Published in final edited form as:

Biochemistry. 2017 August 29; 56(34): 4509–4524. doi:10.1021/acs.biochem.7b00572.

Active sites of O₂-evolving chlorite dismutases probed by halides, hydroxides and new iron-ligand vibrational correlations

Zachary Geeraerts¹, Kenton R. Rodgers^{1,*}, Jennifer L. DuBois², and Gudrun S. Lukat-Rodgers^{1,*}

¹Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102, USA

²Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59715, USA

Abstract

O₂-evolving chlorite dismutases (Clds) fall into two subfamilies, which efficiently convert ClO₂[−] to O₂ and Cl[−]. The Cld from *Dechloromonas aromatica* (*Da*Cld) represents the chlorite-decomposing homopentameric enzymes found in perchlorate and chlorate respiring bacteria. The Cld from the Gram-negative, human pathogen *Klebsiella pneumoniae* (*Kp*Cld) is representative of the second subfamily, comprising homodimeric enzymes having truncated N-termini. Here steric and nonbonding properties of the *Da*Cld and *Kp*Cld active sites have been probed via kinetic, thermodynamic and spectroscopic behaviors of their fluorides, chlorides and hydroxides. Cooperative Cl[−] binding to *Kp*Cld drives formation of a hexacoordinate, high-spin aqua heme, whereas *Da*Cld remains pentacoordinate and high-spin under analogous conditions. Fluoride coordinates to the heme iron in *Kp*Cld and *Da*Cld, exhibiting $\nu(\text{Fe}^{\text{III}}\text{--F})$ bands at 385 and 390 cm^{−1}, respectively. Correlation of these frequencies with their CT1 energies reveals strong H-bond-donation to the F[−] ligand, indicating that atoms directly coordinated to heme iron are accessible by distal H-bond donation. New vibrational frequency correlations between either $\nu(\text{Fe}^{\text{III}}\text{--F})$ or $\nu(\text{Fe}^{\text{III}}\text{--OH})$ and $\nu(\text{Fe}^{\text{II}}\text{--His})$ of Clds and other heme proteins are reported. These correlations orthogonalize proximal and distal effects on the bonding between iron and exogenous π -donor ligands. The axial Fe–X vibrations and the relationships between them illuminate both similarities and differences in the H-bonding and electrostatic properties of the distal and proximal heme environments in pentameric and dimeric Clds. Moreover, they provide general insight into

Corresponding Authors: gudrun.lukat-rodgers@ndsu.edu; tel: 701-231-8834; fax: 701-231-8831. kent.rodgers@ndsu.edu; tel: 701-231-8746; fax: 701-231-8831.

Supporting Information. Additional data referred to in the text: Figures S1–S10 and Tables S1–S2 include UV-vis of *Kp*Cld with various ions (Fig S1), spectrophotometric titration of *Kp*Cld with Br[−] (Fig S2), low frequency rR spectra of *Kp*Cld with various ions (Fig S3), spectrophotometric titration of *Da*Cld with Cl[−] (Fig S4), size exclusion chromatography for *Kp*Cld in the presence of various anions (Fig S5), Michaelis-Menton fit parameters for *Kp*Cld chlorite decomposition reaction in the presence of various anions (Fig S6, Table S1), high frequency rR spectra of *Kp*Cld–F[−], *Da*Cld–F[−], and *Da*Cld(R183Q)–F[−] (Fig S7), cross-eye stereo view of *Nw*Cld nonbonding interactions between $\alpha 5'$ and $\beta 4'$ (Fig S8), Fe–X stretching frequencies used to generate correlation plots (Table S2), placement of *Tf* trHBs on $\nu(\text{Fe}^{\text{III}}\text{--F})/\nu(\text{Fe}^{\text{II}}\text{--His})$ correlation plot (Fig S9), placement of *Tf* and *Mt* trHBs on $\nu(\text{Fe}^{\text{III}}\text{--OH})/\nu(\text{Fe}^{\text{II}}\text{--His})$ correlation plot (Fig S10). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interests.

the structural basis of reactivity toward substrates in heme-dependent enzymes and their mechanistic intermediates, especially those containing the ferryl moiety.

TOC image

Keywords

heme; chlorite dismutase; chloride; fluoride; hydroxide; *Klebsiella*; resonance Raman

The formation of an O–O bond is achieved in a unique manner by chlorite dismutases (Clds) that rapidly and efficiently catalyze the decomposition of a single chlorite ion (ClO_2^-) to O_2 and Cl^- .^{1, 2} Clds are soluble enzymes containing heme *b*, the sole cofactor required for catalyzing its O–O bond-forming reaction. The simplicity of this system derives from its intramolecular redox nature, which does not require coupling to a proton pump. This is in sharp contrast to the complexity of O_2 production, in the water-splitting reaction catalyzed by the tetramanganese center in the oxygen-evolving complex of photosystem II (PSII), a key step in the pathway for photosynthetic fixation of CO_2 into carbohydrates.³

Chlorite dismutases have been identified in almost all bacterial phyla and in many archaea. However, the vast majority of them occur in organisms that do not utilize perchlorate as a respiratory oxidant.^{4, 5} Three major subdivisions of the Cld protein family have been identified in phylogenetic studies.^{6–10} Two of the subfamilies, referred to as functional Clds, catalyze the decomposition of ClO_2^- and differ in subunit size and oligomeric state. The first of these consists predominantly of the respiratory Clds that are pentameric and found mainly in proteobacteria. The second subfamily is populated by Clds from non-perchlorate-respiring species; their subunit size is significantly smaller than that of the respiratory Clds due to a truncated N-terminus. The third Cld subfamily contains Cld-like proteins,¹¹ which catalyze the oxidative decarboxylation of coproheme in the terminal step of heme *b* biosynthesis in Gram-positive bacteria and, potentially, in some Archaea.^{12–16} The encoding gene for these coproheme decarboxylases is *hemQ*.¹²

In perchlorate-respiring bacteria, the ClO_2^- decomposing reaction constitutes the terminal step in perchlorate (ClO_4^-) or chlorate (ClO_3^-) respiration; Cld catalyzes the reaction to efficiently detoxify the ClO_2^- produced by catalytic ClO_3^- reduction.¹⁷ The Cld from *Dechloromonas aromatica* (*DaCld*), a representative of Clds from ClO_4^- and ClO_3^- -respiring bacteria, turns over >20,000 equivalents of chlorite per heme, with catalytic efficiency nearing the diffusion limit, before it is irreversibly inactivated ($k_{\text{cat}}/K_{\text{M}} = 3.2$

(± 0.4) $\times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 5.2, 4 °C).¹⁸ The proposed mechanism for O₂ formation involves initial transfer of an oxygen atom from ClO₂⁻ to the ferric heme in a two- or one-electron oxidation to yield, either a Fe^{IV}=O porphyrin π -cation radical (Compound I),¹⁹ or a Fe^{IV}=O heme (Compound II), respectively.^{20, 21} It and the resulting OCl fragment, either hypochlorite (OCl⁻) or chlorine monoxide (OCl), are maintained as a geminate pair in the distal pocket, which comprises a positively charged Arg residue and a number of hydrophobic residues.^{6, 22–24} The “trapped” OCl fragment is thought to be poised for nucleophilic attack on the electron-deficient ferryl oxygen atom to generate a putative ferric peroxyhypochlorite (OOCl⁻) intermediate that decomposes to yield Cl⁻, O₂ and the resting enzyme.

Although members of the second subfamily are efficient catalysts for ClO₂⁻ decomposition ($k_{\text{cat}}/K_M \approx 10^6 \text{ M}^{-1} \text{ s}^{-1}$, pH 5.0–6.0, 20–30 °C),^{6, 10, 11} their function is not definitively understood. For the Cld from *Klebsiella pneumoniae* strain MGH 78578 (*KpCld*), a Gram-negative, non-perchlorate-respiring bacterium, studies of a *cld* knockout strain suggest that it may function in the detoxification of chlorite produced endogenously by nitrate reductases, from ClO₄⁻ or ClO₃⁻.¹⁰ *KpCld* and perhaps Clds from other non-perchlorate-respiring species may therefore be part of a system that protects against the effects of environmental ClO₄⁻ or ClO₃⁻, each of which has minute but ubiquitous natural sources.^{1, 25, 26}

The heme environments of the two ClO₂⁻-decomposing Cld subfamilies consist of similar active site residues, including a highly conserved proximal histidine, through which the heme cofactor is bound to the enzyme,^{22, 23} and a conserved distal arginine (Figure 1).^{24, 27, 28} Spectroscopic data and crystal structures reveal that the distal arginine has two main conformations: one in which its guanidinium group points toward the heme and one where the side chain is directed toward the main access channel into the heme pocket.^{6, 22, 24, 29} Substantial loss of chlorite degrading activity upon mutation of the distal Arg indicates that it is catalytically important but not essential for activity.^{24, 27, 28} One role of the distal Arg is confining ClO⁻ generated during ClO₂⁻ decomposition in the heme pocket.³⁰ In the case of *DaCld*, ClO⁻ is not detected during catalytic turnover (although it may contribute to inactivation of the enzyme oxidative destruction of the heme).^{18, 31} In contrast, ClO⁻ escape from the active sites of *KpCld* and the Cld from *Candidatus Nitrospira defluvii* (*NdCld*), a pentameric Cld, has been detected as HOCl.^{10, 30} This HOCl is responsible for the limited turnover observed for *KpCld* (6700 $\pm$ 300 turnovers/heme) relative to *DaCld*.¹⁰ This suggests that although the general heme site characteristics of Clds are similar, there are subtle variations in their active site structures and/or dynamics that modulate accessibility of the active site to bulk solvent.

In addition to varying turnover numbers, physical differences have been reported between the active sites of *KpCld* and *DaCld*. Notably, Trp227 of *DaCld*, located on the periphery of the heme (Figure 1A), is highly conserved among known perchlorate respirers but replaced by a Glu in dimeric Clds (Figure 1B).⁵ Also, both the rR core size marker frequencies and rhombicity differences in the HS EPR spectra indicate differing ligation states in the resting, ferric *DaCld* and *KpCld*.¹⁰ Since the distal heme environment is a major determinant of heme enzyme activity, numerous spectroscopic methods using physiological and

nonphysiological heme ligands have been developed to probe the distal landscape of the heme pocket. Here we examine ferric Cld complexes with chloride, fluoride, and hydroxide to probe the heme environments of various Clds with the goal of identifying structural elements that differentially modulate the ClO_2^- -degrading activities of the two functional subfamilies. New vibrational frequency correlations between either $\nu(\text{Fe}^{\text{III}}\text{--F})$ or $\nu(\text{Fe}^{\text{III}}\text{--OH})$ and $\nu(\text{Fe}^{\text{II}}\text{--His})$ for the *trans* (proximal) His of ferric Clds and other heme proteins are reported. These correlations orthogonalize the effects of proximal and distal heme environments on the bonding between iron and exogenous π -donor ligands. The Fe-axial ligand vibrations and the relationships between them illuminate both similarities and differences in the H-bonding and electrostatic properties of the distal and proximal heme environments in pentameric and dimeric Clds.

EXPERIMENTAL METHODS

Growth, purification, and characterization of Da and KpCld enzymes

DNA containing the full-length coding region of chlorite dismutase from *Klebsiella pneumoniae* MGH 78578 (accession no. CP000650.1) was PCR-amplified and cloned into the pET-15b (Merck/Novagen) expression vector for production of protein with a N-terminal His-tag as previously described.¹⁰ The resulting vector was mutated to replace the thrombin protease site (CTG GTG CCG CGC GGC AGC) with a TEV protease site (GAA AAC CTG TAT TTT CAG GGC) for removal of the 6-His tag post purification. A Q5 Site-Directed Mutagenesis Kit was used with primers TEV_F (5'-t t t c a g g g c C A T A T G A A T A C A C G A T T A T T T A C G T T C G-3') and TEV_R (5'-t a c a g g t t c G C C G C T G C T G T G A T G A T G-3'). Mutated plasmid was transformed in Tuner DE3 *E. coli* cells and *KpCld* was expressed, isolated, and purified as previously described.¹⁰ The N-terminal His-tag of *KpCld* was removed by incubation with S219V TEV (at 1 mol TEV: 10 mol Cld) in 50 mM NaPhos pH 8.0, 2 mM DTT, and 0.5 mM EDTA overnight at 4 °C.³³ The proteolysis mixture was passed over a 5 mL HisTrap column equilibrated in 20 mM imidazole, 100 mM NaPhos pH 7.4. A linear gradient (20–500 mM imidazole) was used to separate cleaved *KpCld* from tagged *KpCld* during elution. Imidazole was removed with a PD-10 desalting column and exchanged into 100 mM sodium phosphate pH 6.8. Purity and cleavage were verified by SDS-PAGE.

Plasmids for the over-expression of WT *DaCld*, *DaCld*(R183Q), and *DaCld*(W227F) were available from previous work.^{27, 31, 34} These *DaClds* were expressed, isolated, and purified using published protocols.¹⁸ All Cld concentrations are given as heme-bound monomer, where [Cld] was determined using reported extinction coefficients. The activity with ClO_2^- was routinely monitored to confirm the competency of each enzyme preparation.^{10, 18} Molecular weights for both ferric *DaCld* and *KpCld* were determined by gel filtration on a 26×1200 mm S-200 Sephacryl HR column in 0.1 M sodium phosphate pH 6.8 at 0.7 mL / min. The gel filtration column was calibrated with horse heart cytochrome *c* (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), yeast alcohol dehydrogenase (150 kDa), sweet potato β -amylase (200 kDa), and blue dextran (2,000 kDa). Chromatograms were recorded for each marker and Cld by tracking absorbance at 280 nm or 254 nm. After each chromatographic run, UV-Vis absorbance

spectra were recorded for relevant fractions corresponding to eluted markers/proteins. *KpCld* gel filtration was also performed in the presence of 10 mM imidazole and 200 mM sodium chloride on separate chromatographic runs.

