Published in final edited form as:

Biochemistry. 2018 March 06; 57(9): 1501–1516. doi:10.1021/acs.biochem.7b01278.

Distinguishing active site characteristics of chlorite dismutases with their cyanide complexes

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Abstract

O₂-evolving chlorite dismutases (Clds) efficiently convert chlorite (ClO₂[−]) to O₂ and Cl[−]. *Dechloromonas aromatica* Cld (*DaCld*) is a highly active chlorite-decomposing homopentameric enzyme, typical of Clds found in perchlorate and chlorate respiring bacteria. The Gram-negative, human pathogen *Klebsiella pneumoniae* contains a homodimeric Cld (*KpCld*) that also decomposes ClO₂[−], albeit with a 10-fold lower activity and a lower turnover number compared to *DaCld*. The interactions between the distal pocket and heme ligand of the *DaCld* and *KpCld* active sites have been probed via kinetic, thermodynamic and spectroscopic behaviors of their cyanide complexes for insight into active site characteristics that are deterministic for chlorite decomposition. At 4.7×10^{-9} M, the K_D for *KpCld*–CN[−] is two orders of magnitude smaller than that of *DaCld*–CN[−] and indicates an affinity for CN[−] that is greater than that of most heme proteins. The difference in CN[−] affinity between *Kp* and *DaClds* is predominantly due to differences in k_{off} . The kinetics of cyanide binding to *DaCld*, *DaCld*(R183Q) and *KpCld* between pH 4 and 8.5 corroborate the importance of distal Arg183 and a $pK_a \sim 7$ in stabilizing complexes of anionic ligands, including substrate. The Fe–C stretching and FeCN bending modes of *DaCld*–CN[−] ($\nu_{\text{Fe-C}}$, 441 cm^{−1}; δ_{FeCN} , 396 cm^{−1}) and *KpCld*–CN[−] ($\nu_{\text{Fe-C}}$, 441 cm^{−1}; δ_{FeCN} , 356 cm^{−1}) reveal differences in their FeCN angle, which suggest different distal pocket interactions with their

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Notes

The authors declare no competing financial interests.

Supporting Information. Additional data referred to in the text: Figures S1–S7 and Tables S1–S4 include UV-vis spectra of *Kp* and *DaCld*–CN[−] (Fig S1), representative kinetic data for the formation of *KpCld*–CN[−] (Fig S2), comparison of low frequency window of rR of ferric and ferric *KpCld*–CN[−] (Fig S3), rate constants as a function of pH for the CN[−] reaction with *KpCld*, *DaCld*, *DaCld*(R183Q) (Table S1), rR of isotopically labeled alkaline *Kp* and *DaCld*–CN[−] (Fig S4 & S6), comparison of rR isotope-sensitive frequencies for cyano-ferric Cld complexes as obtained by curve fitting analysis (Table S2) effect of increasing the Fe–C stretching force constant at an FeCN bond angle of 155° on the potential energy distribution and frequency of the stretching and bending normal modes (Fig S5), comparison of resonance Raman isotope-sensitive frequencies for cyano-ferric Cld complexes as obtained by curve fitting analysis (Table S3), comparison of *KpCld*–CN[−] and NP1–CN[−] vibrational frequencies and isotope shifts (Table S4), and active site structure of *NdCld*–CN[−] complex (Fig S7). This material is available free of charge via the Internet at <http://pubs.acs.org>.

bound cyanide. Conformational differences in their catalytic sites are also reported by the single ferrous *KpCld* carbonyl complex, which is in contrast to the two conformers observed for *DaCld*-CO.

Keywords

heme; chlorite dismutase; cyanide; *Dechloromonas*; *Klebsiella*; resonance Raman

INTRODUCTION

O₂-evolving chlorite dismutases (Clds) catalyze the rapid decomposition of a single chlorite ion (ClO₂⁻) to O₂ and Cl⁻ with heme *b* as the sole cofactor required for this unique O–O bond-forming reaction.^{1, 2} Unlike the water-oxidation reaction catalyzed by the tetramanganese center in the oxygen-evolving complex of photosystem II,³ Cld catalyzes the reaction without being coupled to generation of a proton gradient. Instead, the Cld-catalyzed reaction serves to detoxify chlorite generated from respiratory reduction of perchlorate or chlorate.⁴

The Cld protein family consists of three major subfamilies identified in phylogenetic studies.^{5–9} Although members of two of these subfamilies differ in subunit size and oligomeric state, they are referred to as functional Clds because they catalyze the decomposition of ClO₂⁻. The first subfamily consists predominantly of the respiratory pentameric Clds found mainly in Proteobacteria.¹⁰ Dimeric Clds from non-perchlorate-respiring species constitute the second subfamily; their subunit size is significantly smaller than that of the respiratory Clds due to a truncated N-terminus.^{9, 11} The third Cld subfamily contains coproheme decarboxylases.¹² While these enzymes share structural similarities with the first two subfamilies, they catalyze a completely different reaction, the oxidative decarboxylation of coproheme as the terminal step of heme biosynthesis in Gram-positive bacteria.^{12–16}

In perchlorate-respiring bacteria like *Dechloromonas aromatica*, Cld's function is the efficient detoxification of the ClO₂⁻ produced by metabolic reduction of ClO₄⁻.⁴ *Dechloromonas aromatica* Cld (*DaCld*) is capable of turning over >20,000 equivalents of ClO₂⁻ per heme prior to irreversible inactivation ($k_{\text{cat}}/K_{\text{M}} = 3.2(\pm 0.4) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 5.2, 4 °C).¹⁷ Although members of the second subfamily are efficient catalysts for ClO₂⁻ decomposition ($k_{\text{cat}}/K_{\text{M}} = 10^6 \text{ M}^{-1} \text{ s}^{-1}$, pH 5.0–6.0, 20–30 °C)^{5, 9, 11}, their *in-vivo* function is not clear. Growth studies of a *cld* knockout strain of *Klebsiella pneumoniae* strain MGH 78578, a Gram-negative, non-perchlorate-respiring bacterium, suggest that its Cld (*KpCld*) may have a role in detoxification of ClO₂⁻ produced endogenously by nitrate reductases, acting upon ClO₄⁻ or ClO₃⁻.⁹ It has been hypothesized that a general function of Clds from non-perchlorate-respiring species may be protection against the effects of environmental ClO₄⁻ or ClO₃⁻.^{1, 18, 19}

The heme active sites of representatives of the two ClO₂⁻-decomposing Cld subfamilies are shown in Figure 1. In both representative enzymes the heme is bound to the protein through a highly conserved proximal histidine,^{20, 22} and the distal pocket contains a conserved arginine.^{23–25} The distal arginine side chain position is flexible resulting in the accessibility

of two conformations: one in which its guanidinium group is oriented toward the heme and one where the guanidinium group is directed toward the main access channel into the heme pocket.^{5, 20, 23, 26} The catalytic importance of the distal Arg is illustrated by substantial loss of chlorite degrading activity upon its mutation to Ala or Gln.^{23–25} Recent evidence suggests that one role of the distal Arg is confining ClO^- generated during ClO_2^- decomposition to the heme pocket.²⁷ In *DaCld* the Arg is quite effective in this role as ClO^- is not detected during catalytic turnover.^{17, 28} When ClO^- escapes from the heme pocket, as observed for *KpCld* and the Cld from *Candidatus Nitrospira defluvii* (*NdCld*), a pentameric Cld, it is readily detected as HOCl .^{9, 27} Enzyme degradation by HOCl limits *KpCld* to a significantly lower number of turnovers (6700 ± 300 equivalents $\text{ClO}_2^-/\text{heme}$) relative to *DaCld*.⁹ Thus, although the general heme site characteristics of Clds are similar, subtle variations in their active site structures and/or dynamics appear to modulate their ClO_2^- -decomposing activity and turnover.

The resting axial ligation states of five-coordinate high spin (5cHS) and 5cHS/six-coordinate high spin (6cHS) heme in ferric *DaCld* and *KpCld*, respectively, also point to differences in their active sites.⁹ Ligand binding to heme proteins is sensitive to various factors such as the identity of the heme proximal ligand, amino acid composition of the distal pocket and the ability of distal residues to stabilize exogenous heme ligands with H-bonding, electrostatic, or steric interactions. Numerous spectroscopic methods using nonphysiological heme ligands have been developed to probe the distal landscape of the heme pocket to ascertain its influence on enzymatic activity. The reaction of cyanide with ferric heme proteins is widely used to probe their active site structure.^{25, 29–35} Since it does not oxidize the heme iron center subsequent to coordination and its binding can readily be monitored spectrophotometrically to obtain k_{on} , k_{off} and equilibrium constants, cyanide can be used as an anionic reporter ligand. Additionally, subtle structural differences in heme- CN^- complexes due to different distal heme environments are discernible via rR spectroscopy.^{36–43} Here we examine ferric Cld complexes with cyanide to probe the distal environments of Clds from the two functional subfamilies with the goal of identifying structural elements that differentially modulate their ClO_2^- -degrading activities.

