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Structure and Reactivity of Chlorite Dismutase Nitrosyls

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Abstract

Ferric nitrosyl ($\{\text{FeNO}\}^6$) and ferrous nitrosyl ($\{\text{FeNO}\}^7$) complexes of the chlorite dismutases (Cld) from *Klebsiella pneumoniae* and *Dechloromonas aromatica* have been characterized using UV-visible absorbance and Soret-excited resonance Raman spectroscopy. Both of these Clds form kinetically stable $\{\text{FeNO}\}^6$ complexes and they occupy a unique region of $\nu(\text{Fe-NO}) / \nu(\text{N-O})$ correlation space for proximal histidine liganded heme proteins, characteristic of weak Fe-NO and N-O bonds. This location is attributed to admixed $\text{Fe}^{\text{III}}\text{-NO}^\bullet$ character of the $\{\text{FeNO}\}^6$ ground state. Cld $\{\text{FeNO}\}^6$ complexes undergo slow reductive nitrosylation to yield $\{\text{FeNO}\}^7$ complexes. The affects of proximal and distal environment on reductive nitrolylation rates for these dimeric and pentameric Clds are reported. The $\nu(\text{Fe-NO})$ and $\nu(\text{N-O})$ frequencies for Cld $\{\text{FeNO}\}^7$ complexes reveal both six-coordinate (6c) and five-coordinate (5c) nitrosyl hemes. These 6c and 5c forms are in a pH dependent equilibrium. The 6c and 5c $\{\text{FeNO}\}^7$ Cld frequencies provided positions of both Clds on their respective $\nu(\text{Fe-NO})$ vs $\nu(\text{N-O})$ correlation lines. The 6c $\{\text{FeNO}\}^7$ complexes fall below (along the $\nu(\text{Fe-NO})$ axis) the correlation line that reports hydrogen-bond donation to N_{NO} , which is consistent with a relatively weak Fe-NO bond. Kinetic and spectroscopic evidence is consistent with the 5c $\{\text{FeNO}\}^7$ Clds having NO coordinated on the proximal side of the heme, analogous to 5c $\{\text{FeNO}\}^7$ hemes in proteins known to have NO sensing functions.

Graphical Abstract

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflict of interest/disclosure

None

Keywords

chlorite dismutase; heme; Raman; NO; reductive nitrosylation

1. Introduction

Chlorite dismutases (Clds) are heme *b* containing enzymes that catalyze the unimolecular decomposition of chlorite (ClO_2^-) to chloride (Cl^-) and molecular oxygen (O_2). [1–2] These enzymes belong to the CDE structural superfamily which contain α/β domain proteins that have a strictly conserved histidine residue responsible for coordinating to the heme iron. [3] The Cld protein family consists of two functional Cld subfamilies. [4–5] Both Cld subfamilies contain a conserved distal pocket comprised of four residues: phenylalanine, leucine, threonine, and arginine. Despite having a conserved heme pocket, the two Cld subfamilies differ in their subunit size and quaternary structure. One Cld subfamily contains pentameric enzymes like the Cld from *Dechloromonas aromatica* (*DaCld*) which are primarily from perchlorate respiring Proteobacteria. [3] These Clds, found in the periplasmic space, catalyze the detoxification of ClO_2^- , the terminal step in (per)chlorate respiration. [6] The second subfamily includes dimeric Clds with a truncated *N*-terminus; they are found in non-perchlorate respiring Proteobacteria and Cyanobacteria. [7–8] These Clds are hypothesized to be located within the cytoplasm, due to their lack of a signal peptide for transfer to the periplasmic space, but have no definitive *in vivo* function. [8] It has been proposed that the dimeric Cld from *Klebsiella pneumoniae* (*KpCld*) protects against environmental perchlorate and chlorate, whose reduction to ClO_2^- is catalyzed by nitrate reductase. [7]

The resting axial ligation states of five-coordinate high spin (5cHS) and 5cHS/six-coordinate high spin (6cHS) heme in ferric *DaCld* and *KpCld*, respectively, indicate differences in their active sites. [7] The catalytic importance of the conformationally flexible, conserved, and cationic distal arginine is confirmed by substantial loss of ClO_2^- degrading activity upon its mutation to alanine or glutamine. [9–10] In *DaCld* the arginine is quite effective in confining hypochlorite (ClO^-) within the pocket as it is not detected during catalytic turnover. [7] In contrast, during the ClO_2^- reaction with *KpCld* and the Cld from *Candidatus Nitrospira defluvii* (*NdCld*), a pentameric Cld, ClO^- escapes from the heme pocket and is readily detected as HOCl . [7,11]

While bacteria containing dimeric Clds lack any (per)chlorate respiration genes, *K. pneumoniae* specifically has the genes to metabolize nitrogen via both nitrification and

denitrification pathways.[12] Common nitrogen metabolites found in both pathways are nitric oxide (NO) and nitrite (NO_2^-). NO is an important signaling molecule in the bacterial processes of biofilm formation, quorum sensing, and symbiosis.[13] Bacteria like *K. pneumoniae* experience high concentrations of NO during the eukaryote response to infection.[14] Bacterial detoxification of high concentrations of NO is achieved by dissimilatory pathways involving reactions catalyzed by various NO and NO_2^- reductases. [15–18]

Many heme proteins are known to interact with NO resulting in a number of reported ligation and oxidations for their respective complexes. For example, NO sensors like mammalian soluble guanyl cyclase (sGC), bacterial nitric oxide sensing (NosP), and heme-nitric oxide / oxygen binding (H-NOX) proteins from *Shewanella oneidensis* form five-coordinate (5c) ferrous NO complexes[19–21] while H-NOX from *Nostoc* sp. and *Thermoanaerobacter tengcongensis* form six-coordinate (6c) ferrous NO adducts with a histidine trans to the NO.[22–23] *Nostoc punctiforme* and *Legionella pneumophila* H-NOX ferrous NO complexes are a temperature-dependent mixture of 5c and 6c species.[24] This temperature dependence on coordination number has also been observed for *SmFixL*. [25] In contrast, 6c ferric NO adducts are important in the function of nitrophorin (NP) NO donor proteins, cytochrome *cd*₁ nitrite reductase (NO trans to histidine) and fungal nitric oxide reductase (NO trans to cysteine).[26–28]

The oxidation states of heme nitrosyls are herein distinguished by Enemark-Feltham notation which takes the form of $\{\text{MNO}\}^n$ where M is the metal center and n is the sum of d -electrons from the metal and π antibonding electrons from the NO.[29] Ferric heme nitrosyls, $\{\text{FeNO}\}^6$ complexes, are generally thermodynamically unstable with respect to reductive nitrosylation, forming ferrous heme nitrosyls, $\{\text{FeNO}\}^7$ complexes. The general reaction mechanism involves nucleophilic attack on the nitrosyl nitrogen by a base (commonly hydroxide) followed by the dissociation of nitrous acid leaving behind a reduced pentacoordinate iron center, which is rapidly trapped by any excess NO. The conjugate base of the released HNO_2 , NO_2^- , can serve as a nucleophile for subsequent rounds of reductive nitrosylation wherein dinitrogen trioxide (N_2O_3) is released and hydrolyzed to yield two molecules of HNO_2 . [30–31] While the $\{\text{FeNO}\}^6$ complex of the histidine-ligated heme in NPs are reported to be resistant to reductive nitrosylation[32], the $\{\text{FeNO}\}^6$ complexes of many other heme proteins such as myoglobin (Mb), hemoglobin (Hb), and cytochrome *c* undergo reductive nitrosylation.[31] The heme in some of these histidine-ligated proteins undergo *trans* labilization of the proximal iron-histidine bond upon coordination of NO resulting in a five-coordinate $\{\text{FeNO}\}^7$ complex. In some proteins like cytochrome *c'* (Cyt *c'*) [33], sGC [19], and H-NOX [20], the final 5c $\{\text{FeNO}\}^7$ complex has NO bound on the proximal side of the heme. Although, a definitive physiological role for proximally bound heme nitrosyls remains elusive,[34] it has been suggested that they enable a kinetic trap mechanism that could facilitate release of the rapidly rebinding NO ligand from the heme pocket.[35]

In light of questions about *KpCld* function, a role for it in NO storage or transport is investigated by examining whether its heme pocket has characteristics for such activity. This study examines the reactivity of dimeric *KpCld* and the pentameric *DaCld* with NO. Heme

pocket variants R128Q *KpCld* and R183Q *DaCld* are used to address the role of the conserved distal arginine in the formation and reactivities of the various NO complexes. Additionally, the Cld–NO complexes reveal differences in the *KpCld* and *DaCld* active sites. These differences manifest in various active site ligation and reactivity and are discussed in light of known NO heme protein complexes.

2. Materials and Methods

2.1. Protein Expression and Purification

DaCld and R183Q *DaCld* were expressed and purified as previously described [9,36] with the following modification. A 1:1 stoichiometric equivalence of hemin chloride was added to *DaCld* after purification by anion exchange chromatography and allowed to incubate for 12 hours at 4 °C in 0.1 M potassium phosphate pH 6.8 buffer. The resulting mixture was purified over a S–200 HR gel filtration column (26 × 750 mm). Protein and hemin quantification were determined by Bradford[37] and hemochromogen[38] assays, respectively, which verified a 1:1 stoichiometric equivalence between bound hemin and protein. Specific activity measurements of *DaCld* showed a linear increase in O₂ evolution with hemin loaded protein between as isolated and constituted *DaCld*.