Quantification of halide binding to Clds by UV-visible spectrophotometric titrations

Titration of Clds with F⁻ (pH 5.8, 100 mM phosphate), Cl⁻ (pH 6.2, 100 mM phosphate) and Br⁻ (pH 6.0, 100 mM phosphate) ligands (as aqueous NaX solutions) were carried out using 6 – 8 μM enzyme and [NaX] ranging from 10 μM to 0.5 M; ligand solutions were added to 800 μL enzyme samples in aliquots ranging from 1 to 5 μL. UV-visible absorbance (UV-vis) spectra were acquired after each addition until the samples reached equilibrium. The titration was considered complete when no spectral changes were observed upon addition of a 5 μL aliquot of concentrated ligand stock. Individual spectra were corrected for sample dilution; these corrected spectra were used to generate difference spectra for obtaining r values. The fraction of enzyme with the ligand bound (r) was represented by A/A_{∞} where $A = A_f - A_x$, $A_{\infty} = A_f - A_i$, A_f is the initial absorbance, A_x is the absorbance at a given ligand concentration, and A_i is the final absorbance (*i.e.* at saturating ligand concentrations). Plots of r versus free ligand concentration ($[X^-]_F$) were constructed for absorbance data at the wavelength of maximal absorption change and fit by nonlinear regression to equation 1 for a single binding site and to equation 2 to consider cooperative ligand binding:

$$r = \frac{[X^-]_F}{K_D + [X^-]_F} \quad [1]$$

$$r = \frac{[X^-]_F^n}{K_D + [X^-]_F^n} \quad [2]$$

In the experiments described here, $[X^-]_T$ was always $\gg$ [heme-X], such that $[X^-]_T \approx [X^-]_F$.

Chlorite-decomposing activity in the presence of coordinating and non-coordinating anions

Initial rates of chlorite-decomposing activity by *KpCld* were determined by monitoring O₂ evolution with a luminescence-based probe with varying substrate (ClO₂⁻) concentrations and NaCl as a potential inhibitor with concentrations fixed at 1, 5, 10, 100, or 200 mM. These measurements were carried out under pseudo-first order conditions with 2.0×10⁻⁸ M enzyme and [ClO₂⁻] concentrations ranging from 0.1 mM to 2.0 mM in 100 mM sodium phosphate, pH 6.0. Concentrations of freshly prepared stock NaClO₂ solutions were determined via iodometric titration or spectrophotometrically by measuring absorbance at 260 nm using $\epsilon_{260} = 155 \text{ M}^{-1} \text{ cm}^{-1}$.³⁵ *KpCld* samples were equilibrated with Cl⁻ prior to the assay. Reactions were initiated by introducing the enzyme solution with a 10 μL syringe, and kinetic runs, done in quadruplicate, were carried out by recording probe luminescence at

0.1 s intervals for 60 s. Analogous initial rate measurements were performed with *KpCld* in 100 mM NaClO₄, *KpCld*-F (50 mM NaF) and *KpCld*-CN (50 μM KCN).

Vibrational characterization of Cld halide and hydroxide complexes

Resonance Raman (rR) scattering was excited with either 406.7-nm or 413.1-nm emission from a Kr⁺ laser, or 441.6-nm emission from a HeCd laser, using the 135° backscattering geometry for collection of Raman scattered light. The spectrometer was calibrated against Raman frequencies of toluene, dimethylformamide, acetone, and methylene bromide. Spectra were recorded at ambient temperature from samples in spinning, 5-mm NMR tubes. Laser power at samples ranged from 5 to 10 mW; no spectral artifacts due to photoinduced chemistry were observed with these irradiation powers. UV-visible spectra were recorded from the rR samples before and after spectral acquisition to assess whether sample integrity had been compromised by exposure to the laser beam.

Both WT Clds were examined in the presence of varying chloride ion concentrations in potassium phosphate buffer at pH 5.7 or 6.0 which are below the kinetic p*K*_a of *DaCld*, and pH 7.5, which is above the kinetic p*K*_a of *DaCld* and below the p*K*_as for heme-OH formation in both *DaCld* and *KpCld*. Resonance Raman spectra of WT Clds in the presence of 100 mM sodium sulfate and sodium perchlorate were acquired as control experiments. Ferric *KpCld*-F, *DaCld*-F, *DaCld*(W227F)-F samples for rR spectroscopy were prepared 100 mM potassium phosphate buffer at pH 5.8.

RESULTS

Chloride binding to *KpCld* perturbs the heme pocket in favor of a heme-aqua complex

The sensitivity of the *KpCld* active site to its Cl⁻ product has been examined by UV-vis and rR spectroscopic methods. Figure 2A shows the spectral changes in the UV-vis absorbance spectrum of ferric *KpCld* at pH 6.2 in response to increasing [Cl⁻]. In the absence of Cl⁻, ferric *KpCld* exhibits a B band maximum at 403 nm with an intense shoulder at ~380 nm, Q bands at 504 and 540 nm, and a charge transfer (CT1) band at 645 nm. Upon addition of Cl⁻, the B-band shifts to the red and sharpens while its high-energy shoulder disappears. The CT1 band shifts from 645 to 638 nm. However, the positions of the Q bands are not measurably altered. These spectral characteristics are similar to those observed for aqua complexes of metMb and metHb (*c.f.* Sperm Whale aquametMb pH 6.0, 409.5, 505, and 635 nm; Horse aquametMb pH 6.4, 408, 502, and 630 nm; aquametHb pH 6.4, 405, 500, and 631 nm) or the 6cHS *Coprinus cinereus* peroxidase-benzohydroxamic acid complex (CIP-BHA 407, 503, 638 nm).^{36–38} Since the changes observed in the UV-vis spectra of the Cl⁻ titration are consistent with formation of an aqua-heme complex, it suggests that the Cl⁻ may bind in or near the heme pocket so as to favor water binding to the heme in resting *KpCld*.

Isosbestic points observed in the *KpCld* spectra upon titration with Cl⁻ (348, 402 and 432 nm) suggest an equilibrium between two heme species (Figure 2A). However, consistent with the sigmoidal shape of the titration curve (inset, Figure 2A), attempts to fit it to a single binding site model (dashed red line) did not converge. The data were well modeled by the

Hill function with $n = 2.3 \pm 0.1$, suggestive of positive cooperativity in the binding of Cl^- to the enzyme; the same fit yields a composite K_D for the cooperative binding of Cl^- to $KpCld$ of $1.4(\pm 0.3) \times 10^{-3}$ M (solid blue line, Figure 2A inset). The isosbestic points reflect conversion of the five-coordinate high spin (5cHS) ferric heme in the resting enzyme to a six-coordinate high spin (6cHS) aqua complex with the relative amounts of 5cHS and 6cHS heme being influenced by Cl^- binding.

To address the possibility that this behavior is a general ionic strength effect rather than being specific to Cl^- , UV-visible and rR spectra of ferric $KpCld$ with NaCl, KCl, KBr, NaClO_4 , or Na_2SO_4 were recorded. UV-vis spectra obtained for $KpCld$ in 100 mM NaClO_4 or Na_2SO_4 have features comparable to ferric $KpCld$ (Figure S1). The UV-vis spectral signature of $KpCld$ in the presence of Cl^- was insensitive to whether the counter ion was Na^+ or K^+ . Spectrophotometric titration of resting $KpCld$ with Br^- yielded spectral changes similar to those observed upon titration with Cl^- ; albeit for a single binding site and with $K_D = 1.22(\pm 0.03) \times 10^{-2}$ M, approximately ten-fold greater than that for Cl^- (Figure S2). The radii of the anions used to probe this effect increase in the order Cl^- (180 pm) < Br^- (198 pm) < SO_4^{2-} (242 pm) $\approx$ ClO_4^- (241 pm).³⁹ Thus, the conversion of the active-site heme state to 6cHS under the influence of anions depends upon their ionic radii with the upper limit being ~ 200 pm. An upper limit on the size of the anions that induce hexacoordination is consistent with steric constraints on access to the sites whose interactions with the anions drives the change in heme coordination number. This ionic radius effect raises the question of whether the smaller anions bind inside or outside the heme pocket. This question is discussed below following presentation of the rR results.

Under acidic conditions, the Soret-excited rR spectrum of heme in ferric $KpCld$ exhibited a broad ν_3 band, a coordination and spin-state state marker centered at 1490 cm^{-1} . This feature is consistent with the presence of an equilibrium mixture of 5cHS and 6cHS water-bound heme states.¹⁰ The presence of chloride ion favors the 6cHS heme, as judged by a shift in ν_3 to 1484 cm^{-1} and the growth of the 1515 cm^{-1} band corresponding to the in-plane ν_{38} mode (Figure 2B). Neither perchlorate nor sulfate exert this effect on the coordination number. The aforementioned frequencies are comparable to those reported for other aqua heme complexes, such as aqua metMb at pH 6.4 (ν_3 , 1482 ; ν_{38} , 1512 cm^{-1}).⁴⁰ For comparison purposes, $KpCld\text{-F}$ with its ν_3 at 1477 cm^{-1} and ν_{38} at 1511 cm^{-1} is an example of a 6cHS complex of $KpCld$.

No $\nu(\text{Fe-Cl})$ band is observed in the low frequency spectrum of $KpCld$ in the presence of 100 mM Cl^- . The apparent lack of a LMCT band in the visible region of $[\text{Fe}(\text{por})\text{Cl}]$ absorbance spectra⁴¹ precludes identification of a $\nu(\text{Fe-Cl})$ band via selective excitation of Raman scattering by that mode (*c.f.* $\nu(\text{Fe-F})$). Thus, the question of whether Cl^- coordinates to the heme iron must be addressed by other means. A prominent band appears at 325 cm^{-1} in the Soret-excited rR spectrum and the feature around 344 cm^{-1} broadens in the presence of saturating $[\text{Cl}^-]$, suggesting at least two bands within that envelope (Figure S3). This broad peak likely includes the ν_8 band at 347 cm^{-1} and a band near 338 cm^{-1} . These new features are very similar to those observed in the spectrum of $KpCld\text{-F}$ for which bands at 323 and 338 cm^{-1} have been tentatively assigned to γ_{16} and γ_6 , respectively, based on analogy to Mb. These are out-of-plane B_{2u} and A_{2u} modes, respectively, that correspond to

pyrrole tilting.⁴² Their frequencies are consistent with the presence of Cl^- favoring a 6cHS heme complex.

Interestingly, the 6cHS *KpCld* species in the presence of Cl^- exhibits rR features of acidic ferric *KpCld* at low temperature in the absence of chloride. At -29°C the rR spectrum of *KpCld* at pH 6.0 exhibits ν_3 , ν_2 , ν_{10} , and ν_{37} bands at 1483, 1565, 1612, 1584 cm^{-1} ,¹⁰ respectively, consistent with a 6cHS aqua complex.⁴² Additionally, the shoulder at 323 cm^{-1} becomes well defined, as it does in the presence of chloride ion.¹⁰ These data, together with those presented above, suggest that chloride does not bind to the heme iron but that it interacts with the enzyme to alter the active site conformation in such a way that it favors the 6cHS aqua heme complex.

DaCld heme coordination number is insensitive to Cl^-

Spectrophotometric titration of *DaCld* with chloride ion revealed only subtle spectral changes within the chloride concentration range of 0–400 mM. Changes in the ferric *DaCld* UV-vis spectrum (392 nm B-band, 509 and 540 nm Q bands, and 645 nm CT) upon titration with Cl^- at pH 6.0 were limited to a shift of the B-band to 395 nm with a 2.5% change in B-band extinction but constant band width; the Q- and CT bands were unchanged (Figure S4). Consistent with lack of a significant effect of Cl^- on the *DaCld* UV-vis spectrum, no changes were detected in the *DaCld* rR spectrum with Cl^- concentrations below 100 mM. Difference spectra generated by subtracting the ferric *DaCld* spectrum from those recorded at high $[\text{Cl}^-]$ revealed features consistent with the generation of some 6cHS heme (ν_3 , 1483 cm^{-1} ; ν_{38} , 1515 cm^{-1} ; ν_{10} , 1618 cm^{-1} Figure 3A). These 6cHS features are quite small *i.e.* ν_3 for the 5cHS enzyme remains the dominant feature between 1480 and 1510 cm^{-1} . This is in contrast to *KpCld*, whose heme is completely converted to a 6cHS complex at much lower $[\text{Cl}^-]$ with its spectral features being invariant between 100 and 500 mM NaCl (Figures 2A & 3B).

The rR spectrum of *DaCld*(R183Q) reveals the same insensitivity of its coordination number to Cl^- as WT *DaCld* (data not shown). However, the heme in ferric *DaCld*(R183Q) is a mixture of 5cHS and 6cHS heme,²⁷ analogous to the Cl^- -free ferric *KpCld*. In the two former cases, the aqua complex is thermodynamically favored over the 5cHS complex that dominates the speciation of WT *DaCld* at pH 6.0.²⁹ Thus, replacement of the cationic Arg side chain by the shorter, neutral side chain of Gln appears to allow coordination of water in the acidic form of *DaCld*(R183Q). Given that the binding of other anionic ligands is less favorable without the native distal Arg, it seems unlikely that its replacement with Gln would facilitate Cl^- binding to the heme. Yet the rR spectra clearly report the formation of a hexacoordinate heme. Another possibility is that chloride forms an ion pair with the distal guanidinium group, thereby neutralizing the distal charge (*vis-à-vis* *DaCld*(R183Q)) and allowing water to occupy the open heme coordination site. If this were the case, two influences to the alkaline form of WT *KpCld* would be anticipated. First, neutralization of the positive charge would be expected to drive the pK_a to higher values. The pK_a was, in fact, shown by spectrophotometric titration to increase to 9.0. However, this is likely a general anion effect, as it is observed for Cl^- , ClO_4^- and SO_4^{2-} (data not shown). Second, because the low $\nu(\text{Fe}-\text{OH})$ frequencies in the Clds are attributable to the non-bonded

interaction between the OH⁻ ligand and the distal Arg, neutralization of the distal positive charge would very likely shift $\nu(\text{Fe}-\text{OH})$ to higher frequency. The Soret-excited rR spectra of WT *KpCld* in the presence and absence of Cl⁻ are indistinguishable at pH 10.5 (data not shown). As the evidence at hand supports neither the binding of Cl⁻ to the heme nor its presence in the heme pocket and, given the consistency of UV-vis and rR spectral signatures with *KpCld*-OH₂ in the presence of Cl⁻, the possibilities for its binding outside the heme pocket were examined.

