

# Peroxidase-Type Reactions Suggest a Heterolytic/Nucleophilic O–O Joining Mechanism in the Heme-Dependent Chlorite Dismutase

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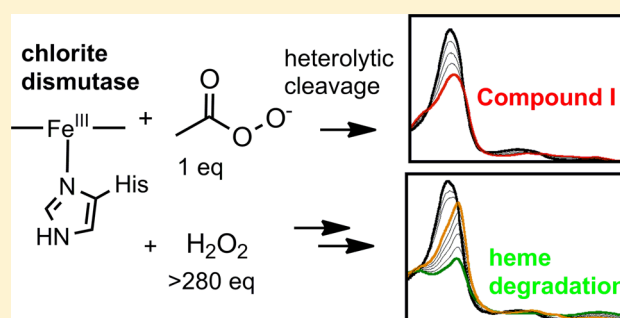
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## S Supporting Information

**ABSTRACT:** Heme-containing chlorite dismutases (Clds) catalyze a highly unusual O–O bond-forming reaction. The O–O cleaving reactions of hydrogen peroxide and peracetic acid (PAA) with the Cld from *Dechloromonas aromatica* (DaCld) were studied to better understand the Cl–O cleavage of the natural substrate and subsequent O–O bond formation. While reactions with H<sub>2</sub>O<sub>2</sub> result in slow destruction of the heme, at acidic pH heterolytic cleavage of the O–O bond of PAA cleanly yields the ferryl porphyrin cation radical (compound I). At alkaline pH, the reaction proceeds more rapidly, and the first observed intermediate is a ferryl heme. Freeze-quench EPR confirmed that the latter has an uncoupled protein-based radical, indicating that compound I is the first intermediate formed at all pH values and that radical migration is faster at alkaline pH. These results suggest by analogy that two-electron Cl–O bond cleavage to yield a ferryl-porphyrin cation radical is the most likely initial step in O–O bond formation from chlorite.



O–O bond breaking processes are fundamental to biological reactions involving O<sub>2</sub> and its aqueous reduction products. O–O bond joining, formally the microscopic reverse, is much rarer. Chlorite dismutases (Clds) are heme-dependent enzymes that catalyze a biologically unusual O–O bond joining reaction, converting a single chlorite ion (ClO<sub>2</sub><sup>−</sup>) to O<sub>2</sub> and Cl<sup>−</sup>.<sup>1–7</sup> This reaction is of interest as a surrogate for understanding the only other well-described O–O bond joining process in biology, catalyzed by photosystem II, as well as efforts to convert water or other reduced species to O<sub>2</sub> using synthetic catalysts.

Clds are widespread across bacterial and archaeal species,<sup>8,9</sup> with only a handful known or expected to catalyze efficient chlorite decomposition, a reaction normally associated with bacterial respiration of perchlorate.<sup>10</sup> Clds are frequently described and annotated as peroxidases, though the biochemical capabilities and biological roles of these presumably nonchlorite-reactive Clds are not entirely clear. Structurally, the Clds are part of a larger protein superfamily containing the heme-binding dye-decoloring peroxidases (DyPs) and their EfeB sequence relatives, both of which bear striking similarities to Clds (Figure 1).<sup>7–9,11–15</sup> Well-characterized DyPs and EfeBs are highly active heme-dependent peroxidases, facilitating cleavage of the O–O bond of H<sub>2</sub>O<sub>2</sub> via generation of high-valent Fe(IV)=O intermediates that serve as reactive oxidants.<sup>11–13,16</sup> The structural similarity between Clds and these proteins suggests that Clds, particularly those from

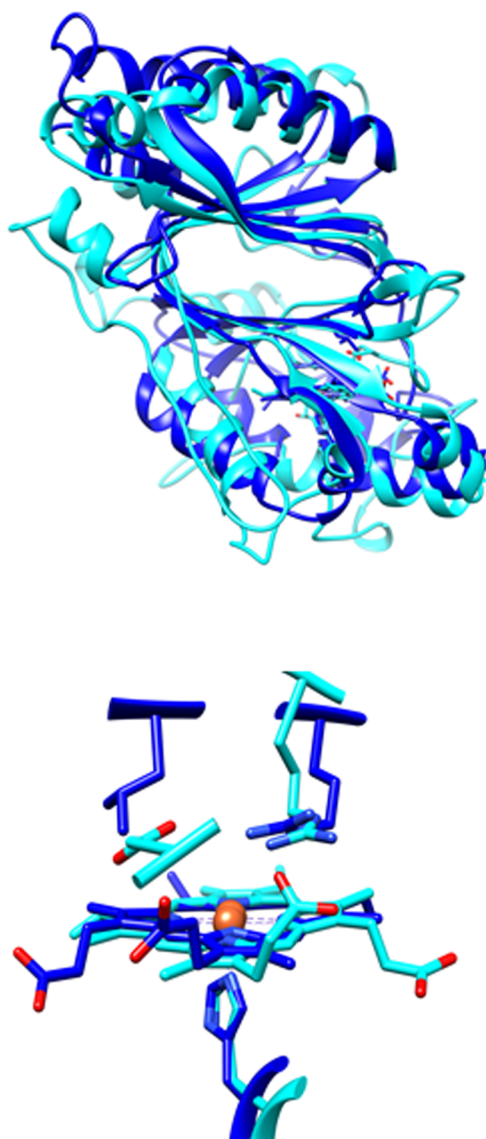
nonperchlorate respiring bacteria and archaea, may likewise react with H<sub>2</sub>O<sub>2</sub>, organic peroxides, or peracids (ROOH), which are also much more biologically common substrates than chlorite. If such reactivity were avid, it might suggest that members of this large family of proteins serve as novel peroxidases or catalases. The reactivity of Clds with alternate substrates, however, has not previously been examined in detail.

Here, we describe steady-state, transient kinetic, and initial spectroscopic studies of the reaction of the *Dechloromonas aromatica* Cld (DaCld) with H<sub>2</sub>O<sub>2</sub> and peracetic acid (PAA, CH<sub>3</sub>(CO)OOH, pK<sub>a</sub> = 8.2). These studies both reflect the Clds' potential biological roles as peroxide-directed catalysts and give insights into the unusual O–O bond joining reaction catalyzed by this enzyme. The use of these alternate oxidants has been especially important for achieving the latter objective because O<sub>2</sub> generation by DaCld is extraordinarily rapid,<sup>17</sup> preventing the direct observation of reaction intermediates via conventional stopped-flow UV/vis transient kinetic methods or allied freeze-quench techniques. We have therefore studied surrogate reactions with peroxide and peracids in order to gain an indirect understanding of how O<sub>2</sub> is formed by Clds. These studies draw on the insight, originally put forward by Babcock

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**Figure 1.** *DaCld* monomer and active site structures (dark blue) shown overlaid with the structure of the representative dye-decoloring peroxidase from *Rhodococcus jostii* (*RjDypB*, cyan). (top) Monomers of *RjDypB* (PDB 3QNR) and *DaCld* (PDB 3QNR) are shown as cartoons. Hemes are shown as sticks in the N-terminal domains. (bottom) Heme-containing active sites are overlaid, showing the different orientations of the heme and catalytically important residues. *RjDypB* has a proximal His226 and distal Arg-Asp pair (Arg244, Asp153). *DaCld* likewise has a proximal His ligand to the heme (His170) and an Arg-Leu pair (Arg183, Leu185). A propionic acid side chain in DyPs is in hydrogen bonding contact with the distal Arg. This interaction is not present in chlorite dismutases, due to the heme's flipped position in the active site.

and others, that biologically relevant O–O cleaving mechanisms are the microscopic reverse of O–O joining.<sup>18</sup> The proposed radical and nucleophilic joining mechanisms for *DaCld* are indeed analogous in a reverse sense to the homolytic and heterolytic mechanisms for breaking the O–O of peracid or peroxide (Scheme 1), and the high-valent heme intermediates formed in either O–O joining or scission are identical. As a consequence, we expect that the identity of the intermediates in the *DaCld* reaction with the reduced oxygen species should report on the types of intermediates this enzyme

preferentially stabilizes and therefore the likely mechanism by which it converts chlorite to O<sub>2</sub>.

The results reported here support a heterolytic O–O bond cleaving mechanism for PAA over the entire range of pH for which the enzyme is stable. This suggests, by analogy, a mechanism for O<sub>2</sub> production from chlorite involving compound I ([Fe(IV)=O]P<sup>•+</sup>) formation followed by nucleophilic recombination of the hypochlorite leaving group (Scheme 1). Movement of the exchange coupled radical away from the porphyrin and onto the protein accelerates at higher pH, kinetically destabilizing the compound I intermediate. Slow turnover with substrates and bleaching or degradation of the heme cofactor by H<sub>2</sub>O<sub>2</sub> suggest that peroxidase chemistry is biologically unlikely for *DaCld* and closely related Clds that lack an active site base; however, the protein is an avid peroxidase if an already-deprotonated peracid is used. These results are discussed in the context of both the likely O<sub>2</sub>-generating mechanisms and their implications for the larger superfamily.