Recombinant *KpCld* and R128Q *KpCld* both with an N-terminal His₆-tag (pET15-b, Merck/Novagen) and a tobacco etch virus (TEV) protease site for His₆-tag removal were expressed in Tuner DE3 *E. coli* cells grown in Terrific Broth supplemented with ampicillin (100 mg/mL). *KpCld* was expressed and purified as previously described.[7,39] His₆-tagged TEV protease S219V was expressed and isolated according to literature procedures [40] with the following modifications: no glycerol used during chromatographic purification steps and no polyetheleneimine used during lysis. The His₆-tag of *KpCld* was removed by incubation with S219V TEV (at 1 mol TEV: 10 mol Cld) in 50 mM sodium phosphate pH 8.0, 2 mM dithiothreitol, and 0.5 mM ethylenediaminetetraacetate overnight at 4 °C. These conditions resulted in greater than 90% tag-free *KpCld*. The proteolysis reaction mixture was passed over a 5 mL Nickel-nitrilotriacetate sepharose column and a linear gradient (20–500 mM imidazole) was used to separate tag-free *KpCld* from tagged *KpCld* and TEV protease S219V. The cleaved *KpCld* was passed over a PD–10 desalting column and exchanged into 0.1 M sodium phosphate pH 6.8 buffer to remove imidazole. Purity and cleavage were verified by sodium dodecylsulfate polyacrylamide gel electrophoresis. Purified cleaved *KpCld* was then frozen in liquid nitrogen in aliquots and stored at –80 °C. The same procedure was used for the R128Q *KpCld* variant.

Enzyme activity for all Clds was verified by measuring chlorite-decomposition activity. The luminescence-based NEOFOX® system from Ocean Optics, Inc. was used to measure O₂ evolution during Cld-catalyzed ClO₂[–] decomposition under steady state conditions. The probe was calibrated to at least three known O₂(aq) concentrations. Samples were 100 nM in *KpCld*, R128Q *KpCld*, R183Q *DaCld* or 10 nM in *DaCld*, and 2 mM in ClO₂[–] in 100 mM phosphate (pH 6.8). The probe was equilibrated in the buffered ClO₂[–] solution for 5 minutes prior to initiation of the reaction by introduction of Cld. Kinetic traces were recorded at 0.1 second intervals for 2 minutes.

2.2. Preparation of Nitrosyl Complexes

The $\{\text{FeNO}\}^6$ complexes were generated by equilibrating ferric Cld in $\text{N}_2(\text{g})$ for 30 – 60 minutes prior to the addition of 1 atm $\text{NO}(\text{g})$. Natural abundance $\text{NO}(\text{g})$ was purified by passage over solid NaOH pellets and through 1 M $\text{NaOH}(\text{aq})$. Labeled $^{15}\text{NO}(\text{g})$ was obtained directly from a lecture bottle (98%+ purity) without further purification. The $\{\text{FeNO}\}^7$ complexes were generated by either reducing $\{\text{FeNO}\}^6$ samples directly with sodium dithionite (2 – 5 mM) or by reducing $\text{N}_2(\text{g})$ equilibrated ferric Cld samples followed by exposure to 1 atm $\text{NO}(\text{g})$. Stock sodium dithionite solutions (50 – 100 mM) were generated under $\text{N}_2(\text{g})$ in 0.1 M potassium phosphate pH 8 or 0.1 M glycine pH 9.6 buffers.

2.3. Vibrational spectroscopy of Cld Nitrosyl Complexes

The resonance Raman (rR) spectra of nitrosyl complexes were recorded on a locally built spectrometer. Resonance Raman scattering was excited with either 406.7- or 413.1-nm emission from a Kr^+ laser. The laser beam was focused to a line at the sample using a cylindrical lens. Scattered light was collected in the 135° backscattering geometry using a $f/1$ lens to collect and colimate the light. Rayleigh scattered light was attenuated with a holographic notch filter and the remaining light passed through a polarization scrambler. Collection was f -matched to a single $f/4.7$ 0.67-m Czerny-Turner spectrograph fitted with a 120×140 mm 1200 groove/mm holographic grating and operated with a $10 \mu\text{m}$ (0.74 cm^{-1}) slit. The spectrograph was fitted with a LN_2 -cooled CCD detector having a 26.8 mm (1340 pixel) $\times$ 8.0 mm (400 pixel) image area. The spectrometer was calibrated using known absolute Raman shifts of toluene and dimethylformamide. Unless otherwise noted, all spectra were recorded under ambient conditions from samples contained in 5-mm NMR tubes spinning at $\sim 20 \text{ Hz}$ using a locally fabricated spinner. Laser powers ranged from 1 to 2 mW at the sample. All Cld samples for rR spectroscopy ranged from 25 – 50 μM (heme) in 0.1 M buffer (potassium phosphate and glycine) at indicated pH values. Samples were examined spectrophotometrically before and after laser exposure to monitor sample integrity. For a given set of solution conditions, care was taken to generate natural-abundance and ^{15}N -enriched NO complexes from aliquots of the same Cld solutions in order to match their concentrations. Spectra of pairs of isotopolog samples were recorded with identical acquisition parameters (number of integrations and integration times) and in temporal proximity to one another on the same day to minimize spectrometer alignment artifacts in the isotope difference spectra.

Raman difference spectra in which either the isotopolog bands of a given mode overlapped, or there were clearly multiple overlapping isotope-sensitive bands were subjected to peak fitting in order to identify FeNO frequencies of the isotopologs. In all cases, the difference spectra were fit with positive bands for spectra of the natural abundance samples and negative bands for the ^{15}NO -enriched samples. Gaussian band shapes were used with the full width at half maximum constrained to the same value ($10\text{--}25 \text{ cm}^{-1}$) for a given pair of isotopologs. Goodness of fit was based on minimization of χ^2 using a commercial implementation of the Levenberg-Marquardt method.

Fourier-transform infrared absorbance (FTIR) spectra were recorded for both $Kp\text{Cld}$ and $Da\text{Cld}$ $\{\text{FeNO}\}^6$ complexes in a 30 mm CaF_2 liquid cell. Cld concentrations ranged from

400 – 1000 μM . A 0.1 M potassium phosphate buffer spectrum recorded under identical sample conditions was used to manually subtract the water background such that the baseline between 1750 – 2000 cm^{-1} was flat.

2.4. Spectrophotometric tracking and kinetic analysis of reductive nitrosylation reactions

Buffered Cld $\{\text{FeNO}\}^6$ solutions were prepared as described in §2.2. Heme concentration was 1.0×10^{-6} M in all reactions. Conversion of $\{\text{FeNO}\}^6$ complexes to their $\{\text{FeNO}\}^7$ counterparts was monitored by UV-visible absorbance (UV-vis) spectrophotometry at 20.0 °C. Reactions were tracked for 14 to 22 hours. During these reactions there were no effects attributable to O_2 incursion, which would result in the formation of the ferric nitrito complex and the appearance of its ligand to metal charge transfer band at 627 nm.[41] The t_∞ spectrum was recorded after driving the formation of $\{\text{FeNO}\}^7$ to completion by adding enough buffered ascorbate stock solution to make it 2 mM in ascorbate. The kinetic traces of fraction $\{\text{FeNO}\}^6$ reduced vs time were modeled by thermodynamically two-term exponential decay function shown in equation [1].

$$\Delta A_t = \Delta A_{\infty 1} \exp(-k_{\text{obs}1}t) + \Delta A_{\infty 2} \exp(-k_{\text{obs}2}t) \quad [1]$$

The apparent rate constant for the $[\text{OH}^-]$ dependent pathway, $k_{\text{obs}1}$, is given by equation [2].

$$k_{\text{obs}1} = k_{\text{OH}^-}[\text{OH}^-] + k_{\text{H}_2\text{O}} \quad [2]$$

Therefore, the second-order rate constant (k_{OH^-}) was determined from the slope of the plot $k_{\text{obs}1}$ vs $[\text{OH}^-]$ and the pH insensitive first order rate constant ($k_{\text{H}_2\text{O}}$) was determined by the y-intercept.

2.5. Spectrophotometric tracking of NO-dependent conversion of 6c $\{\text{FeNO}\}^7$ to 5c $\{\text{FeNO}\}^7$ Cld

All ferric Cld samples ranged from 5 – 10 mM and were equilibrated with $\text{N}_2(\text{g})$ prior to being reduced with a 5 – 10 fold excess of sodium dithionite. Aqueous NO solutions were prepared by saturating anaerobic buffer solutions with $\text{NO}(\text{g})$ at ambient pressure. Concentrations of stock $\text{NO}(\text{aq})$ solutions were determined using the Henry's Law constant $K_{\text{H}} = 1.9 \times 10^{-3} \text{ M} \cdot \text{atm}^{-1}$ at ambient temperature.[42] The resulting NO solution was added in appropriate measure to ferrous Cld samples. UV-vis spectra were recorded as a function of time with a double beam, scanning spectrophotometer for each substoichiometric NO addition to ferrous Cld.

2.6 Spectrophotometric determination of pH dependence for $6\text{c} \rightleftharpoons 5\text{c} \{\text{FeNO}\}^7$ Clds

Multiple anaerobic ferric Cld samples were prepared at the same concentration in the following buffers: potassium phosphate (pH 6.0, 7.0, and 8.0), Tris (pH 8.6), and glycine (pH 9.0, 9.6, and 10). Ferrous Cld samples were prepared with sodium dithionite as described above and then converted to their respective $\{\text{FeNO}\}^7$ forms by addition of 1 atm $\text{NO}(\text{g})$. UV-vis spectra of each $\{\text{FeNO}\}^7$ sample was recorded with a double beam, scanning spectrophotometer several times to ensure complete complex formation.

3. Results

3.1. Ferric Clds form {FeNO}⁶ complexes

Solutions of ferric *KpCld*, *DaCld*, or their distal pocket variants, R128Q *KpCld* and R183Q *DaCld*, equilibrated with NO exhibit UV visible spectra with the Soret band at 416–418 nm and α/β bands at 565 and 531 nm (Fig. 1A). Spin-state marker bands ν_2 , ν_3 , and ν_{10} in their rR spectra are observed at 1580–1583, 1510, and 1644 cm⁻¹ (Fig. 1B), respectively. These spectral features are consistent with hexacoordinate low-spin (6cLS) {FeNO}⁶ heme complexes.[43–44] Since ν_3 and ν_{10} bands shift to ~1501 and ~1632 cm⁻¹, respectively, upon reduction of the 6cLS {FeNO}⁶ to 6cLS {FeNO}⁷, they are useful spectral signatures for monitoring for reductive nitrosylation. In fact, the shoulder observed around 1501 cm⁻¹ on the ν_3 band for {FeNO}⁶ *KpCld* is due to some {FeNO}⁷ which is formed at an accelerated rate upon exposure to laser light (Figs. S1 and S2).