Given the difference in sensitivity of the heme spectroscopic signatures of *KpCld* and *DaCld* to the presence of Cl⁻, the oligomeric state of *KpCld* in the absence and presence of Cl⁻ was evaluated to determine whether conformational changes induced by Cl⁻ influence subunit interactions. *KpCld* eluted from the S200 size exclusion column as a single band with an elution volume of 330 mL under both sets of conditions (Figure S5). The theoretical mass of dimeric *KpCld* is 42.6 kDa and, like the short Clds from *Nitrobacter winogradskyi* (*NwCld*)⁶ and *Cyanothece* sp. PCC7425 (*CCld*)¹¹, the data in Figure S5 are consistent with *KpCld* being a dimer in solution, whether Cl⁻ is present, or not. Therefore, the conformational and coordination changes that occur in the heme pocket when Cl⁻ is present do not affect the oligomeric state of the enzyme.

KpCld chlorite decomposition activity is not inhibited by water coordination to its resting high spin heme

The effect of Cl⁻ on the ClO₂⁻ decomposition reaction was assessed by measuring the rate of O₂ evolution as a function of [Cl⁻]. Plots of initial rate versus [ClO₂⁻] at [Cl⁻] ranging from 0 to 200 mM were constructed and fit to the Michaelis-Menten function to determine k_{cat} and K_{M} (Table S1). As their values at various [Cl⁻] were all within statistical uncertainty of one another, only the data set at 200 mM [Cl⁻] is shown in Figure S6. Values of $2910 \pm 70 \text{ s}^{-1}$ and $3.8 (\pm 0.2) \times 10^{-4} \text{ M}$ for k_{cat} and K_{M} , respectively, in the presence of saturating Cl⁻ were both quite close to the k_{cat} and K_{M} of ferric *KpCld* in the absence of chloride ($2600 \pm 100 \text{ s}^{-1}$ and $3.0 (\pm 0.4) \times 10^{-4} \text{ M}$). The effect of Cl⁻ on the ClO₂⁻ decomposing activity of *KpCld*, as revealed here by activity assays, indicates comparable enzyme efficiency in the presence and absence of Cl⁻; $k_{\text{cat}}/K_{\text{M}}$ values under both conditions are within the statistical uncertainties of the determination (Table S1). If Cl⁻ facilitates formation of a 6cHS *KpCld*-OH₂ complex, the coordination of water does not inhibit the enzyme measurably. Thus, replacement of the water ligand by an incoming substrate ion is not the rate limiting step in the decomposition reaction.

In the *KpCld*-F case, both k_{cat} and K_{M} are also on the same order of magnitude as those of the halide-free, resting enzyme, although $k_{\text{cat}}/K_{\text{M}}$ decreased by a factor of 2 relative to halide-free enzyme, suggesting a small decrease in enzyme efficiency (Table S1). As in the case with coordinated water, coordinated F⁻ is not a significant inhibitor of chlorite decomposition, even under conditions where the 6cHS species account for >99.9 % of the heme at equilibrium.

Probing the Cld distal pocket with ferric heme–F complexes

Fluoride complexes of heme proteins are sensitive probes of distal H-bond donation to atoms directly bonded with the heme iron atom. A strong linear correlation is observed between the Fe–F stretching ($\nu(\text{Fe–F})$) frequency and the CT1 transition energy. The position of a heme–fluoride complex on this correlation line has been shown to be a general spectroscopic indicator of the extent of H-bond donation from its distal pocket. Strong H-bond donation, such as that in peroxidases (distal His and Arg) or the bacterial truncated hemoglobin from *Thermobifida fusca* (Tf-trHb) (distal Trp and Tyr), is revealed by a low $\nu(\text{Fe–F})$ frequency and a red-shifted CT1 band. Weaker H-bonding environments found in Mb or dehaloperoxidase (DHP) exhibit higher $\nu(\text{Fe–F})$ frequencies.^{43, 44} The only possible endogenous distal H-bond donor in the Cld pocket is the conserved Arg side chain. Here, *DaCld* and *KpCld* fluoride complexes are used to evaluate whether their distal Arg is indeed capable of providing H-bond stabilization to a ligand atom bonded directly to the heme iron atom.

EPR data suggest that Trp227, which is conserved among pentameric Clds from known perchlorate respirers but not in dimeric Clds,^{5, 6, 10} plays an important role in differentiating the heme environments of *KpCld* and *DaCld*. *KpCld* has Glu in the position analogous to Trp227 of *DaCld*. Under acidic conditions, constraints on the heme of *DaCld*(W227F) are relaxed such that its rhombicity is lost and it presents an axial EPR signal, analogous to that of *KpCld*.¹⁰ Since mutation at this peripheral position of the heme does not abolish F[–] binding to *DaCld*(W227F) ($K_D = 3.8 \times 10^{-3}$ M),³⁴ *DaCld*(W227F)–F was characterized and compared to WT *DaCld*–F and *KpCld*–F to determine how the distal pocket interactions in *DaCld*(W227F) compare to those in the WT Clds.

Titration of *KpCld* with F[–] revealed its K_D to be $3.3(\pm 0.2) \times 10^{-3}$ M (Figure 4). The UV-visible spectrum of the *KpCld*–F complex at pH 5.8 exhibits a B band at 403 nm and a weak Q band at 491 nm. The CT1 [$a_{2u}(\pi) \rightarrow e_g(d_\pi)$] and CT2 [$a'_{2u}(\pi) \rightarrow a_{1g}(d_{22})$] bands observed at $16,347 \text{ cm}^{-1}$ (611.7 nm) and $22,150 \text{ cm}^{-1}$ (451.5 nm), respectively, (Figure 5 Inset) are assigned based on comparison to spectra of typical 6cHS complexes.⁴³ The high frequency rR spectrum shows ν_4 at 1369 cm^{-1} and ν_3 at 1477 cm^{-1} , consistent with a 6cHS F[–] complex (Figure 2B). Although the K_D for WT *DaCld* with F[–] is 1.5×10^{-2} M, about 5-fold greater than that for *KpCld*, the UV-visible (Figure 5B & 5C Insets) and rR (Figure S7) spectra for WT *DaCld*–F and *DaCld*(W227F)–F have characteristics similar to those described for *KpCld*–F. Comparison of low frequency, Soret-excited rR spectra of ferric Cld–F complexes facilitated assignment of the $\nu(\text{Fe–F})$ frequencies (Figure 5). The Fe–F stretching mode is observed in the spectra of the Cld–F complexes, but is absent in the corresponding ligand-free ferric enzymes. Assignment of the $\nu(\text{Fe–F})$ bands at 385, 390, and 393 cm^{-1} for *KpCld*–F, *DaCld*–F, and *DaCld*(W227F)–F, respectively, was facilitated by Raman excitation within both the Soret and CT2 transitions using 406.7- and 441.6-nm light, respectively. Resonance enhancement of scattering by the z-polarized $\nu(\text{Fe–F})$ modes of heme fluorides has been reported with excitation at the frequency of the x,y-polarized CT1 transition, where scattering by $\nu(\text{Fe–F})$ gains resonance enhancement in hemes exhibiting equilibrium out-of-plane distortion.⁴⁵ More recently, scattering by the $\nu(\text{Fe–F})$ modes has been achieved via excitation within the z-polarized CT2 transition, whose energy

lies between the B and Q(0,1) transitions (Figure 5, insets).⁴³ There are a couple advantages to this approach. First, even though the molar absorptivities of the CT2 and CT1 bands are similar, scattering efficiency in the blue region of the spectrum is substantially higher than in the red. Second, since the hemes in proteins and enzymes are typically distorted from planarity, it is usually possible to mark the appearance of any candidate $\nu(\text{Fe-F})$ bands in rR spectra excited within the in-plane Soret transition by comparison with the spectrum in the absence of F^- . As $\nu(\text{Fe-F})$ modes have been shown to be isolated (i.e. they behave as diatomic oscillators⁴⁵), the compositions of the other normal heme modes are sufficiently unperturbed by F^- binding that they are easily correlated between the spectra recorded in the absence and presence of F^- . Finally, by moving the exciting wavelength away from the B band and toward or within the z -polarized CT2 absorbance band, the z -polarized $\nu(\text{Fe-F})$ band gains resonance enhancement while the relative intensities of the in-plane polarized heme bands are diminished. This behavior facilitates assignment of the $\nu(\text{Fe-F})$ band and is easily seen by comparing the three rR spectra in each panel of Figure 5. The only complication of this approach is that precise determination of the $\nu(\text{Fe-F})$ frequency requires fitting of the overlapping $\nu(\text{Fe-F})$ and heme bands. However, the clear correlation of heme bands between the spectra (Figure 5) defines the number of bands to use in the fit. So, as long as different initial frequencies converge to the same best fit frequencies for a given spectrum, the possibility of errors due to degenerate solutions is easily minimized or eliminated. With attention paid to these nuances, the widespread availability of fitting routines makes this determination of $\nu(\text{Fe-F})$ frequencies reliable and straight-forward.

Variability along the positive correlation between the $\nu(\text{Fe-F})$ frequency and the CT1 energy reflects the H-bond donor ability of the distal environment, as revealed by the plot shown in Figure 6. Points with low $\nu(\text{Fe-F})$ frequency and CT1 energy are consistent with strong distal H-bond donation to the coordinated F^- ligand, like those known for *THb* and horseradish peroxidase C (HRPC).^{43, 46} Weaker distal H-bond donation is manifested in increased $\nu(\text{Fe-F})$ and CT1 energies. The position of WT *DaCld-F* and *KpCld-F* on the correlation plot is consistent with strong H-bond donations from the distal cavity to the coordinated fluoride. By analogy to HRPC-F where its distal Arg38 and water have been reported to form a H-bond network to F^- ,⁴³ the *DaCld-F* and *KpCld-F* data suggest that the distal Arg, with the possible participation of a water molecule, is responsible for H-bond donation to the coordinated F^- ligand. *DaCld-F* complexes at pH 5.8 and 7.9 fall at a similar location on the correlation line, consistent with comparable H-bonding interactions over this pH range. This insensitivity of the $\nu(\text{Fe-F})$ frequency to pH on either side of the kinetic pK_a of 6.5 is in strong agreement with the earlier conclusion that Arg183 is not deprotonated above the pK_a .²⁷ Fluoride ion has been shown to displace hydroxide in *DaCld* even at pHs greater than 9.0. It was speculated that this is attributable to direct interaction with a well-positioned H-bond-donating distal Arg183.²⁹

For *DaCld*(W227F)-F, its $\nu(\text{Fe-F})$ /CT1 energy correlation, i.e. its position on the correlation plot (Figure 6), reveals that distal H-bond donation to the coordinated F^- ligand in *DaCld*(W227F) is weaker than in both *DaCld* and *KpCld*. Despite the lack of direct hydrogen bonding between Trp227 and the heme (Figure 1A), the distinct positions of WT *DaCld* and *DaCld*(W227F) on the $\nu(\text{Fe-F})$ /CT1 correlation plot suggest that Trp227 plays a

remote role in maintaining the electrostatic and H-bonding environment of the distal heme pocket, where the substrate, ClO_2^- , binds.

Distal $\nu(\text{Fe}^{\text{III}}-\text{F})$ and proximal $\nu(\text{Fe}^{\text{II}}-\text{His})$ frequencies are inversely correlated for Clds and other heme proteins

The scatter of points about the positive correlation line discussed above (Figure 6) is greater than the uncertainty with which the frequencies can be experimentally determined. This suggests that $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequency is likely influenced by factors other than simply the CT1 energy. One clear candidate is the nature of bonding between the heme iron atom and its proximal His ligand as a reporter of the proximal H-bonding environment. Given that $\nu(\text{Fe}^{\text{III}}-\text{F})$ modes in heme fluorides behave essentially as diatomic oscillators,⁴⁵ a simple way to assess whether proximal (i.e. *trans*) $\text{Fe}^{\text{III}}-\text{His}$ bonding affects systematic influence on distal $\text{Fe}^{\text{III}}-\text{F}$ bond strength is to plot $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequencies versus $\nu(\text{Fe}^{\text{III}}-\text{His})$ frequencies for a series of hemeprotein fluorides. However, $\nu(\text{Fe}^{\text{III}}-\text{His})$ frequencies for HS hemins are not generally available. Hence, as is typically done, their $\nu(\text{Fe}^{\text{II}}-\text{His})$ counterparts were used here as proxies for distal environmental effects on the $\text{Fe}^{\text{III}}-\text{His}$ bond in hemin fluorides. The validity of this proxy finds support in EXAFS data on resting HRP-C and metMb, which, in accord with their respective 5cHS $\nu(\text{Fe}^{\text{II}}-\text{His})$ frequencies of 244⁴⁷ and 220 cm^{-1} ,⁴⁸ provide proximal $\text{Fe}^{\text{III}}-\text{His}$ bond lengths of 1.92 Å and 2.09 Å.⁴⁹ Thus, the $\nu(\text{Fe}^{\text{II}}-\text{His})$ frequencies for 5cHS ferrous heme proteins were used as reporters of proximal H-bond accepting factors known to be deterministic for σ -donor strength of the proximal His ligand.⁵⁰ The question is whether *trans*- $\text{Fe}^{\text{III}}-\text{F}$ bonding, as reported by $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequencies in the corresponding ferric hemin fluorides is responsive to environmental modulations of proximal $\text{Fe}-\text{His}$ bonding.⁵⁰ Plots of $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequency versus $\nu(\text{Fe}^{\text{II}}-\text{His})$ frequency for the Clds studied here, along with a variety of other heme proteins for which literature data are available, (Figure 7A and Table S2) reveal linear and negative correlations between them. These correlations constitute experimental evidence for the effects predicted previously by DFT calculations⁵¹ and provide more detailed insight into the roles of both distal and proximal heme environments on $\text{Fe}^{\text{III}}-\text{F}$ bonding. Although all the heme proteins on this plot have proximal His ligands, the protein environments that dictate their nonbonded interactions with the distal and proximal axial heme ligands vary considerably.