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.⁹ 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 TTT CAG GGC CAT ATG AAT ACA CGA TTA TTT ACG TTC G-3') and TEV_R (5'-TA CAG GTT TTC GCC GCT GCT GTG ATG ATG-3'). Mutated plasmid was transformed in Tuner DE3 *E. coli* cells and *KpCld* was expressed, isolated, and purified as previously described.⁹ The N-terminal His-tag of *KpCld* was removed by incubation with S219V TEV (at 1 mol TEV: 10 mol Cld) in

50 mM sodium phosphate pH 8.0, 2 mM DTT, and 0.5 mM EDTA overnight at 4 °C.⁴⁴ The proteolysis mixture was passed over a 5 mL HisTrap column equilibrated in 20 mM imidazole, 100 mM sodium phosphate pH 7.4. A linear gradient (20–500 mM imidazole) was used to separate cleaved *Kp*Cld from tagged *Kp*Cld 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 *Da*Cld and *Da*Cld(R183Q) were available from previous work.^{24, 28, 45} These *Da*Clds were expressed, isolated, and purified using published protocols.¹⁷ All Cld concentrations are given as heme-bound monomer, where [Cld] was determined using reported extinction coefficients. The activity with chlorite was routinely monitored to confirm the competency of each enzyme preparation.^{9, 17} Initial rates of chlorite-decomposing activity by Clds were determined by monitoring O₂ evolution with a luminescence-based probe under pseudo-first order conditions with 20 nM enzyme and [ClO₂⁻] concentrations from 0.1 mM – 2.0 mM in 100 mM sodium phosphate pH 6.0 at 25 °C. Concentrations of freshly prepared stock chlorite solutions were determined via iodometric titration or spectrophotometrically by measuring absorbance at 260 nm using $\epsilon_{260} = 155 \text{ M}^{-1}\text{cm}^{-1}$.⁴⁶

Equilibrium binding of cyanide to Cld

Potassium cyanide (KCN) stock solutions were prepared anaerobically under nitrogen in 0.2 M potassium phosphate pH 5 – 8 and 0.2 M glycine pH > 8. Stock KCN concentrations were determined analytically via titration against a known silver nitrate (AgNO₃) solution in KOH with *p*-dimethylaminobenzylidene rhodanine as the indicator.^{47, 48} Titrations were done anaerobically to insure that the cyanide concentration did not change during the course of the experiment due to oxidation.⁴⁹

For each spectrophotometric titration, a 4 – 8 μM Cld sample was prepared in a 1 mL volume of anaerobic buffer. Stock KCN solution was added in 1–2 μL aliquots. Spectra were repeatedly measured after each addition until the reaction mixtures had reached equilibrium. At the end of the titration, a cyanide stock of 10-fold higher concentration was added in 10 μL aliquots to insure a clear endpoint had been reached. Difference spectra were generated from spectra which had been corrected for sample dilution. The wavelength of maximum absorbance change was used to construct a plot of ΔA versus $[\text{L}]_{\text{T}}$ (total concentration of added ligand) and for tight binding ligands such as cyanide, where the binding of ligand to the enzyme (E) affects the overall concentration of ligand in solution, the data were fit to the quadratic form of the one-to-one titration equation:^{34, 50}

$$\Delta A = \Delta A_{\infty} ([\text{E}] + [\text{L}]_{\text{T}} + K_{\text{D}} - \{([\text{E}] + K_{\text{D}} + [\text{L}]_{\text{T}})^2 - 4[\text{E}][\text{L}]_{\text{T}}\}^{1/2}) / (2[\text{E}]) \quad [1]$$

Transient kinetic reactions with KCN

Rapid kinetic measurements were made using a Hi-Tech SF-61DX2 stopped-flow system. Spectra were measured from 320 – 700 nm with a diode array detector. Reactions were

carried out under pseudo-first order conditions (10-fold ligand excess over heme) by rapidly mixing *Da*Cld, *Kp*Cld, or *Da*Cld(R183Q) (1–10 μ M final concentrations) with varied final concentrations of potassium cyanide (10 μ M –100 mM for WT and R183Q *Da*Cld and 5–200 μ M for *Kp*Cld). Both the enzyme and ligand were diluted into the same buffer prior to binding experiments.

Observed rate constants (k_{obs}) were calculated by fitting A time courses at either 420 nm (*Da*Cld), or 405 and 419 nm (*Kp*Cld) to the single-exponential function:

$$\Delta A_t = \Delta A_{\text{tot}} e^{-k_{\text{obs}} t} \quad [2]$$

Values for the second-order association rate constant (k_{on}) and the first-order dissociation constant (k_{off}) were determined from fitting plots of k_{obs} versus [KCN] to the following:

$$k_{\text{obs}} = k_{\text{on}}[\text{KCN}] + k_{\text{off}} \quad [3]$$

Dissociation constants for the *Da*Cld–CN[−] complexes were determined from the ratio of rate constants:

$$K_D = \frac{k_{\text{off}}}{k_{\text{on}}} \quad [4]$$

In the case of *Kp*Cld, values for k_{off} extrapolated to negative values near the origin. In each case, k_{off} is on the order of 10% or less of the corresponding value for k_{on} . We interpret these results as indicative of a small k_{off} across the pH range. Since the uncertainties in the intercepts for *Kp*Cld–CN[−] are larger than the k_{off} itself, K_D s were measured using spectrophotometric titrations and then used to calculate k_{off} using equation 4.

The rate constants k_{on} and k_{off} were measured in citrate-phosphate buffers as a function of pH from 4 to 8.5, as described above. The log₁₀ values of these rate constants were computed and plotted versus pH. The data were then fit to either a one (Equation 5) or two (Equation 6) K_a model, depending on whether single or dual inflection points were observed in the data. These fits in turn yielded values for acid dissociation constants:

$$\log(k) = \log \left[\frac{c}{1 + \frac{[\text{H}^+]}{K_a}} \right] \quad [5]$$

$$\log(k) = \log \left[c \times \frac{1 + \frac{[\text{H}^+]}{K_{a1}}}{1 + \frac{[\text{H}^+]}{K_{a2}}} \right] \quad [6]$$

All data were plotted using KaleidaGraph. Fits to equation 1 were made using the KinetAssyst software from Tgk Scientific. Fits to equations 2–5 were generated using the least squares fitting program in KaleidaGraph. All values for k_{obs} were based on measurements made in triplicate or more. The reported uncertainties are based on the standard deviations of k , which were determined from these multiple runs.

Vibrational characterization of Cld cyanide and CO complexes

Resonance Raman (rR) spectra were recorded with 413.1-nm emission from a Kr⁺ 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 2 mW for CO complexes to 5–12 mW for CN[−] complexes; 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, and they confirmed that sample integrity had not been compromised by exposure to the laser beam.

The vibrational parameters for Cld cyanide complexes were examined at pH 5.8, 6.8, 8.8, and 10.0 using ¹³C and ¹⁵N isotopologs of cyanoferric Clds (40 μM enzyme and 25–50 mM cyanide). Isotopically labeled potassium cyanides K¹³CN (99 atom % ¹³C), KC¹⁵N (98 atom % ¹⁵N) and K¹³C¹⁵N (99 atom % ¹³C; 98 atom % ¹⁵N) were used. For the spectral range of 600–250 cm^{−1} no baseline corrections were applied. The original spectra were simulated using Origin® to calculate frequencies, intensities and widths of overlapping Gaussian bands. The number of bands and their widths were held constant whenever possible during peak fitting of the spectra for the four cyanide isotopolog complexes for a given Cld while the frequencies were allowed to vary. The simulated spectra were then subtracted from one another and the resulting simulated difference spectra were compared by superposition to the experimental difference spectra.

Enzyme samples in ²H₂O were prepared from concentrated stock protein solutions. Samples were exchanged into 100 mM sodium phosphate buffer prepared in ²H₂O (99.9% ²H) at the desired pH by a 25-fold dilution with the buffer followed by concentration of the sample back to its original volume. This procedure was performed three times to obtain a final solution enrichment of 99 % ²H₂O.

The Fe–C and C–O stretching frequencies were assigned for ferrous *Kp*Cld–CO using the ¹³C isotopolog. Ferrous *Kp*Cld was generated anaerobically by reduction of ferric *Kp*Cld with an excess of buffered stock sodium dithionite solution. The corresponding CO complexes were prepared by flushing the ferrous proteins with natural abundance CO (¹²CO) or ¹³CO (99 atom % ¹³C). Protein concentration was 40 μM *Kp*Cld.

RESULTS

Comparison of kinetic parameters for ClO₂[−] decomposition by Clds

Activities have been reported for a number of Clds.^{5, 9, 17, 23, 26, 51–53} Nevertheless, the kinetic parameters for the various Clds are difficult to compare because the pHs, buffers,

ionic strengths, temperatures, and detection methods used in these assays differ among the literature reports. The *DaCld* and *KpCld* used here were assayed near the peaks of their pH activity profiles and under identical solution conditions to support comparisons of their kinetic parameters (Table S1). These data show that the enzyme efficiencies differ only by a factor of about four (k_{cat}/K_M : *DaCld* $(4\pm1)\times10^7 \text{ M}^{-1}\cdot\text{s}^{-1}$; *KpCld* $(9\pm1)\times10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$), while their activities differ by an order of magnitude (k_{cat} : *DaCld* $(2.3\pm0.4)\times10^4 \text{ s}^{-1}$; *KpCld* $(2.6\pm0.1)\times10^3 \text{ s}^{-1}$).

Low spin ferric Cld-CN as a probe of the catalytic site

Conversion of high spin ferric *Da*- and *Kp*Clds to six-coordinate, low spin (6cLS) heme complexes upon cyanide binding is readily monitored by the changes in their UV-vis spectra, as shown in Figure S1. Their cyanide complexes exhibit UV-vis spectra (B band at 418–420 nm, Q bands at 540 and 565 nm)^{24, 26} comparable to those reported for *NaCld* (420 nm, 540 nm)²⁵ and the dimeric Cld from *Cyanothece* sp. PCC7425 (CCld) (421 nm, 538 nm).⁵⁴ The UV-vis spectra of the three 6cLS Cld–CN complexes examined here do not change with pH.

Kinetics of Cyanide Association and Dissociation as a Function of pH—

Changes in absorbance at 419 nm upon binding of cyanide to the ferric form of all three proteins at each pH and $[\text{CN}^-]$ examined were monophasic with time, yielding high quality fits to a single-term exponential decay function (Equation 1, Figure S2) having R^2 values 0.98. The resulting values for k_{obs} were plotted versus $[\text{KCN}]$ and used to determine k_{on} , k_{off} , and K_D (Equations 3 and 4). Above pH 8.5, the binding events became too fast to track with rapid-mixing techniques, reflected by linear changes in absorbance over time. The k_{on} , k_{off} , and K_D values over the pH range of 4.0 to 8.5 are tabulated in Table S2.