R128Q *KpCld* is the only Cld examined here that exhibits an additional ν_3 band at 1483 cm⁻¹ upon exposure to 1 atm NO; such a ν_3 frequency is typically indicative of 6cHS heme. This suggests that a small fraction of the R128Q *KpCld* has not bound NO. Ferric R128Q *KpCld* exhibits some characteristics that make it distinct from the wild-type (WT) Clds and the R183Q *DaCld* variant. While the heme resting state is 5cHS in WT *DaCld* and a mixture of 5cHS and 6cHS in R183Q *DaCld* and WT *KpCld*,[7,9,41] ferric R128Q *KpCld* presents as a mixture of 6cHS and 6cLS with ν_3 band frequencies of 1483 and 1506 cm⁻¹, respectively. This 6cHS/6cLS mixture is not dependent on pH over the range of 7 to 12 (Fig. S3A). Similar lack of pH dependence between pH 6.8 and 10 has also been reported for R183Q *DaCld* in both its spectral characteristics and activity.[9] At pH 5.5 the *KpCld* variant is converted completely to a 6cHS species. The UV-vis pH titration reports a p*K*_a of approximately 6.3 associated with this speciation change (Fig. S3B). Mixtures of 6cHS/6cLS heme are observed for the hydroxide complexes of both WT Clds. However, the p*K*_as for their hydroxide formations are higher (p*K*_a=8.3±0.1, *KpCld*; p*K*_a=8.7±0.1, *DaCld*)[7,41] than the one observed here for speciation change of R128Q *KpCld*. To determine if the 6cHS/6cLS R128Q *KpCld* is due a hydroxide complex in a thermal spin state equilibrium like those known for the WT enzymes, its Soret-excited rR spectra in H₂O, H₂¹⁸O, and ²H₂O are examined for isotope-sensitive bands indicative of a heme-hydroxide complex (Fig. S4). Since no ²H- or ¹⁸O-sensitive bands are observed, even at pH 12, the 6cHS/6cLS species are due to some heme ligand other than hydroxide. The identity of the sixth ligand is currently unknown. In R128Q *KpCld*, the 6cHS/6cLS mixture may be attributable to the glutamine 128 providing either its amide O atom (HS) or N atom (LS) as the iron ligand. Such a 6cLS species due to a distal glutamine binding to the heme *b* of coproheme decarboxylase has been reported.[45] The sixth ligand appears to compete effectively with NO coordination to the ferric iron.

Formation of {FeNO}⁶ Clds are confirmed by identification of three ¹⁵NO isotope sensitive bands for each complex using rR and FTIR spectroscopy (Fig. 2). These bands are assigned to the FeNO bending mode, δ (FeNO), Fe–NO stretch, ν (Fe–NO), and N–O stretch, ν (N–O) of {FeNO}⁶ Clds (Table 1) in order of increasing frequency based on nuclear resonance vibrational spectroscopy assignments for nitrosyl complexes of iron porphyrinates[46] and

rR assignments for $\{\text{FeNO}\}^6$ complexes of Mb[47], NP[48], and FixL from *Sinorhizobium meliloti* (*SmFixL*).[49] While both $\delta(\text{FeNO})$ and $\nu(\text{Fe-NO})$ bands are observed in the rR spectra for all $\{\text{FeNO}\}^6$ Clds, they overlap substantially, resulting in $^{14}\text{NO} - ^{15}\text{NO}$ difference features whose maxima and minima do not correspond directly to the mode frequencies. Since the $\nu(\text{N-O})$ band was only observed in the rR spectrum of R183Q *DaCld* $\{\text{FeNO}\}^6$, the $\nu(\text{N-O})$ frequencies for *KpCld* and *DaCld* complexes were assigned using FTIR spectroscopy. The lack of a $\nu(\text{N-O})$ band in the WT Cld rR spectra is due to poor resonance enhancement with 413-nm excitation; enhancement for this mode occurs at 244 nm via resonance with the ligand to metal charge transfer transition.[50] The respective $\nu(\text{N-O})$ frequencies observed for *KpCld* and *DaCld* are similar at 1896 and 1898 cm^{-1} . Mutation of the distal arginine to a glutamine in *DaCld* causes a 15 cm^{-1} increase in the $\nu(\text{N-O})$ frequency; FTIR and rR spectra of R183Q *DaCld* $\{\text{FeNO}\}^6$ both contain bands at 1911 cm^{-1} (Fig. S5 and Fig. 2).

3.2. Reductive nitrosylation of Clds

All Cld $\{\text{FeNO}\}^6$ complexes studied here undergo slow reductive nitrosylation at ambient temperature and pressure to yield $\{\text{FeNO}\}^7$. Rates of reductive nitrosylation increase with pH and were tracked spectrophotometrically as a function of time. Due to the exceedingly long time required to affect complete conversion from $\{\text{FeNO}\}^6$ to $\{\text{FeNO}\}^7$, ascorbate was added after the first kinetic phase to drive the $\{\text{FeNO}\}^7$ formation to completion. These effective t_{∞} spectra are isosbestic in the Q-band region with all the spectra recorded during the natural time course of the reaction. The spectrum of the ascorbate-treated reaction mixture did not share the isosbestic point in the Soret region of the spectrum due to small ascorbate absorbance in the near-UV. This demonstrates that the $\{\text{FeNO}\}^7$ produced during reductive nitrosylation is the same as the product of reduction with ascorbate. Changes in absorbance as a function of time were best modeled by the two-term exponential function shown in Equation 1 (Fig. 3C). The rate constants obtained from this biphasic behavior are k_{obs1} (faster and pH dependent) and k_{obs2} (slower and pH independent). Amplitudes of the two phases varied, with the slow phase accounting for more than half the total amplitude. This eliminates the possibility of parallel reductive nitrosylation pathways. The kinetic behavior is consistent with the presence of an unreactive form of $\{\text{FeNO}\}^6$ that is converted to a reactive form at a rate slower than reductive nitrosylation. This is effectively a slowly established equilibrium followed by a fast product-forming reaction step. Considering that isosbestic behavior is retained throughout the time course (Fig. 3A & Fig. 3B), it can be concluded that both pathways yield the same product and that the reactive and unreactive $\{\text{FeNO}\}^6$ complexes are spectroscopically indistinguishable. Given that the slow phase was independent of $[\text{OH}^-]$, our working hypothesis is that the unreactive form is a kinetic trap and that it reverts only very slowly to the reactive form. *SmFixL** exhibits similar behavior. [49]

Previously published studies of $\{\text{FeNO}\}^6$ reductive nitrosylation have revealed the rate limiting step of the reaction to be nucleophilic attack of the coordinated N atom of the NO ligand.[31] Reaction mixtures contain three nucleophiles, solvent water, hydroxide and the nitrite produced in the reaction. Thus, the rate law could comprise up to three terms, each showing dependence on one of the nucleophile concentrations. Since the data were well

modeled without considering any NO_2^- -catalyzed reductive nitrosylation, this nucleophile was not considered in the kinetic analyses reported herein. In order to differentiate the two observed rate constants, reductive nitrosylation of Cld $\{\text{FeNO}\}^6$ complexes were monitored at pH 6.8, 8.0, 9.0, and 9.6. These data revealed that k_{obs1} was $[\text{OH}^-]$ -dependent (Fig. 3D) and was modeled by Equation 2 to obtain values for $k_{\text{H}_2\text{O}}$ and k_{OH^-} (Table 2). Both $k_{\text{H}_2\text{O}}$ and k_{OH^-} values reported here are several orders of magnitude lower than those reported for HbA, Mb, trHb, but similar to *SmFixL**. The pH independent rate constant (k_{obs2}) ranged between 2×10^{-5} and $5 \times 10^{-5} \text{ s}^{-1}$ for all three Cld enzymes. At pH 6.8, k_{obs1} and k_{obs2} were kinetically indistinguishable and A vs time was well modeled by a single exponential function for each enzyme. Considering both WT *DaCld* and *KpCld* bind OH^- under alkaline conditions[7,41], we hypothesized that the slow phase could be rate limited by OH^- dissociation. However, the pH independence of the slow phase and the lack of spectral features associated with a Cld- OH^- complex in the $\{\text{FeNO}\}^6$ solutions eliminate this mechanistic possibility from further consideration. Moreover, the R183Q *DaCld* variant does not bind OH^- [9] and its reductive nitrosylation is also biphasic. These behaviors are consistent with the hypothesis that Cld $\{\text{FeNO}\}^6$ complexes form rapidly upon treatment of ferric Clds with excess NO to yield two spectrally indistinguishable $\{\text{FeNO}\}^6$ complexes, one that is reactive towards reductive nitrosylation and one that is not. The nonreactive Cld $\{\text{FeNO}\}^6$ converts to the reactive form on the time scale dictated by k_{obs2} .

3.3. The coordination state of $\{\text{FeNO}\}^7$ Cld complexes is pH dependent

All Cld $\{\text{FeNO}\}^6$ complexes form $\{\text{FeNO}\}^7$ complexes upon addition of dithionite. Under alkaline conditions (pH 9.6), the spectroscopic signatures of *KpCld* $\{\text{FeNO}\}^7$ (UV-vis: Soret, 418 nm; α/β bands, 574 nm and 542 nm (Fig. S6); rR: ν_3 , 1501 cm^{-1} and the sharpness of the ν_{10} and $\nu(\text{C}=\text{C})$ region (Fig. 4)) are consistent with formation of a 6cLS complex. The two isotopically sensitive bands observed for *KpCld* $\{\text{FeNO}\}^7$ at 1609 and 548 cm^{-1} are assigned as $\nu(\text{N}-\text{O})$ and $\nu(\text{Fe}-\text{NO})$, respectively, and are also characteristic of a 6cLS complex.[25,47] At pH 6.0 the *KpCld* $\{\text{FeNO}\}^7$ UV-vis spectrum has its Soret at 401 nm, a shoulder near 397 nm, α/β bands at 566 nm and 535 nm, and a charge transfer (CT) band at 483 nm (Fig. S6). The acidic *KpCld* $\{\text{FeNO}\}^7$ has a broad, asymmetric ν_3 band centered at 1506 cm^{-1} ; the width of this band at half height ($\nu_{1/2} = 16 \text{ cm}^{-1}$) is large compared to its ν_3 band at pH 9.6 ($\nu_{1/2} = 12 \text{ cm}^{-1}$), suggesting that it comprises multiple unresolved components, consistent with a mixture of species contributing to ν_3 intensity. Three isotope sensitive bands are observed and assigned as follows: 1680 cm^{-1} , 5c $\nu(\text{N}-\text{O})$; 527 cm^{-1} , 5c $\nu(\text{Fe}-\text{NO})$; and 555 cm^{-1} , 6c $\nu(\text{Fe}-\text{NO})$ (Fig. 5). Observation of two $\nu(\text{Fe}-\text{NO})$ frequencies is consistent with two species suggested by the broad ν_3 band and lends support to *KpCld* being a mixture between 5c and 6c $\{\text{FeNO}\}^7$ at acidic pH.