Distal environments—The Clds and a variant of the bacterial hemin trafficking protein HmuT have single distal Arg residues.⁵² Myoglobin, Hb and dehaloperoxidase (DHP)⁶⁴ have single distal His residues while the distal pocket of HRP contains a single His *and* a single Arg.⁶⁵ The $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequencies of metHb and metMb fluorides are sensitive to pH, which has been reported to result from protonation of the distal His under acidic conditions with consequent donation of a H bond to the bound F^- ligand.⁵³ On the correlation plot in Figure 7A, the increased H-bond donation from the distal, cationic imidazolium moiety places the acidic metMb and metHb fluorides well below their neutral counterparts. The vertical dispersion of these complexes along the $\nu(\text{Fe}^{\text{III}}-\text{F})$ axis is attributable to the range in distal charge and number of hydrogen bond donors for a set of heme pockets having the same proximal environment. Thus, for a given proximal environment, $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequencies reveal differences in the protonation states and charges of their distal H-bond donors.

Inspection of the entire graph reveals three correlation lines whose heights on the $\nu(\text{Fe}^{\text{III}}\text{--F})$ axis reflect a range of H-bond donor environments. Each line comprises proteins having the same amino acid-based distal H-bond donor, with the highest line corresponding to neutral His, and those for HisH^+ and Arg being negatively displaced by offsets that reflect increasing H-bond donation. According to this analysis, the distal Arg of the Clds constitutes a stronger H-bond donor environment than either the neutral or protonated distal His in the globins, likely due to its ability to donate two hydrogen bonds to the coordinated F^- . In general, for a given proximal environment, $\nu(\text{Fe}^{\text{III}}\text{--F})$ frequencies appear to reveal differences in the number of distal hydrogen bonds donated to the coordinated F^- ligand. The heights of these new correlation lines on the $\nu(\text{Fe}^{\text{III}}\text{--F})$ axis in Figure 7A are related to positions of the corresponding proteins along the $\nu(\text{Fe}^{\text{III}}\text{--F})/\text{CT1}$ correlation line in Figure 6.

Proximal environments—In addition to the distal H-bonding effects on $\nu(\text{Fe}^{\text{III}}\text{--F})$ described above, the plots in Figure 7A reveal an inverse correlation between $\nu(\text{Fe}^{\text{III}}\text{--F})$ and $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies for a given number of distal hydrogen bonds to F^- . Insofar as it is reasonable to relate the trend in $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequency to a trend in $\text{Fe}^{\text{III}}\text{--His}$ bond strength, the inverse correlation lines revealed in Figure 7A can be classified as a *trans* effect. The *trans* effect of the proximal ligand on the $\text{Fe}^{\text{III}}\text{--F}$ bond strength is reported by the negative slopes of the individual correlation lines. This behavior is consistent with a *trans* effect that weakens the $\nu(\text{Fe}^{\text{III}}\text{--F})$ force constant as that for $\nu(\text{Fe}^{\text{II}}\text{--His})$ increases. For the Clds reported here, the largest *trans* effect is observed for *KpCld*; so even though *Da* and *KpCld*s have the same distal amino acid-based H-bond donors, the $\text{Fe}\text{--F}$ bond strength in *KpCld*– F is less than in *DaCld*– F .

Interestingly, ferric heme proteins such as Mb and Hb at pH 7 and DHP, which exhibit only weak or no distal H-bond donation to a coordinated F^- ligand, fall on a nearly horizontal correlation line (slope = -0.2 ± 0.3). Among the possible explanations for this behavior is that distal H-bond donation somehow activates the *trans* effect (*i.e.* there is no clear *trans* effect on $\nu(\text{Fe}^{\text{III}}\text{--F})$ in the absence of at least a single distal H-bond donor). Although such a hydrogen-bond switch is difficult to rationalize, it is reasonable to consider the possibility of one or more effects on $\nu(\text{Fe}^{\text{III}}\text{--F})$ whose trends offset that of the *trans* ligand. A considerable database of proximal $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies for 5cHS ferrous hemes is consistent with high $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies being correlated with the presence of an anionic H-bond acceptor in the proximal heme pocket. Proteins having anionic carboxylate acceptors from Asp or Glu generally exhibit the highest $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies, which is attributed to the imidazolate character of the proximal His, whose σ donor strength is greater than neutral imidazole.^{66, 67} The accumulation of negative charge on the axial F ligand, which might otherwise result from its competition with the proximal imidazole for the d_{22} orbital, is moderated by increased π donation from p_π orbitals of F^- to the iron d_π orbitals. In other words, there is synergy between axial valence electron delocalization through the σ and π pathways that minimizes the separation of charge with increased anionic character of the proximal ligand. This self-balancing of the σ and π contributions to $\text{Fe}\text{--F}$ bond strength renders it relatively insensitive to the trend in σ *trans* effect of the proximal His ligand. This

behavior accounts for the very small negative slope of the correlation line representing heme pockets providing little or no H-bond donation (top line in Figure 7A).

By contrast, in heme pockets that provide strong H-bond donation to the coordinated F^- ligand, the $Fe-F$ π electron density is polarized toward the F atom, thereby diminishing the π character of the $Fe-F$ bond. Accordingly, distal H-bond donation diminishes the ability of the system to compensate, through increased $Fe-F$ π bonding, for *trans-induced* diminution of $Fe-F$ σ bonding. This H-bond induced decoupling of σ and π donation by the F^- ligand is manifested in a steeper negative slope of the *trans-effect* correlation lines for heme proteins having distal imidazolium and guanidinium H-bond donors. Interestingly, the slopes of those lines are the same within experimental uncertainty (-0.91 ± 0.05 and -0.90 ± 0.07 , respectively). The common slope of the imidazolium and guanidinium lines is consistent with the *trans* effect on $Fe-F$ σ bonding being independent of or insensitive to the distal effect on its π bonding. Dispersion of proteins having a given number of distal hydrogen bonds along the $\nu(Fe^{III}-F)$ axis is attributable to variation in the strength of the $Fe-F$ σ bond as the proximal ligand takes on more or less imidazolate character. These plots orthogonalize proximal and distal contributions to the exogenous $Fe^{III}-X$ bond strength, as revealed by trends in the $\nu(Fe^{III}-F)$ frequencies. Thus, the plots in Figure 7A provide direct insight into the heme pocket properties of the *resting ferric* states of the proteins. In the case of the Clds, these are the properties that govern their interactions with the anionic substrate, chlorite.

The additive proximal and distal effects revealed in Figure 7A synergistically weaken the $Fe^{III}-X$ bonds in 6cHS π -donor complexes of the Clds investigated here. Both F^- and water form such 6cHS complexes with resting *KpCld*. The kinetic barrier to dissociation of these ligands is expected to be low with high dissociation rates, as has been reported for HRP ($k_{off} = 4.2 \times 10^2 \text{ s}^{-1}$).⁶⁸ Based on this reasoning, the labilization of these ligands is expected to render them poor competitive inhibitors of Cld activity. Indeed, the K_I for inhibition by F^- and H_2O is too large to measure (Figure S6).

Distal $\nu(Fe^{III}-OH)$ and proximal $\nu(Fe^{II}-His)$ frequencies are inversely correlated for Clds and other heme proteins

Like the fluorides of *DaCld* and *KpCld*, their hydroxides are also enzymatically active.^{10, 29} Also like the fluorides, the stabilities of their hydroxides are highly dependent on the distal Arg. *DaCld* variants R183Q and R183A do not bind OH^- under conditions where the WT enzyme is completely converted to the heme hydroxide.²⁷ To test our understanding of the proximal and distal effects described above and its general applicability to π donor ligand complexes of heme proteins, $\nu(Fe^{III}-OH)$ frequencies were plotted versus $\nu(Fe^{II}-His)$ frequencies for the Clds studied here, along with a variety of other heme proteins for which literature data are available (Figure 7B and Table S2). The hydroxide complexes of numerous heme proteins, including the Clds, exist as equilibrium mixtures of 6cLS and 6cHS forms. Under conditions where heme hydroxides form, distal His residues are expected to have neutral imidazole side chains whereas Arg maintains its positively charged guanidinium side chain throughout the pH range over which the protein fold is stable. As illustrated in Figure 7B, the 6cHS hydroxide complexes exhibit trends similar to the 6cHS

fluoride complexes in Figure 7A. Specifically, the points fall on two correlation lines. One comprises proteins whose distal pockets contain a neutral His and, like its F^- counterpart, is nearly horizontal. The other contains proteins whose distal pockets donate H-bonds from Arg and has a negative slope. As in the case of the heme fluorides, the vertical offset of the individual lines report the dependence of the Fe–OH bond strength, as reported by the $\nu(Fe^{III}\text{--}OH)$ frequency, on the number of amino acid-based distal hydrogen bonds to the coordinated OH^- . The slope of the correlation lines (-0.04 ± 0.04 for HS–OH with neutral distal His and -2.3 ± 0.9 for HS–OH with Arg) can be explained in the same terms as heme fluorides. The slope of the $\nu(Fe^{III}\text{--}OH)/\nu(Fe^{II}\text{--}His)$ Arg line in Figure 7B is steeper than the corresponding F^- line in Figure 7A, ostensibly because OH^- is a weaker π donor ligand than F^- , making its susceptibility to Fe–OH bond weakening by the *trans* effect greater than that in the heme fluorides. Note that there is no line for distal imidazolium because the pK_a of imidazolium lies far below that for heme hydroxide formation.

The $\nu(Fe^{III}\text{--}OH)$ frequencies of LS heme hydroxides are also sensitive to both proximal and distal environments. The top of Figure 7B shows that, like the HS heme hydroxides and fluorides, the LS hydroxides for which frequencies are available, from this study and literature sources (Table S2), fall on two $\nu(Fe^{III}\text{--}OH)/\nu(Fe^{II}\text{--}His)$ correlation lines. Their positions along the $\nu(Fe^{III}\text{--}OH)$ axis distinguish between distal pockets comprising neutral His (higher line) and Arg (lower line) as single and double H-bond donors, respectively. In contrast to the HS cases, both lines correlating the $\nu(Fe^{III}\text{--}OH)$ and $\nu(Fe^{II}\text{--}His)$ frequencies have similar negative slopes (slopes of the correlation lines are -1.3 ± 0.5 and -1.8 ± 0.2 , respectively, Figure 7B). Thus, the *trans* effect on the Fe–OH σ bonding is the dominant factor in modulating the overall Fe–OH bond strength, as reported by the $\nu(Fe^{III}\text{--}OH)$ frequency, even for proteins in which the distal H-bond donor is a charge-neutral His. The most likely reason for this is that, even though hydroxide is a weak π donor ligand, the filled d_π orbitals preclude significant π bonding in the LS hydroxides.

DISCUSSION

The O_2 -evolving Clds constitute an unusual example of rapid and efficient catalytic O–O bond-formation. Vibrational signatures of halide and hydroxide complexes with the resting ferric enzyme are sensitive probes of the heme environment. They have been used here to compare and contrast the anion complexes of *KpCld* and *DaCld*, having low and high turnover numbers, respectively, in order to gain insight into the structural properties crucial to high-fidelity O_2 generation.

Differential sensitivity of Cld active sites to the presence of halides reveals distinctions between the nonbonding interactions that shape their heme pockets

The binding of Br^- and the cooperative binding of chloride to *KpCld* drives formation of a 6cHS heme complex. There are two ways this could occur: coordination of the halides to the heme iron or binding to one or more non-heme sites, inducing a conformational change that favors coordination of a water molecule. There are a few examples of Cl^- coordination to the iron center in heme-dependent enzymes. As bacterial dye-decolorizing peroxidases (DyPs) belong to the CDE (Clds, DyPs, EfeB) superfamily, the most relevant examples are several

distal pocket DypB variants in which Cl^- coordinates to their heme iron.⁶⁹ However, a $\nu(\text{Fe}-\text{Cl})$ band is not observed in the low frequency spectrum of *KpCld* at $[\text{Cl}^-]$ where the formation of 6cHS heme is known, by spectrophotometric titration and the high-frequency rR spectrum, to be complete. In contrast, there are examples in which Cl^- binds to heme enzymes at sites removed from the iron. For example the distal heme pockets of lactoperoxidase and myeloperoxidase contain Cl^- binding sites that accommodate the halide without its direct coordination to the iron.^{70, 71} The spectral similarities among the 6cHS *KpCld* complex at saturating $[\text{Cl}^-]$ with other heme aqua complexes in globins and peroxidases is consistent with Cl^- binding at a site removed from the heme iron and inducing a conformational change that facilitates water binding at the heme. The crystal structure of *NwCld* (Figure 1B), a dimeric enzyme like *KpCld*, shows a water molecule coordinated to the heme iron. Several steps of the published isolation procedure expose *NwCld* to $[\text{NaCl}] = 150 \text{ mM}$.⁶ Thus, if sensitivity to Cl^- binding is a general characteristic of dimeric Clds, a 6cHS aqua complex of *NwCld* would be anticipated in the presence of high $[\text{Cl}^-]$. Furthermore, solutions of *NwCld* isolated in the absence of Cl^- are hypothesized to contain at least some 5cHS heme.