## EXPERIMENTAL PROCEDURES

**Reagents.** Stocks of reagent grade hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (35% Acros) and peracetic acid (PAA) (Sigma-Aldrich) were freshly made and their concentrations determined by iodometric titration. Briefly, to 100  $\mu$ L of a solution of stock was added 10  $\mu$ L of 12 M HCl and  $\sim$ 10  $\mu$ L of a solution of KI (7 mM final concentration). The resulting I<sub>2</sub> that formed (yellow solution) was titrated by addition of 2–200  $\mu$ L volumes of a 1 mM sodium thiosulfate standard (Titristar) in the presence of 2  $\mu$ L of a starch indicator (Ricca Chemicals/1.0% w/v) until the blue color due to I<sub>2</sub> disappeared. For each mole of peroxide or peracid initially present, 2 mol of sodium thiosulfate is consumed. Reductants used were guaiacol (99%, Acros, TCI-Ace) and L-ascorbic acid (Sigma).

**Protein Preparation and Analysis.** *DaCld* expression and purification were carried out as previously described for both the wild-type and R183Q mutant.<sup>19</sup> All data are referenced to the concentration of heme-bound protein subunit as determined by the pyridine hemochrome assay of Berry and Trumpower.<sup>20</sup>

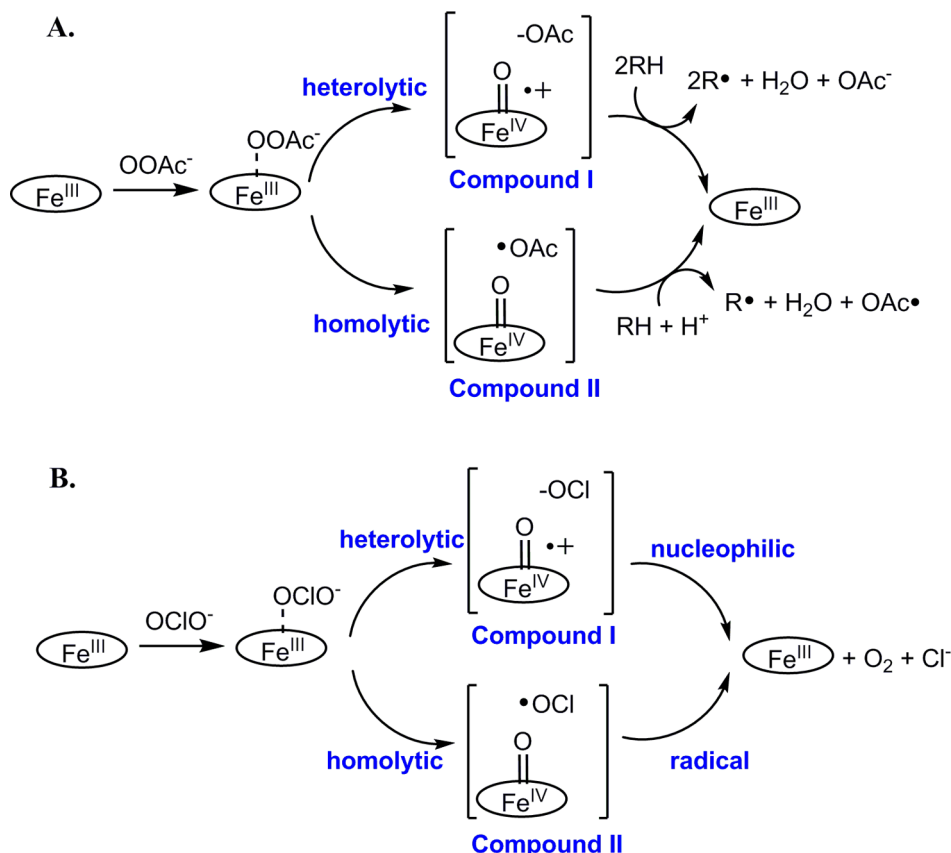
**Chlorite Dismutase Steady-State Assay.** Continuous oxygen production from chlorite decomposition was routinely measured using a Clark-type O<sub>2</sub> electrode. The electrode was equilibrated at 4  $^{\circ}$ C and calibrated using the calculated O<sub>2</sub> concentration of air-saturated water as a reference. The specific activity of the enzyme was defined as micromoles of O<sub>2</sub> produced per minute per milligram of enzyme (4  $^{\circ}$ C, 0.1 M phosphate buffer, pH 6.8, 2 mM chlorite).

**Buffers.** For pH-dependent studies, the following buffers were used: 0.1 M citrate–phosphate (pH 4–8) and 0.1 M borate–phosphate or 0.2 M glycine (pH 8–10).

**Steady-State Kinetics.** Peroxidase activity was studied by measuring the initial rates for the oxidation of guaiacol using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or peracetic acid (PAA). Reactions were monitored spectroscopically at 25  $^{\circ}$ C in a 1 cm path length quartz cuvette using a Varian Cary 50 spectrometer with temperature control from a Peltier cooler. Guaiacol oxidation was monitored at 460 nm ( $\epsilon_{470}$  = 26.6 mM<sup>–1</sup> cm<sup>–1</sup>). Reactions were initiated by the addition of 1–2  $\mu$ M *DaCld*.

Catalase activity was continuously monitored via oxygen production using a Clark-type O<sub>2</sub> electrode. Equilibration and calibration were as described above. Reactions were carried out in a 1.5 mL chamber in which buffer alone was equilibrated for

**Scheme 1. Proposed Reaction Mechanisms for Cl–O/O–O Bond Cleavage and O–O Bond Formation in *DaCld*, Illustrating the Relationships between the Two Pathways and Their Proposed Microscopic Steps<sup>a</sup>**



<sup>a</sup>(A) Heterolytic O–O bond cleavage from the heme–PAA (OOAc<sup>-</sup>) complex (top pathway) yields compound I and an acetate (OAc<sup>-</sup>) leaving group. Compound I is two electron equivalents above Fe(III) and therefore can catalyze two H-atom transfers from substrate (RH). Homolytic bond cleavage (bottom pathway) is an alternative, leading to compound II and acetyl radical (OAc•). Compound II can catalyze one H-atom transfer, returning the heme to its ferric form. (B) Heterolytic bond cleavage from the analogous ferric–chlorite complex leads to compound I and the chlorite leaving group. Homolytic bond cleavage leads to compound II and chlorine monoxide. Recombination of the leaving group and high valent heme intermediate by a nucleophilic (top pathway) or radical (bottom pathway) process could yield a ferric–peroxyhypochlorite complex that would in turn proceed to decompose to chloride and dioxygen.

5 min under N<sub>2</sub> gas, bringing the starting O<sub>2</sub> concentration to 0–50 μM. Background O<sub>2</sub> consumption by the probe was measured prior to addition of saturating H<sub>2</sub>O<sub>2</sub> via a gas-tight Hamilton syringe. *DaCld* (1–2 μM) was added via gas-tight syringe to initiate the reactions.

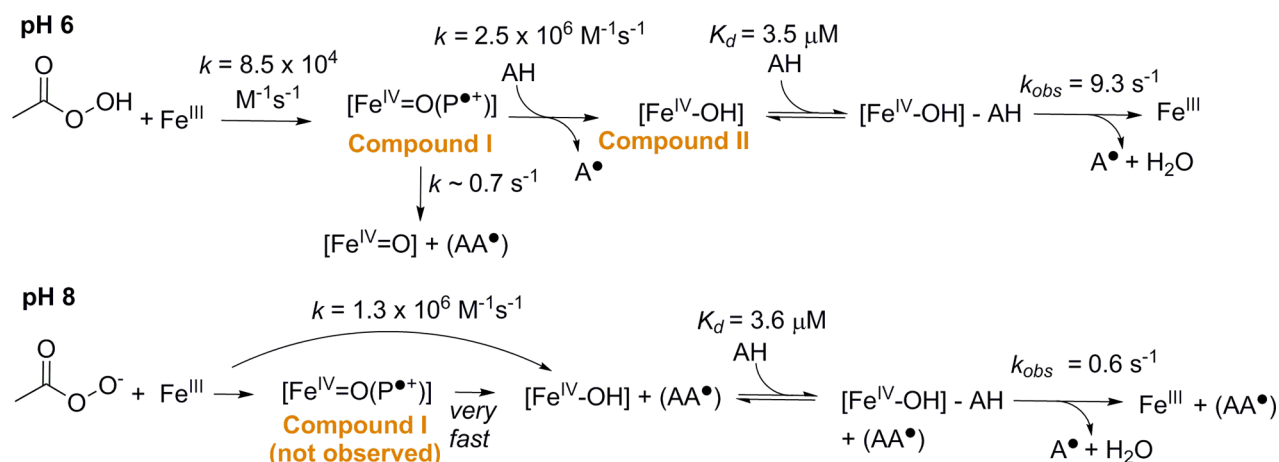
**Transient Kinetics.** Reactions with H<sub>2</sub>O<sub>2</sub> and PAA were monitored using a Hi-Tech SF-61DX2 stopped-flow system in single mixing mode by rapidly mixing the enzyme (~1–10 μM) with varying concentrations of PAA or H<sub>2</sub>O<sub>2</sub>. Apparent rate constants (*k*<sub>obs</sub>) were calculated by fitting single-exponential curves using the KinetAssyst software from Tgk Scientific. Second-order rate constants were determined under pseudo-first-order conditions from the slope and intercepts of *k*<sub>obs</sub> versus PAA or H<sub>2</sub>O<sub>2</sub> concentrations. Both enzyme and oxidant were dissolved in the same buffer prior to experiments, and the pH was confirmed routinely.