Values of $\log k_{\text{on}}$ measured for CN^- and WT *DaCld*, *DaCld*(R183Q), and WT *KpCld* are plotted as a function of pH (Figure 2A). In every case, the $\log k_{\text{on}}$ increased roughly linearly with pH, having slopes ≈ 1 , consistent with affinity for CN^- being modulated by a single deprotonation event. For *DaCld* at the highest pH values, the data plateau, suggesting a nearby $\text{p}K_a$. Fitting the data to a single- $\text{p}K_a$ model (Equation 5) yielded 8.1 ± 0.2 , which is an approximation since values for k_{on} above pH 8.5 were too fast to be measured. The R183Q mutant showed a similar pH dependence for k_{on} , albeit with values ~ 500 -fold smaller than their WT counterparts. Fitting the data to Equation 5 reflects both the expected linearity of the data and the slope ≈ 1 ; however, the $\text{p}K_a$ was too high to be determined and is therefore designated as >8.5 . Finally, the values and pH-dependent trends in $\log k_{\text{on}}$ for *KpCld* were similar to those of WT *DaCld*. Given the inflection in its plot (Figure 2A), the data were fit to a two $\text{p}K_a$ model (Equation 6) which yielded $\text{p}K_a$ values of 7.7 ± 0.1 and 4.4 ± 0.2 . The lower $\text{p}K_a$ corresponds to irreversible protein denaturation, which makes it an apparent $\text{p}K_a$. A similar pH profile of k_{on} for CN^- binding to dimeric CCld has been recently reported.⁵⁴

Values of $\log k_{\text{off}}$ for *DaCld*, *DaCld*(R183Q), and *KpCld* cyanide complexes are plotted as a function of pH in Figure 2B. The k_{off} for *DaCld* is approximately four orders of magnitude smaller than its k_{on} at the lowest pH where rates were measured. The $\log k_{\text{off}}$ remains unchanged until $\sim \text{pH } 6.5$, whereupon it begins to rise with a slope near 1. Fitting the curve to Equation 5 yields $\text{p}K_a = 6.7 \pm 0.3$. By contrast, k_{off} for the R183Q variant appeared to be

unaffected by pH, remaining small ($<1 \text{ s}^{-1}$) at all pH values measured. Finally, the k_{off} values for *KpCld* are approximately two orders of magnitude smaller than those for *DaCld*. As a function of pH, they exhibit the similar behavior as those for *DaCld*, unchanging until $\sim \text{pH } 7$ followed by a linear rise; a fit of the $\log k_{\text{off}}$ versus pH to equation 5 reveals a $\text{p}K_{\text{a}} = 7.6 \pm 0.2$.

Comparison of Cyanide Affinities—Under alkaline conditions (pH 8.5) *DaCld* and *DaCld*(R183Q) had similar binding affinities for cyanide ($K_{\text{D}} \approx 3 \times 10^{-6} \text{ M}$) while *KpCld* exhibited a much larger cyanide affinity ($K_{\text{D}} \approx 1 \times 10^{-8} \text{ M}$) (Table S2). This difference in cyanide affinity is clearly revealed by spectrophotometric titrations of *KpCld* and *DaCld* with cyanide (Figure 3).

The difference in cyanide affinity is attributed to differences in the k_{off} values, as k_{on} for *KpCld* and *DaCld* were essentially the same across the pH range examined. Trends in K_{D} with pH reflected the trends observed independently in k_{on} and k_{off} . Diminution followed by some degree of flattening in K_{D} values was observed for *KpCld*, *DaCld*, and *DaCld*(R183Q) dominated by the much larger magnitude linear increases in $\log k_{\text{on}}$, relative to $\log k_{\text{off}}$, over much of the measurable range of pH. The K_{D} data were not well modelled by either the single or double $\text{p}K_{\text{a}}$ functions of Equations 2 and 3, respectively.

The spectrophotometrically determined K_{D} for *DaCld*– CN^- of $(6.0 \pm 0.5) \times 10^{-7} \text{ M}$ at pH 7.0 (Figure 3) is in excellent agreement with K_{D} calculated from kinetic constants using equation 4 (Table S2). The previously reported spectrophotometrically determined K_{D} for *DaCld*(R183Q)– CN^- of $8.4 \times 10^{-5} \text{ M}$ ²⁴ is close to the $6.0 \times 10^{-5} \text{ M}$ calculated here using kinetic constants (Table S2). Agreement between the kinetically and spectroscopically determined K_{D} values was taken as an indication that use of our spectrophotometrically determined K_{D} values for *KpCld*– CN^- to calculate k_{off} is reasonable.

Identification of the FeCN vibrational modes—The low frequency range of the Soret-excited rR spectra of *KpCld*– CN^- and *DaCld*– CN^- at pH 5.8 are shown in Figures 4A and 4C. The ferric Cld– CN^- complexes were generated with cyanide isotopologs $^{12}\text{C}^{14}\text{N}^-$, $^{13}\text{C}^{14}\text{N}^-$, $^{12}\text{C}^{15}\text{N}^-$ and $^{13}\text{C}^{15}\text{N}^-$ to identify bands arising from the modes involving distortions of the $\text{Fe}^{\text{III}}\text{CN}$ fragment. As these frequencies and isotope shift patterns are responsive to properties of the distal heme pocket, they constitute a probe of its structural properties. Since several strong heme deformation modes are observed in the $270\text{--}500 \text{ cm}^{-1}$ region, the difference spectra shown in Figures 4B and 4D were generated by digital subtraction of various pairs of Cld– CN^- isotopolog spectra to facilitate identification of the isotope-sensitive modes. Peak-fitting analyses of the experimental spectra identified the bands responsible for features in the difference spectra; multiple isotope-sensitive bands were observed and their Raman shifts are listed in Table S3. The frequencies indicated at the minima and maxima of the difference spectra (Figures 4B and 4D) differ from the actual Raman shifts obtained from the fits of the experimental data (Figures 4A and 4C) because the isotope shifts are less than the widths of the $^{13}\text{CN}^-$ - and C^{15}N^- -sensitive bands. The FeCN angle in cyanide complexes of synthetic iron porphyrinates is typically near 180° . Distortions that alter the Fe–C–N angle can be imposed by electronic and/or steric factors in the heme pocket of proteins, lending both stretching ($\nu_{\text{Fe-C}}$) and bending (δ_{FeCN}) character

to the $^{13}\text{CN}^-$ and C^{15}N^- -sensitive normal modes. The FeCN fragment exhibits a $\nu_{\text{Fe-C}}$ band that shifts monotonically to lower frequency with increasing total mass of the cyanide ligand and a δ_{FeCN} band whose frequency shifts in a “zigzag” pattern upon isotopic substitution of the carbon (in order: $^{12}\text{C}^{14}\text{N}^-$, $^{13}\text{C}^{14}\text{N}^-$, $^{12}\text{C}^{15}\text{N}^-$, $^{13}\text{C}^{15}\text{N}^-$, the frequency decreases, increases, and then decreases). In heme protein cyanide complexes where there are small equilibrium distortions, the nearly linear FeCN fragments exhibit a $\nu_{\text{Fe-C}}$ frequency that is greater than the δ_{FeCN} frequency (Table 1). For bent FeCN fragments ($\angle\text{FeCN}$ between ~ 160 – 130°), the relative frequencies of $\nu_{\text{Fe-C}}$ and δ_{FeCN} are less clear, possibly because of the effect of lower force constants for the Fe–C stretching ($f_{\text{Fe-C}}$) and FeCN bending (f_{FeCN}) internal coordinates relative to those for a linear FeCN fragment (vide infra).⁴³

FeCN geometry and vibrational signatures—The $\nu_{\text{Fe-C}}$ frequencies for *KpCld*– CN^- and *DaCld*– CN^- at pH 5.8 are observed at 439 and 441 cm^{-1} , respectively. Both bands shift monotonically (Figures 4A and 4C) by 3 cm^{-1} (*KpCld*) and 5 cm^{-1} (*DaCld*) between $^{12}\text{C}^{14}\text{N}^-$ and $^{13}\text{C}^{15}\text{N}^-$ complexes. These assignments are further supported by the difference spectra of Cld– CN^- isotopolog complexes which have differing total cyanide mass but carbon atoms of equal mass; in these difference spectra, the features arising from the stretching vibrations are predominant and, since the bending vibrations occur at the same frequencies, they are removed by the subtraction process. The difference spectra of [$^{12}\text{C}^{14}\text{N}^-$ – $^{12}\text{C}^{15}\text{N}^-$] and [$^{13}\text{C}^{14}\text{N}^-$ – $^{13}\text{C}^{15}\text{N}^-$] in Figure 4B and 4D exhibit a prominent feature corresponding to a single stretching mode in each Cld. The observed shifts are considerably smaller than the ~ 11 cm^{-1} calculated for a linear FeCN;⁴² the myoglobin cyanide complexes, which have “nearly linear” FeCN geometries ($\angle\text{FeCN} = 166^\circ$ for sperm whale Mb– CN^- ; pdb code: 1EBC),⁶¹ exhibit isotope shifts of 8–10 cm^{-1} .⁴¹ The smaller isotope sensitivity of *KpCld*– CN^- relative to *DaCld*– CN^- and cyanometMb is consistent with delocalized modes, attributable to the FeCN triad of *KpCld*–CN having the greater deviation from linearity.

