and W409H proteins as shown in Fig. 8 and Table 2. Based on our previous work on Cyt P450cam (see Ref. [23]), it is expected that removal of the hydrogen bonds that stabilize the axial thiolate (Cysteinate) ligand should lead to a decrease of the energy of the S(Cys) → Fe(III) σ CT transition, due to the destabilization of the sulfur 3p donor orbitals. This trend is indeed fulfilled for the W409F and W409Y mutants, where the CT band is observed at 27,740 (W409F) and 27,651 cm⁻¹ (W409Y) relative to wt (27,841 cm⁻¹). Note that the overall magnitude of this energy shift (<200 cm⁻¹) is similar to the observations for Cyt P450cam and Cys pocket mutants, indicating once again that the hydrogen bond to the thi-olate has only a limited effect on the strength of the Fe–S bond in the hs ferric state of {heme-thiolate} active sites. It is interesting to note that the decrease in energy for the S(Cys) → Fe(III) σ CT transition is different for W409F and W409Y, yet both of these amino acid substitutions are expected to remove the relatively strong W409–S(Cys) hydrogen bond (see Fig. 2). This implies that other factors could also play a role for the energy of the CT transition in NOS, which are absent in Cyt P450cam. In particular, W409 shows a strong π stacking interaction with the heme, which is likely altered in the W409F and W409Y mutants. This could lead to slight changes in heme conformation and/or Fe-porphyrin orbital energies, which would explain why W409F and W409Y do not show similar S(Cys) → Fe(III) σ CT transition energies. This is different in Cyt P450cam, where the residues participating in hydrogen bonding to the axial thiolate ligand are not directly interacting with the heme.

Further support for the idea that the changes observed in the S(Cys) → Fe(III) σ CT energies in W409 mutants in NOS are attributed to additional factors besides changes in the hydrogen bonding network of the axial Cys ligand is provided by the data of the W409H mutant. In this case, it is believed that the hydrogen bond between the His side chain and the axial Cys ligand is actually stronger compared to wt enzyme. On the other hand, the His residue shows differences in π -stacking with the heme, at least in the case of iNOS where structural data are available [36]. Based on the increase in hydrogen bonding, the S(Cys) → Fe(III) σ CT energy in W409H would be expected to shift to higher energy, but instead, the CT band shifts to lower energy and is observed at 27,608 cm⁻¹. Clearly, there are other factors at work in NOS influencing the energy of the S(Cys) → Fe(III) σ CT transition, with π stacking to the heme being a possible contributor. It is also possible that the His group in W409H recruits additional water molecules into the proximal pocket. Without crystallographic data for nNOSoxy, this point remains speculative.

Further comparison can be made with reported redox potentials for the W mutants for bacterial NOS from *Staphylococcus aureus* (here the equivalent residue is W56) [37]. The potentials of the Fe³⁺/Fe²⁺ couples are: –303 mV for W56H, –334 mV for wt, and –419/–436 mV for W56F/Y [37]. These results are in agreement with our observation that the F and Y mutants are very similar, whereas the H mutant is different (but keep in mind that these results were reported for *S. aureus* NOS, whereas our results were obtained for rat nNOS). Interestingly, the change in redox potential of W56H (relative to wt) would support the idea that the H-bond between His and the axial Cys ligand is somewhat stronger compared to that of Trp with Cys in wt enzyme, leading to a somewhat weaker Fe–S

interaction in the W56H mutant. Our spectroscopic results for nNOS, on the other hand, do not support the idea of a significantly weaker Fe–S bond in the H mutant. This implies that either the positive shift in redox potential in W56H originates from other factors (like electrostatic potential in the active site, etc.) or that the energy of the S(Cys) to Fe(III) CT band is influenced by other factors like changes in π stacking, etc., as discussed above.

In any case, the observed energy shifts of the S(Cys) $\rightarrow$ Fe(III) σ CT transition in NOS upon alteration of the hydrogen bonding network of the axial Cys ligand are small, around 200 cm⁻¹ and below, as observed for Cyt P450cam [23]. This again emphasizes the fact that the hydrogen bonds to the proximal Cys ligand in the hs ferric state of {heme-thiolate} active sites have only a minor effect on the absolute strength of the Fe–S bond. This is in agreement with the resonance Raman results (see “Introduction”), which also show small shifts of only a few wavenumbers in the Fe(II)–CO stretches of the ferrous CO adducts in the W409 variants [39]. In the case of nNOSoxy, studies on the ferric NO adduct show the Fe–NO stretch at 546 cm⁻¹ [33]. In this case, an increase in Fe–S(Cys) bond strength should lead to a shift of the Fe–NO stretch to lower energy, due to a *trans* interaction between the axial thiolate(Cys) and formally NO⁺ ligands [52–54]. Correspondingly, the W409F and W409Y mutants result in a shift of this mode to 539 cm⁻¹ ($\nu(\text{Fe–NO}) = 7 \text{ cm}^{-1}$) [33], in agreement with a slight strengthening of the Fe–S(Cys) bond in these cases.