Sequential-mixing stopped-flow analysis was used to measure the rates of reduction of intermediates or to determine how many equivalents of reductant were required to regenerate the ferric starting material (Scheme 1). Intermediates were prepared by rapidly mixing enzyme with 1 equiv of PAA and aged (based on kinetics obtained from single mixing experiments) before subsequent mixing with a solution containing

varying concentrations of reductant (ascorbate). All measurements were carried out at 20 °C. Kinetic data were subjected to global nonlinear least-squares analyses (SPECFIT program) to obtain best fit kinetic parameters and component spectra (i.e., reactant, intermediate, and product spectra).

**Resonance Raman Spectroscopy.** For RR spectroscopy, the final Cld concentrations in the PAA reaction ranged between 55 and 70 μM. Resonance Raman spectra were obtained with 406.7 nm excitation from a Kr<sup>+</sup> laser, using the 135° backscattering geometry. The spectrometer was calibrated against Raman frequencies of toluene, dimethyl formamide, and acetone. Data were collected at ambient temperature from samples in spinning 5-mm NMR tubes. Laser power at the samples ranged from 10 to 15 mW. Spectra were collected over time in 15 s acquisitions.

**Freeze-Quench EPR.** Reaction mixtures of 30–100 μM *DaCld* and peracetic acid (5–20-fold excess) in 0.2 M phosphate buffer at pH 5.8 or 8.0 were freeze-quenched in millisecond and second time regimes. Freeze quenching in the millisecond range was accomplished with a commercial rapid-mix freeze-quench system. Room temperature reaction mixtures were sprayed into an isopentane slush at –120 °C and then packed into quartz tubes for examination by EPR

Scheme 2. Proposed Reactions between Ferric *DaCld* and Peracetic Acid at pH 6 and 8 with the Proposed Intermediates Indicated

 Table 1. Steady State Kinetic Constants for the Reactions of *DaCld* and Peroxidases with Phenolic Cosubstrates

enzyme/oxidant	cosubstrate	pH	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_{\text{M}}(\text{oxidant})$ ( $\text{M}^{-1} \text{s}^{-1}$ )	ref
<i>DaCld</i> /ClO <sub>2</sub> <sup>a</sup>	none	5.2	$2.0(0.6) \times 10^4$	$3.2(0.4) \times 10^7$	21
		7.6	$3.0(0.8) \times 10^3$	$6.9(0.8) \times 10^6$	21
<i>DaCld</i> /PAA <sup>b</sup>	guaiacol	6	$2.8(0.50)$	$9.3(1.8) \times 10^3$	this work
		8	$0.3(0.01)$	$3.1(0.2) \times 10^4$	this work
<i>DaCld</i> /H <sub>2</sub> O <sub>2</sub> <sup>b</sup>	guaiacol	6	$0.2(0.02)$	$4.1(1.5) \times 10^1$	this work
		8	$0.2(0.01)$	$4.8(0.9) \times 10^2$	this work
HrP-C/H <sub>2</sub> O <sub>2</sub> <sup>c</sup>	guaiacol	7	$376(30.0)$	$4.5 \times 10^6$	50
DyP-A/H <sub>2</sub> O <sub>2</sub>	pyrogallol	4.5	$0.31(0.01)$	$4.9(0.3) \times 10^2$	16
DyP-A/H <sub>2</sub> O <sub>2</sub> <sup>d</sup>	ABTS	4.5	$68.0(2.00)$	$1.7(0.1) \times 10^4$	16
DyP-B/H <sub>2</sub> O <sub>2</sub>	pyrogallol	4.5	$2.08(0.04)$	$7.9(0.2) \times 10^4$	16
DyP-B/H <sub>2</sub> O <sub>2</sub> <sup>d</sup>	ABTS	4.5	$14.1(0.20)$	$2.1(0.1) \times 10^5$	16

<sup>a</sup>Buffer = 100 mM citrate–phosphate, 4 °C. <sup>b</sup>Guaiacol at saturating (8.35 mM) concentrations; buffer = 100 mM citrate–phosphate, 20 °C. <sup>c</sup> $K_{\text{M}}$  determined from Lineweaver–Burke plots; buffer = 10 mM sodium phosphate, 25 °C. <sup>d</sup>50 mM sodium acetate.

spectroscopy. Reactions quenched on the second time scale were mixed at 4 °C, put into quartz tubes, and flash frozen in an acetone slush bath (−95 °C). Uncertainty in the quench times for these samples is estimated at  $\pm 1$  s. X-band EPR spectra were recorded at 77 K using a Varian E9 spectrometer. The spectra were referenced to 2,2-diphenyl-1-picrylhydrazyl (DPPH) in a frozen toluene glass. Spin quantification was carried out with microwave power of 20  $\mu\text{W}$ , where neither the DPPH standard nor the protein radical signals were saturated. Integrated intensities were determined from normalized double integrals of the derivative EPR spectra.

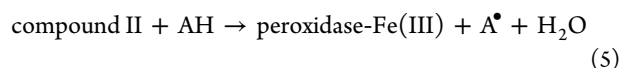
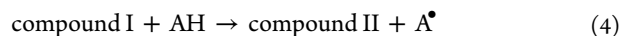
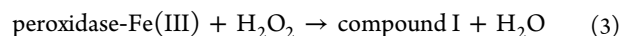
## RESULTS

**pH Dependence of Steady State and Transient Reactions.** The steady state reaction of *DaCld* with chlorite was previously studied over a wide range of pH,<sup>21</sup> though the transient reaction with chlorite is too fast to observe directly.<sup>22</sup> The steady state and transient kinetics of the *DaCld* reactions with H<sub>2</sub>O<sub>2</sub> and PAA were studied here at several pH values over which the enzyme is stable (pH 6–10). Though it was not possible to obtain an exact  $pK_{\text{a}}$ , the reactions with PAA change dramatically around neutral pH (*vide infra*). Several results are therefore presented at pH 6 and 8, because these values illustrate the behavior of *DaCld* over the entire active range of acidic and alkaline pHs, respectively (Scheme 2).

**Catalase activity of *DaCld*.** Rates of O<sub>2</sub> production from *DaCld*/H<sub>2</sub>O<sub>2</sub> were measured in the presence of 20 mM H<sub>2</sub>O<sub>2</sub>

from pH 4 to 10. In this pH range, the values obtained were not significantly above background and well below rates measured for typical catalases.<sup>23,24</sup> Based on comparison of these data, *DaCld* does not exhibit significant catalase activity.

**Steady-State Kinetics of *DaCld*, H<sub>2</sub>O<sub>2</sub> or Peracetic Acid, and Guaiacol.** The peroxidase reaction (Scheme 1, top) begins with peroxide activation followed by two sequential one-electron oxidations (hydrogen atom transfers) of the substrate AH:



Compound II is a ferryl heme,  $[\text{Fe}^{\text{IV}}=\text{O}]$ , with no porphyrin or protein-based radical. The steady-state reaction of *DaCld* with H<sub>2</sub>O<sub>2</sub> and the peroxidase cosubstrate guaiacol (2-methoxyphenol) was studied at pH 6 and 8. At both pH values, *DaCld* is an inefficient catalyst for the oxidation of guaiacol by H<sub>2</sub>O<sub>2</sub>, yielding values for  $k_{\text{cat}} \sim 10^3$ -fold and  $k_{\text{cat}}/K_{\text{M}}(\text{H}_2\text{O}_2) \sim 10^4$ – $10^5$ -fold smaller than those measured for highly efficient horseradish peroxidase-C (HrP-C) at pH 7 (Table 1). The  $k_{\text{cat}}/K_{\text{M}}(\text{H}_2\text{O}_2)$  increases only slightly ( $\sim 10$  fold) with pH. By contrast, *DaCld* exhibits significantly greater peroxidase activity with PAA as the oxidizing agent, with  $k_{\text{cat}}/$



$K_M(\text{PAA})$  2–3 orders of magnitude higher than  $k_{\text{cat}}/K_M(\text{H}_2\text{O}_2)$ .

The  $k_{\text{cat}}$  for the  $\text{O}_2$  producing reaction with chlorite is larger still (Table 1), possibly because this reaction depends on the formation of a single enzyme–substrate complex rather than sequential reactions with three separate substrate molecules (oxidant, HA, HA). The magnitude of  $k_{\text{cat}}/K_M(\text{oxidant})$  is largely due to the efficiency of formation of the initial enzyme–substrate complex and appears to be greater for anionic oxidants. Chlorite is effectively deprotonated over the pH range studied ( $\text{HClO}_2 \rightleftharpoons \text{ClO}_2^- + \text{H}^+$ ;  $\text{p}K_a = 1.86$ )<sup>25</sup> and  $k_{\text{cat}}/K_M(\text{chlorite})$  is close to the limit imposed by diffusion. By contrast, the relatively high  $\text{p}K_a$  of  $\text{H}_2\text{O}_2$  (11.6) suggests that it is present overwhelmingly in its neutral form over the pH range studied,<sup>26</sup> while PAA's  $\text{p}K_a$  (and  $k_{\text{cat}}/K_M$ ) is intermediate between the two ( $\text{CH}_3(\text{CO})\text{OOH} \rightleftharpoons \text{CH}_3\text{COO}^- + \text{H}^+$ ;  $\text{p}K_a = 8.2$ ).