In *KpCld*– CN^- at pH 5.8, there are two possibilities for the δ_{FeCN} bending mode; bands at 356 and 372 cm^{-1} exhibit small, but clearly discernable, zigzag patterns in their cyanide isotopolog frequencies. The band at 356 cm^{-1} is tentatively assigned to the δ_{FeCN} mode because it is absent in the ferric *KpCld* spectrum whereas a 371 cm^{-1} band, assigned to a propionate bending mode, $\delta(\text{C}_\beta\text{C}_\epsilon\text{C}_\delta)$, is present (Figure S3). The zigzag pattern due to the isotope sensitivity of the 356- cm^{-1} band yields a difference feature with a maximum at 361 cm^{-1} and a minimum at 353 cm^{-1} in [$^{12}\text{C}^{14}\text{N}^-$ – $^{13}\text{C}^{14}\text{N}^-$], [$^{12}\text{C}^{14}\text{N}^-$ – $^{13}\text{C}^{15}\text{N}^-$], and [$^{12}\text{C}^{15}\text{N}^-$ – $^{13}\text{C}^{15}\text{N}^-$] difference spectra (Figure 4B). A similar difference feature for the 372- cm^{-1} band (max. @ 377 cm^{-1} , min. @ 368 cm^{-1}) is tentatively attributed to kinematic or vibrational coupling of a FeCN coordinate, involving displacement of the iron along a vector having a nonzero projection on the mean porphyrin plane, and in-plane porphyrin coordinates.⁴¹ The 372 cm^{-1} difference feature is not observed in the *DaCld*– CN^- spectra (Figure 4D), suggesting that the kinematic coupling is activated by a bent FeCN geometry in *KpCld*– CN^- . One additional isotope-sensitive band at 306 cm^{-1} , attributed to kinematic coupling of the FeCN and Fe–His distortions,⁴² is observed for *KpCld*– CN^- at pH 5.8.

The δ_{FeCN} mode is assigned for *DaCld*– CN^- pH 5.8 at 396 cm^{-1} based on its zigzag isotope shift pattern (Figure 4C). The difference feature due to this zigzag pattern has a maximum at

401 cm^{-1} and a minimum at $\sim 390 \text{ cm}^{-1}$ in the $[^{12}\text{C}^{14}\text{N}^- - ^{13}\text{C}^{14}\text{N}^-]$, $[^{12}\text{C}^{14}\text{N}^- - ^{13}\text{C}^{15}\text{N}^-]$, and $[^{12}\text{C}^{15}\text{N}^- - ^{13}\text{C}^{15}\text{N}^-]$ difference spectra (Figure 4D). One additional band at 309 cm^{-1} is isotope-sensitive with shifts of $\sim 2 \text{ cm}^{-1}$ to lower frequency upon substitution of natural abundance CN^- (hereinafter designated by $^{12}\text{C}^{14}\text{N}^-$) with $^{13}\text{C}^{15}\text{N}^-$. A similar isotope-sensitive mode has been reported for other ferric heme protein cyanides and, like the 306 cm^{-1} band in *KpCld*- CN^- , is attributed to vibrational coupling between the local Fe-His stretching coordinate and the FeCN distortion coordinates.^{42, 43}

Two major differences are observed when comparing vibrational data for cyanide complexes of *DaCld*(R183Q), shown in Figure 4E and 4F, and WT *DaCld*. First, although the $\nu_{\text{Fe-C}}$ frequency at 441 cm^{-1} for the mutant is the same as that of the WT, the line width of the band increases by a factor of approximately 1.5 (from 12 to 17 cm^{-1}); this is visualized by the greater difference between maxima/minima in the difference spectra; for example, in the $[^{12}\text{C}^{14}\text{N}^- - ^{13}\text{C}^{15}\text{N}^-]$ spectrum the maximum and minimum for WT appear at 444 and 433 cm^{-1} while for the R183Q mutant they are observed at 448 and 429 cm^{-1} . This band width increase is consistent with greater conformational inhomogeneity in the distal pocket of the mutant. It suggests that the Arg side chain constrains the FeCN fragment to a narrower distribution of geometries (FeCN angles) than the amide side chain of Gln. Secondly, the δ_{FeCN} frequency (403 cm^{-1}) is 7 cm^{-1} higher than its WT counterpart. This is consistent with some contribution from a less strained FeCN unit. Several additional isotope sensitive bands are observed in the *DaCld*(R183Q)- CN^- spectra. An isotope sensitive band at 305 cm^{-1} appears comparable to that observed in WT at 309 cm^{-1} due to coupling between the Fe-His stretching and FeCN distortions.^{42, 43} Isotope shifts of 1–2 cm^{-1} are observed for bands at 375 and 361 cm^{-1} ; sensitivity of these modes is similar to that reported for cyanide complexes of Mb and Hb and are likely due to kinematic coupling of in-plane coordinates having Fe- $\text{N}_{\text{pyrrole}}$ stretching character with the Fe-CN stretching coordinate.⁴¹ Although the shifts in these bands are small, the bands overlap with other bands, some of which are also isotope sensitive, and they appear in the difference spectra as features around 381 and 350 cm^{-1} .

FeCN geometry and ($\nu \delta$)—In contrast to bent FeCN of peroxidases, for which the δ_{FeCN} frequency is greater than the $\nu_{\text{Fe-C}}$ frequency, the *KpCld*- CN^- $\nu_{\text{Fe-C}}$ frequency is higher than its δ_{FeCN} frequency (Table 1). These cyanide-sensitive normal vibrational modes comprise displacements along both the $\nu_{\text{Fe-C}}$ and δ_{FeCN} coordinates, with their frequencies depending upon both the FeCN geometry and force constants of the Fe-C stretching ($f_{\text{Fe-C}}$) and FeCN bending (f_{FeCN}) internal coordinates. Each of these frequencies has a potential energy distribution that includes contributions from internal Fe-C stretching, FeCN bending and Fe-His_{prox} stretching coordinates. The relative contributions of displacements along these internal coordinates determine which of the normal modes comprises more stretch than bend or more bend than stretch as illustrated in Figure S5.⁴³ As $f_{\text{Fe-C}}$ increases, a point is reached where the Fe-His_{prox} stretching contribution changes dramatically and the relative frequencies of the predominantly stretching and bending normal modes reverse. The value of $f_{\text{Fe-C}}$ at which this reversal occurs depends on the equilibrium FeCN angle; for angles of 170° and 155°, reversal occurs near $f_{\text{Fe-C}} = 1.80 \text{ mdyn/\AA}$ and 1.45 $\text{mdyn/\AA}$, respectively.⁴³ *KpCld*- CN^- has observed frequencies and isotopic sensitivities comparable to the nonlinear

form of NP1–CN[−]. In fitting the vibrational data of NP1–CN[−], a $f_{\text{Fe–C}}$ of 1.55 mdyn/Å and an $\angle\text{FeCN}$ of 155.0° reproduced the observed isotope shift pattern (Table S4).³⁹ Thus, the occurrence of the Fe–C stretch at a higher frequency than the FeCN bend in the spectra of *KpCld*–CN[−] and NP1–CN[−] (Table 1) indicates that $f_{\text{Fe–C}}$ for these two cyanide complexes are greater than 1.45 mdyn/Å. By contrast, the bent cyanide complexes of other heme proteins (Table 1, Figure S5) exhibit lower values of $f_{\text{Fe–C}}$ (< 1.45 mdyn/Å), thereby pushing their δ_{FeCN} modes to higher frequencies than their $\nu_{\text{Fe–C}}$ modes.⁴³

The frequency separations between modes of predominantly $\nu_{\text{Fe–C}}$ and δ_{FeCN} character are 83, 45, and 38 cm^{−1} at pH 5.8 for *KpCld*–CN[−], *DαCld*–CN[−] and *DαCld*(R183Q)–CN[−], respectively. Since the frequency difference between $\nu_{\text{Fe–C}}$ and δ_{FeCN} modes is larger for bent FeCN geometries (65–106 cm^{−1}) than for the “nearly linear” forms (11–58 cm^{−1}) of cyano heme proteins,^{37–39, 41} these frequency separations together with isotope sensitivities (vide supra) support a bent geometry for *KpCld*–CN[−] (~155°). Nearly linear geometries, wherein $\angle\text{FeCN}$ lies between ~166° and 176°, are observed for *DαCld*–CN[−] and *DαCld*(R183Q)–CN[−]. The smaller (ν – δ) for *DαCld*(R183Q)–CN[−] is consistent with the largest $\angle\text{FeCN}$ of the three cyanide complexes probably due to the distal Gln exerting less off-axis force on the coordinated CN[−] than the native Arg side chain.

pH sensitivity of FeCN modes—As the pH of *KpCld*–CN[−] is increased from 5.8 to 8.0, the bands assigned to $\nu_{\text{Fe–C}}$ and δ_{FeCN} modes both sharpen with line widths decreasing by ~2 cm^{−1}, while their frequencies increase slightly from 439 cm^{−1} to 440 cm^{−1} and from 356 cm^{−1} to 359 cm^{−1}, respectively (Figures 4A, 4B, and S4). Isotope shifts of 1–3 cm^{−1} were observed upon substitution of ¹²C¹⁴N[−] by ¹³C¹⁵N[−]. As already noted for the pH 5.8 case, these observed shifts are considerably smaller than the calculated 11 cm^{−1} for a linear FeCN.⁴² Under alkaline conditions, the isotope-sensitive $\nu_{\text{Fe–C}}$ and δ_{FeCN} bands both exhibit a zigzag isotope shift pattern. Zigzag patterns for the band predominantly due to the stretch have on occasion been observed in proteins where the FeCN has a bent geometry and the $\nu_{\text{Fe–C}}$ and δ_{FeCN} internal coordinates both contribute to the isotope-sensitivity of the frequencies. Thus the band assigned to the “stretching” mode has a significant contribution from the bending coordinate and vice versa.³⁶

Comparison of the *KpCld*–CN[−] spectra at pH 5.8 and 8.0 suggests that increasing pH causes subtle changes in the heme environment which alter the contributions of the stretching and bending coordinates to the isotope-sensitive bands. The frequency separation between the predominantly $\nu_{\text{Fe–C}}$ and δ_{FeCN} -dominated modes (ν – δ) for *KpCld*–CN[−] at pH 8.0 remains large at 82 cm^{−1}. Large ν – δ , the small isotope shifts, and the zigzag shift pattern for the band at 440 cm^{−1} support the conclusion that the overall geometry of *KpCld*–CN[−] remains bent over the pH range examined in this study.