Alkaline *DaCld* and R183Q *DaCld* $\{\text{FeNO}\}^7$ complexes exhibit essentially identical UV-vis spectra (Soret, 415 nm; shoulder, 397 nm; α/β bands, 574 nm and 542 nm; CT band, 483 nm) (Fig. S6); their rR spectra exhibit broad ν_3 bands at 1503 cm^{-1} ($\nu_{1/2} = 16 \text{ cm}^{-1}$) and 1504 cm^{-1} ($\nu_{1/2} = 17 \text{ cm}^{-1}$), respectively, (Fig. 4) suggest a mixture of 5cLS and 6cLS heme. In addition, *DaCld* has two ^{15}NO sensitive bands in the high frequency window, assigned to 6c $\nu(\text{N}-\text{O})$ (1599 cm^{-1}) and 5c $\nu(\text{N}-\text{O})$ (1673 cm^{-1}). In the low frequency window, the $^{14}\text{NO} - ^{15}\text{NO}$ difference spectra have a single broad difference feature (544/539

cm^{-1}). While the difference features for 6c *KpCld* $\{\text{FeNO}\}^7 \nu(\text{Fe-NO})$ fit with bandwidths of 10 cm^{-1} , both *Da* enzymes required 25 cm^{-1} bandwidths to obtain reasonable fits. Given the 5c and 6c $\nu(\text{N-O})$ frequencies differ by approximately 19 cm^{-1} (Table 1), the broadness of the *DaCld* difference feature is consistent with overlap of 5c and 6c $\nu(\text{Fe-NO})$ bands such that they are not resolved in the difference spectra. This, together with the $\nu(\text{N-O})$ and ν_3 , indicates the WT and R183Q *DaCld* $\{\text{FeNO}\}^7$ complexes are a mixture of 5cLS and 6cLS heme nitrosyls under alkaline conditions.

Under slightly acidic conditions (pH 6.8), speciation of *DaCld* and R183Q *DaCld* $\{\text{FeNO}\}^7$ complexes favors the 5c states based on their UV-vis ($\lambda_{\text{maxB}} = 397 \text{ nm}$; $\lambda_{\text{maxQ}} = 566, 535 \text{ nm}$; $\lambda_{\text{maxCT}} = 483 \text{ nm}$) and rR spectra (ν_3 for 5c $\{\text{FeNO}\}^7$, 1509 cm^{-1}) (Fig. 5). Two prominent isotope sensitive bands are observed in their rR spectra. They have been assigned as 5c $\nu(\text{N-O})$ at $1683/1684 \text{ cm}^{-1}$ and to 5c $\nu(\text{Fe-NO})$ at 525 cm^{-1} for WT and R183Q *DaClds*, respectively.

Cld $\{\text{FeNO}\}^7$ samples were prepared over a range of pHs for examination by UV-vis spectrophotometry to characterize the pH-dependent transition between the 5c and 6c $\{\text{FeNO}\}^7$ complexes of *DaCld* and *KpCld* (Fig. 6). This transition is reported by the shift in the Soret maximum as a function of pH. Unfortunately *DaCld* $\{\text{FeNO}\}^7$ could not be completely converted to the 6c form at alkaline pH, and *KpCld* did not form a pure 5c $\{\text{FeNO}\}^7$ under acidic conditions. Therefore, the pK_a associated with the speciation change could only be estimated; qualitatively the pK_a for the 5c to 6c transition is higher for *DaCld* than *KpCld*. This is consistent with the rR data where at low-pH *KpCld* $\{\text{FeNO}\}^7$ is a mixture of 5c and 6c species and *DaCld* $\{\text{FeNO}\}^7$ is predominantly 5c while at high-pH the *KpCld* complex is 6c and the *DaCld* is a mixture between 5c and 6c species.

3.4. Formation of 5c $\{\text{FeNO}\}^7$ Clds is $[\text{NO}]$ dependent

Conversion from 6c to 5c $\{\text{FeNO}\}^7$ was tracked at pH 6.8 after addition of sub-stoichiometric amounts of aqueous NO to ferrous Clds (Fig. 7). The spectra do not exhibit clean isosbestic behavior, and spectral features for three heme species are discernable: 5cHS ferrous Cld, 6c $\{\text{FeNO}\}^7$, and 5c $\{\text{FeNO}\}^7$. As ferrous Cld concentration decreased (evidenced by decreasing absorbance at 433 nm), spectral features attributable to 6c $\{\text{FeNO}\}^7$ appeared (418 nm) and disappeared as those for 5c $\{\text{FeNO}\}^7$ grew (400 nm). This slow conversion has also been observed for AXCP under stoichiometric conditions.[53] The interconversion between 6c and 5c $\{\text{FeNO}\}^7$ Clds was too fast to be observed in the presence of saturating $[\text{NO}]$. These data support NO-catalyzed conversion of 6c $\{\text{FeNO}\}^7$ to 5c $\{\text{FeNO}\}^7$ wherein the final product has NO coordinated on the proximal side of the heme, as reported for cyt c' [33,53], H-NOX [20,54] and sGC.[19,55]

4. Discussion

4.1. Fe-NO and N-O bonds of $\{\text{FeNO}\}^6$ Clds are exceptionally weak under the influence of the distal arginine cation in closed heme pocket conformations.

Fig. 8 shows the locations of Cld $\{\text{FeNO}\}^6$ complexes, along with other proximal histidine-ligated heme proteins, on the $\nu(\text{Fe-NO})$ vs $\nu(\text{N-O})$ backbonding correlation plot. The two

correlation lines with slopes close to -1 are consistent with the positions of $\{\text{FeNO}\}^6$ complexes along these lines being modulated by the extent of $\text{Fe}^{\text{II}} \rightarrow \text{NO}^+ \pi$ backbonding. [47] The offsets of the two backbonding correlation lines along the $\nu(\text{N-O})$ axis has been explained in terms of Lewis acid-base interactions in the distal pocket of the Mb variants. [47,56] The higher $\nu(\text{N-O})$ frequency line is attributed to Lewis acid-base interactions between the NO^+ ligand and either a distal histidine or an exogenous water molecule. The lower $\nu(\text{N-O})$ frequency line represents the backbonding correlation in the absence of Lewis donation to the coordinated NO^+ in systems having solvent-inaccessible distal pockets and no endogenous distal bases.[56] One $\{\text{FeNO}\}^6$ conformer was observed for each Cld complex reported here. Both open and closed conformers have been observed for the isoelectronic $\{\text{FeCO}\}^6$ complexes of *Da*Cld [41] and R183Q *Da*Cld[9], and a single open conformer has been reported for that of *Kp*Cld[39]. However, since none of the $\{\text{FeNO}\}^6$ Clds reported here fall on the higher $\nu(\text{N-O})$ frequency correlation line in Fig. 8, the heme pockets are concluded to be solvent inaccessible and in their closed conformations.

Interestingly, the $\{\text{FeNO}\}^6$ complexes of WT *Kp*Cld and *Da*Cld define the low-frequency extreme of $\nu(\text{N-O})$, $\nu(\text{Fe-NO})$ correlation space for $\{\text{FeNO}\}^6$ heme proteins having proximal imidazole ligands reported to date. This suggests that distal $\{\text{FeNO}\}^6$ interactions in the Clds exert their influence on FeNO bonding and vibrational dynamics in a way that is distinct from other proteins in Fig. 8. The very low $\nu(\text{N-O}) / \nu(\text{Fe-NO})$ frequencies for WT *Da*Cld and *Kp*Cld are attributed to the Lewis acidity (or positive charge) of their distal arginine side chains. The nucleophilicity of Lewis bases, such as imidazole and water, favor $\text{Fe}^{\text{II}}\text{-NO}^+$ character of the $\{\text{FeNO}\}^6$ moiety with its formal NO bond order of three, as revealed by their $\nu(\text{N-O})$ frequencies ranging from ~ 25 to 50 cm^{-1} higher than that of free NO (bond order = 2.5, $\nu(\text{N-O}) = 1876 \text{ cm}^{-1}$). Distal Lewis acids, such as the guanidinium side chain of arginine, are expected to destabilize the $\text{Fe}^{\text{II}}\text{-NO}^+$ form due to repulsive electrostatic interactions with NO^+ . In a qualitative sense, the growing electrostatic repulsion between the approaching guanidinium group of a distal arginine and the NO^+ ligand could be mitigated by delocalizing the nitrosonium charge away from the distal positive charge. Electron transfer from Fe(II) to NO^+ would yield a $\text{Fe}^{\text{III}}\text{-NO}^\bullet$ state in which the positive charge has been fully delocalized to the iron center, thereby maximizing the distance between the protein-based positive charge and that localized on the $\{\text{FeNO}\}^6$ moiety. Based on DFT computational studies of $\{\text{FeNO}\}^6$ porphyrinates, it has been reported that the ground and excited states have $\text{Fe}^{\text{II}}\text{-NO}^+$ and $\text{Fe}^{\text{III}}\text{-NO}^\bullet$ character, respectively.[64] The low $\nu(\text{N-O})$ and $\nu(\text{Fe-NO})$ frequencies of WT $\{\text{FeNO}\}^6$ Clds reported here reveal substantially weakened Fe-NO and N-O bonds relative to other $\{\text{FeNO}\}^6$ heme proteins. Furthermore, their $\nu(\text{N-O})$ frequencies are shifted toward that of free NO relative to other proteins with similar $\nu(\text{Fe-NO})$ frequency, consistent with assignment of considerable $\text{Fe}^{\text{III}}\text{-NO}^\bullet$ character to the $\{\text{FeNO}\}^6$ moiety. Finally, because σ donation from their proximal ligands is not particularly high compared to $\{\text{FeNO}\}^6$ HRP with its proximal imidazolate ligand, these weakened FeNO bonds are unlikely to be attributable to proximal effects. Therefore, the results reported here support the conclusion that these WT Clds are the first examples of $\{\text{FeNO}\}^6$ heme proteins in which distal factors drive stabilization of the $\text{Fe}^{\text{III}}\text{-NO}^\bullet$ excited state. It seems unlikely that the nitrosonium and neutral NO radical states are fully inverted, as the $\nu(\text{N-O})$ frequencies remain $\sim 20 \text{ cm}^{-1}$ above that of free NO. However,

since the frequencies are quite low and only one set of $\nu(\text{Fe-NO})$ and $\nu(\text{N-O})$ bands is observed, it is most reasonable to suggest that the presence of distal positive charge induces an admixture of the nitrosonium and neutral radical states having an intermediate NO bond order, as illustrated in Scheme 1.