**Transient Reaction with PAA at pH 6.** Following reaction with 3–5 equiv of PAA, the ferric species converts isospectically to an apparent compound I species based on the diminished Soret band and the band energies (395 nm, shoulder at 341 nm; visible bands: 525, 550, 600, and 645 nm). A second-order rate constant ( $k = 1.9 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) for its formation was determined (Figure S1, Supporting Information).

Compound I formed at pH 6 with fewer than 5 equiv of PAA decays to a mixture of two species within 15 s, consistent with an Fe(III) high spin heme similar to the resting enzyme ( $\nu_3$  1494  $\text{cm}^{-1}$ ,  $\nu_4$  1372  $\text{cm}^{-1}$ ) and a ferryl species ( $\nu_3$  1508  $\text{cm}^{-1}$ ,  $\nu_4$  1378  $\text{cm}^{-1}$ ) (Figure S2, Supporting Information).<sup>27–34</sup> The 77 K X-band EPR spectrum of a sample prepared in a similar manner and frozen in liquid  $\text{N}_2$ /acetone has a  $g = 2.005$  signal with a peak-to-peak line width of 18 G (Figure S3, Supporting Information). Because this signal is readily observed at 77 K, it is concluded to arise from an organic, amino-acid-based radical ( $\text{AA}^\bullet$ ) that is not exchange coupled with the ferryl:  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$ . The isoelectronic, exchange-coupled intermediates  $[\text{Fe}^{\text{IV}}=\text{O}(\text{AA}^\bullet)]$  or  $[\text{Fe}^{\text{IV}}=\text{O}(\text{P}^\bullet)]$ , by contrast, would both be expected to exhibit very broad EPR signals at 10 K that would be undetectable at 77 K.<sup>35–37</sup> The lack of exchange coupling with the iron suggests the Fe and  $\text{AA}^\bullet$  are separated by  $>10 \text{ \AA}$ . Based on current data, it is not possible to determine the location or locations of the amino acid radical, which may be inhomogeneous, or the heme oxidation state ( $\text{Fe}^{\text{III}}$  or  $\text{Fe}^{\text{IV}}=\text{O}$ ) with which the protein-based radical is associated.

Gradual decay of the  $[\text{Fe}^{\text{IV}}=\text{O}]$  to ferric Cld or  $\text{Fe}^{\text{III}} + (\text{AA}^\bullet)$  or both, which are indistinguishable by RR, is observed in the RR spectra via increasing intensity of the 1494  $\text{cm}^{-1}$   $\nu_3$  and a corresponding decrease in the 1508  $\text{cm}^{-1}$   $\nu_3$  intensity. These data (Figure S2, Supporting Information) indicate that the conversion of  $[\text{Fe}^{\text{IV}}=\text{O}]$  back to resting enzyme is slow in the absence of a large excess of oxidant. Over a period of minutes, the heme-based chromophore observed for the 15 s intermediate bleaches completely, indicative of heme destruction.

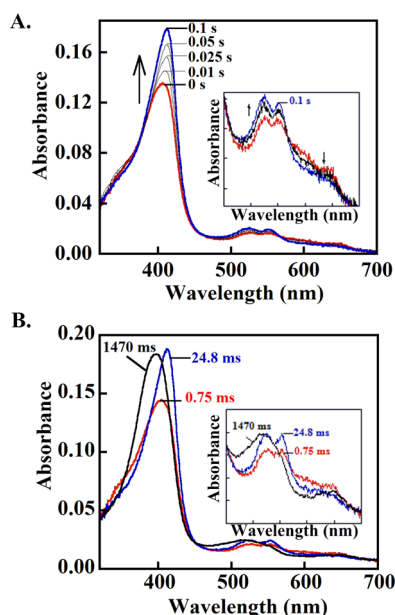
The reaction of ferric DaCld with higher concentrations ( $>5$  equiv) of PAA yields compound I more rapidly. Its conversion to a second intermediate and subsequent heme destruction (occurring over several minutes) are more clearly observable. The second intermediate has a UV/vis spectrum consistent with either compound II, the one-electron reduction product of compound I, or  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$ , which is isoelectronic with compound I (Soret at 410 nm;  $\alpha/\beta$  bands 525, 555 nm). Both

intermediates have indistinguishable UV/vis and RR spectra in CcP and similar peroxidases.<sup>38</sup> Their Soret band extinction of  $\sim 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  reflects intact aromaticity in the porphyrin ring and contrasts with the diminished absorbance of the non-aromatic compound I. The rate of conversion of compound I to the second intermediate is independent of PAA concentration (100–500  $\mu\text{M}$  or 10–50 equiv), occurring via a single exponential process with  $k = 0.95 \text{ s}^{-1}$  (Figure S5, Supporting Information) and maximizing at  $\sim 8.7 \text{ s}$  after compound I forms. PAA concentration independence indicates that PAA does not act as a reducing agent toward compound I and is consistent with a unimolecular process in which the porphyrin radical from  $[\text{Fe}^{\text{IV}}=\text{O}(\text{P}^\bullet)]$  moves onto an amino acid to form an uncoupled radical. The 77 K EPR spectrum measured for a 15 s sample is similar in appearance to that described above for the reaction of DaCld with  $<5$  equiv of PAA. Spin quantification of the EPR signal indicates a protein-based radical in  $\sim 36\%$  of the total enzyme.

**Transient Reactions of Compound I with Ascorbate (pH 6).** Sequential mixing experiments with PAA and ascorbate were carried out to study the reactivity of the intermediate. The putative compound I was generated by mixing a slight stoichiometric excess of PAA with the enzyme and aging for 2 s. It was then mixed with either 1 or 2 equiv of ascorbate and the reaction to form 1  $\text{e}^-$  oxidized semidehydroascorbate followed via stopped-flow spectrophotometry. One-electron donation from ascorbate (which can in principle act as either a 1 or 2  $\text{e}^-$  donor) is the observed pathway in plant peroxidases; semidehydroascorbate is thought to dimerize over time.<sup>39,40</sup> Following mixing with 1 equiv of ascorbate, a species consistent with compound II, the expected 1  $\text{e}^-$  reduction product, forms (Soret band at 412 nm;  $\alpha/\beta = 525, 555 \text{ nm}$ ). This species persists for several minutes with concurrent, gradual bleaching of the chromophore. It is important to note that, while this species is electronically consistent with assignment as compound II, a definitive assignment will require EPR characterization of the intermediate. Reacting compound I with 2 equiv of ascorbate results in the initial formation of an apparent compound II, maximizing after  $\sim 0.0025 \text{ s}$ . This intermediate subsequently converts isospectically back to the ferric form in 1.5 s (Figure 2B).

The reaction of compound I with pseudo-first-order concentrations of ascorbate ( $\geq 10$  equiv) was examined. Rate constants for the single-exponential conversion of compound I to compound II were fit at 415 nm, yielding a second-order rate constant of  $2.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  (Figure S3, Supporting Information). The subsequent conversion of compound II to the ferric species (400 nm) occurred as a single phase. The resulting  $k_{\text{obs}}$  has a hyperbolic dependence on ascorbate concentration with a maximum of  $9.3 \text{ s}^{-1}$  and an apparent  $K_d = 3.5 \mu\text{M}$  (Figure S6, Supporting Information).

**Transient Reaction with PAA at pH 8.** Ferric DaCld at pH 8.0 has a Soret band at 396 nm (slightly red-shifted relative to pH 6) and charge-transfer bands at 510 and 645 nm (unchanged with pH).<sup>21</sup> Following reaction with  $\geq 3$  equiv of PAA, the ferric enzyme converts isospectically to an apparent ferryl species (Soret band at 415 nm with greater absorptivity than ferric; shoulder at 350 nm;  $\alpha/\beta$  bands = 525, 555 nm; Figure 3B, Table 2). The enzymatic intermediate could be  $[\text{Fe}^{\text{IV}}=\text{O}]$ , the product of direct, homolytic cleavage of the PAA O–O bond (Scheme 1, top). Alternatively, it could be  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$ , which would form via radical migration away from a preceding, undetected compound I and which

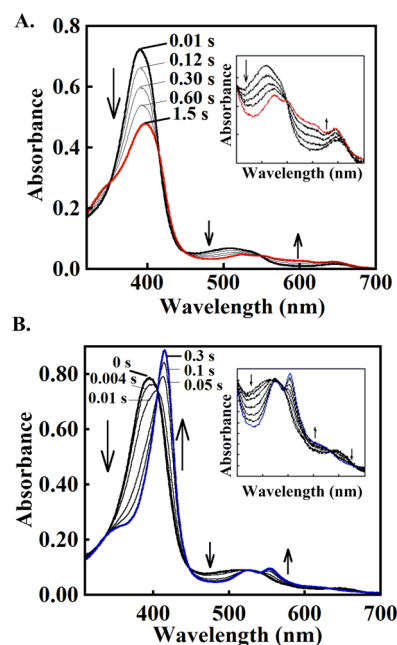


**Figure 2.** *DaCld* compound I converts to (A) ferryl and (B) ferryl and ferric species after mixing with 1 and 2 equiv of ascorbate, respectively, exhibiting a complete peroxidase cycle at pH 6. (A) *DaCld* (10  $\mu\text{M}$ , 2.5  $\mu\text{M}$  final) was mixed with a slight stoichiometric excess of peracetic acid and aged for  $\sim 2$  s. The compound I species that forms (red spectrum) was then mixed with 1 equiv of ascorbate (2.5  $\mu\text{M}$  final), forming the ferryl species (blue spectrum) after  $\sim 0.1$  s. This species is tentatively assigned as compound II. (B) Compound I (red) was formed as in panel A and subsequently mixed with 2 equiv of ascorbate (5  $\mu\text{M}$  final). An intermediate with a spectrum resembling compound II or  $[\text{Fe}(\text{IV})=\text{O}] + (\text{AA}^\bullet)$  forms after 2.5 ms and converts to the ferric enzyme after 1.5 s (black). Insets, visible bands (450–700 nm) shown on an expanded scale.

would have an identical UV/vis spectrum. Because the intermediate forms with apparent isosbestic points from the ferric species, conversion of  $[\text{Fe}^{\text{IV}}=\text{O}(\text{P}^\bullet)]$  to  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$  would have to be very fast relative to the rate of  $[\text{Fe}^{\text{IV}}=\text{O}(\text{P}^\bullet)]$  formation. Values for  $k_{\text{obs}}$  fit at 415 nm depended linearly on PAA concentration ( $k = 1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ) (Figure S7, Supporting Information). The intermediate persists for several seconds before the heme chromophore bleaches. Bleaching occurs at a faster rate at higher PAA concentration.