The FeCN modes of *DαCld*–CN[−] also exhibit very small shifts with increasing pH. The $\nu_{\text{Fe–C}}$ frequency decreases from 442 to 441 cm^{−1} while that of δ_{FeCN} increases from 396 to 398 cm^{−1} going from pH 5.8 to 8.8 (Figures 4C and S6). Thus, no dramatic changes in FeCN geometry occur as the pH is raised. However, several low frequency porphyrin modes (309, 336, and 347 cm^{−1}) exhibit band width increases of 2 to 4 cm^{−1} and 1–2 cm^{−1} shifts in frequency as the pH is increased. These changes are the source of the features observed in

the [pH 8.8 – pH 5.6] rR difference spectra (Figure 5A). By analogy to previously assigned modes, these bands correspond to $\nu_{\text{Fe-His}}$, γ_6 , and ν_8 .^{42, 62} In contrast, neither the FeCN nor the out of plane porphyrin modes of *DaCld*(R183Q)–CN[−] are altered by increases in pH, consistent with the lack of features in the *DaCld*(R183Q)–CN[−] [pH 8.0 – pH 5.6] difference spectrum (Figure 5B). Since these pH-dependent shifts for WT are not observed for *DaCld*(R183Q)–CN[−], they may be correlated with the kinetically observed pK_a of 6.7 for *DaCld*–CN[−].

To probe for H-bonding interactions between bound cyanide and the distal pocket, rR spectra of the cyanide complexes of *DaCld* at pH 5.6 and 8.8 and *DaCld*(R183Q) at pH 5.6 and 8.0 were recorded in H₂O and in ²H₂O (Figure 5). These [H₂O – ²H₂O] difference spectra indicate that no significant perturbation occurs in the $\nu_{\text{Fe-C}}$ and δ_{FeCN} frequencies of either enzyme upon isotope substitution, regardless of pH. A similar lack of H₂O/²H₂O sensitivity is observed for *KpCld*–CN[−] (data not shown). Although the lack of deuterium isotope shifts in $\nu_{\text{Fe-C}}$ and δ_{FeCN} frequencies does not eliminate the possibility of hydrogen bonding between the bound cyanide and residues in the distal pocket,^{36, 42, 63} it is consistent with a bound cyanide that does not have strong hydrogen bonding along the Fe–C–N axis. This suggests that the distal Arg is not positioned for optimal hydrogen bonding to the cyanide nitrogen.

Low spin ferrous Cld–CO as a probe of the catalytic site

The Soret-excited rR spectra of *KpCld*–CO (Figure 6) reveal three isotope-sensitive bands at 495, 588, and 1944 cm^{−1}. They are assigned to $\nu_{\text{Fe-C}}$, δ_{FeCO} , and $\nu_{\text{C-O}}$ modes, respectively. These data report a single form of *KpCld*–CO; its position on the $\nu_{\text{Fe-C}}/\nu_{\text{C-O}}$ correlation plot (Figure 6) is consistent with little or no non-bonded interaction between the distal Arg and the bound CO ligand. The CO complexes of WT *DaCld* and *DaCld*(R183Q) have open and closed conformers, likely corresponding to different orientations of the distal Arg side chain.²⁴ In contrast, *KpCld*–CO exhibits a single form, having a Raman fingerprint comparable to the open form of WT *DaCld*–CO.

DISCUSSION

The O₂-evolving Clds constitute an unusual example of rapid and efficient catalytic O–O bond-formation. Despite the similarities in protein folds and active sites in the catalytic domains of enzymes in both the homodimeric and homopentameric Cld subfamilies, there is variability in their catalytic activities, enzyme efficiencies, and turnover numbers. Cyanide complexes, which are sensitive probes of the heme environment, have been used here to evaluate the active sites of the *KpCld* dimer and *DaCld* pentamer as a means of gaining insight into the structural and electronic features responsible for their high-fidelity O₂ generation.

Anion binding to Clds and catalytic efficiency

Cyanide is known to be a strong, competitive inhibitor of *KpCld*⁶⁵ and *Azospira oryzae* strain GR-1 Cld.⁵¹ This is not unexpected for the strong field CN[−] ligand, which readily forms stable 6cLS complexes with the ferric Clds, thereby blocking access of the substrate

to the exogenous (i.e. distal) ligand site of the catalytic heme center. In the pH-dependent reactions between resting Clds and CN^- , the CN^- ligand can be considered in some ways a non-reactive surrogate for ClO_2^- ($\text{p}K_a = 1.96$; $\text{HClO}_2 \rightleftharpoons \text{H}^+ + \text{ClO}_2^-$). The pH-dependent behavior of the chlorite reaction with *Da*Cld was previously studied,²⁶ and interconversions associated with two $\text{p}K_a$ s were previously shown to modulate its activity and spectroscopic properties. The lowest $\text{p}K_a$ of 6.5 involves the conformation reorganization of distal Arg183,²⁶ which is strictly conserved among all O_2 -evolving Clds.^{10, 23} Activity, spectroscopic, crystallographic, and molecular dynamics studies suggest that the Arg side chain can rotate from a catalytically optimal position in a closed conformer to a less active position in an open conformer.^{20, 23, 26, 66} Conversion of the most active 5cHS heme to a 6cLS heme hydroxide occurs with a $\text{p}K_a$ of 8.7.²⁶ The analogous $\text{p}K_a$ s for the distal heme pocket conformation and heme hydroxide formation in *Kp*Cld are 7.0 and 8.3.⁹ Based on the pH profiles of their chlorite decomposing activities, these Clds are most active in their acidic form, which has been hypothesized to have a heme-substrate complex wherein the side chain of the conserved distal Arg points inward over the heme plane (closed conformation).

In contrast to the chlorite reaction, the second-order rate constant for CN^- binding, k_{on} , increased with pH for both *Kp*Cld and *Da*Cld, having values of $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ near the estimated $\text{p}K_a$ s of 7.7 and ~8.1, respectively. These $\text{p}K_a$ values are consistent with k_{on} being limited by the deprotonation of HCN to yield the anion ($\text{p}K_a = 9.2$, 25 °C), exchange of the heme OH^- ligand^{9, 26} for CN^- , or both. The similarity in magnitudes of k_{on} and k_{cat}/K_M at their respective pH maxima, however, suggests that values of k_{on} near $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ define the limiting rate for *Kp*Cld and *Da*Cld complexation with anions. Moreover, based on the similarity among values of k_{on} reported for CN^- binding to other Clds,^{11, 23, 67, 54} rapid anion binding appears to be characteristic of Clds (Table 2).

In *Da*Cld(R183Q), where the side chain of the distal Gln bears no overall charge, k_{on} diminishes by 2 to 3 orders of magnitude (Table S2). Similar decreases in k_{on} relative to WT enzyme have been reported for R173Q, R173K, and R173A mutants (*Nd* distal Arg numbering) of *Nd*Cld (Table 2).^{23, 25} Notably, k_{on} does not exhibit a $\text{p}K_a$ near 6.5, attributed to the impact of the open/closed transition on k_{cat}/K_M , for either ferric WT *Da*Cld or its R183Q mutant.²⁶ This suggests that closing of the Arg “gate” is not important for forming the Fe–CN bond in the *Da* enzymes but for a subsequent step in the catalytic cycle. Comparison of k_{off} values at pH 7.0 for pentameric Cld– CN^- s and their distal Arg mutants (Table 2) reveals that these mutations have a $\sim 10^2$ fold smaller effect on k_{off} relative to their effect on k_{on} .

The pH dependence of k_{off} , in contrast, yields $\text{p}K_a$ s for *Da*Cld and *Kp*Cld, which are very close to those reported for these Clds and their chlorite decomposition reaction. Based on X-ray structures of pentameric Cld ligand complexes,^{20, 22, 53} a closed conformation of the charged distal Arg appears to be readily accessible in *Da*Cld where its interaction with the bound CN^- imparts maximum stability and activity under acidic conditions. Above the $\text{p}K_a$ k_{off} increases; the coordinated CN^- is more readily lost from *Da*Cld suggesting that an open orientation is more accessible as conditions become more alkaline. In contrast, X-ray structures for dimeric Clds, *Nw*Cld and *Cc*Cld, show the distal Arg held in an open conformation by H-bonding to a Gln residue (Figure 1).^{5, 54} In turn, the Arg is H-bonded to

a water molecule that is within H-bonding distance of heme ligands such as hydroxide and fluoride.⁵⁴ Examination of a *KpCld* homology model reveals a similar Gln residue poised to facilitate a comparable open conformer. In such an open conformer of *KpCld*-CN, the Fe-C bond would be exposed to considerably less influence of Coulombic or steric interaction with Arg. The distancing of the Arg charge from the Fe-C bond stabilizes the bond against dissociation; this is supported by the two orders of magnitude slower dissociation rate observed for *KpCld* relative to pentameric Clds. In addition stabilization of an open conformation via H-bonding between the distal Arg and a conserved Gln in dimeric Clds is an attractive explanation for the decreased activity and chlorite turnover number of *KpCld* relative to *DaCld* which lacks a H-bond partner in the comparable position.

FeCN vibrational distortions as indicators of FeCN geometry and distal pocket landscape

Open and closed conformers of the ferrous *DaCld*-CO complex, forming with Arg183 in its open and closed configuration, respectively, have been observed.²⁴ Their positions on the π backbonding correlation plot are consistent with different distal H-bond donor strengths.^{24, 26} It was anticipated, that in *DaCld*-CN⁻, the open conformer would lack H-bonding between Arg183 and CN⁻ while the closed conformer, with its inward-oriented guanidinium group, would interact more strongly with the coordinated CN⁻ through H-bond donation. However, only one *DaCld*-CN⁻ conformer was observed. Since the polar non-bonding interactions between distal H-bond donors and the anionic CN⁻ ligand are expected to be stronger than their CO counterparts,^{36, 38} one explanation for the single *DaCld*-CN⁻ conformer is that the stronger non-bonding interaction coupled with the ionic interaction between the anionic ligand and the cationic Arg result in a closed conformation.