Although Cl^- and Br^- drive a change in the coordination number of the HS iron from 5c to 6c in *KpCld*, its chlorite decomposition activity remains essentially unchanged (Table S1). So Cl^- formed during the chlorite decomposition reaction is not an inhibitor of *KpCld*. For the pentameric *DaCld* and the Cld from *Magnetospirillum sp.* (*MaCld*), Cl^- has been shown to be a weak mixed hyperbolic inhibitor: $K_I = 225 \text{ mM}$ and $K'_I = 95.6 \text{ mM}$ for *DaCld*¹⁸ and $K_I = 460 \text{ mM}$ and $K'_I = 480 \text{ mM}$ for the *MaCld*.⁷² These K_I values are approximately two orders of magnitude larger than the composite K_D of 1.44 mM for cooperative Cl^- binding to *KpCld*. This is consistent with the observation that the *DaCld* heme remains 5cHS at Cl^- concentrations that drive *KpCld* to be 6c (Figure 3). Thus *DaCld* is more resistant to chloride-induced conformational changes than *KpCld*. A possible explanation for this is revealed by comparing the crystal structures of pentameric and dimeric Clds, as shown in Figure 8. In the *DaCld* structure, the heme pocket is formed by hydrophobic interactions between residues on the alpha helix (α_9) and the beta sheets (β_5 , β_8 , and β_9), shaping the heme pocket: L205/L233, F208/I145, M212/I147, M212/W227 (Figure 8A). These hydrophobic residues are not strongly driven to interact with ions like Cl^- . Additionally, access to these residues is blocked because they are located near the subunit interface in the pentamers. In dimeric *NwCld*, the heme pocket is shaped by ionic interactions between residues on the alpha helix (α_5') and on the beta sheets (β_4' and β_1'): E156/R178, R163/D176, R163/E174, and W168/E174 (Figure S8). Similar pairwise ionic interactions are also observed in the homology structure for *KpCld*: E155/R181, R166/D179, R166/E177, and W171/E177. An additional interacting pair of residues (N159/R181) along this face of the heme pocket is noted in the *KpCld* homology structure (Figure 8B). Any of these ionic interactions could be disrupted or perturbed by the incursion of Cl^- . The cooperative nature of Cl^- binding ($n = 2.3/\text{heme}$) suggests that its energetically coupled participation in more than one of these ionic interactions results in conformational changes that, as discussed above, drive formation of a heme-OH₂ complex in *KpCld*.

Crystal structures of pentameric *DaCld* and *MaCld* indicate that the conserved Trp227, one of the residues lining the heme pocket, is within 4.3 to 5 Å of a heme vinyl group.^{22, 73}

Thus, although Trp227 is not directly H-bonded to the heme, the participation of its indole side chain in hydrophobic stacking interactions with Met212 and His224 contributes to stabilization of tertiary structure involving $\alpha 9$, $\beta 8$ and $\beta 9$, which shape the back of the *Da*Cld heme pocket. Subtle disruption of that triad is achieved by mutation of Trp227 to Phe, likely due to the difference in the extents of their π systems. Based on the position of *Da*Cld(W227F) on the $\nu(\text{Fe}^{\text{III}}\text{--F})/\text{CT1}$ correlation plot (Figure 6), one might conclude that this peripheral perturbation manifests as weakened distal H-bond donation. However, Figure 7 reveals that this disruption in peripheral hydrophobic interactions actually diminishes the *trans* effect on both $\nu(\text{Fe}^{\text{III}}\text{--F})$ and $\nu(\text{Fe}^{\text{III}}\text{--OH})$. Interestingly, in dimeric Clds the residue at this position is Glu (E177 in *Kp*Cld; E174 in *Nw*Cld). This natural substitution contributes to stabilization of the heme pocket in *Kp*Cld by ionic interactions. The positions of *Da* and *Kp*Cld on the $\nu(\text{Fe}^{\text{III}}\text{--F})/\text{CT1}$ correlation plot (Figure 6), suggest that the ionic interactions between Arg166, Glu177, and Trp171 in *Kp*Cld are effective in maintaining a distal heme environment similar to that shaped by the corresponding hydrophobic interactions in *Da*Cld. In this case, the fact that, both enzymes fall on the same $\nu(\text{Fe}^{\text{III}}\text{--F})/\nu(\text{Fe}^{\text{II}}\text{--His})$ and $\nu(\text{Fe}^{\text{III}}\text{--OH})/\nu(\text{Fe}^{\text{II}}\text{--His})$ lines in Figure 7 also supports similarity in their distal H-bonding environments, albeit under the influence of different *trans* environments.

All reported subfamily 1 Clds contain a signal peptide which designates them to the periplasm. The lack of a signal peptide in subfamily 2 suggests that these enzymes are localized to the cytoplasm.¹¹ Thus, different subcellular localizations of Clds from subfamilies 1 and 2 exposes them to different Cl^- concentrations. A $[\text{Cl}^-]$ gradient is maintained such that cytoplasmic $[\text{Cl}^-]$ at 10 to 100 mM is less than in the extracellular fluid. Since the outer membrane of Gram-negative bacteria contains porins, which allow for passive diffusion of small anions, such as Cl^- , the periplasm has a similar Cl^- concentration as the extracellular fluid.⁷⁴ Therefore, subfamily 1 Clds are likely exposed to higher $[\text{Cl}^-]$ than their counterparts in subfamily 2. The distinct sensitivities of *Da* and *Kp*Clds to $[\text{Cl}^-]$ reflect the different subcellular Cl^- concentrations. Measurable loss of chlorite-decomposing activity in the presence of Cl^- is observed for *Da*Cld, at concentrations up to 200 mM.¹⁸ Despite the measurable activity loss, heme spectroscopic features reveal that the cofactor speciation in *Da*Cld is not significantly altered in the presence of excess Cl^- . This is in stark contrast to *Kp*Cld which exhibits no measurable activity loss when exposed to Cl^- . Interestingly, however, the affinity of its heme for water as a sixth ligand is increased in the presence of Cl^- . Therefore, the physiologically relevant form of the heme in resting *Kp*Cld may be 6cHS, as opposed to the 5cHS heme in resting *Da*Cld.²⁹ Thus, distribution of the heme between 5c and 6c states in *Kp*Cld is likely to be strongly dependent on the cytosolic Cl^- concentration over the physiological range of 10 to 100 mM.⁷⁵

Strong H-bond donation to the coordinating atom of exogenous heme ligands is characteristic of Clds

The affinity of pentameric Clds for F^- (K_D : *Da*Cld, 15 mM;²⁹ *Nd*Cld, 5.9 mM⁷⁶) is comparable to that determined here for dimeric Clds (K_D : *Kp*Cld, 3.3 mM). That *Da*Cld distal pocket mutants R183Q, R183K, and R183A and analogous *Nd*Cld distal pocket variants do not bind F^- points to an important role of the distal Arg in stabilizing heme-anion complexes in Clds.^{27, 76} The similar positions of *Kp*Cld and *Da*Cld on the plot in

Figure 6, which shows the correlation of $\nu(\text{Fe}-\text{F})$ frequency with CT1 energy, suggests similarity among their distal H-bond donation to the coordinated F^- ligands. The more detailed view provided by the correlations in Figure 7 reveal that *Da* and *KpCld* fall on the same low $\nu(\text{Fe}^{\text{III}}-\text{X})/\nu(\text{Fe}^{\text{II}}-\text{His})$ correlation lines, where X is either F^- or OH^- , consistent with the distal Arg residue in each enzyme providing two H-bonds to the bound X^- ligand. Therefore, the two H-bond donating distal environments in these enzymes are expected to contribute similarly to the stabilities of exogenous anion complexes, including putative ferryl intermediates. The Clds examined here all fall on correlation lines that reveal inverse relationships between $\text{Fe}^{\text{III}}-\text{F}^-$ and $\text{Fe}^{\text{III}}-\text{His}$ bond strengths. Analogous trends are seen for HS and LS heme hydroxides. A similar inverse correlation between $\nu(\text{Fe}^{\text{IV}}=\text{O})$ and $\nu(\text{Fe}^{\text{II}}-\text{His})$ has been reported for Mb and a number of peroxidases.⁵⁰ These four inverse correlations illustrate the importance of variability in the *trans* effect on the exogenous $\text{Fe}-\text{X}$ bond strength for a given number of hydrogen bonds donated by the distal pocket. Interestingly, that variability, as reported by the slopes of the correlation lines, is similar for the aforementioned $\nu(\text{Fe}^{\text{IV}}=\text{O})/\nu(\text{Fe}^{\text{II}}-\text{His})$ correlation⁵⁰ and the LS $\nu(\text{Fe}^{\text{III}}-\text{OH})/\nu(\text{Fe}^{\text{II}}-\text{His})$ correlation reported here. The *KpCld* complexes, being lower (more to the right) on their correlation lines (Figure 7), reveal a greater *trans-effect* of their proximal His on their $\text{Fe}-\text{X}$ bonds than *DaCld*. However, neither experiences a *trans* effect as large as the $\text{Fe}^{\text{III}}-\text{OH}$ bond in peroxidases, as illustrated by their position well to the right of the Clds. Indeed, Clds are poor peroxidases¹⁹ because, in addition to lacking a distal catalytic base, they lack sufficient “push” because their proximal His residues are weaker σ donors than the imidazoles of the peroxidases (Figure 7). Moreover, the polarizing cationic side chain of the distal Arg in Clds is sufficiently close to the heme that it donates two hydrogen bonds to the coordinating atom in $\text{Fe}-\text{X}^-$ complexes. This arrangement is unlikely to be optimal for polarizing the peroxo bond toward the heterolytic $\text{O}-\text{O}$ bond cleavage characteristic of peroxidases. By contrast, strong polarization of the $\text{O}-\text{Cl}$ bond in the $\text{Cld}-\text{OCIO}^-$ complex is likely less critical to heterolytic bond cleavage, as that bond is already slightly polar. Rather, the distal Arg in the Clds appears to facilitate formation of the enzyme substrate complex and maintaining the $\text{Fe}^{\text{IV}}=\text{O}/\text{OCl}$ geminate pair after the putative $\text{O}-\text{Cl}$ bond scission.

$\nu(\text{Fe}^{\text{III}}-\text{X})/\nu(\text{Fe}^{\text{II}}-\text{His})$ correlations are general reporters of ferric heme pocket properties

Given that two of the distal H-bond donors considered in Figure 7A are cationic, the importance of charge in displacement along the $\nu(\text{Fe}^{\text{III}}-\text{F})$ axis must be considered. To test the general applicability of the $\nu(\text{Fe}^{\text{III}}-\text{F})/\nu(\text{Fe}^{\text{II}}-\text{His})$ correlation plots, a set of *TftrHb*- F^- variants representing a range of charge-neutral distal H-bonding environments were placed on the plot. The active site of *TftrHb* contains distal Trp and Tyr residues known to stabilize complexes of exogenous ligands with a second Tyr providing indirect H-bonding to the heme ligand via a bridging water molecule.^{44, 46, 77} Figure S9 shows that the vertical distribution of the $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequencies for these proteins on the correlation plot is consistent with the number of H-bonds predicted by molecular dynamics simulations to be donated to the coordinated F^- ligand. While charge appears to have some systematic effect on vertical displacement along the $\nu(\text{Fe}^{\text{III}}-\text{F})$ axis, $\nu(\text{Fe}^{\text{III}}-\text{F})$ frequencies cluster in ranges corresponding to the number of H-bonds donated to the F^- ligand.

Support for the HS OH⁻ line with 2 H-bond come from the *Mt* trHbN and *Mt* trHbO proteins that have been reported to undergo a conformational change upon spin state change. The LS OH⁻ complexes of these proteins exhibit little to no H-bonding to OH⁻ in their distal pocket, and they are positioned near the LS OH⁻ weak H-bonding line. The conformational change accompanying a spin state change to HS results in their OH⁻ complexes falling on or close to the HS OH⁻ 2-H bond line. The H-bond donors are for trHbN and trHbO are Tyr/Gln and Tyr/Trp, respectively.⁷⁸ This indicates that the trends illustrated in the correlation plot are general in nature and not just applicable to distal pocket H-bond donation from amino acid side chains containing nitrogen (His, Arg, Trp versus Tyr).⁷⁸

An enzyme's position on these correlation plots yields insight into the extent to which it weakens π bonding between Fe and the π -donor ligands, F⁻ and OH⁻, through distal H-bond donation and σ bonding via the *trans* effect (*vide supra*). As such, it may provide insight into why and whether π -donor ligands, such as F⁻ and OH⁻, and OH₂ are poor competitive inhibitors of enzymes whose substrate binding site is an axial coordination site on the heme iron center. Specifically, such ligands that accept multiple distal H-bonds and lie to the high-frequency end of the $\nu(\text{Fe}^{\text{II}}\text{--His})$ axis are likely to exhibit low barriers to Fe–L bond dissociation, a necessary prelude to formation of the enzymesubstrate complex. Worthy of note is that *KpCld*–F and *KpCld*–OH lie to the right of their *DaCld* analogues on the respective correlation lines. On that basis, it is hypothesized that *KpCld* will be inhibited to a lesser extent by F⁻, OH⁻, and OH₂ than is *DaCld*.