Resonance Raman data were measured for *DaCld* at 15 s intervals following reaction of the enzyme with  $\sim 8$  equiv of PAA at pH 8 (Figure 4A). The resting enzyme contains a five-coordinate high-spin heme, similar to the enzyme at pH 6.0 ( $\nu_3$  1494  $\text{cm}^{-1}$ ,  $\nu_4$  1371  $\text{cm}^{-1}$ ). Following addition of PAA, the enzyme converts to a species with a RR spectrum consistent with a ferryl ( $\nu_3$  at 1508  $\text{cm}^{-1}$ ,  $\nu_4$  at 1377  $\text{cm}^{-1}$ ).<sup>27,28,30,31,34,41,42</sup> This spectrum is very similar to that measured for the  $[\text{Fe}(\text{IV})=\text{O}] + (\text{AA}^\bullet)$  intermediate that follows compound I in the pH 6.0 *DaCld*/PAA reactions (Figure S2, inset, Supporting Information). The intermediate persists for 15–30 s, after which some ferric enzyme begins to be detected (intensity at 1494  $\text{cm}^{-1}$  and a shift in the  $\nu_4$  frequency back toward 1371  $\text{cm}^{-1}$ ). A plot of  $\nu_4$  versus time illustrates conversion of the ferryl back to the resting ferric under low PAA concentration, when the bleaching process is relatively slow (Figure S4, Supporting Information).

The protein/PAA mixture was freeze-quenched at 77 K at varying time points following mixing and examined by EPR



**Figure 3.** *DaCld* forms distinct intermediates at (A) acidic and (B) alkaline pH following rapid mixing with 3 equiv of PAA. (A) Compound I forms at pH 6. *DaCld* ( $\sim 15 \mu\text{M}$ ; 7.5  $\mu\text{M}$  final) was mixed with 50  $\mu\text{M}$  peracetic acid (25  $\mu\text{M}$  final) in 0.2 M citrate–phosphate buffer (20  $^\circ\text{C}$ ). The initial spectrum is shown in black, and the spectrum for the intermediate formed after  $\sim 1.5$  s is shown in red. Intervening spectra measured at the given times are gray. (B) Compound II or  $[\text{Fe}(\text{IV})=\text{O}] + (\text{AA}^\bullet)$  forms as the initial intermediate under similar conditions but at pH 8. The initial spectrum is shown in black, and the spectrum for the ferryl intermediate that formed after  $\sim 300$  ms is shown in blue. Intervening spectra measured at the given times gray. Insets, visible bands (450–700 nm) shown on an expanded scale.

(Figure 4B). The poorly resolved fine structure and the temporal dependence of the spectral amplitude, which increases over tens of milliseconds, are consistent with a protein-based radical (e.g., Tyr $^\bullet$  or Trp $^\bullet$ ) or mixture of radicals. Comparison of the shape of the EPR spectrum at 50 ms (Figure 4B, top) with ones after 15–60 s of reaction clearly reveals changes (Figure 4B, bottom). Spin quantification of the EPR signal showed  $\sim 46\%$  of the enzyme in the radical form at 50 ms and 38% at 15 s. The existence of radical species at 77 K in a significant fraction of the sample suggests that it is  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$  rather than  $[\text{Fe}^{\text{IV}}=\text{O}]$ . The time dependence of the spectral signature is consistent with changing populations of radical sites due to radical migration. Assignments of the radical sites will require multifrequency EPR spectroscopy of WT, mutant, and isotopically labeled (Trp- and Tyr-deuterated) enzymes. These studies are ongoing and will be reported separately (A. Ivancich, personal communication).

The Soret-excited RR spectra were recorded for the same set of freeze-quenched intermediate samples at 77 K, pH 8 (data not shown). The RR data are very similar to the 22  $^\circ\text{C}$  spectra obtained at 15 s, showing only the ferryl species and no Fe(III). Because the EPR data indicated the presence of a substoichiometric population of radicals in the aged reactions, the freeze-quench samples must contain a mixture of  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$  and an EPR-silent species:  $[\text{Fe}^{\text{IV}}=\text{O}]$  or exchange coupled  $[\text{Fe}^{\text{IV}}=\text{O}(\text{AA}^\bullet)]$ .

**Table 2. UV/Visible Absorbances for Stable and Intermediate States of *DaCld* and Well-Characterized Heme Peroxidases**

species	Soret <sup>b</sup>	CT1	$\beta$	$\alpha$	CT2	ref
<i>DaCld</i> ferric, pH 6	392 (99)	645 (2.2)			510 (9.7)	this work
<i>DaCld</i> compound I, pH 6	395 (66.7)	600, 645 sh (3.3)	525 (6.9)	550 sh		this work
<i>DaCld</i> compound II, pH 6 <sup>a</sup>	412		525	555		this work
<i>DaCld</i> [Fe <sup>IV</sup> =O(P) + (AA <sup>•+</sup> )], pH 6	410	none	525	555	none	this work
<i>DaCld</i> ferric, pH 8	396 (109)	645 (3.3)	535 sh		510 (12.5)	this work
<i>DaCld</i> compound 0, pH 8	408 (112.5)	none	535 (11.7)	575 sh	none	this work
<i>DaCld</i> [Fe <sup>IV</sup> =O(P) + (AA <sup>•+</sup> )], pH 8	415 (125)	none	525 (12.5)	555 (13.5)	none	this work
ferric HRP	402.5 (102)	643 (3.23)	530 (8.4)	580 (2.8)	498 (11.25)	23
HRP ferrous-O <sub>2</sub> (compound III)	417 (108)	none	544 (11.5)	580 (10.0)	none	23
HRP compound I	400 (53.8)	651, 622 (6.6) sh	525 sh	577 (7.4)		23
HRP compound II	410 (105)		527 (9.5)	555 (9.65)		23
ferric DypB	404	634			503	16
DypB compound I	397	648		580, 613 sh		16
ferric DypA	408	632			502	16
DypA compound II	419	619	528	557		16
ferric EfeB	406	660			485	76
EfeB compound I	414	603	530	555		76

<sup>a</sup>This species forms following reaction of compound I with 1 equiv of ascorbate. <sup>b</sup>Numbers in parentheses are extinction coefficients (mM<sup>-1</sup> cm<sup>-1</sup>). The indicated band has a shoulder = sh.

**Transient Reactions of the Ferryl Intermediate with Ascorbate (pH 8).** The presumed [Fe<sup>IV</sup>=O] + (AA<sup>•</sup>) species that initially forms at pH 8 was prepared and reacted with 1 equiv of ascorbate in a sequential mixing experiment. The intermediate converted completely and with isosbestic points to the starting ferric enzyme (Figure 5). These data are consistent with the intermediate either being one electron equivalent above the ferric (compound II) or being an [Fe<sup>IV</sup>=O] + (AA<sup>•</sup>) species (isoelectronic with compound I) in which only the Fe<sup>IV</sup>=O reacts with ascorbate. In the latter case, the lack of reactivity between ascorbate/protein radical may be due to the radical's inaccessibility, which might result if it migrates away from the active site to one or more locations on the protein (see below).

The sequential reaction was then carried out under pseudo-first-order conditions. The resulting plot of  $k_{\text{obs}}$  versus ascorbate concentration is hyperbolic with a maximum of 0.6 s<sup>-1</sup> and apparent  $K_d = 3.6 \mu\text{M}$  (Figure S8, Supporting Information). These numbers are similar to those measured for the ascorbate-mediated conversion of compound II to the ferric at pH 6.0.