The ²H₂O insensitivity of the FeCN modes of the nearly linear *DaCld*-CN⁻ leaves the question of H-bonding in the distal pocket unresolved.^{63, 77} Insensitivity of the $\nu_{\text{Fe-C}}$ frequencies to ²H₂O suggests that either there is no hydrogen bond or the hydrogen bond is so weak as to be undetectable by a shift of the $\nu_{\text{Fe-C}}$ frequencies in ²H₂O because its geometry is not optimal for polarizing electron density in the FeCN unit.^{36, 42, 63}

The FeCN angle in heme protein cyanides is quite sensitive to distal steric interactions. In and out movement of the distal Arg in *DaCld* would certainly alter steric interactions between CN⁻ and the distal heme pocket. Modulation of distal steric interactions with coordinated CN⁻ could be directly associated with dynamics of the distal Arg183 side chain or indirectly, through its influence on the side chain conformations of other distal residues, including Phe200, Thr198 and Leu185. In either case, the $\angle\text{FeCN}$ could be influenced by the position of the distal Arg. As judged by its $\nu_{\text{Fe-C}}$ and δ_{FeCN} frequencies, the *DaCld*-CN⁻ $\angle\text{FeCN}$ is unaffected by pH; this also supports a single conformation for the cyanide complex. There is, however, a pH-dependent broadening of $\nu_{\text{Fe-His}}$ and ν_8 rR bands indicating some conformational inhomogeneity of the heme pocket. This decrease in homogeneity could correspond to the kinetic pK_a estimated from k_{off} . These data support the hypothesis that at low pH, where k_{off} for *DaCld*-CN⁻ is small, the active site is in a closed conformation while at alkaline pH open Arg conformational states become more accessible, lowering the barrier to CN⁻ dissociation with a corresponding increase in k_{off} values. However, it is likely that, even in the absence of distal H-bond interactions, the closed

conformation dominates the rR spectrum of the ferric cyanide complex due to the ionic interaction between the cationic Arg side chain and the negatively charged cyanide ligand. Such a salt bridge-type interaction is not available to *DaCld*-CO with its neutral CO ligand, resulting in CO complexes mostly in the open Arg conformation.

Although the low pK_a governing the Arg183 gating of the active site in ferric WT *DaCld* is not observed kinetically in the R183Q mutant, two forms of its heme carbonyl have been reported and interpreted as this mutant accessing two conformations analogous to those of the WT enzyme, one with the Gln183 side chain oriented towards the heme and a second in which it is oriented away.²⁴ Although WT *DaCld*-CN⁻ and *DaCld*(R183Q)-CN⁻ exhibited similar $\nu_{\text{Fe-C}}$ frequencies, the broadened $\nu_{\text{Fe-C}}$ band of the R183Q mutant reveals a decrease in structural homogeneity of the FeCN unit analogous to that seen for alkaline WT enzyme (*vide infra*). The smaller $(\nu-\delta)$ for *DaCld*(R183Q)-CN⁻ relative to WT enzyme indicates that its FeCN geometry is less constrained. This suggests that lack of charge in the R183Q distal pocket or the difference in Gln side chain size and/or its hydrogen bond donor strength result in a weaker distal interaction with the bound cyanide in the mutant than in WT. Conformational inhomogeneity and the less constrained FeCN unit of *DaCld*(R183Q)-CN⁻ indicate that the open conformer is accessible. However, based on the lack of pH sensitivity of the k_{off} for *DaCld*(R183Q)-CN⁻ and its similarity to the WT *DaCld*-CN⁻ k_{off} under acidic conditions, it is likely that an active-site conformer of *DaCld*(R183Q)-CN⁻ analogous to the WT closed conformer is also accessible. Taken together, these lines of evidence suggest why *DaCld*(R183Q) retains a modicum of its chlorite-decomposing activity;²⁴ while its closed conformation is responsible for the mutant's residual activity, its diminished ability to bind and retain substrate in the open conformation significantly attenuates its activity.

In contrast to *DaCld* and its R183Q mutant, a single conformer is observed for *KpCld*-CO; its position on the $\nu_{\text{Fe-C}}/\nu_{\text{C-O}}$ correlation plot indicates that there is only weak interaction between heme-CO and distal pocket of *KpCld* (Figure 6). By analogy to its CO complex, *KpCld*-CN⁻ would be expected to exist as a single conformer with a minimal H bonding environment around the CN⁻. The insensitivity of FeCN frequencies in *KpCld*-CN⁻ to ²H₂O substitution could be consistent with this; however, only very small isotope shifts are anticipated for H bonding to the cyanide making it difficult to detect. One FeCN geometry with very subtle frequency differences on either side of the pK_a is observed for *KpCld*-CN⁻, suggesting that, like *DaCld*-CN⁻, its distal Arg is in a single conformation. Interestingly, *NdCld*-CO exhibits a single open conformation in which its distal Arg does not interact with the CO (Figure 6).⁶⁴ Molecular dynamics simulations for ferrous *NdCld* predict that its distal Arg is in the open position 62% of the time, while in ferric *NdCld*-CN⁻ the distal Arg is predominantly in the closed position (85% of the time). This suggests that, while the open conformation is observed for *KpCld*-CO, the form of *KpCld*-CN⁻ observed spectroscopically is mostly likely closed due to nonbonding interactions comparable to those described above for *DaCld*.

Distal Arg versus Gln – effects of charge and steric bulk—The small differences between the FeCN frequencies of the *DaCld* and *DaCld*(R183Q) cyanides suggest that the structural features influencing the force constants and reduced masses of these modes are

similar in the WT and mutant enzymes. Increased breadth of the mutant $\nu_{\text{Fe-C}}$ band relative to that of WT (Figure 4) reflects greater structural flexibility (*i.e.* inhomogeneity) in the heme pocket structure. This points toward the Arg charge being important in controlling its side chain conformation.

Interestingly, several heme proteins having a distal Gln residue exhibit $\nu_{\text{Fe-C}}$ frequencies comparable to those of the three Cld-CN⁻ complexes reported here (Table 1). Increases in these $\nu_{\text{Fe-C}}$ frequencies have been attributed to removal of constraints on the FeCN unit based on “strapped” heme cyanide model complexes with (CH₂)_n chains over the heme, where lengthening or eliminating the strap hindering the bound CN⁻ caused the $\nu_{\text{Fe-C}}$ to shift to higher frequencies (n=13 to 15, 445–447 cm⁻¹; no strap, 451 cm⁻¹).⁷⁸ A similar trend is observed in heme protein cyanide complexes. For example, the crystal structure of *Chlamydomonas* Hb-CN⁻ (pdb code:1DLY) revealed that its FeCN angle is 130° due to a H-bonding network involving a Tyr and two Gln in its distal pocket.⁷⁹ Disruption of this H-bonding network resulted in a less hindered FeCN (*i.e.* more linear FeCN moiety), which was reported by the $\nu_{\text{Fe-C}}$ of *Chlamydomonas* Hb-CN⁻ shifting from 440 cm⁻¹ to 454 cm⁻¹ upon mutation of its distal Gln to Gly.⁴² Replacing the distal His in HRP-CN⁻ with Gln elicits a downshift of its $\nu_{\text{Fe-C}}$ frequency from 453 to 443 cm⁻¹, consistent with steric reorganization of the distal heme pocket accompanying loss of the H-bond donation to the terminal N atom of the CN⁻ ligand.⁵⁹ A similar $\nu_{\text{Fe-C}}$ frequency (444 cm⁻¹)³⁸ reported for HRP at pH 12.5 (*c.f.* pK_a of ~14 for ImH of aqueous histidine) is probably due to loss of the H bond to the CN⁻ ligand in addition to relaxing structural factors influencing the FeCN angle. Another possibility is that in the absence of H-bonding from the distal His, the distal Arg of HRP interacts with the coordinated CN⁻ in a manner comparable to the distal Gln in the examples described here. This idea is supported by the fact that the WT Cld-CN⁻ complexes with their distal Arg residues exhibit similar $\nu_{\text{Fe-C}}$ frequencies (439–441 cm⁻¹). Steric interactions dictated by Gln or Arg in these distal pockets appear to provide comparable nonbonding environments, which restrict the FeCN moiety, in their respective heme cyanides.

Relative insensitivities of $\nu_{\text{Fe-C}}$ frequencies in the Cld-CN⁻s to Arg/Gln mutation and H₂O/²H₂O substitution suggest that neither the distal Arg nor distal Gln serve as significant H-bond donors to the terminal N atom of coordinated CN⁻. Interestingly, the distal Arg does donate two hydrogen bonds to coordinated F⁻.⁶⁵ Therefore, the apparent lack of hydrogen bonding with coordinated CN⁻ leads to the conclusion that heme pocket structure precludes it. In other words, the Cld heme pockets are organized to favor hydrogen bond donation to the coordinated atom of exogenous ligands.⁶⁵ This is the first experimental evidence that supports a structurally based explanation for the poor peroxidase activity²⁸ of the ClDs. Specifically, their heme pocket structure disfavors peroxidase and catalase reactions by disfavoring the terminal hydrogen bond necessary to polarize the peroxo bond, which is responsible, in part, for its heterolytic cleavage in the conversion of compound 0 to compound I in heme peroxidases.

While *Dα*ClDs and other heme proteins like peroxidases, Mb and Hb, have comparable affinities for cyanide ($K_D \sim 10^{-6}$ M), their k_{on} and k_{off} values vary significantly (Table 2). The k_{on} values for cyanide binding to ClDs are 1 to 2 orders of magnitude greater than those

for cytochrome *c* peroxidase (CcP) and HRP, which have distal pockets containing His and Arg, and 4 orders of magnitude greater than Mb and Hb with a His in their distal pockets. The distal Arg in Cld is partially responsible for these greater k_{on} values, which drop from 10^6 to $10^3 \text{ M}^{-1}\cdot\text{s}^{-1}$ upon its replacement with Gln. The series of *NdCld* Arg mutants and *DaCld*(R183Q) still have greater k_{on} values than Mb or Hb indicating that factors intrinsic to Clds other than their distal Arg, such as the hydrophobicity of the distal pocket, contribute to their low kinetic barriers to cyanide binding.