Interestingly, the only other protein in Fig. 8 having a distal arginine residue is the O_2 -sensing domain FixLN from *Sinorhizobium meliloti*. The heme environment in FixLN differs from those of the Clds in a couple of ways. First, its Fe^{II} -His bond is relatively weak based on its $\nu(\text{Fe}^{\text{II}}\text{-His})$ frequency of 212 cm^{-1} [65] and, by virtue of its position on the Lewis donor correlation line in Fig 8, likely has an arginine-stabilized water molecule in its distal heme pocket.

Consistent with the cause-and-effect relationship between distal positive charge and very low FeNO frequencies, when the cationic side chain of Arg183 is replaced by the charge-neutral amide of glutamine, its $\{\text{FeNO}\}^6$ complex falls at the low- $\nu(\text{Fe-NO})$ extreme of the donor-free correlation line. That R183Q *DaCld* $\{\text{FeNO}\}^6$ falls on the correlation line is consistent with re-establishment of dominant $\text{Fe}^{\text{II}}\text{-NO}^+$ character in the $\{\text{FeNO}\}^6$ moiety and the susceptibility of its backbonding to environmental interactions.

Given the similarity in the distal environments of *DaCld* and *KpCld*, it seems reasonable to consider whether their relative positions in Fig. 8 are attributable to differential proximal (*trans*) effects. Their respective $\nu(\text{Fe}^{\text{II}}\text{-His})$ frequencies of 222 cm^{-1} and 229 cm^{-1} reveal more proximal imidazolate character in *KpCld*. [7,41] Its correspondingly stronger σ donation is expected to have a greater *trans* effect, resulting in the lower $\nu(\text{Fe-NO})$ and $\nu(\text{N-O})$ frequencies, which is observed, along with a smaller $\angle\text{FeNO}$ in *KpCld* than *DaCld*. An inverse relationship between *trans* donor strength and $\angle\text{FeNO}$ was also predicted by previous computational studies[66–67] and recently corroborated experimentally with *trans*-thiolate $\{\text{FeNO}\}^6$ compounds.[68] Based on the greater proximal imidazolate character in *KpCld*, this relationship would predict it to have the smaller $\angle\text{FeNO}$. However the difference between its $\nu(\text{Fe-NO})$ and $\delta(\text{FeNO})$ frequencies is smaller than that of *DaCld* $\{\text{FeNO}\}^6$ ($\nu = 10\text{ cm}^{-1}$ for *KpCld* and 15 cm^{-1} for *DaCld*), consistent with its $\angle\text{FeNO}$ being the larger of the two WT enzymes.[48]. Therefore, the relative FeNO frequencies and bond angles cannot be explained in terms of the *trans* effect alone. Earlier computational studies of this *trans* effect, varied *trans* structural and electronic factors independently in order to establish the aforementioned relationship between donor strength and $\angle\text{FeNO}$. Here, there are both distal and proximal (i.e. *trans*) forces at play and, as suggested by the apparent inconsistency described above, both influence FeNO bond strengths and angles in combinatorial ways that remain to be clarified.

4.2. Structural basis of slow reductive nitrosylation of $\{\text{FeNO}\}^6$ Clds

Heme proteins with a proximal histidine and a distal arginine (i.e. Clds and FixL) have $k_{\text{H}_2\text{O}}$ and k_{OH^-} of similar magnitudes (Table 2). This contrasts with heme proteins having a proximal histidine, and either a distal histidine or tyrosine, whose $k_{\text{H}_2\text{O}}$ and k_{OH^-} values are several orders of magnitude larger (i.e. Hb, Mb, trHb, and LegHb).[50–52] While tempting to attribute the sluggish rates of reductive nitrosylation for Clds to the positive charge from their distal arginine, the similarity in $k_{\text{H}_2\text{O}}$ and k_{OH^-} values of R183Q*DaCld* to those of the

WT Cld enzymes indicates that this is not the case. A possible explanation is that the WT Clds and the R183Q *Da*Cld are characterized by slow conformational dynamics dominated by one or more closed conformers in which access of nucleophiles to the coordinated NO is blocked. In the Cld case either a distal arginine or glutamine can serve this role of slowly and alternately blocking and providing access in the {FeNO}⁶ liganded state. Access of a nucleophile to coordinated NO is likely only possible for the trace fraction of the open conformer present at equilibrium. In this case, pocket access by a reactive nucleophile would be rate limiting for reductive nitrosylation. This would likely be the case, even if there is a water molecule bridging the distal arginine and the reactive N_{NO}⁺ atom, as suggested above for *Sm*FixLN.

Both WT Cld enzymes and R183Q *Da*Cld exhibit biphasic reductive nitrosylation similar to *Sm*FixL*.[49] The cause of the slow phase remains elusive. However, based on the kinetic behavior of these systems, it may involve a conformation that is kinetically distinct in its lack of reactivity toward reductive nitrosylation. One possible explanation for the slow phase was incomplete formation of {FeNO}⁶ due to competition from hydroxide coordination. This was ruled out as cause for the slow kinetic phase, as there is no evidence from UV-vis or rR spectroscopy of the ferric heme hydroxides in alkaline {FeNO}⁶ solutions. Thus, it appears that the slow phase is attributable to a kinetically trapped and unreactive fraction of the enzyme that is either intrinsic to the purified enzyme or that forms upon reaction with NO. This trapped state is illustrated in Scheme 2. In either case, the UV-vis absorbance spectrum of the {FeNO}⁶ complex is not sensitive to the structural differences that govern their reactivity, suggesting that these differences are structurally remote from the heme.

4.3. Hydrogen bonding in the 6c {FeNO}⁷ Clds is directed towards the nitrogen of the NO ligand.

Fig. 9 shows the $\nu(\text{Fe-NO})$ vs $\nu(\text{N-O})$ correlation plots for 5c and 6c {FeNO}⁷ complexes of the Clds alongside other histidine-ligated heme proteins. The 6c {FeNO}⁷ complexes fall on two distinct $\nu(\text{Fe-NO})$ vs $\nu(\text{N-O})$ correlation lines (Fig. 9A). One reveals a positive correlation between $\nu(\text{Fe-NO})$ and $\nu(\text{N-O})$ frequencies, for {FeNO}⁷ hemes having hydrogen-bond donation to their N_{NO} atoms. The second shows a negative correlation among complexes having hydrogen-bond donation to the O_{NO} atoms of their NO ligands. [56] Both 6c{FeNO}⁷ WT *Da*Cld and *Kp*Cld complexes fall slightly below the positive correlation line, consistent with hydrogen-bond donation from their distal arginine side chains to N_{NO} in both enzymes. This behavior is in agreement with data on their fluoride and hydroxide complexes, in which hydrogen bond donation from their distal positively charged arginine residues to the coordinated atom of the ligand is clear from their low $\nu(\text{Fe-F})$ and $\nu(\text{Fe-OH})$ frequencies, respectively.[69] As hydrogen-bond donation to N_{NO} increases along the positive correlation line, the systematic lowering of their $\nu(\text{Fe-NO})$ and $\nu(\text{N-O})$ frequencies reveals weakening of both the Fe-NO and N-O bonds. Thus, *Da*Cld, having lower $\nu(\text{Fe-NO})$ and $\nu(\text{N-O})$ frequencies than *Kp*Cld, exhibits stronger hydrogen-bond donation to N_{NO}. The stronger hydrogen-bonding to the NO ligand in the substrate binding site of *Da*Cld relative to *Kp*Cld is consistent with greater ability of the *Da*Cld distal pocket to polarize the substrate O-Cl bond and lower the kinetic barrier to its heterolytic scission in catalytic ClO₂⁻ decomposition.

4.4. The pH dependence of 5c:6c {FeNO}⁷ Clds is correlated with acid-base properties of the proximal histidine.

The two 5c {FeNO}⁷ *Da*Cld complexes reported here are co-located on the backbonding correlation plot (Fig. 9B), consistent with the similarity expected for their NO environments in the proximal *Da*Cld heme pocket. While some effort has been made to distinguish proximal from distal NO binding based on spectroscopic signatures[78–79], position on the backbonding correlation plot alone has yet to provide clear insight into the heme face occupied by NO. Proximal 5c {FeNO}⁷ heme proteins that are known NO sensors (e.g. sGC and H-NOX) respond to changes in [NO] by forming their 5c sensor-on states via two [NO]-dependent steps: NO binding directly to the open distal heme face to yield transient 6c {FeNO}⁷ followed by [NO]-dependent isomerization to give 5c {FeNO}⁷ wherein NO is coordinated to the proximal face.[19,54–55] These two steps support a dampened response to changes in [NO] which might otherwise be physiologically too potent. While a physiological ClO₂[−] detoxification function is known for the periplasmic *Da*Cld enzyme it has only been proposed for the cytoplasmic *Kp*Cld.[5] Hence, it is interesting to consider whether the cytoplasmic potential in *K. pneumoniae*, could support formation of the proximal 5c {FeNO}⁷ complex *in vivo*, as that would suggest the possibility of an NO sensing function.