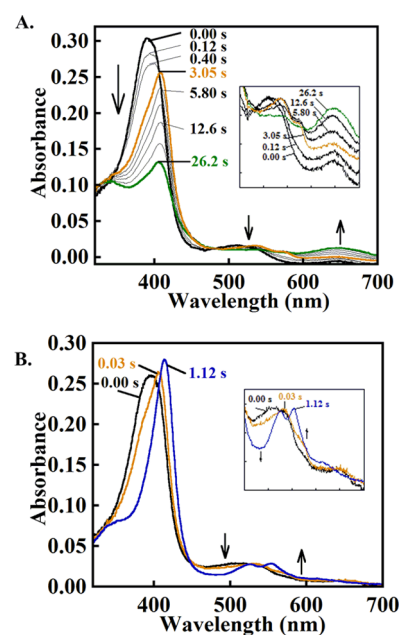
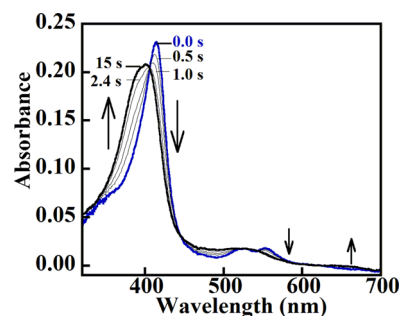
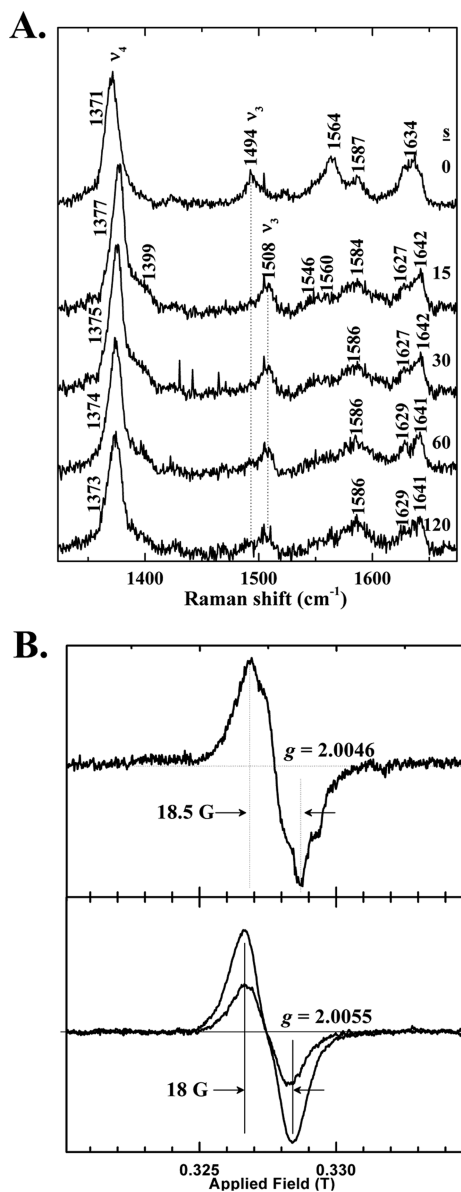
**Transient Reaction of *DaCld* with H<sub>2</sub>O<sub>2</sub> at pH 6 and 8.** Reaction of peroxidases with peroxide in the absence of reducing equivalents results in heme destruction via heme oxygenase-like pathways that have been described in several cases.<sup>17,37,43–50</sup> The heme in *DaCld* degrades following incubation with ~500 equiv of H<sub>2</sub>O<sub>2</sub>. Rapid mixing of *DaCld* with H<sub>2</sub>O<sub>2</sub> (10–500 equiv) results in little interpretable change in its UV/vis spectrum. Reaction with a large excess of H<sub>2</sub>O<sub>2</sub> (2800 equiv) at pH 6 converts the ferric enzyme slowly (within 2 s) to a species with a Soret band at 408 nm and visible bands at 535, 575 nm (Table 2). This spectrum is similar to that measured in peroxidases for the ferric–anion complexes, compound 0 (ferric–hydroperoxide) or compound III (ferric–superoxide).<sup>37,43,50,51</sup> The latter intermediate forms following the reaction of ferric peroxidases or catalases with multiple equivalents of H<sub>2</sub>O<sub>2</sub> and proceeds via more than one documented pathway.<sup>43</sup> Kinetic traces corresponding to compound 0/III formation (at 408 nm) fit well to single exponential curves (second-order rate constant  $k = 96 \text{ M}^{-1} \text{ s}^{-1}$ ) (Figure S9, Supporting Information). The chromophore for the

compound 0/III intermediate converts without isosbestic behavior to a verdoheme-like spectrum with a broad band at 650 nm and concomitant bleaching of the Soret ( $k = 0.1 \text{ s}^{-1}$ ) (Figure 6A).

A similar compound 0/III-like species forms more readily at pH 8 from the reaction of *DaCld* and an order of magnitude less (280 equiv) H<sub>2</sub>O<sub>2</sub> ( $1.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , Figure 6B and Figure S10, Supporting Information). This species converts to an apparent ferryl (Soret maximum at 415 nm;  $\alpha$ ,  $\beta$  bands = 525, 555 nm) via a single exponential process. Associated  $k_{\text{obs}}$  values depend hyperbolically on H<sub>2</sub>O<sub>2</sub> concentration (maximum  $k_{\text{obs}} = 7.9 \text{ s}^{-1}$ ,  $K_d = 1.5 \text{ mM}$  (Figure S10, Supporting Information)).

A hallmark of peroxidases is the rapid reaction of H<sub>2</sub>O<sub>2</sub> with the ferric heme to form compound I, occurring with second-order rate constants up to  $10^7$ – $10^8 \text{ M}^{-1} \text{ s}^{-1}$ .<sup>23,52,53</sup> The second-order rate constants for the *DaCld*/H<sub>2</sub>O<sub>2</sub> reaction are much smaller ( $1.2 \times 10^2$  to  $1.6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , pH 6–8) and comparable to those measured for the reaction of H<sub>2</sub>O<sub>2</sub> and ferric (met) myoglobin ( $10^2$ – $10^3 \text{ M}^{-1} \text{ s}^{-1}$ ) or for some peroxidases in which the distal histidine has been mutated to a hydrophobic residue (an active site configuration resembling *DaCld*'s).<sup>37,54</sup> The first detectable intermediate for the *DaCld*/H<sub>2</sub>O<sub>2</sub> reaction at either pH is likewise not a peroxidase-like compound I but resembles compound 0 or III, which have very similar UV/vis spectra.<sup>37,50,51,55</sup> Accumulation of compound 0 would suggest that deprotonation of H<sub>2</sub>O<sub>2</sub> to form the hydroperoxy adduct, while slow relative to compound I formation in a peroxidase, is faster than the subsequent bond cleavage steps. Compound III can form at high H<sub>2</sub>O<sub>2</sub> concentration via reaction of the enzyme with multiple equivalents of H<sub>2</sub>O<sub>2</sub>. The need for high equivalents to form the compound 0/III intermediate at pH 6 and its conversion to a species resembling verdoheme (though blue-shifted) suggests that the intermediate may be compound III, which can form via compound I, compound II, or [Fe<sup>IV</sup>=O] + (AA<sup>•</sup>) as intermediates. At pH 8, several factors suggest that the first intermediate is compound 0: the progression from the putative compound 0/III to a ferryl; the lack of any observed O<sub>2</sub> release from H<sub>2</sub>O<sub>2</sub>, which would accompany conversion of compound





**Figure 4.** Spectroscopic characterization of the pH 8 intermediate shows that it is likely a breakdown product of compound I. (A) High-frequency resonance Raman spectroscopy confirms the presence of a ferryl intermediate in the reaction of PAA and *Da*Cld at pH 8. Final concentrations of *Da*Cld and peracetic acid following mixing were 58  $\mu$ M and 480 mM, respectively ( $\sim$ 8 equiv of PAA). Spectra were measured prior to mixing and via 15 s acquisitions subsequently. Data were acquired at ambient temperature with 406.7 nm excitation and 12 mW power at the sample. Spectra initiated at 0, 15, 30, 60, and 120 s after mixing are plotted. (B) X-band EPR spectra of *Da*Cld/PAA reaction mixtures measured at 77 K indicate an uncoupled  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$  species. (top) *Da*Cld was reacted with 5 equiv of PAA (50.8  $\mu$ M *Da*Cld final concentration) at 22  $^\circ\text{C}$ . The sample was freeze-quenched into a liquid  $\text{N}_2$ /isopentane slush after a 50 ms reaction time. Instrument parameters were 200 G sweep width, 0.5 G modulation amplitude, 2.0 mW power, 100 kHz modulation frequency, and 77 K. (bottom) *Da*Cld (22  $\mu$ M, final concentration) was reacted with 9 equiv of PAA at 22  $^\circ\text{C}$ . Samples were subsequently frozen in an acetone/liquid nitrogen bath at 14 and 60 s (less intense signal). Instrument parameters were 200 G sweep width, 4 G modulation amplitude, 2.0 mW power, 100 kHz modulation frequency, and 77 K.