The significantly higher affinity of *KpCld* for CN^- relative to *DaCld*, driven by the much smaller k_{off} value in the former, is surprising. An affinity for CN^- of similar magnitude has been reported for the truncated Hb from *Campylobacter jejuni* (*Cj*trHbP). However, its distal heme pocket is quite different than that of *KpCld*. In *Cj*trHbP, a Tyr and a Trp (pdb code: 2IG3) provide direct H bonds to stabilize bound CN^- .⁸⁰ How the pocket characteristics that promote the high CN^- affinity of *KpCld* are relevant to its function is currently unclear.

Differing FeCN geometries in *DaCld* and *KpCld*—The stretching and bending frequencies reported here for *DaCld* and *KpCld* cyanides are compared to those of other cyanoferric heme proteins in Table 1. The frequencies for WT *DaCld* and its R183Q mutant are similar to other nearly linear ($166^\circ - 179^\circ$) cyanide complexes, especially those of HRP.^{38, 43} While the $\nu_{\text{Fe-C}}$ and δ_{FeCN} frequencies of *KpCld*- CN^- are close to those of bent NP1- CN^- , the Raman shift patterns of their $^{13}\text{CN}^-$ and C^{15}N^- isotopologs reveal that their $\nu_{\text{Fe-C}}$ and δ_{FeCN} frequencies are reversed from those for bent peroxidase, catalase, and P450 cyanides. The bent FeCN geometry of the *KpCld*- CN^- indicates that there are interactions in the distal pocket that are strong enough to generate large off-axis distortions of the FeCN unit that are absent in *DaCld*. The presence of one or two water molecules in the *KpCld* active site, as is the case in dimeric ferric *NwCld* and *CCld* and pentameric *NdCld*- CN^- ,^{5, 23, 54} could provide the steric interactions and possibly H bonds responsible for the off-axis distortions in *KpCld*- CN^- that contribute in slower k_{off} relative to *DaCld*- CN^- . This is also consistent with the “nearly linear” *DaCld*- CN^- having an active site that is less accessible to water than the *KpCld* heme pocket.^{9, 26} Given that the chlorite decomposition activity of these enzymes differs by an order of magnitude and assuming that both enzymes react via similar intermediates, it appears that the factors responsible for the differences in FeCN geometry could play a significant role in their oxygen-evolving activity. The presence of water in the *KpCld* heme pocket would weaken the ion pair presumably formed between the distal Arg and ClO^- , possibly explaining the decrease in activity. This may also be the reason that *KpCld* leaks ClO^- more readily than *DaCld*, resulting in oxidative damage to the enzyme, lowering its turnover number.

While crystal structures of various Cld-ligand complexes have been reported,^{5, 20, 22, 23, 53, 54, 81} only one cyanide complex structure is available.²³ In the *NdCld*- CN^- crystal structure (3NN2), $\angle\text{FeCN}$ ranges from 140.9 to 177.0° with an O atom of a glycerol molecule within H-bonding distance of the terminal cyanide nitrogen atom. The guanidinium group of the distal Arg is pointed away from the heme and lies beyond H-bonding distance from the cyanide ligand (Figure S7).²³ It is unclear how the glycerol in the heme pocket of the *NdCld*- CN^- structure influences the geometry of the FeCN unit or the orientation of the distal Arg. It does illustrate that a wide range of $\angle\text{FeCN}$ can be

accommodated. Since there is no glycerol present in the experiments described here and a bent FeCN unit is also observed for *KpCld*-CN⁻, it is possible that 1) its Arg is in a position analogous to the glycerol, pointed into the pocket toward the cyanide or 2) a water molecule can occupy the glycerol position and provide a bridge between the Arg and the cyanide.

CONCLUSIONS

KpCld has one of the highest affinities for CN⁻ reported for heme proteins. Its particularly small K_D of 4.7×10^{-9} M for CN⁻ dissociation is attributable to slow dissociation kinetics. While the rates of CN⁻ association with WT *DaCld* and *KpCld* are similar to one another, their k_{on} values are at least an order of magnitude larger than those reported for peroxidases and four orders of magnitude greater than those of Mbs. The distal Arg is an important factor in the high CN⁻ association rates, of both Cld subfamilies. Substitution of Arg with Gln reveals that the charge of the distal residue is important in controlling the homogeneity of the ligand-bound pentameric enzyme conformation and the pK_a of ~7 is important in stabilizing the CN⁻ complex. Differing FeCN geometries between *DaCld* (nearly linear) and *KpCld* (bent) reveal that their distal pocket environments, and possibly those between the subfamilies they represent, are distinct. Factors responsible for the bent FeCN geometry in *KpCld*, such as the possibility of water being positioned between the ligand and the distal Arg in the active site, could also affect the stability of the intermediates and/or transition states. Associated differences in kinetic barriers likely account for *KpCld*'s 10-fold lower oxygen-evolving activity relative to *DaCld*. That larger kinetic barrier is also likely the reason that escape of enzyme-inactivating HOCl from the catalytic heme pocket under turnover conditions competes with product formation, thereby limiting the turnover number. Whether these distal pocket characteristics are conserved within the Cld subfamilies remains to be determined.

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.).

ABBREVIATIONS

Cld	chlorite dismutase
<i>KpCld</i>	<i>Klebsiella pneumoniae</i> Cld
<i>DaCld</i>	<i>Dechloromonas aromatica</i> Cld
<i>NwCld</i>	<i>Nitrobacter winogradskyi</i> Cld
<i>NdCld</i>	<i>Candidatus Nitrospira defluvii</i> Cld
CCld	Cld from <i>Cyanothece</i> sp. PCC7425

HRP	horseradish peroxidase
Mb	myoglobin
Hb	hemoglobin
NP1	<i>Rhodnius prolixus</i> Nitrophorin 1
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

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Figure 1. Active site structures of a pentameric and a dimeric Cld

Heme environments for A) the ferric nitrite complex of the respiration-associated, pentameric *DaCld* (PDB ID **3Q08**)²⁰ and B) ferric aqua complex of *NwCld* (**3QPI**),⁵ which like *KpCld* is a dimeric enzyme, are shown. Heme carbons are cyan, protein 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. The two distal pockets differ in the orientation of the distal Arg side chain. In the *DaCld* structure (A), a MES buffer molecule (shown in black, yellow and red) is H-bonded to Arg183. A Gln, the H-bond partner to the distal Arg in *NwCld* (B), stabilizes the Arg in its open conformation. The structures are displayed using UCSF Chimera.²¹

Figure 2. Kinetics of formation and dissociation for cyano-complex formation with ferric *DaCld* (closed circles, blue), *DaCld*(R183Q) (open circles, blue), and *KpCld* (closed circles, red) Values for (A) $\log k_{on}$ and (B) $\log k_{off}$ are plotted as a function of pH. Lines represent the fit of equations 5 and 6 to the data, as described in the text.

Figure 3. Spectrophotometric determination of K_D values for the reaction of ClDs with cyanide
 A) UV-visible spectra of 2.97 μM *KpCld* in 0.2 M potassium phosphate titrated with KCN at pH 7.0. Starting spectrum, blue; final spectrum, red; spectra at intermediate cyanide concentrations, gray. $[\text{CN}^-] = 0, 1.59, 1.99, 2.39, 2.78, 3.17, 3.57, 4.35, 5.52,$ and $9.38 \mu\text{M}$. Inset: A spectra generated from spectra in A by subtraction of starting ferric *KpCld* spectrum from each spectrum for subsequent CN^- addition. B) Spectrophotometric titration of 6.8 μM *DaCld* in 0.2 M potassium phosphate with KCN at pH 7.0. Color scheme is the same as in A. $[\text{CN}^-] = 0, 0.86, 1.73, 2.59, 3.02, 3.45, 3.88, 4.73, 5.58, 6.43, 6.85, 7.69, 8.95, 17.1, 21.1,$ and $26.9 \mu\text{M}$. Inset: A spectra generated from spectra in B by subtraction of starting ferric *DaCld* spectrum from each spectrum for subsequent CN^- addition. C) Plots of

fraction of protein bound with cyanide versus total [KCN] (M); $KpCld$, red squares; $DaCld$ blue circles. Since cyanide binding to both enzymes is quite strong, the fraction bound (A_{obs}/A_{max}) is a quadratic function of the total [KCN]. Nonlinear least squares regression was used to fit A_{422} vs $[CN^-]$ to equation 1.

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Figure 4. A) Low-frequency resonance Raman spectra of isotopically labeled cyanoferric *KpCld* pH 5.8

The cyanoferric *KpCld* complexes were 20 μ M *KpCld* and 25 mM cyanide in 100 mM sodium phosphate buffer. *KpCld*-CN⁻ $\nu_{\text{Fe-C}}$ and δ_{FeCN} frequencies are consistent with a bent FeCN unit. **B) Isotope difference spectra for *KpCld*-CN⁻.** Difference spectra generated by digital subtraction of the spectra shown in A. **C) Low-frequency resonance Raman spectra of isotopically labeled cyanoferric *DaCld* pH 5.8.** The cyanoferric *DaCld* complexes were 20 μ M *DaCld* and 25 mM cyanide in 100 mM sodium phosphate buffer. *DaCld*-CN⁻ $\nu_{\text{Fe-C}}$ and δ_{FeCN} frequencies are consistent with a larger FeCN angle than that of *KpCld*-CN⁻. **D) Isotope difference spectra for *DaCld*-CN⁻.** Difference spectra generated by digital subtraction of the spectra shown in C. **E) Low-frequency resonance Raman spectra of isotopically labeled cyanoferric *DaCld*(R183Q) pH 5.8.** The cyanoferric *DaCld*(R183Q) complexes were 25 μ M *DaCld*(R183Q) and 25 mM cyanide in 100 mM Tris/sulfate buffer. *DaCld*(R183Q)-CN⁻ $\nu_{\text{Fe-C}}$ and δ_{FeCN} are consistent with a nearly linear FeCN unit. **F) Isotope difference spectra for *DaCld*(R183Q)-CN⁻.** Difference spectra generated by digital subtraction of the spectra shown in E. The excitation

wavelength was 413.1 nm; laser power at the sample was 12 mW. Spectra were acquired at 20 °C. Original spectral data are shown in black, bands used to fit the spectra are grey, and the calculated spectra are shown in red. Difference spectra generated from subtraction of spectral data are shown in black; the fit difference spectra are shown in red. Fits for the difference spectra were obtained by generating fits for the experimental spectra and then subtracting those best-fit spectra.