Intersecting or overlapping correlation lines—Heme hydroxides of the trHbs are similarly dispersed along the $\nu(\text{Fe}^{\text{III}}\text{--OH})$ frequency axis in accord with the number of H-bonds to the O atom of coordinated OH⁻. As seen in Figure S10, the HS and LS *Tf* and *Mycobacterium tuberculosis* (*Mt*) trHbs fall remarkably close to the HS and LS correlation lines established in Figure 7B. Interestingly, the LS *Tf* trHb hydroxides that are predicted by MD simulations to have three distal hydrogen bonds^{46, 79} lie, as these new correlations would predict, lower on the $\nu(\text{Fe}^{\text{III}}\text{--OH})$ axis than those having two hydrogen bonds. Noteworthy is that these LS heme hydroxides fall near the line for HS hydroxides having only weak or no H-bond donors. Hence, even though the position of a heme protein hydroxide on this correlation plot can appear to indicate a particular spin state and number of hydrogen bonds for a given $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequency, the $\nu(\text{Fe}^{\text{III}}\text{--OH})$ frequency alone may not be sufficient to assign these two parameters because, as seen in Figure S10, correlation lines can intersect due to offsetting effects of spin state and number of hydrogen bonds. Therefore, using these correlations to assign number of hydrogen bonds and properties of the proximal ligand may require assignment of the spin state by an independent means at the same temperature, such as high frequency rR, NMR or EPR spectroscopy.

Clarification of ambiguous assignments—This approach has the potential to clarify assignments that may be ambiguous on the basis of $\nu(\text{Fe}^{\text{III}}\text{--OH})$ frequency alone. For example, for HRP enzymes whose distal pockets comprise both Arg and His, the positions of HRP-A1 and HRP-C⁶¹ on the $(\text{Fe}^{\text{III}}\text{--OH})/\nu(\text{Fe}^{\text{II}}\text{--His})$ plot suggest that these two amino acids contribute differently to distal H-bonding in these isozymes. The HRP-C variant HRP-C(H42L), which has no distal His, leaves only the distal Arg to stabilize anion coordination

by H-bond donation. Although the core-size marker region of the rR spectrum indicates an equilibrium mixture of HS and LS heme hydroxides, only a single $\nu(\text{Fe}-\text{OH})$ band is detected in the low-frequency rR spectrum. This single the $(\text{Fe}^{\text{III}}-\text{OH})$ band was assigned to the HS hydroxide based on $\nu(\text{Fe}^{\text{III}}-\text{OH})$ frequency similarity with Hb.⁸⁰ The $\nu(\text{Fe}^{\text{III}}-\text{OH})$ and $\nu(\text{Fe}^{\text{II}}-\text{His})$ frequencies for HRP-C(H42L) place it close to the intersection of the LS correlation line comprising Clds that also have only a distal Arg and the correlation line for HS hydroxides with little to no H-bond donation from the distal pocket. This raises the question of whether this is a LS heme hydroxide under the influence of a distal Arg, or a HS hydroxide in which the mutation precludes H-bond donation by the distal Arg. Since the distal Arg in peroxidases is important to the H-bond network in the distal cavity, essential for stabilizing peroxidase anion complexes, and stabilizes the enzymes at alkaline pH,⁸¹ distal Arg interaction with hydroxide in HRP-C(H42L) is reasonable. If the Arg were a H-bond donor to the HS HRP-C(H42L)-OH, the correlation line for HS hydroxide heme in strongly H-bond donating environments predicts that its $\nu(\text{Fe}^{\text{III}}-\text{OH})$ would be lower than those observed for HS *Da*- and *Kp*Cld-OHs; this was not observed. In light of the $(\text{Fe}^{\text{III}}-\text{OH})/\nu(\text{Fe}^{\text{II}}-\text{His})$ correlation plot, the possibility of the $\nu(\text{Fe}^{\text{III}}-\text{OH})$ band arises from a LS hydroxide with significant H-bond donation from the distal Arg cannot be eliminated and its assignment to the HS heme hydroxide should be considered tentative.

Sensitivity of the $\nu(\text{Fe}^{\text{III}}-\text{X})$ frequencies to proximal and distal effects—Table S2 shows the relationship between the $\nu(\text{Fe}^{\text{III}}-\text{F})$ and $\nu(\text{Fe}^{\text{III}}-\text{OH})$ frequencies and the respective bond lengths, calculated in accord with Badger's Rule.^{82, 83} The estimated equilibrium bond lengths ($r_e(\text{Fe}-\text{X})$) within a set of heme fluorides or hydroxides on the same correlation line fall within a range of only ~3% and ~5% of the average values, respectively. Thus, these $\nu(\text{Fe}^{\text{III}}-\text{X})$ frequency trends reflect changes in heme environment that elicit changes in $r_e(\text{Fe}-\text{X})$ that are on the cusp of being too small to be reliably reported by other structural methods. Insofar as catalysis in heme-dependent oxidoreductases is linked to the facility with which bonds between the heme iron and exogenous ligands are made and broken, understanding the structural basis of Fe-X bond strength sensitivity to differences between or changes in heme environments is a necessary prelude to understanding the structural basis of mechanism. The relationships described herein constitute an exquisitely sensitive reporter of such mechanistically relevant structural changes.

CONCLUSIONS

Examination of halide and hydroxide complexes of Clds highlights both similarities and differences between dimeric *Kp* and pentameric *Da*Cld active sites. Strong H-bond donation occurs from the distal Arg residue to the atom directly bonded to the heme iron in Clds from subfamilies 1 and 2. This is apparent from their similar positions on the plot showing the direct correlation between the $\nu(\text{Fe}^{\text{III}}-\text{F})$ and CT1 frequencies.

Inverse correlations, reported here for the first time, between $\nu(\text{Fe}^{\text{III}}-\text{X})$, where X = F⁻ or OH⁻, and $\nu(\text{Fe}^{\text{II}}-\text{His})$ provide a more detailed account of the proximal and distal environments and their effects on the Fe-X bond strength. The positions of *Da*Cld-X and *Kp*Cld-X on those lines clearly shows that the effects of their distal environments on Fe-X

bonding are strikingly similar. Where the two enzymes differ is in their proximal environments, which impose *trans* effects of different magnitudes on the Fe–X bond. These inverse correlations hold for other His-ligated heme proteins that donate different numbers of distal hydrogen bonds of various strengths to the bound X[−] ligand. They reveal that the H bond donating character in the distal pocket increases in the order: neutral His < protonated His < Arg. Thus, these correlations reveal additive effects of distal H-bond donation and *trans* (i.e. proximal) ligand influence on the Fe–X bond strength, as reported by $\nu(\text{Fe}^{\text{III}}\text{--X})$ frequencies for π -donor ligands. As such, these two effects can combine to labilize ligands, such as F[−], OH[−], and OH₂ toward ligand exchange with heme-binding substrates like ClO₂[−] in the case of Clds. Consequently, these ligands do not strongly inhibit *Kp*Cld activity.

The proximal ligands in the Clds, Mb and most heme-dependent peroxidases are the imidazole side chains of His residues and proximal $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies are governed by the same environmental factors. Thus, it is reasonable to hypothesize that the *trans* effect on distal Fe–OH and Fe=O bond strengths would be similar. This hypothesis is supported by similarity between the slopes of the $\nu(\text{Fe}^{\text{III}}\text{--OH})/\nu(\text{Fe}^{\text{II}}\text{--His})$ and $\nu(\text{Fe}^{\text{IV}}\text{=O})/\nu(\text{Fe}^{\text{II}}\text{--His})$ frequency correlations (−1.8±0.2 and −1.2±0.5, respectively), which characterize the *trans* effect. Using the inverse correlation between the $\nu(\text{Fe}^{\text{III}}\text{--OH})$ and $\nu(\text{Fe}^{\text{II}}\text{--His})$ frequencies as a gauge for the *trans* effects on $\nu(\text{Fe}^{\text{IV}}\text{=O})$ frequencies for *Kp* and *Da*Clds, they are predicted to fall between those for Mb and HPRC.⁵⁰ This suggests that the Fe^{IV}=O bond strength required for chlorite decomposing activity is lower than that in heme peroxidases, consistent with a lowered barrier for Fe^{IV}=O bond scission in an oxo-atom transfer step with the putative OCl fragment. This may lend kinetic facility to the penultimate O–O bond forming step of ClO₂[−] decomposition hypothesized to yield an Fe^{III}–OOCl intermediate.³¹

*Kp*Cld binds Cl[−] cooperatively ($n = 2.3 \pm 0.1$), likely at or near salt bridges remote from the heme and deterministic for heme pocket conformation. Although binding of anionic π -donor ligands is not specific to Cl[−], Cl[−] is the only one that was observed to bind cooperatively. Because cooperativity places the steepest region of the Cl[−] binding curve for *Kp*Cld in the physiological [Cl[−]] range (Figure 2A) of the cytosol, the heme coordination number is a steep function of that Cl[−] concentration. While cooperative binding of Cl[−] is expected to affect the coordination state of cytosolic, dimeric Clds, the coordination state of periplasmic, pentameric Clds remains insensitive to Cl[−]. This is consistent with a role for Cl[−] in modulating ligand/substrate access to the *Kp*Cld heme pocket in this pathogenic organism.¹⁰

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support is gratefully acknowledged from the National Institutes of Health Grants GM114787 (to G. S. L.-R.) and GM090260 (to J. L. D.). The authors wish to thank Garrett Moraski for his helpful suggestions during the preparation of this manuscript.

ABBREVIATIONS

Cld

chlorite dismutase

KpCld

Klebsiella pneumoniae Cld

DaCld

Dechloromonas aromatica Cld

NwCld

Nitrobacter winogradskyi Cld

NdCld

Candidatus Nitrospira defluvii Cld

HRPC

horseradish peroxidase C

Mb

myoglobin

Hb

hemoglobin

DHP

dehaloperoxidase

Tf trHb

Thermobifida fusca truncated Hb

Mt trHbO and Mt trHbN

Mycobacterium tuberculosis truncated Hb

FixL

bacterial two-component heme-based O₂ sensing protein from *Sinorhizobium meliloti*

HmuT

hemin trafficking protein from *Corynebacterium diphtheriae*

rR

resonance Raman

EPR

electron paramagnetic resonance

WT

wild type

HS

high spin

LS

low spin

6cHS

six coordinate high spin

5cHS

five coordinate high spin

6cLS

six coordinate low spin

References

1. DuBois JL, Ojha S. Production of dioxygen in the dark: dismutases of oxyanions. *Met Ions Life Sci.* 2015; 15:45–87. [PubMed: 25707466]
2. Schaffner I, Hofbauer S, Krutzler M, Pirker KF, Furtmueller PG, Obinger C. Mechanism of chlorite degradation to chloride and dioxygen by the enzyme chlorite dismutase. *Arch Biochem Biophys.* 2015; 574:18–26. [PubMed: 25748001]
3. Tagore R, Crabtree RH, Brudvig GW. Oxygen Evolution Catalysis by a Dimanganese Complex and Its Relation to Photosynthetic Water Oxidation. *Inorg Chem.* 2008; 47:1815–1823. [PubMed: 18330972]
4. Maixner F, Wagner M, Lueker S, Pelletier E, Schmitz-Esser S, Hace K, Spieck E, Konrat R, Le Paslier D, Daims H. Environmental genomics reveals a functional chlorite dismutase in the nitrite-oxidizing bacterium '*Candidatus Nitrospira defluvii*'. *Environ Microbiol.* 2008; 10:3043–3056. [PubMed: 18459973]
5. Goblirsch B, Kurker RC, Streit BR, Wilmot CM, DuBois JL. Chlorite dismutases, DyPs, and EfeB: 3 microbial heme enzyme families comprise the CDE structural superfamily. *J Mol Biol.* 2011; 408:379–398. [PubMed: 21354424]
6. Mlynek G, Sjoeblohm B, Kostan J, Fuereder S, Maixner F, Gysel K, Furtmueller PG, Obinger C, Wagner M, Daims H, Djinoovic-Carugo K. Unexpected diversity of chlorite dismutases: a catalytically efficient dimeric enzyme from *Nitrobacter winogradskyi*. *J Bact.* 2011; 193:2408–2417. [PubMed: 21441524]
7. Melnyk RA, Engelbrektson A, Clark IC, Carlson HK, Byrne-Bailey K, Coates JD. Identification of a perchlorate reduction genomic island with novel regulatory and metabolic genes. *Appl Environ Microbiol.* 2011; 77:7401–7404. [PubMed: 21856823]
8. Clark IC, Melnyk RA, Engelbrektson A, Coates JD. Structure and evolution of chlorate reduction composite transposons. *mBio.* 2013; 4:e00379–00312. [PubMed: 23919996]
9. Hofbauer S, Schaffner I, Furtmueller PG, Obinger C. Chlorite dismutases - a heme enzyme family for use in bioremediation and generation of molecular oxygen. *Biotechnol J.* 2014; 9:461–473. [PubMed: 24519858]
10. Celis AI, Geeraerts Z, Ngmenterebo D, Machovina MM, Kurker RC, Rajakumar K, Ivancich A, Rodgers KR, Lukat-Rodgers GS, DuBois JL. A Dimeric Chlorite Dismutase Exhibits O₂-Generating Activity and Acts as a Chlorite Antioxidant in *Klebsiella pneumoniae* MGH 78578. *Biochemistry.* 2015; 54:434–446. [PubMed: 25437493]
11. Schaffner I, Hofbauer S, Krutzler M, Pirker KF, Bellei M, Stadlmayr G, Mlynek G, Djinoovic-Carugo K, Battistuzzi G, Furtmueller PG, Daims H, Obinger C. Dimeric chlorite dismutase from the nitrogen-fixing cyanobacterium *Cyanothece* sp. PCC7425. *Mol Microbiol.* 2015; 96:1053–1068. [PubMed: 25732258]
12. Dailey HA, Gerdes S, Dailey TA, Burch JS, Phillips JD. Noncanonical coproporphyrin-dependent bacterial heme biosynthesis pathway that does not use protoporphyrin. *Proc Natl Acad Sci U S A.* 2015; 112:2210–2215. [PubMed: 25646457]