**Figure 6.** *Da*Cld forms a transient compound 0 or III-like intermediate (ferric–(hydro)peroxide), rather than compound I following reactions with excess peroxide. (A) *Da*Cld was mixed with a large excess (>2800 equiv) of  $\text{H}_2\text{O}_2$  at pH 6: 7  $\mu\text{M}$  enzyme, 3.5  $\mu\text{M}$  final mixed with 20 mM  $\text{H}_2\text{O}_2$ , 10 mM final in 0.2 M citrate–phosphate buffer at pH 6, 20 °C. The initial ferric spectrum is shown in black, the putative compound 0 or compound III intermediate formed after ~3 s in orange, and a spectrum suggesting verdoheme formation at 26 s in green. Intervening spectra are shown at the indicated time points in gray. The absence of isosbestic points indicates that simple conversion of one species to another is unlikely. (B) *Da*Cld forms a transient compound 0 or III-like intermediate spectrum after reaction with a smaller excess (>280 equiv) of  $\text{H}_2\text{O}_2$  at pH 8. *Da*Cld (7  $\mu\text{M}$ , 3.5  $\mu\text{M}$  final) was mixed with 2 mM hydrogen peroxide (1 mM final) in 0.2 M citrate–phosphate buffer at pH 8 and 20 °C. The initial spectrum is shown in black, the putative compound 0 intermediate that formed after 0.03 s in orange, and the following apparent ferryl spectrum that formed after 1.1 s in blue. Intervening spectra are omitted for clarity. (top) Complete spectra; (bottom) visible bands shown on an expanded scale. Note the similarity in the spectra for the first intermediates, colored orange, at pH 8 and 6.



III to compound II, in the steady state; the more rapid formation of this intermediate than the initial intermediate at pH 6 even at 10-fold lower  $\text{H}_2\text{O}_2$ ; the lack of an active site base to facilitate deprotonation of  $\text{H}_2\text{O}_2$  (a factor normally limiting capture of compound 0). Further study is needed to definitively characterize these intermediates.

## DISCUSSION

The reactions of *DaCld* with  $\text{H}_2\text{O}_2$  and PAA have been comprehensively studied in order to understand the relationships between the O–O cleaving (from  $\text{H}_2\text{O}_2$ , PAA) and the highly unusual O–O bond forming reactions catalyzed by several proteins in the chlorite dismutase family. The data presented here indicate that reaction with the peracetate ion proceeds rapidly with heterolytic O–O cleavage as the sole pathway, while the much less acidic  $\text{H}_2\text{O}_2$  reacts sluggishly and leads to destruction of the heme. These results have important implications for the mechanism of  $\text{O}_2$  formation from chlorite and set distinct boundaries for the probable biological functions of *DaCld*'s diverse sequence relatives.

PAA reacts rapidly with resting *DaCld* (Scheme 2) to form high-valent intermediates that differ at low and high pH (Figure 3). Under acidic conditions (pH 6), the ferric species converts neatly to the product of heterolytic bond cleavage (compound I,  $[\text{Fe}^{\text{IV}}=\text{O}(\text{P}^{\bullet})]$ ) following reaction with 3 equiv of PAA. Compound I reacts rapidly and with overall second order ( $k = 2.6 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ ) kinetics with ascorbate, suggesting that peroxidase reactivity is not limited by the inability of an organic substrate to dock into and react at the active site. The resulting ferryl species subsequently converts to the ferric form after reaction with a second molecule of ascorbate ( $K_{\text{d}}(\text{apparent}) = 3.5 \text{ }\mu\text{M}$ ;  $k_{\text{max}} = 9.3 \text{ s}^{-1}$ ), indicating a complete peroxidase catalytic cycle (Figure 2).

The reaction between *DaCld* and 3 equiv of PAA even at mildly alkaline pH (8) results, by contrast, in the complete conversion of the ferric starting material to what appears via UV/vis to be a ferryl species without compound I's porphyrin cation radical. UV/vis suggests that the heme in this species returns completely to the ferric form following reaction with 1 equiv of ascorbate. This change can be interpreted in one of two ways. First, the reaction mechanism could undergo a pH-dependent shift from heterolytic to homolytic cleavage of the O–O bond (Scheme 1). Such a shift would be supported by structural rearrangement of the distal arginine (Arg183) side chain (Figure 1), which we have previously proposed goes from a heme-directed (closed) to a solvent-directed (open) conformation with a  $\text{p}K_{\text{a}}$  near 6.6.<sup>21</sup> Structural shifts in distal arginine residues have been documented in other heme systems, including peroxidases and the sensor protein FixL.<sup>56–60</sup> A heme-oriented, positively charged Arg183 would promote heterolytic bond cleavage by polarizing the O–O bond of PAA. The closed Arg183 would likewise be optimally situated to stabilize an anionic acetate leaving group. The open orientation of the side chain predominating at alkaline pH, on the other hand, would render the already hydrophobic heme pocket even more so, stabilizing a neutral acetyl radical as the leaving group and supporting homolytic O–O cleavage. This reaction would generate compound II as the first intermediate. Homolytic Cl–O cleavage was indeed predicted as the preferred mechanism in a recent computational study of water-soluble, synthetic porphyrin models of Cld.<sup>61</sup> A pH-dependent switch from heterolytic to homolytic bond cleavage with a near-neutral  $\text{p}K_{\text{a}}$  is consequently consistent with both

the data and proposed models for small molecule and enzymatic function.

The stopped flow data can also be explained by heterolytic formation of compound I and an acetate leaving group at all pHs. The rates of compound I formation and of its conversion to an isoelectronic  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^{\bullet})$  species would both have to accelerate with pH. The first observable intermediate under alkaline conditions would then be  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^{\bullet})$  (or equivalently,  $[\text{Fe}^{\text{IV}}-\text{OH}] + (\text{AA}^{\bullet})$ ), which is indistinguishable via UV/vis and RR from  $[\text{Fe}^{\text{IV}}=\text{O}]$  alone. Migration of compound I's porphyrin cation radical onto various redox-active amino acid side chains to form  $(\text{AA}^{\bullet})$ , typically oxidized tryptophans or tyrosines, occurs as part of the regular catalytic cycle in some peroxidases and as an unwanted side reaction in others.<sup>24,35,36</sup> The *DaCld* crystal structure shows three highly conserved tryptophan and two tyrosine residues within 5 Å of the heme (Scheme 2), any or all of which might in principle provide a conduit for radical migration.<sup>62</sup> The observed reaction of  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^{\bullet})$  with a single equivalent of ascorbate to yield a ferric species would then most likely be due to selective one-electron reduction of the ferryl iron to the ferric. This might occur if the protein radical species, which one would expect to have proceeded out of the active site, were inaccessible to the reductant.

To distinguish these two possibilities, the acidic and alkaline intermediates were examined by RR and EPR (Figure 4 and Supporting Information). RR confirmed that the entire sample converts to a ferryl species following reaction with PAA irrespective of pH, and low-temperature (77 K) EPR confirmed the presence of a large population of protein-based radicals uncoupled from the ferryl heme under basic conditions. These data suggest that compound I is indeed the initially produced intermediate at all pH values examined here. Radical migration away from the heme to form an uncoupled amino acid radical is rapid in the absence of a peroxidase-like reducing substrate, particularly at high pH when the distal arginine is in its open form. The rapidity of this reaction is potentially responsible for the observation of some protein-based radicals even at low pH, where the initially observed compound I intermediate may be imperfectly trapped.

If the reactions between *DaCld* and PAA or chlorite are analogous, cleavage of the  $\text{Fe}(\text{III})\text{O}-\text{ClO}$  bond should be heterolytic, forming a compound I/hypochlorite pair (Scheme 1). Nucleophilic attack of the hypochlorite upon compound I would then yield a transient  $\text{Fe}(\text{III})-\text{OOCl}^-$  intermediate, breaking down to produce the ferric resting enzyme,  $\text{O}_2$ , and  $\text{Cl}^-$ . However, while PAA and chlorite are both anionic, oxygen-donating oxidants that react with ferric heme, they are different substrates, and one may ask how closely the PAA/chlorite analogy can be expected to hold. Linear free energy relationship (LFER) analyses have given insight into the kinetics and mechanisms of bond cleavage by oxidation catalysts. These analyses have shown that rate constants for  $\text{RO}-\text{O}(\text{H})$  bond heterolysis ( $k_{\text{RO}-\text{O}(\text{H})}$ ) depend linearly on the  $\text{p}K_{\text{a}}$  of the leaving group ( $\text{RO}^- + \text{H}^+ \rightleftharpoons \text{ROH}$ ).<sup>63,64</sup> Leaving groups that are stronger acids (and hence relatively stable anions) are associated with faster heterolytic bond scission. As leaving group  $\text{p}K_{\text{a}}$ 's increase, however, the downward linear trend in  $k_{\text{RO}-\text{O}(\text{H})}$  values abruptly ceases and the plot becomes horizontal. In the horizontal region of the  $k_{\text{RO}-\text{O}(\text{H})}$  versus  $\text{p}K_{\text{a}}$  plot, the competing homolytic pathway with its neutral radical leaving group ( $\text{RO}^{\bullet}$ ) predominates. Heterolytic scission of the  $\text{CH}_3(\text{CO})\text{O}-\text{O}(\text{H})$  bond of PAA results in compound I and

the relatively acidic acetate leaving group ( $pK_a = 4.8$ ), pointing clearly toward kinetically favorable O–O heterolysis for PAA.