In summary, the LT MCD data presented here nicely corroborate our previous studies on Cyt P450cam, and in particular, show that the weak Soret features and the intense (negative) band originating from the S(Cys) $\rightarrow$ Fe(III) σ CT transition previously observed for Cyt P450cam and variants are in fact characteristics of hs ferric heme sites with axial Cys coordination. This is only the second report that describes these spectral features. The discovery of this intense S(Cys) $\rightarrow$ Fe(III) σ CT band that is readily observed via LT MCD enables a completely new approach for the identification and potentially classification of {heme-thiolate} sites in proteins (in the hs ferric state). Additional research on other related enzymes will be conducted in the future to further investigate this point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Proximal hydrogen bonding network of Cyt P450cam (PDB 2CPP). The *yellow* and *blue atoms* represent the sulfur atom of Cys and the main chain amide nitrogen atoms of Leu358, Gly359, and Gln360, respectively. The *dashed lines* represent the NH...S hydrogen bonds with the backbone. The iron center is hidden in the heme macrocycle. The indicated distances are between the corresponding heavy atoms, since the hydrogens are invisible in the crystal structure

Fig. 2.

Left Crystal structure of the ferrous rat nNOS active site showing the *b*-type heme with bound Cys415, L-arginine substrate, and the key tryptophan 409 residue. *Right* Hydrogen bonding between the bound Cys415 and Trp409. Note that the Trp side chain also π stacks with the heme. The images were generated using PyMOL and PDB entry 2G6H. The indicated distance is between the corresponding heavy atoms, since the hydrogens are invisible in the crystal structure

Fig. 3.

Comparison of the MCD spectra of wild-type (*black*), W409Y (*blue*), W409F (*green*), and W409H (*purple*) nNOSoxy, measured at 2 K/7 T in 100 mM bis-tris propane buffer with ~50 % (v/v) ethylene glycol added as a glassing agent. Spectra were measured in the presence of H₄B but the absence of L-Arg, the (+H₄B, -L-Arg) form, leading to a six-coordinate 1s ferric heme at cryogenic temperatures. *Right* a zoomed in overlay of the Q band region of the MCD spectra

Fig. 4.

Electronic spectra of ferric L-arginine-bound wild-type nNOSoxy. *Top* UV-visible absorption spectrum measured at room temperature. *Bottom* MCD spectrum measured at 2 K/7 T in 100 mM bis-tris propane buffer containing 5 mM L-arginine with ~50 % (v/v) ethylene glycol added as a glassing agent. The *blue lines* represent a correlated Gaussian fit of these data (Table 1). The experimental spectra are shown in *black* and the fit is shown in *green*

Fig. 5.

Electronic spectra of ferric L-arginine-bound nNOSoxy W409F. *Top* UV–visible absorption spectrum measured at room temperature. *Bottom* MCD spectrum measured at 2 K/7 T in 100 mM bis–tris propane buffer containing 5 mM L-arginine with ~50 % (v/v) ethylene glycol added as a glassing agent. The *blue lines* represent a correlated Gaussian fit of these data (Table S1). The experimental spectra are shown in *black* and the fit is shown in *green*

Fig. 6. Electronic spectra of ferric L-arginine-bound nNOSoxy W409Y. *Top* UV-visible absorption spectrum measured at room temperature. *Bottom* MCD spectrum measured at 2 K/7 T in 100 mM bis-tris propane buffer containing 5 mM L-arginine with ~50 % (v/v) ethylene glycol added as a glassing agent. The *blue lines* represent a correlated Gaussian fit of these data (Table S2). The experimental spectra are shown in *black* and the fit is shown in *green*

Fig. 7.

Electronic spectra of ferric L-arginine-bound nNOSoxy W409H. *Top* UV-visible absorption spectrum measured at room temperature. *Bottom* MCD spectrum measured at 2 K/7 T in 100 mM bis-tris propane buffer containing 5 mM L-arginine with ~50 % (v/v) ethylene glycol added as a glassing agent. The *blue lines* represent a correlated Gaussian fit of these data (Table S3). The experimental spectra are shown in *black* and the fit is shown in *green*

Fig. 8. Comparison of the S(Cys) $\rightarrow$ Fe(III) σ CT MCD band of wild-type (*black*), W409Y (*blue*), W409F (*green*), and W409H (*purple*) nNOSoxy measured at 2 K/7 T in 100 mM bis-tris propane buffer containing 5 mM L-arginine with ~50 % (v/v) ethylene glycol added as a glassing agent

Table 1

Correlated fit of the UV–visible absorption and MCD spectra of ferric L-arginine bound wild-type nNOSoxy, measured at 2 K/7 T

No	MCD		UV–Vis	
	Energy (cm ⁻¹)	ϵ (mM ⁻¹ cm ⁻¹)	Energy (cm ⁻¹)	ϵ (mM ⁻¹ cm ⁻¹)
1	15,447	0.053		
2	16,553	−0.068	16,560	3
3	17,389	−0.0589	17,378	2
4	18,135	−0.219	18,450	9
5	20,058	0.083	19,974	11
6	21,157	0.185	21,119	5
7	22,228	0.347	22,031	11
8	23,512	−0.27	23,317	24
9	24,211	0.649	24,382	54
10	25,481	−0.131	25,481	74
10a	–	–	26,754	45
11	27,841	−0.589	28,100	39
12	30,891	0.2181	30,924	25

Table 2

Comparison of the energies and intensities (at 2 K/7T) of the S(Cys) $\rightarrow$ Fe(III) σ charge transfer band (band 11) in the MCD spectra of wt nNOSoxy and the corresponding W409 mutants

Protein	Energy (cm ⁻¹)	ϵ (mM ⁻¹ cm ⁻¹)
Wild-type	27,841	-0.589
W409F	27,740	-1.00
W409Y	27,651	-0.70
W409H	27,608	-0.34