13. Dailey HA, Gerdes S. HemQ: An iron-coproporphyrin oxidative decarboxylase for protoheme synthesis in Firmicutes and Actinobacteria. *Arch Biochem Biophys*. 2015; 574:27–35. [PubMed: 25711532]
14. Dailey TA, Boynton TO, Albetel AN, Gerdes S, Johnson MK, Dailey HA. Discovery and Characterization of HemQ: an essential heme biosynthetic pathway component. *J Biol Chem*. 2010; 285:25978–25986. [PubMed: 20543190]
15. Celis AI, Streit BR, Moraski GC, Kant R, Lash TD, Lukat-Rodgers GS, Rodgers KR, DuBois JL. Unusual Peroxide-Dependent, Heme-Transforming Reaction Catalyzed by HemQ. *Biochemistry*. 2015; 54:4022–4032. [PubMed: 26083961]
16. Lobo SAL, Scott A, Videira MAM, Winpenny D, Gardner M, Palmer MJ, Schroeder S, Lawrence AD, Parkinson T, Warren MJ, Saraiva LM. Staphylococcus aureus haem biosynthesis: characterisation of the enzymes involved in final steps of the pathway. *Mol Microbiol*. 2015; 97:472–487. [PubMed: 25908396]
17. Coates JD, Achenbach LA. Microbial perchlorate reduction: rocket-fueled metabolism. *Nat Rev Microbiol*. 2004; 2:569–580. [PubMed: 15197392]
18. Streit BR, DuBois JL. Chemical and Steady-State Kinetic Analyses of a Heterologously Expressed Heme Dependent Chlorite Dismutase. *Biochemistry*. 2008; 47:5271–5280. [PubMed: 18422344]
19. Mayfield JA, Blanc B, Rodgers KR, Lukat-Rodgers GS, DuBois JL. Peroxidase-Type Reactions Suggest a Heterolytic/Nucleophilic O–O Joining Mechanism in the Heme-Dependent Chlorite Dismutase. *Biochemistry*. 2013; 52:6982–6994. [PubMed: 24001266]
20. Sun S, Li ZS, Chen SL. A dominant homolytic O–Cl bond cleavage with low-spin triplet-state Fe(IV)=O formed is revealed in the mechanism of heme-dependent chlorite dismutase. *Dalton Trans*. 2014; 43:973–981.
21. Su JX, Chen HL. Significant electron transfer in heme catalysis: The case of chlorite dismutase. *J Catal*. 2017; 348:40–46.
22. Goblirsch BR, Streit BR, DuBois JL, Wilmot CM. Structural features promoting dioxygen production by Dechloromonas aromatica chlorite dismutase. *J Biol Inorg Chem*. 2010; 15:879–888. [PubMed: 20386942]
23. de Geus DC, Thomassen EAJ, Hagedoorn PL, Pannu NS, van Duijn E, Abrahams JP. Crystal Structure of Chlorite Dismutase, a Detoxifying Enzyme Producing Molecular Oxygen. *J Mol Biol*. 2009; 387:192–206. [PubMed: 19361444]
24. Kostan J, Sjoebloom B, Maixner F, Mlynek G, Furtmueller PG, Obinger C, Wagner M, Daims H, Djinojic-Carugo K. Structural and functional characterisation of the chlorite dismutase from the nitrite-oxidizing bacterium “Candidatus Nitrospira defluvii”: Identification of a catalytically important amino acid residue. *J Struct Biol*. 2010; 172:331–342. [PubMed: 20600954]
25. Catling DC, Claire MW, Zahnle KJ, Quinn RC, Clark BC, Hecht MH, Kounaves S. Atmospheric origins of perchlorate on Mars and in the Atacama. *J Geophys Res, [Planets]*. 2010:E00E11/01–E00E11/15. American Geophysical Union.
26. Rao B, Hatzinger PB, Bohlke JK, Sturchio NC, Andraski BJ, Eckardt FD, Jackson WA. Natural Chlorate in the Environment: Application of a New IC-ESI/MS/MS Method with a Cl18O3- Internal Standard. *Environ Sci Technol*. 2010; 44:8429–8434. [PubMed: 20968289]
27. Blanc B, Mayfield JA, McDonald CA, Lukat-Rodgers GS, Rodgers KR, DuBois JL. Understanding How the Distal Environment Directs Reactivity in Chlorite Dismutase: Spectroscopy and Reactivity of Arg183 Mutants. *Biochemistry*. 2012; 51:1895–1910. [PubMed: 22313119]
28. Hofbauer S, Gysel K, Bellei M, Hagmueller A, Schaffner I, Mlynek G, Kostan J, Pirker KF, Daims H, Furtmueller PG, Battistuzzi G, Djinojic-Carugo K, Obinger C. Manipulating conserved heme cavity residues of chlorite dismutase: Effect on structure, redox chemistry, and reactivity. *Biochemistry*. 2014; 53:77–89. [PubMed: 24364531]
29. Streit BR, Blanc B, Lukat-Rodgers GS, Rodgers KR, DuBois JL. How Active-Site Protonation State Influences the Reactivity and Ligation of the Heme in Chlorite Dismutase. *J Am Chem Soc*. 2010; 132:5711–5724. [PubMed: 20356038]
30. Hofbauer S, Gruber C, Pirker KF, Suendermann A, Schaffner I, Jakopitsch C, Oostenbrink C, Furtmueller PG, Obinger C. Transiently produced hypochlorite is responsible for the irreversible inhibition of chlorite dismutase. *Biochemistry*. 2014; 53:3145–3157. [PubMed: 24754261]

31. Lee AQ, Streit BR, Zdilla MJ, bu-Omar MM, DuBois JL. Mechanism of and exquisite selectivity for O-O bond formation by the heme-dependent chlorite dismutase. *Proc Natl Acad Sci U S A*. 2008; 105:15654–15659. [PubMed: 18840691]
32. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera—A visualization system for exploratory research and analysis. *J Comput Chem*. 2004; 25:1605–1612. [PubMed: 15264254]
33. Tropea, JEC., Scott, Waugh, David, S. Expression and purification of soluble His6-tagged TEV protease. In: Doyle, SA., editor. *Methods in Molecular Biology: High Throughput Protein Expression and Purification*. Humana Press; Totowa, NJ: 2009. p. 297-306.
34. Blanc B, Rodgers KR, Lukat-Rodgers GS, DuBois JL. Understanding the roles of strictly conserved tryptophan residues in O₂ producing chlorite dismutases. *Dalton Trans*. 2013; 42:3156–3169.
35. Philippi M, dos Santos HS, Martins AO, Azevedo CM, Pires M. Alternative spectrophotometric method for standardization of chlorite aqueous solutions. *Anal Chim Acta*. 2007; 585:361–365. [PubMed: 17386686]
36. Antonini, E., Brunori, M. *Frontier in Biology; Hemoglobin and Myoglobin in Their Reactions with Ligands*. North-Holland Publishing Co; Amsterdam: 1971.
37. Indiani C, Feis A, Howes BD, Marzocchi MP, Smulevich G. Benzohydroxamic Acid-Peroxidase Complexes: Spectroscopic Characterization of a Novel Heme Spin Species. *J Am Chem Soc*. 2000; 122:7368–7376.
38. Indiani C, Santoni E, Becucci M, Boffi A, Fukuyama K, Smulevich G. New Insight into the Peroxidase-Hydroxamic Acid Interaction Revealed by the Combination of Spectroscopic and Crystallographic Studies. *Biochemistry*. 2003; 42:14066–14074. [PubMed: 14636075]
39. Marcus Y. Ionic radii in aqueous solutions. *Chem, Rev*. 1988; 88:1475–1498.
40. Rwere F, Mak PJ, Kincaid JR. Resonance Raman determination of vinyl group disposition in different derivatives of native myoglobin and its heme-disoriented form. *J Raman Spectrosc*. 2014; 45:97–104.
41. Paulat F, Praneeth VKK, Naether C, Lehnert N. Quantum Chemistry-Based Analysis of the Vibrational Spectra of Five-Coordinate Metalloporphyrins [M(TPP)Cl]. *Inorg Chem*. 2006; 45:2835–2856. [PubMed: 16562940]
42. Hu S, Smith KM, Spiro TG. Assignment of protoheme resonance Raman spectrum by heme labeling in myoglobin. *J Am Chem Soc*. 1996; 118:12638–12646.
43. Droghetti E, Nicoletti FP, Bonamore A, Sciamanna N, Boffi A, Feis A, Smulevich G. The optical spectra of fluoride complexes can effectively probe H-bonding interactions in the distal cavity of heme proteins. *J Inorg Biochem*. 2011; 105:1338–1343. [PubMed: 21867665]
44. Nicoletti FP, Droghetti E, Boechi L, Bonamore A, Sciamanna N, Estrin DA, Feis A, Boffi A, Smulevich G. Fluoride as a Probe for H-Bonding Interactions in the Active Site of Heme Proteins: The Case of *Thermobifida fusca* Hemoglobin. *J Am Chem Soc*. 2011; 133:20970–20980. [PubMed: 22091531]
45. Asher SA, Schuster TM. Differences in iron-fluoride bonding between the isolated subunits of human methemoglobin fluoride and sperm whale metmyoglobin fluoride as measured by resonance Raman spectroscopy. *Biochemistry*. 1981; 20:1866–1873. [PubMed: 7225362]
46. Droghetti E, Nicoletti FP, Bonamore A, Boechi L, Arroyo MP, Estrin DA, Boffi A, Smulevich G, Feis A. Heme pocket structural properties of a bacterial truncated hemoglobin from *Thermobifida fusca*. *Biochemistry*. 2010; 49:10394–10402. [PubMed: 21049911]
47. Teraoka J, Kitagawa T. Structural implication of the heme-linked ionization of horseradish peroxidase probed by the iron-histidine stretching Raman line. *J Biol Chem*. 1981; 256:3969–3977. [PubMed: 7217068]
48. Kitagawa T, Nagai K, Tsubaki M. Assignment of the iron-nitrogen (His F8) stretching band in the resonance Raman spectra of deoxymyoglobin. *FEBS Letters*. 1979; 104:376–378. [PubMed: 478002]
49. Chance M, Powers L, Kumar C, Chance B. X-ray absorption studies of myoglobin peroxide reveal functional differences between globins and heme enzymes. *Biochemistry*. 1986; 25:1259–1265. [PubMed: 3964675]

50. Oertling WA, Kean RT, Wever R, Babcock GT. Factors affecting the iron-oxygen vibrations of ferrous oxy and ferryl oxo heme proteins and model compounds. *Inorg Chem.* 1990; 29:2633–2645.
51. Zhao J, Moretto J, Le P, Franzen S. Measurement of Internal Substrate Binding in Dehaloperoxidase–Hemoglobin by Competition with the Heme–Fluoride Binding Equilibrium. *J Phys Chem B.* 2015; 119:2827–2838. [PubMed: 25611370]
52. Draganova EB, Akbas N, Adrian SA, Lukat-Rodgers GS, Collins DP, Dawson JH, Allen CE, Schmitt MP, Rodgers KR, Dixon DW. Heme Binding by *Corynebacterium diphtheriae* HmuT: Function and Heme Environment. *Biochemistry.* 2015; 54:6598–6609. [PubMed: 26478504]
53. Asher SA, Adams ML, Schuster TM. Resonance Raman and absorption spectroscopic detection of distal histidine-fluoride interactions in human methemoglobin fluoride and sperm whale metmyoglobin fluoride: measurements of distal histidine ionization constants. *Biochemistry.* 1981; 20:3339–3346. [PubMed: 7260037]
54. Asher SA, Schuster TM. Resonance Raman examination of axial ligand bonding and spin-state equilibria in metmyoglobin hydroxide and other heme derivatives. *Biochemistry.* 1979; 18:5377–5387. [PubMed: 518843]
55. Jeyarajah S, Kincaid JR. Resonance Raman studies of hemoglobins reconstituted with mesoheme. Unperturbed iron-histidine stretching frequencies in a functionally altered hemoglobin. *Biochemistry.* 1990; 29:5087–5094. [PubMed: 2378867]
56. Lukat-Rodgers GS, Rexine JL, Rodgers KR. Heme Speciation in Alkaline Ferric FixL and Possible Tyrosine Involvement in the Signal Transduction Pathway for Regulation of Nitrogen Fixation. *Biochemistry.* 1998; 37:13543–13552. [PubMed: 9753440]
57. Feis A, Marzocchi MP, Paoli M, Smulevich G. Spin State and Axial Ligand Bonding in the Hydroxide Complexes of Metmyoglobin, Methemoglobin, and Horseradish Peroxidase at Room and Low Temperatures. *Biochemistry.* 1994; 33:4577–4583. [PubMed: 8161513]
58. Lansky IB, Lukat-Rodgers GS, Block D, Rodgers KR, Ratliff M, Wilks A. The Cytoplasmic Heme-binding Protein (PhuS) from the Heme Uptake System of *Pseudomonas aeruginosa* Is an Intracellular Heme-trafficking Protein to the δ -Regioselective Heme Oxygenase. *J Biol Chem.* 2006; 281:13652–13662. [PubMed: 16533806]
59. Belyea J, Belyea CM, Lappi S, Franzen S. Resonance Raman Study of Ferric Heme Adducts of Dehaloperoxidase from *Amphitrite ornata*. *Biochemistry.* 2006; 45:14275–14284. [PubMed: 17128967]
60. Franzen S, Roach MP, Chen YP, Dyer RB, Woodruff WH, Dawson JH. The Unusual Reactivities of *Amphitrite ornata* Dehaloperoxidase and *Notomatus lobatus* Chloroperoxidase Do Not Arise from a Histidine Imidazolate Proximal Heme Iron Ligand. *J Am Chem Soc.* 1998; 120:4658–4661.
61. Sitter AJ, Shifflott JR, Turner J. Resonance Raman spectroscopic evidence for heme iron-hydroxide ligation in peroxidase alkaline forms. *J Biol Chem.* 1988; 263:13032–13038. [PubMed: 3417650]
62. Smulevich G, Feis A, Focardi C, Tams J, Welinder KG. Resonance Raman Study of the Active Site of *Coprinus cinereus* Peroxidase. *Biochemistry.* 1994; 33:15425–15432. [PubMed: 7803406]
63. Lukat-Rodgers GS, Rodgers KR. Spin-state equilibria and axial ligand bonding in FixL hydroxide: a resonance Raman study. *J Biol Inorg Chem.* 1998; 3:274–281.
64. McCombs NL, D'Antonio J, Barrios DA, Carey LM, Ghiladi RA. Nonmicrobial Nitrophenol Degradation via Peroxygenase Activity of Dehaloperoxidase-Hemoglobin from *Amphitrite ornata*. *Biochemistry.* 2016; 55:2465–2478. [PubMed: 27070125]
65. Smulevich G, Feis A, Howes BD. Fifteen years of Raman spectroscopy of engineered heme containing peroxidases: What have we learned? *Accounts of Chemical Research.* 2005; 38:433–440. [PubMed: 15895981]
66. Teraoka J, Job D, Morita Y, Kitagawa T. Resonance Raman study of plant tissue peroxidases. Common characteristics in iron coordination environments. *Biochim Biophys Acta, Protein Struct Mol Enzymol.* 1983; 747:10–15.
67. Desbois A, Mazza G, Stetzkowski F, Lutz M. Resonance Raman spectroscopy of protoheme-protein interactions in oxygen-carrying hemoproteins and in peroxidases. *Biochim Biophys Acta, Protein Struct Mol Enzymol.* 1984; 785:161–176.