The equilibrium between 6c and 5c {FeNO}⁷ appears to be controlled by the relative proximal Fe–His bond strengths in the Clds. Although the pH dependence alone does not reveal the localization of the controlling acid-base reaction, we note that the enzymes likely have different Fe–His bond strengths, as suggested by their different $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies. The extent of the thermodynamic NO *trans* effect on proximal histidine dissociation is expected to correlate inversely with the strength of the Fe–His bond. Thus, greater susceptibility of the proximal histidine to *trans* labilization by NO is expected for the Cld having the least proximal imidazolate character. Based on $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies, that enzyme is *Da*Cld. Consistent with this expectation, the observed susceptibility of the proximal histidine in *Da*Cld to *trans* labilization by NO, as judged by the extent of conversion to 5c {FeNO}⁷ at pH 6, is greater than that for *Kp*Cld. Thus, the pH sensitivity of the 5c:6c population ratio is attributed to the acid-base properties of the distal histidine ligand.

4.5. Relevance of these results to catalytic ClO₂[−] decomposition

The behavior of the {FeNO}⁶ Clds is consistent with their established penchant for closed heme pocket conformations in liganded states, which has been suggested to be part of the reason that *Da*Cld has such high catalytic efficiency and specificity. Upon formation of the enzyme substrate complex, the decomposition reaction is sequestered, which prevents escape of reactive chlorine species from the active site[7,36] and diversion of the key intermediates along unproductive or damaging pathways.[80] Especially in the {FeNO}⁶ Clds, the closed solvent-inaccessible and otherwise hydrophobic distal heme pocket supports strong polarization of Fe–L_{axial} moieties by distal positive charge, as revealed by the heretofore unobserved effects of their Fe^{III}–NO[•] / Fe^{II}–NO⁺ admixture on the bond strengths and charge distribution in the {FeNO}⁶ moiety. This is consistent with facilitation of polarization-dependent O–Cl^{III} bond scission in the first reaction step of the enzyme-

substrate complex (Cld–ClO₂[−]). Such polarizing capacity of distal arginine in the heme peroxidases is well established to be crucial in lowering the kinetic barrier to heterolytic cleavage of O–O bond in compound 0 to yield compound I and water.[81] The strong polarizing capacity of the distal arginine revealed here for the Cld {FeNO}⁶ complexes suggests that analogous two-electron coupled heterolytic cleavage of the O–Cl^{III} bond to yield compound I and ClO[−] warrants further consideration.

5. Conclusion

This study contains the first reported vibrational characterization of both {FeNO}⁶ and {FeNO}⁷ complexes of dimeric and pentameric Clds. Both *Da*Cld (pentameric) and *Kp*Cld (dimeric) form kinetically stable {FeNO}⁶ complexes and have the lowest correlated $\nu(\text{Fe}-\text{NO})$ / $\nu(\text{N}-\text{O})$ frequencies on record for heme nitrosyls with proximal histidine ligands. These low frequencies are attributed to Fe^{III}–NO[•] character of their ground states, in which both NO bond order and π backbonding are both diminished relative to the intrinsic Fe^{II}–NO⁺ ground state. Reductive nitrosylation reactions of the {FeNO}⁶ Clds are slow and biphasic. The closed heme pocket of these ligated states is concluded to be responsible for the slow rates.

Both Clds form mixtures of 6c and proximal 5c {FeNO}⁷ complexes whose equilibrium 5c populations depend on the σ donor strengths of their proximal histidine ligands. The Clds reported here are among the few heme proteins known to form proximal 5c {FeNO}⁷ complexes. Proximal NO coordination in 5c {FeNO}⁷ appears to be intrinsic to the Clds investigated here. Whether this penchant for coordination chemistry heretofore associated with NO sensing speaks to a possible sensor role is an interesting and open question.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Cld	chlorite dismutase
<i>Kp</i>Cld	Cld from <i>Klebsiella pneumoniae</i> Cld
Cld from <i>Da</i>Cld	<i>Dechloromonas aromatic</i>
Mb	myoglobin
Hb	hemoglobin
trHb	truncated Hb

FixL	bacterial two-component heme-based O ₂ sensing protein from <i>Sinorhizobium meliloti</i>
Np	nitrophorin
AXCP	cytochrome <i>c'</i> from <i>Alcaligenes xylosoxidans</i>
RCCP	cytochrome <i>c'</i> from <i>Rhodobacter capsulatus</i>
rR	resonance Raman
WT	wild type
HS	high spin
LS	low spin
6c	six-coordinate
5c	five-coordinate

References

- [1]. DuBois JL; Ojha S Met Ions Life Sci 15 (2015) 45–87. [PubMed: 25707466]
- [2]. Schaffner I; Hofbauer S; Krutzler M; Pirker KF; Furtmueller PG; Obinger C Arch. Biochem. Biophys 574 (2015) 18–26. [PubMed: 25748001]
- [3]. Goblirsch B; Kurker RC; Streit BR; Wilmot CM; DuBois JL J. Mol. Biol 408 (2011) 379–398. [PubMed: 21354424]
- [4]. Hofbauer S; Schaffner I; Furtmueller PG; Obinger C Biotechnol. J 9 (2014) 461–473. [PubMed: 24519858]
- [5]. Celis AI; DuBois JL Arch. Biochem. Biophys 574 (2015) 3–17. [PubMed: 25778630]
- [6]. Coates JD; Achenbach LA Nat. Rev. Microbiol 2 (2004) 569–580. [PubMed: 15197392]
- [7]. Celis AI; Geeraerts Z; Ngmenterebo D; Machovina MM; Kurker RC; Rajakumar K; Ivancich A; Rodgers KR; Lukat-Rodgers GS; DuBois JL Biochemistry 54 (2015) 434–446. [PubMed: 25437493]
- [8]. Schaffner I; Hofbauer S; Krutzler M; Pirker KF; Bellei M; Stadlmayr G; Mlynek G; Djinovic-Carugo K; Battistuzzi G; Furtmüller PG; Daims H; Obinger C Mol. Microbiol 96 (2015) 1053–1068. [PubMed: 25732258]
- [9]. Blanc B; Mayfield JA; McDonald CA; Lukat-Rodgers GS; Rodgers KR; DuBois JL Biochemistry 51 (2012) 1895–1910. [PubMed: 22313119]
- [10]. Hofbauer S; Gysel K; Bellei M; Hagmueller A; Schaffner I; Mlynek G; Kostan J; Pirker KF; Daims H; Furtmueller PG; Battistuzzi G; Djinovic-Carugo K; Obinger C Biochemistry 53 (2014) 77–89. [PubMed: 24364531]
- [11]. Hofbauer S; Gruber C; Pirker KF; Suendermann A; Schaffner I; Jakopitsch C; Oostenbrink C; Furtmueller PG; Obinger C Biochemistry 53 (2014) 3145–3157. [PubMed: 24754261]
- [12]. Pal RR; Khardenavis AA; Purohit HJ Funct. Integr. Genomics 15 (2015) 63–76. [PubMed: 25326064]
- [13]. Williams DE; Boon EM J. Innate Immun 11 (2019) 205–215. [PubMed: 30557874]
- [14]. Tsai WC; Strieter RM; Zisman DA; Wilkowski JM; Bucknell KA; Chen G-H; Standiford TJ Infect. Immun 65 (1997) 1870–1875. [PubMed: 9125574]
- [15]. Poole RK; Hughes MN Mol. Microbiol 36 (2000) 775–783. [PubMed: 10844666]
- [16]. Gardner AM; Helmick RA; Gardner PR J. Biol. Chem 277 (2002) 8172–8177. [PubMed: 11751865]
- [17]. Stevanin TM; Moir JWB; Read RC Infect. Immun 73 (2005) 3322–3329. [PubMed: 15908358]