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Figure 5. Low-frequency resonance Raman spectra of A) cyanoferric *DaCld* and B) cyanoferric *DaCld(R183Q)* as a function of pH and $^2\text{H}_2\text{O}$.

The parent spectra at pH 8.8, 8.0, and 5.6 in H_2O (black) and in $^2\text{H}_2\text{O}$ (red) were used to generate the $[\text{H}_2\text{O} - ^2\text{H}_2\text{O}]$ difference spectra (blue). Difference spectra highlighting changes that occur upon increased pH were obtained by subtraction of spectra of acidic enzymes from those under alkaline conditions (grey).

Figure 6. Resonance Raman characterization of ferrous CO complex of *KpCld* reports on its proximal ligands and distal environments

A) Soret-excited rR spectra of the isotopomers of CO complexes of *KpCld*-CO at pH 8.0. Spectra were acquired with 413.1 nm excitation and 2 mW power at the sample. ¹²CO complex, black; ¹³CO complex, red; difference spectra ¹²CO-¹³CO, blue. **B) Backbonding correlation plot of ν_{Fe-C} versus ν_{C-O} for ferrous carbonyls of heme proteins shows the dependences of their positions on axial ligation and distal pocket properties.** The black line correlates ν_{Fe-C} with ν_{C-O} for six-coordinate FeCO adducts in which the sixth ligand is histidine (neutral imidazole). Solid symbols represent ClDs: dimeric ClDs, stars; pentameric ClDs, squares and diamonds. Data for *Da*ClDs, *Nd*ClDs and *CCld* are from references 24, 64 and 54, respectively. Raman shifts for other heme carbonyls are tabulated in Table S1 of reference 24 and their plot positions are shown as open symbols.

Stretching (ν) and bending (δ) frequencies (cm^{-1}) for the FeCN fragment in the cyanide complex of various ferric heme proteins with proximal histidine ligation.

Table 1

protein ^a	“nearly linear” FeCN		“bent” Fe-CN		Potential distal H-bond donors to CN ⁻	references		
	$\nu_{\text{Fe-C}}$	δ_{FeCN}	$\nu_{\text{Fe-C}}$	δ_{FeCN}				
Chlorite dismutases								
<i>KpCld</i> pH 5.8			439	356	Arg/H ₂ O	This work		
<i>KpCld</i> pH 8.0			440	359	Arg/H ₂ O	This work		
<i>DxCl</i> d pH 5.8	441	396	45		Arg	This work		
<i>DxCl</i> d pH 8.8	441	398	43		Arg	This work		
<i>DxCl</i> d(R183Q)	441	403	38		Gln	This work		
Nitrophorin								
<i>Rhodnius prolixus</i> Nitrophorin 1	454	397	57	443 ^b	357 ^c	86	H ₂ O	39
Globins								
TrHbC				440 ^d	nd ^e	nd ^e	Gln/Tyr/Gln	42
trHbC E7Gln→Gly	452	nd ^e	nd ^e				Tyr/Gln	42
<i>Tf</i> -trHb				439	380	59	Trp/Tyr	55
Horse Mb pH 8.0	452	441	11				His	41
Human HbA, pH 8.0	452	434	18				His	41
<i>Chironomus</i> Hb	453	412	41				His	56
<i>C. jejuni</i> single domain Hb	440	403	37	353	417	-64	Gln/Tyr	57
Peroxidases								
HRP pH 11.6	444	405	35	355	420	-65	Arg	38
HRP pH 5.5	453	405	48	360	422	-62	His/Arg	38, 58
HPR(H42Q) pH 7.0	443	nd ^e	nd ^e				Gln/Arg	59
LPO, pH 7.0				360	453	-93	His/Arg	60
MPO				361	453	-92	His/Arg	43

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^a Hb, hemoglobin; Mb, myoglobin; *TfHb*, *Thermobifida fusca* truncated Hb II; *trHbC*, *Chlamydomonas euganetos* trHb-I; *Chironomus* Hb, *Chironomus thummi thummi* Hb; *C. jejuni* single domain Hb, *Campylobacter jejuni* single domain Hb; HRP, horseradish peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase.

^b Normal mode calculations indicate that band has contributions from modes $\nu(\text{Fe-CN})$, $\delta(\text{FeCN})$, and $\nu(\text{Fe-His})$.

^c Normal mode calculations indicate that band has contributions from $\delta(\text{FeCN})$, $\delta(\text{His-Fe-C})$, and $\nu(\text{Fe-His})$.

^d Assignment made based on comparison to $\nu(\text{Fe-CN})$ frequencies for Mb and Hb. Isotopic shifts from $^{12}\text{C}^{14}\text{N}^-$ to $^{13}\text{C}^{15}\text{N}^-$ were comparable to those observed for $K\alpha\text{Cld-CN}^-$.

^e not determined

Table 2
Kinetic and thermodynamic parameters for cyanide binding to selected heme proteins with varying distal pockets

Protein	conditions	$k_{on}/M^{-1}s^{-1}$	k_{off}/s^{-1}	K_D/M	reference
<i>KpCld</i>	20 °C, pH 7.0	$(1.15 \pm 0.01) \times 10^6$	$(5.8 \pm 3.2) \times 10^{-3a}$	$(4.7 \pm 2.8) \times 10^{-9b}$	This work
<i>DαCld</i>	20 °C, pH 7.0	$(1.30 \pm 0.02) \times 10^6$	$(8 \pm 1) \times 10^{-1}$	$(6.1 \pm 0.4) \times 10^{-7c}$	This work
<i>CCld</i>	25 °C, pH 7.0	1.6×10^5	1.4	8.6×10^{-6}	11
<i>NuCld</i>	25 °C, pH 7.0	1.0×10^6	2.4	2.4×10^{-6}	67
<i>NdCld</i>	25 °C, pH 7.0	2.57×10^6	9.3	3.6×10^{-6}	23
CcP	25 °C, pH 7.0	1.1×10^5	9.0×10^{-1}	8×10^{-6}	68
CcP(H52L)	25 °C, pH 7.0	7.0×10^3	1.5×10^{-1}	6.3×10^{-6}	34
HRP	25 °C, pH 7.05	9.8×10^4	2.8×10^{-1}	2.9×10^{-6}	35
<i>C'utHbpd</i>	20 °C, pH 7.0	2×10^4	1×10^{-4}	5.8×10^{-9}	69, 70
<i>MtutHbN^d</i>	20 °C, pH 7.0	3.80×10^2	6.8×10^{-4}	1.8×10^{-6}	69, 70
<i>MtutHbO^d</i>	20 °C, pH 7.0	3.20×10^2	3.5×10^{-4}	1.1×10^{-6}	69, 70
Human Mb	20 °C, pH 7.0	2.30×10^2	3.8×10^{-4}	1.67×10^{-6}	71, 72
SW Mb	20 °C, pH 7.0	3.20×10^2	4.0×10^{-4}	1.25×10^{-6}	71, 72
Human Mb(H64A)	20 °C, pH 7.0	1.1×10^2	4.6×10^{-4}	4.17×10^{-6}	72
Human Mb(H64Q)	20 °C, pH 7.0	1.8×10^2	1.0×10^{-5}	5.6×10^{-8}	71, 72
<i>BjFixLH^d</i>	25 °C, pH 7.0	1.8×10^1	1.2×10^{-4}	5.2×10^{-6}	73
<i>SmtFixLH^d</i>	25 °C, pH 7.0	1.4×10^1	1.7×10^{-4}	1.04×10^{-5}	73
<i>SmtFixLN^d</i>	22 °C, pH 7.0	3.2×10^1	14.7×10^{-4}	1.48×10^{-5}	49
Microperoxidase-11	20 °C, pH 7.0	8.9×10^3	1.5×10^{-3}	1.7×10^{-7}	74
<i>DαCld</i> (R183Q)	20 °C, pH 7.0	$(3.0 \pm 0.02) \times 10^3$	$(1.7 \pm 0.2) \times 10^{-1}$	$(6.0 \pm 0.6) \times 10^{-5}$	This work
<i>NdCld</i> (R173Q)	25 °C, pH 7.0	2.3×10^3	5×10^{-1}	2.164×10^{-4}	25
<i>NdCld</i> (R173K)	25 °C, pH 7.0	1.62×10^3	3.0×10^{-1}	1.85×10^{-4}	23
<i>NdCld</i> (R173A)	25 °C, pH 7.0	3.43×10^3	5.01×10^{-1}	1.46×10^{-4}	23
<i>NdCld</i> (R173E)	25 °C, pH 7.0	7.3×10^1	3.0×10^{-1}	3.874×10^{-3}	25

Protein	conditions	$k_{on}/M^{-1} s^{-1}$	k_{off}/s^{-1}	K_D/M	reference
CcP(H52Q)	25 °C, pH 6.0	8.4×10^1	4.9×10^{-2}	6.1×10^{-3}	33
GdII Hb ⁺ d	20 °C, pH 7.0	4.91×10^{-1}	9.82×10^{-8}	2×10^{-7}	75
GdIII Hb ⁺ d	20 °C, pH 7.0	3.02×10^{-1}	2.9×10^{-6}	1×10^{-5}	76
GdIV Hb ⁺ d	20 °C, pH 7.0	1.82	2.2×10^{-5}	1.2×10^{-5}	76

^a Calculated from spectrophotometric K_D and kinetically determined k_{on} .

^b Measured via spectrophotometric titration.

^c Kinetic and spectrophotometric titration yield the same value for DdCld K_D .

^d Cj, *Campylobacter jejuni*; Mt, *Mycobacterium tuberculosis*; Bj, *Bradyrhizobium japonicum*; Sm, *Sinorhizobium meliloti*; Gd, *Glycera dibranchiate*.