68. Ellis WD, Dunford HB. The kinetics of cyanide and fluoride binding by ferric horseradish peroxidase. *Biochemistry*. 1968; 7:2054–2062. [PubMed: 4298219]
69. Singh R, Grigg JC, Armstrong Z, Murphy MEP, Eltis LD. Distal Heme Pocket Residues of B-type Dye-decolorizing Peroxidase: Arginine but not Aspartate is Essential for Peroxidase Activity. *J Biol Chem*. 2012; 287:10623–10630. [PubMed: 22308037]
70. Singh AK, Pandey N, Sinha M, Kaur P, Sharma S, Singh TP. Structural evidence for the order of preference of inorganic substrates in mammalian heme peroxidases: crystal structure of the complex of lactoperoxidase with four inorganic substrates, SCN^- , I^- , Br^- and Cl^- . *Int J Biochem Mol Biol*. 2011; 2:328–339. [PubMed: 22187667]
71. Fiedler TJ, Davey CA, Fenna RE. X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 Å resolution. *J Biol Chem*. 2000; 275:11964–11971. [PubMed: 10766826]
72. Freire DM, Rivas MG, Dias AM, Lopes AT, Costa C, Santos-Silva T, Van Doorslaer S, Gonzalez PJ. The homopentameric chlorite dismutase from *Magnetospirillum sp.* *J Inorg Biochem*. 2015; 151:1–9. [PubMed: 26218477]
73. De Schutter A, Correia HD, Freire DM, Rivas MG, Rizzi A, Santos-Silva T, Gonzalez PJ, Van Doorslaer S. Ligand binding to chlorite dismutase from *Magnetospirillum sp.* *J Phys Chem B*. 2015; 119:13859–13869. [PubMed: 26287794]
74. Benz R, Schmid A, Hancock RE. Ion selectivity of gram-negative bacterial porins. *J Bact*. 1985; 162:722–727.
75. Schultz SG, Wilson NL, Epstein W. Cation Transport in *Escherichia coli* II. Intracellular chloride concentration. *J Gen Physiol*. 1962; 46:159–166. [PubMed: 13909522]
76. Hofbauer S, Howes BD, Flego N, Pirker KF, Schaffner I, Mlynek G, Djjinovic-Carugo K, Furtmueller PG, Smulevich G, Obinger C. From chlorite dismutase towards HemQ—the role of the proximal H-bonding network in haeme binding. *Biosci Rep*. 2016; 36:e00312. [PubMed: 26858461]
77. Nicoletti FP, Droghetti E, Howes BD, Bustamante JP, Bonamore A, Sciamanna N, Estrin DA, Feis A, Boffi A, Smulevich G. H-bonding networks of the distal residues and water molecules in the active site of *Thermobifida fusca* hemoglobin. *Biochim Biophys Acta, Proteins Proteomics*. 2013; 1834:1901–1909.
78. Egawa T, Yeh SR. Structural and functional properties of hemoglobins from unicellular organisms as revealed by resonance Raman spectroscopy. *J Inorg Biochem*. 2005; 99:72–96. [PubMed: 15598493]
79. Nicoletti FP, Bustamante JP, Droghetti E, Howes BD, Fittipaldi M, Bonamore A, Baiocco P, Feis A, Boffi A, Estrin DA, Smulevich G. Interplay of the H- Bond Donor-Acceptor Role of the Distal Residues in Hydroxyl Ligand Stabilization of *Thermobifida fusca* Truncated Hemoglobin. *Biochemistry*. 2014; 53:8021–8030. [PubMed: 25437272]
80. Howes BD, Rodriguez-Lopez JN, Smith AT, Smulevich G. Mutation of Distal Residues of Horseradish Peroxidase: Influence on Substrate Binding and Cavity Properties. *Biochemistry*. 1997; 36:1532–1543. [PubMed: 9063902]
81. Neri F, Indiani C, Welinder KG, Smulevich G. Mutation of the distal arginine in *Coprinus cinereus* peroxidase. Structural implications. *Eur J Biochem*. 1998; 251:830–838. [PubMed: 9490058]
82. Badger RM. The Relation Between the Internuclear Distances and Force Constants of Molecules and Its Application to Polyatomic Molecules. *J Chem Phys*. 1935; 3:710–714.
83. Badger RM. A Relation Between Internuclear Distances and Bond Force Constants. *J Chem Phys*. 1934; 2:128–131.

Figure 1. Active site structures of pentameric and dimeric

Active site environments for A) the respiration-associated, pentameric *DaCld* (PDB ID **3Q08**)²² as a nitrite complex and B) the dimeric *NwCld* (**3QPI**) as an aqua complex,⁶ which like *KpCld* is from proteobacteria that do not respire perchlorate, are shown. Heme carbons are cyan, protein side chain carbons are gray; nitrogen and oxygen atoms are blue and red, respectively. The distal face contains conserved threonine, phenylalanine, leucine, and arginine which form a cage around the sixth coordination site of the heme. Differences between the two active sites are the orientation of the distal Arg side chain and the identity of the residue at the position of W227, which is a conserved glutamate in *NwCld* and other dimeric Clds. In the *DaCld* structure (A), a MES buffer molecule (shown in black, yellow and red) is H-bonded to Arg183. Secondary structure elements are labeled according to the ferredoxin fold using the labeling schemes consistent with those used for *AcCld*²³ in A and *NnCld*²⁴ in B. All of the structures are displayed using UCSF Chimera.³²

Figure 2. In the presence of chloride ion, ferric *KpCld* exhibits a 6cHS species

A) The spectrophotometric titration of *KpCld* in 100 mM sodium phosphate pH 6.2 is shown with the initial spectrum (0 mM Cl^-) in blue and the final spectrum (500 mM Cl^-) in red. Spectra corresponding to intermediate concentrations of chloride are grey. Aliquots of a stock chloride solution prepared in the same buffer were added to the enzyme to increase the chloride ion concentration in 0.010–0.025 M steps. Equilibrium was reached prior to each sequential addition. Arrows indicate the direction of change in the spectra as the chloride concentration increases. Inset: The fraction of *KpCld* with chloride bound ($r = (A_{\text{Cl}} - A_{\text{initial}})/(A_{\text{final}} - A_{\text{initial}})$) calculated using the absorbance data at 412 nm, the wavelength of greatest change in the titration, is plotted as a function of chloride concentration. The dashed line shows the fit obtained using a single chloride binding model (eqn 1); the solid line is the fit to the Hill equation (eqn 2) where the Hill coefficient measured is 2.3 ± 0.1 . B) Resonance Raman spectra of *KpCld* in 100 mM sodium phosphate buffer pH 6 were obtained with 413.1 nm excitation in the presence of the following anions: a) 500 mM sodium fluoride, b) no additional anion (buffer only), c) 100 mM sodium perchlorate, d) 100 mM sodium sulfate, and e) 100 mM sodium chloride. Enzyme concentration is 25 μM . The spectrum of *KpCld*-F is shown for comparison purposes; the complex is 6cHS.

Figure 3. Titration of pentameric and dimeric Clds with chloride

A) Resonance Raman spectrum of *DaCld* with varying $[\text{Cl}^-]$: 0 mM Cl^- , black; 100 mM Cl^- , red; 500 mM Cl^- , green; difference spectrum generated by subtracting the 0 mM Cl^- /*DaCld* spectrum from the 500 mM Cl^- /*DaCld* spectrum, purple; difference spectrum generated by subtracting the 0 mM Cl^- /*DaCld* spectrum from the 100 mM Cl^- /*DaCld* spectrum, blue. *DaCld* concentration was 25 μM in sodium phosphate buffer pH 6.0. B) Resonance Raman spectrum of *KpCld* with varying $[\text{Cl}^-]$. Spectra as described in B. *KpCld* concentration was 40 μM in sodium phosphate buffer pH 6.0. Comparison of A and B shows that *DaCld* is predominantly a 5cHS species even at 500 mM Cl^- while *KpCld* is completely converted to a 6cHS species at 100 mM Cl^- .

Figure 4. Fluoride complex characterized by spectrophotometric titration of *KpCld* with fluoride ion

The spectrophotometric titration of *KpCld* in 100 mM sodium phosphate pH 5.8 is shown with the initial spectrum (0 mM F^-) in blue and the final spectrum (83 mM F^-) in red. Spectra corresponding to intermediate concentrations of fluoride are grey. Aliquots of a stock sodium fluoride solution prepared in the same buffer were added to the enzyme in 0.5–10.0 mM steps. Equilibrium was reached prior to each sequential addition. Arrows indicate the direction of change in the spectra as the fluoride concentration increases. Inset: The fraction of *KpCld* with fluoride bound ($r = (A_F - A_{\text{initial}})/(A_{\text{final}} - A_{\text{initial}})$) calculated using the absorbance data at 403 nm and 612 nm, the wavelengths of greatest change in the titration, are plotted as a function of free fluoride concentration. The solid line (403 nm) and dashed line (612 nm) are fits obtained using a single fluoride binding model (Eqn 1) which both generate a $K_D = 3.3 \pm 0.2$ mM.

Figure 5. Fluoride complexes of Clds compared to ferric Clds

A) WT *KpCld*, B) WT *DaCld*, and C) *DaCld*(W227F). Each panel contains Soret-excited rR spectra of a) ferric Cld and b) Cld-F at pH 5.8 with 406.7 excitation and c) Cld-F with 441.6 nm ($22,645\text{ cm}^{-1}$) excitation for identification of the Fe(III)-F stretch. The data are shown in black while the components of the fit spectrum and the composite fit spectrum are shown in blue and red, respectively and the band assigned to the Fe-F stretch is shown in magenta. Insets: UV-vis spectrum of Cld-F at pH 5.8.

Figure 6. Correlation plot showing the relationship between the $\nu(\text{Fe-F})$ frequency and the CT1 energy

DaCld-F, ▲; *DaCld*(W227F)-F, ■; *KpCld*-F, purple star; data points for other heme proteins (hhMb, horse heart myoglobin; HRPC, horseradish peroxidase isozyme C; DHP, dehaloperoxidase; *Tf*-trHb, truncated hemoglobin from *Thermobifida fusca*), blue ○; a series of *Tf*-trHb mutants, black ○.⁴⁴

Figure 7. Correlation plots illustrating the inverse correlation between $\nu(\text{Fe}^{\text{III}}-\text{X})$ and $\nu(\text{Fe}^{\text{II}}-\text{His})$

A) $\text{X} = \text{F}^-$. Heme proteins with Arg in their distal pocket, red circles; other heme proteins with no H bonding to bound F^- , black squares; other heme proteins with H-bonding to bound F^- , blue triangles.^{43, 48, 52–55} B) $\text{X} = \text{OH}^-$ Low spin heme hydroxide complexes: heme proteins with Arg in their distal pocket, red circles;^{10, 29, 56} heme proteins with distal His, black squares.^{47, 57–62} HRP proteins with His and/or Arg available as H bond donor in the pocket are shown as gray triangles. The HRP-C(H42L) variant contains only a distal Arg. High spin heme hydroxides: same as low-spin hydroxides with open symbols.^{10, 29, 48, 54, 57, 59, 60, 63}

Figure 8. Secondary structure of pentameric and dimeric Clds shaping the heme pocket
 (A) Cross-eyed stereo view of the crystal structure of *DaCld* (3Q08)²² depicting residues with nonbonding interactions (2.9 – 4.2 Å) between $\alpha 9$ and $\beta 5$, $\beta 8$, & $\beta 9$ as indicated by dashed magenta lines; these define a side of the heme pocket. These residues are hydrophobic suggesting their interactions are a mixture between London dispersion forces and the hydrophobic effect. This hydrophobic region is located at one intersubunit interface within the pentameric structure and is therefore not solvent exposed. (B) Cross-eyed stereo view of the Swiss Model homology model of *KpCld* (generated using *NwCld* (3QPI))⁶ as a template) depicting residues with nonbonding interactions (2.5 – 4.6 Å) between $\alpha 5'$ & $\alpha 6'$ and $\beta 4'$ as indicated by dashed magenta lines. Nitrogen and oxygen atoms of the amino acid side chains and heme are shown in blue and red, respectively. The carbons of the heme are shown in cyan and the iron is orange. The heme cofactor is an overlay from *NwCld* onto the *KpCld* homology model. These residues are similar to *NwCld* and are hydrophilic suggesting their interactions are primarily ionic in nature. This region is not located at an intersubunit interface. Secondary structure elements are labeled according to the ferredoxin fold using the labeling schemes consistent with those used for *AcCld*²³ in A and *NdCld*²⁴ in B. All of the structures are displayed using UCSF Chimera.³²