- [18]. Mills PC; Rowley G; Spiro S; Hinton JCD; Richardson DJ *Microbiology* 154 (2008) 1218–1228. [PubMed: 18375814]
- [19]. Martin E; Berka V; Sharina I; Tsai A-L *Biochemistry* 51 (2012) 2737–2746. [PubMed: 22401134]
- [20]. Herzik MA Jr.; Jonnalagadda R; Kuriyan J; Marletta MA *Proc. Natl. Acad. Sci. U. S. A* 111 (2014) E4156–4164. [PubMed: 25253889]
- [21]. Bacon BA; Liu Y; Kincaid JR; Boon EM *Biochemistry* 57 (2018) 6187–6200. [PubMed: 30272959]
- [22]. Karow DS; Pan D; Tran R; Pellicena P; Presley A; Mathies RA; Marletta MA *Biochemistry* 43 (2004) 10203–10211. [PubMed: 15287748]
- [23]. Tsai A-L; Berka V; Martin F; Ma X; van den Akker F; Fabian M; Olson JS *Biochemistry* 49 (2010) 6587–6599. [PubMed: 20572679]
- [24]. Boon EM; Davis JH; Tran R; Karow DS; Huang SH; Pan D; Miazgowicz MM; Mathies RA; Marletta MA *J. Biol. Chem* 281 (2006) 21892–21902. [PubMed: 16728401]
- [25]. Lukat-Rodgers GS; Rodgers KR *Biochemistry* 36 (1997) 4178–4187. [PubMed: 9100012]
- [26]. Walker FA J. *Inorg. Biochem* 99 (2005) 216–236. [PubMed: 15598503]
- [27]. Wasser IM; de Vries S; Moeenne-Loccoz P; Schroeder I; Karlin KD *Chem. Rev* 102 (2002) 1201–1234. [PubMed: 11942794]
- [28]. Daiber A; Shoun H; Ullrich VJ *Inorg. Biochem* 99 (2005) 185–193.
- [29]. Enemark JH; Feltham RD *Coord. Chem. Rev* 13 (1974) 339–406.
- [30]. Fernandez BO; Ford PC *J. Am. Chem. Soc* 125 (2003) 10510–10511. [PubMed: 12940720]
- [31]. Ford PC *Inorg. Chem* 49 (2010) 6226–6239. [PubMed: 20666383]
- [32]. Andersen JF; Ding XD; Balfour C; Shokhireva TK; Champagne DE; Walker FA; Montfort WR *Biochemistry* 39 (2000) 10118–10131. [PubMed: 10956000]
- [33]. Lawson DM; Stevenson CEM; Andrew CR; Eady RR *The EMBO Journal* 19 (2000) 5661–5671. [PubMed: 11060017]
- [34]. Horst BG; Marletta MA *Nitric Oxide* 77 (2018) 65–74. [PubMed: 29704567]
- [35]. Andrew CR; Rodgers KR; Eady RR *J. Am. Chem. Soc* 125 (2003) 9548–9549. [PubMed: 12903995]
- [36]. Streit BR; DuBois JL *Biochemistry* 47 (2008) 5271–5280. [PubMed: 18422344]
- [37]. Bradford MM *Anal. Biochem* 72 (1976) 248–254. [PubMed: 942051]
- [38]. Berry EA; Trumpower BL *Anal. Biochem* 161 (1987) 1–15. [PubMed: 3578775]
- [39]. Geeraerts Z; Celis AI; Mayfield JA; Lorenz M; Rodgers KR; DuBois JL; Lukat-Rodgers GS *Biochemistry* 57 (2018) 1501–1516. [PubMed: 29406727]
- [40]. Tropea JE; Cherry S; Waugh DS In *High Throughput Protein Expression and Purification: Methods and Protocols*; Doyle SA, Ed.; Humana Press: Totowa, NJ, 2009.
- [41]. Streit BR; Blanc B; Lukat-Rodgers GS; Rodgers KR; DuBois JL *J. Am. Chem. Soc* 132 (2010) 5711–5724. [PubMed: 20356038]
- [42]. Schwartz SE; White WH *Adv. Environ. Sci. Eng* 4 (1981) 1–45.
- [43]. Li XY; Czernuszewicz RS; Kincaid JR; Su YO; Spiro TG *J. Phys. Chem* 94 (1990) 31–47.
- [44]. Choi S; Spiro TG; Langry KC; Smith KM; Budd DL; La Mar GN *J. Am. Chem. Soc* 104 (1982) 4345–4351.
- [45]. Milazzo L; Gabler T; Pfanzagl V; Michlits H; Furtmuller PG; Obinger C; Hofbauer S; Smulevich GJ *Inorg. Biochem* 195 (2019) 61–70.
- [46]. Li J; Peng Q; Oliver AG; Alp EE; Hu MY; Zhao J; Sage JT; Scheidt WR *J. Am. Chem. Soc* 136 (2014) 18100–18110. [PubMed: 25490350]
- [47]. Soldatova AV; Ibrahim M; Olson JS; Czernuszewicz RS; Spiro TG *J. Am. Chem. Soc* 132 (2010) 4614–4625. [PubMed: 20218710]
- [48]. Maes EM; Walker FA; Montfort WR; Czernuszewicz RS *J. Am. Chem. Soc* 123 (2001) 11664–11672. [PubMed: 11716723]
- [49]. Rodgers RK; Lukat-Rodgers SG; Tang LJ *Biol. Inorg. Chem* 5 (2000) 642–654.
- [50]. Hoshino M; Maeda M; Konishi R; Seki H; Ford PC *J. Am. Chem. Soc* 118 (1996) 5702–5707.

- [51]. Herold S; Puppo AJ Biol. Inorg. Chem 10 (2005) 946–957.
- [52]. Ascenzi P; di Masi A; Tundo GR; Pesce A; Visca P; Coletta M PLOS ONE 9 (2014) e102811. [PubMed: 25051055]
- [53]. Andrew CR; George SJ; Lawson DM; Eady RR Biochemistry 41 (2002) 2353–2360. [PubMed: 11841228]
- [54]. Wu G; Liu W; Berka V; Tsai A.-I. Biochemistry 52 (2013) 9432–9446. [PubMed: 24351060]
- [55]. Tsai A-L; Berka V; Sharina I; Martin EJ Biol. Chem 286 (2011) 43182–43192.
- [56]. Spiro TG; Soldatova AV; Balakrishnan G Coord. Chem. Rev 257 (2013) 511–527. [PubMed: 23471138]
- [57]. Benko B; Yu NT Proc. Natl. Acad. Sci. U. S. A 80 (1983) 7042–7046. [PubMed: 6580627]
- [58]. Tomita T; Haruta N; Aki M; Kitagawa T; Ikeda-Saito MJ Am. Chem. Soc 123 (2001) 2666–2667.
- [59]. Mukai M; Ouellet Y; Ouellet H; Guertin M; Yeh S-R Biochemistry 43 (2004) 2764–2770. [PubMed: 15005611]
- [60]. Ding XD; Weichsel A; Andersen JF; Shokhireva TK; Balfour C; Pierik AJ; Averill BA; Montfort WR; Walker FA J. Am. Chem. Soc 121 (1999) 128–138.
- [61]. Zaczek MB; Zareba AA; Czernuszewicz RS; Montfort W J. of Porphyrins and Phthalocyanines 10 (2006) 928–933.
- [62]. Wang J; Lu S; Moenne-Loccoz P; Ortiz d. M. P. R. J. Biol. Chem 278 (2003) 2341–2347. [PubMed: 12433915]
- [63]. Pinakoulaki E; Gemeinhardt S; Saraste M; Varotsis CJ Biol. Chem 277 (2002) 23407–23413.
- [64]. Praneeth VKK; Paulat F; Berto TC; DeBeer George S; Nather C; Sulok CD; Lehnert NJ Am. Chem. Soc 130 (2008) 15288–15303.
- [65]. Rodgers KR; Lukat GS; Barron JA Biochemistry 35 (1996) 9539–9548. [PubMed: 8755735]
- [66]. Linder DP; Rodgers KR Inorg. Chem 44 (2005) 1367–1380. [PubMed: 15732977]
- [67]. Linder DP; Rodgers KR J. Biol. Inorg. Chem. 12 (2007) 721–731. [PubMed: 17356871]
- [68]. Hunt AP; Lehnert N Inorg. Chem 58 (2019) 11317–11332. [PubMed: 30912445]
- [69]. Geeraerts Z; Rodgers KR; DuBois JL; Lukat-Rodgers GS Biochemistry 56 (2017) 4509–4524. [PubMed: 28758386]
- [70]. Tsubaki M; Yu NT Biochemistry 21 (1982) 1140–1144. [PubMed: 7074070]
- [71]. Andrew CR; Kemper LJ; Busche TL; Tiwari AM; Kecskes MC; Stafford JM; Croft LC; Lu S; Moëne-Loccoz P; Huston W; Moir JWB; Eady RR Biochemistry 44 (2005) 8664–8672. [PubMed: 15952773]
- [72]. He C; Neya S; Knipp M Biochemistry 50 (2011) 8559–8575. [PubMed: 21866897]
- [73]. Tomita T; Gonzalez G; Chang AL; Ikeda-Saito M; Gilles-Gonzalez MA Biochemistry 41 (2002) 4819–4826. [PubMed: 11939776]
- [74]. Deinum G; Stone JR; Babcock GT; Marletta MA Biochemistry 35 (1996) 1540–1547. [PubMed: 8634285]
- [75]. Tomita T; Ogura T; Tsuyama S; Imai Y; Kitagawa T Biochemistry 36 (1997) 10155–10160. [PubMed: 9254612]
- [76]. Andrew CR; Green EL; Lawson DM; Eady RR Biochemistry 40 (2001) 4115–4122. [PubMed: 11300792]
- [77]. Reynolds MF; Parks RB; Burstyn JN; Shelper D; Thorsteinsson MV; Kerby RL; Roberts GP; Vogel KM; Spiro TG Biochemistry 39 (2000) 388–396. [PubMed: 10631000]
- [78]. Kekilli D; Petersen CA; Pixton DA; Ghafoor DD; Abdullah GH; Dworkowski FSN; Wilson MT; Heyes DJ; Hardman SJO; Murphy LM; Strange RW; Scrutton NS; Andrew CR; Hough MA Chemical Science 8 (2017) 1986–1994. [PubMed: 28451315]
- [79]. Servid AE; McKay AL; Davis CA; Garton EM; Manole A; Dobbin PS; Hough MA; Andrew CR Biochemistry 54 (2015) 3320–3327. [PubMed: 25961377]
- [80]. Lee AQ; Streit BR; Zdilla MJ; bu-Omar MM; DuBois JL Proc. Natl. Acad. Sci. U. S. A 105 (2008) 15654–15659. [PubMed: 18840691]
- [81]. Poulos TL Arch. Biochem. Biophys 500 (2010) 3–12. [PubMed: 20206121]

Synopsis:

A new class of ferric heme protein nitrosyls is reported. Their location in FeNO vibrational correlation space is heretofore unknown in heme proteins and attributed to admixed $\text{Fe}^{\text{III}}\text{-NO}^\bullet$ character of the ground state. Reductive nitrosylation of these complexes is slow and yields pentacoordinate ferrous heme nitrosyls having proximal NO ligands.

Highlights

- NO adducts of chlorite dismutase (Cld) have been studied by resonance Raman spectroscopy.
- A new class of ferric heme nitrosyls, {FeNO}⁶, in Cld is reported.
- {FeNO}⁶ hemes in Clds occupy a new region of FeNO vibrational correlation space.
- Reductive nitrosylation of {FeNO}⁶ Clds to yield ferrous heme nitrosyls {FeNO}⁷ is slow.
- Pentacoordinate {FeNO}⁷ Clds have proximally coordinated NO ligands.

Fig. 1.
 $\{\text{FeNO}\}^6$ Cld complexes form upon reaction of NO with ferric Clds as illustrated by their UV-visible absorbance spectra (A) and their Soret-excited (413.1 nm) rR spectra (B). Spectra for $\{\text{FeNO}\}^6$ Cld complexes in 0.1 M potassium phosphate pH 6.8 under 1 atmosphere NO(g) are shown for a) *DaCld* (red), b) R183Q *DaCld* (blue), c) *KpCld* (black), and d) R128Q *KpCld* (green). * ν_3 attributed to $\{\text{FeNO}\}^7$ formed by photo-induced reduction of $\{\text{FeNO}\}^6$.

Fig. 2.

Assignment of $\delta(\text{FeNO})$, $\nu(\text{Fe-NO})$, and $\nu(\text{N-O})$ via rR and FTIR indicate generation of $\{\text{FeNO}\}_6$ Clds. Soret excited (413.1-nm) resonance Raman spectra of N isotopologs of $\{\text{FeNO}\}_6$ Cld complexes in 0.1 M potassium phosphate pH 6.8 under 1 atmosphere $\text{NO}(g)$. Red, blue, black, and green (smooth) traces: ^{15}NO , ^{14}NO , $^{14}\text{NO} - ^{15}\text{NO}$, and peak fit of $^{14}\text{NO} - ^{15}\text{NO}$, respectively. Inset: High frequency windows for both *KpCld* and *DaCld* are FTIR absorbance spectra. Water vapor rotation-vibration features appear in both *KpCld* and *DaCld* FTIR spectra.

Fig. 3.

{FeNO}⁶ Cld complexes exhibit similar stability with respect to red uctivenitrosylation. Timereolved absorbancespectra of {FeNO}⁶ (red line) converting to {FeNO}⁷ (blue line) over 26 hours and 20 hours for 1.0×10⁻⁵ M (A) *KpCld* and (B) *DaCld* at pH 9.0, respectively. Arrows show direction of change with increasing reaction time. Complete reduction to {FeNO}⁷ was achieved upon addition of 2 mM ascorbate (magenta line). (C) Representative ΔA vs time trace for *KpCld* exhibits biphasic behavior which was fit to a two term exponential function. (D) *k*_{obs1} vs. [OH⁻] for *KpCld*, *DaCld*, and R183Q *DaCld* used to determine values for *k*_{H₂O} and *k*_{OH⁻}.

Fig. 4.

Soret-excited rR spectra of $\{\text{FeNO}\}^7$ Cld complexes reveal their coordination states under alkaline conditions. Soret excited (413.1-nm) resonance Raman Fe–NO and N–O frequency windows of $\{\text{FeNO}\}^7$ Cld complexes in 0.1 M glycine pH 9.6 under 1 atm NO(g). Red spectra = ^{15}NO , blue spectra = ^{14}NO , black spectra = $^{14}\text{NO} - ^{15}\text{NO}$, green (smooth) traces = peak fits to $^{14}\text{NO} - ^{15}\text{NO}$. The ν_3 , $\nu(\text{Fe-NO})$ and $\nu(\text{NO})$ frequencies reveal speciation to be dominated by 6c $\{\text{FeNO}\}^7$ *KpCld* while the two *Da* enzymes are mixtures of 6c and 5c complexes. *Difference features at 1673 and 1642 cm^{-1} for *DaCld* may correspond to subtle speciation changes or $\nu(\text{NO})$ for 5c $\{\text{FeNO}\}^7$.

Fig. 5.

Soret-excited rR spectra of N isotopologs of $\{\text{FeNO}\}^7$ Clds indicate that they are predominantly 5-coordinate in at pH 6.8. Soret excited (406.7-nm) resonance Raman Fe–NO and N–O frequency windows of $\{\text{FeNO}\}^7$ Cld complexes in 0.1 M potassium phosphate pH 6.8 under 1 atm NO(g). Red spectra = ^{15}NO , blue spectra = ^{14}NO , black spectra = $^{14}\text{NO} - ^{15}\text{NO}$, green (smooth) traces = peak fits to $^{14}\text{NO} - ^{15}\text{NO}$. The ν_3 , $\nu(\text{Fe-NO})$ and $\nu(\text{NO})$ frequencies indicate that the dominant form is 5cLS. *Difference features at 555 and 534 cm^{-1} for *KpCld* may correspond to subtle speciation changes or $\nu(\text{Fe-NO})$ for 6c $\{\text{FeNO}\}^7$.

Fig. 6. Spectrophotometric pH dependence on coordination number for $\{FeNO\}^7$ Cld complexes. pH-dependent UV-vis absorbance spectra of A) *KpCld* and B) *DaCld* $\{FeNO\}^7$. Arrows show the direction of absorbance change with increasing pH. Spectra with bluest and reddest Soret maxima correspond to pH 6 and ~10, respectively. Gray spectra represent pH's between the two extremes. Complete conversion to pure 5c or 6c states was not attained over this pH range. Therefore, a pK_a could not be determined.

Fig. 7.

Optical absorbance spectra tracking the reaction of ferrous Clds with substoichiometric [NO] yields 6c {FeNO}⁷ and 5c complexes in sequence. A) *KpCld*, B) *DaCld*, and C) R183Q *DaCld* in 0.1 M sodium phosphate buffer at pH 6.8 with 0.5 molar equivalents of NO. Black spectra show starting ferrous Cld while the blue and red spectra indicate initial and final time points after NO addition, respectively. Arrows show directions of absorbance change with increasing reaction time. Total conversion time took 40 minutes for each enzyme to reach their respective end points.

Fig. 8. Backbonding correlation plot of 6c {FeNO}⁶ complexes reveals closed conformation for {FeNO}⁶ Clds. Both lines were generated from ferric nitrosyl myoglobin mutants[47,56] shown by circles while purple squares correspond to other heme proteins.[25,48,57–63] Proteins falling on the gray line comprise a distal base while those on the black line have none. Blue triangles and red star are *DaCld*s and *KpCld*, respectively.

Fig. 9.

A) Two $\nu(\text{Fe}^{\text{II}}\text{-NO})/\nu(\text{N-O})$ correlation lines for 6c {FeNO}⁷ complexes represent different hydrogen bond orientations. The negative correlation line was generated from ferrous tetraphenylporphyrin with various ligands (gray) while the positive correlation line was generated from ferrous nitrosyl myoglobin mutants (black).[56] The gray line indicates hydrogen bonding directed toward O_{NO} while the black line indicates hydrogen bonding directed toward N_{NO}. Blue triangles and red star are DaClds and KpCld, respectively while purple squares correspond to other heme proteins.[53,57,70–73] B) The $\nu(\text{Fe-NO})$ vs $\nu(\text{N-O})$ backbonding correlation plot of 5c {FeNO}⁷ complexes supports proximal NO binding in Clds. The line was generated from ferrous tetraphenylporphyrin with various ligands (open circles).[56] Blue triangles and red star are DaClds and KpCld, respectively, while purple squares correspond to other heme proteins.[21,25,41,53,72,74–77]

Scheme 1.

Effect of distal arginine on valence electron distribution in {FeNO}⁶ Clds.

Scheme 2.

Proposed mechanistic scheme for reductive nitrosylation of Clds.

Table 1Vibrational frequencies and absorbance maxima of {FeNO}ⁿ Cld complexes

Raman shift (cm ⁻¹)						Absorbance maxima (nm)		
Protein	pH	ν_3	$\delta(\text{Fe-N})$ (¹⁵ NO)	$\nu(\text{Fe-N})$ (¹⁵ NO)	$\nu(\text{N-O})$ (¹⁵ NO)	λ_{Soret}	λ_{α}	λ_{β}
6c {FeNO} ⁶								
<i>KpCld</i>	6.8	1510	577 (7)	589 (5)	1896 (36) ^a	418	565	531
<i>DaCld</i>	6.8	1510	575 (16)	591 (5)	1898 (37) ^a	416	565	531
R183Q <i>DaCld</i>	6.8	1510	574 (8)	587 (5)	1911 (37)	416	565	531
R128Q <i>KpCld</i>	6.8	1509		589 (6)	no ^b	418	565	531
5c {FeNO} ⁷								
<i>KpCld</i>	6.8	1506		527 (14)	1680 (34)	401	566	535
<i>DaCld</i>	6.8	1509	525 (9)	1683 (33)	397	566	535	
R183Q <i>DaCld</i>	6.8	1509	525 (13)	1684 (40)	397	566	535	
R128Q <i>KpCld</i>	6.8	1509	525	no ^b	397	566	535	
6c {FeNO} ⁷								
<i>KpCld</i>	9.6	1501	548 (21)	1609 (26)	418	574	542	
<i>DaCld</i>	9.6	1502	544 (27)	1599 (19)	415	574	542	
R183Q <i>DaCld</i>	9.6	1502	539 (27)	1604 (17)	415	574	542	

^a assigned from FTIR spectra;^b not observed

Table 2

Reductive nitrosylation rate constants at 20 °C for select 5c heme proteins with a proximal histidine.

Protein	[NO] (M)	k_{OH^-} ($\text{M}^{-1} \text{s}^{-1}$)	$k_{\text{H}_2\text{O}}$ (s^{-1})	Distal Residue	Ref
R183Q <i>Dα</i> Cld	1.8×10^{-3}	$6.0 \pm 0.2 \times 10^0$	$9 \pm 1 \times 10^{-6}$	Gln	this work
<i>Dα</i> Cld	1.8×10^{-3}	$8.4 \pm 0.2 \times 10^0$	$1.3 \pm 0.1 \times 10^{-5}$	Arg	this work
<i>Kp</i> Cld	1.8×10^{-3}	$8.8 \pm 0.3 \times 10^0$	$2.5 \pm 0.1 \times 10^{-5}$	Arg	this work
<i>Sm</i> FixL*	1.6×10^{-3}	$7 \pm 1 \times 10^0$	$3 \pm 2 \times 10^{-5}$	Arg	[49]
HbA	2.5×10^{-4}	$3.2 \pm 0.2 \times 10^3$	$1.1 \pm 0.1 \times 10^{-3}$	His	[50]
swMb	2.5×10^{-4}	$3.2 \pm 0.2 \times 10^2$	no	His	[50]
<i>Gm</i> LegHb	1.0×10^{-3}	$3.3 \pm 0.2 \times 10^3$	$3.0 \pm 0.9 \times 10^{-4}$	His	[51]
<i>Mt</i> -trHbN	1.9×10^{-3}	$1.7 \pm 0.2 \times 10^2$	$6.4 \pm 0.7 \times 10^{-4}$	Tyr	[52]
<i>Mt</i> -trHbO	1.9×10^{-3}	$2.4 \pm 0.3 \times 10^2$	$2.9 \pm 0.3 \times 10^{-4}$	Tyr	[52]
<i>Cj</i> -trHbP	1.9×10^{-3}	$9 \pm 1 \times 10^2$	$4.8 \pm 0.5 \times 10^{-4}$	Tyr	[52]