The situation is more ambiguous for chlorite. The aqueous  $\text{HClO}/\text{ClO}^- + \text{H}^+$   $pK_a$  of 7.5 is near the heterolytic/homolytic transition for aqueous model complex reactions.<sup>65</sup> A full LFER analysis for *DaCld* itself was not possible, due first to the protein's lack of reactivity with high- $pK_a$  peroxides and peracids and second to the insolubility of other potentially interesting oxidants in water. The spectroscopy-based model suggests that the active site arginine side chain toggles between in and out conformations with a near-neutral  $pK_a$  of 6.5.<sup>21</sup> The positive charge of this side chain could stabilize the hypochlorite anion, lowering the hypochlorite  $pK_a$  and enhancing the kinetic favorability of heterolytic (O)Cl–O<sup>−</sup> bond cleavage. In the out position, the neutral  $\text{ClO}^\bullet$  leaving group and compound II might be favored. An unambiguous kinetic argument can therefore not be made that Cl–O cleavage, in analogy to O–O cleavage, is heterolytic across all values of pH.

The thermodynamic analogy between chlorite and PAA, by contrast, is both closer and more strongly supportive of heterolytic bond breakage. A simple analysis of driving forces requires estimation of the energetic costs of generating compounds I or II and balancing those against the energy yielded from heterolytic or homolytic scission of the oxidant bonds. The cost of compound I and compound II formation in the ferric heme peroxidase HRP-C has been measured at  $-0.894$  V and  $-0.885$  V at pH 7.0, respectively (all potentials relative to the SHE).<sup>66</sup> The *DaCld* heme environment is more hydrophobic than that of the peroxidases, and the more positively charged heme states should consequently be less stable.<sup>14</sup> Consistent with this expectation, the  $\text{Fe(III)}|\text{Fe(II)}$  potential of *DaCld* is  $-0.023$  V at pH 7, while the same potential for HRP-C is much more negative at  $-0.262$  V.<sup>19,66</sup> If the factors producing the approximately  $+0.230$  V offset in *DaCld*'s  $\text{Fe(III)}|\text{Fe(II)}$  potential yield a comparable shift in its intermediate  $\text{Fe(III)}|\text{Fe(II)}$  potentials, then these should range from  $+1.188$  to  $1.069$  V (compound II  $\text{Fe(III)}$ , pH 6–8) and  $1.168$  to  $0.972$  V (compound III  $\text{Fe(III)}$ , pH 6–8). On the oxidant side, the standard potential (pH 0) for the two-electron PAA/lactic acid ( $\text{HOOAc}/\text{HOAc}$ ) couple corresponding to heterolytic O–O cleavage was recently estimated at  $+1.960$  V,<sup>67</sup> which decreases to  $1.636$  V at pH 6.0 and  $1.570$  V at pH 8.0. Hence, as expected, PAA has the necessary driving force to effect two-electron oxidation of ferric *DaCld* to compound I over the entire pH range investigated here, with overall reaction potentials of  $+0.451$  V at pH 6.0 and  $+0.474$  V at pH 8.0.

The potentials for the analogous two-electron/heterolytic couple for  $\text{ClO}_2^-/\text{HClO}$  (pH 6.0) or  $\text{ClO}_2^-/\text{ClO}^-$  (pH 8.0) are  $1.200$  and  $1.039$  V, respectively. This suggests that heterolytic cleavage of the Cl–O bond by HRP-C at either pH is exergonic, with a driving force of  $+0.245$  to  $0.203$  V. Consistent with this analysis, chlorite has been observed to act as a “shunt” reagent for compound I formation in peroxidases and synthetic porphyrin complexes.<sup>68–71</sup> However, *DaCld* is not as strongly oxidizing as a peroxidase. With the estimated offset in potential described above, the oxidation of ferric *DaCld* to compound I by  $\text{ClO}_2^-$  is weakly exergonic by  $0.015$  V ( $-2.9$  kJ/mol) at pH 6.0 and endergonic by  $0.030$  V ( $+5.8$  kJ/mol) at pH 8.0. It is important to point out that the *DaCld* potentials are estimates based on potential offsets and the assumption that the  $\text{Cl}^{\text{III}}|\text{Cl}^{\text{I}}$  potential in the catalytic site is the same as it is in aqueous solution. Hence, this analysis is qualitatively consistent with

weakly driven compound I formation from chlorite at acidic pH that becomes even more weakly driven at alkaline values.

The estimated homolytic/one-electron reduction potentials suggest more clearly that direct formation of compound II from the *DaCld*/chlorite reaction is thermodynamically unlikely. The potential for the  $\text{HClO}_2/\text{ClO}^\bullet$  couple describing homolytic Cl–O bond cleavage has to our knowledge not been reported. Based on available free energies of formation, we estimate the standard potential of this half-cell to be  $+1.220$  V (pH 0),<sup>26</sup> lowering to  $+0.626$  V at pH 6.0 and  $+0.389$  V ( $-38$  kJ/mol) at pH 8.0 (see Supporting Information for details). One electron oxidation of ferric *DaCld* by  $\text{ClO}_2^-$  to yield the  $\text{ClO}^\bullet$  radical and compound II would therefore be endergonic by  $-0.542$  V ( $+52$  kJ/mol) at pH 6.0 and  $-0.582$  V ( $+56$  kJ/mol) at pH 8.0. Neither the *DaCld*/ $\text{ClO}_2^-$  nor the HRP-C/ $\text{ClO}_2^-$  reactions are therefore likely to proceed via homolytic Cl–O cleavage at any pH investigated here. In sum, the analyses of the one- and two-electron driving forces argue for the first bond-breaking step of the catalytic cycle proceeding by heterolytic O–Cl bond cleavage to yield  $[\text{Fe}^{\text{IV}}=\text{O}(\text{P}^{\bullet})]$  (compound I) and  $\text{ClO}^-$  as a leaving group. Rereaction of the intermediate/leaving group pair would be enforced by the sterically confined distal pocket and the weakly basic hypochlorite ( $\text{ClO}^- + \text{H}^+ \rightleftharpoons \text{HClO}$ ,  $pK_a = 7.5$ ) stabilized by the positive charge on the distal arginine.

The two possible mechanisms for O–O joining proposed here are similar to those proposed for O–O joining in the OEC reaction of photosystem II and synthetic  $\text{O}_2$ -forming complexes, with either nucleophilic or radical rejoining of a sterically confined group occurring with a high-valent metal-oxo complex.<sup>18,72</sup> The complete reaction cycle is even more closely analogous to that proposed for ferric heme models and proteins that isomerize peroxynitrite to nitrate. In that case, both homolytic and heterolytic models for peroxynitrite (ON)O–O<sup>−</sup> bond scission have been proposed, leading to a compound II/ $\text{NO}_2^\bullet$  pair in the first case and compound I/ $\text{NO}_2^-$  in the second. Steric confinement is also likely important for enforcing recombination of the metal-oxo and leaving group to produce nitrate. Interestingly, while the work reported here favors heterolytic bond cleavage followed by nucleophilic attack, recent work with myoglobin suggests compound II/ $\text{NO}_2^\bullet$  as the more likely reaction pathway in that case.<sup>73</sup>

The majority of *Cld* proteins likely do not react with chlorite to generate  $\text{O}_2$  and have been proposed to use peroxide as a substrate. However, the weak  $\text{H}_2\text{O}_2$  reactivity observed here for *DaCld* suggests that peroxide reactivity is biologically unlikely for chlorite dismutases (Table 1) despite very close structural similarity to the very efficient dye decoloring peroxidases (DyPs) and their “EfeB” homologues. The EfeB and YfeX proteins from *Rhodococcus jostii* RH1 (also called DypA and DypB, respectively), for example, react avidly with  $\text{H}_2\text{O}_2$ .<sup>16,74,75</sup> The DypA forms a ferryl (compound II or  $[\text{Fe}^{\text{IV}}=\text{O} + (\text{AA}^\bullet)]$ ) intermediate following reaction with just 1 equiv of  $\text{H}_2\text{O}_2$ , and the DypB converts stoichiometrically to a stable compound I (pH 7.5, 25 °C; half-life = 9 min).<sup>16,74</sup> Apparent compound I intermediates have also been observed in the transient  $\text{H}_2\text{O}_2$  reactions of  $\text{DyP}_{\text{Decl}}$  (from *Thanatephorus cucumeris*) and the EfeB from *Escherichia coli* (also known as YcDB).<sup>11,76</sup> In addition, characterized DyPs exhibit steady state constants with  $\text{H}_2\text{O}_2$  in the range of what one would expect for efficient peroxidases (Table 1).<sup>77</sup> Key active site differences between Dyp and *Cld* family proteins might be responsible for the observed differences in reactivity with  $\text{H}_2\text{O}_2$  (Figure 1). First, an active site base has been viewed as a *sine qua non* of

peroxidase chemistry, since deprotonation of  $\text{H}_2\text{O}_2$  is typically necessary for making a strong complex with ferric heme.<sup>50,78,79</sup> Cld family proteins have a lone cationic residue in their distal pockets that is apparently not able to act as an active site base.<sup>19</sup> The conserved distal aspartic acid found in the DyP and EfeB members of this family, or alternatively the arginine of an Asp-Arg pair, has by contrast been proposed to serve in lieu of the conventional distal histidine base in these proteins.<sup>11,74,75</sup> The Cld-family proteins also exhibit a  $180^\circ$  rotation of the heme around an axis spanning the A and C pyrrole rings relative to the proteins in the EfeB and DyP families.<sup>8</sup> This rotation removes hydrogen bonding connections between the distal arginine and one of the propionic side chains and appears to allow for conformational mobility of the arginine residue in the DaCld distal pocket.<sup>19</sup>

## CONCLUSIONS

The reactions of DaCld with  $\text{H}_2\text{O}_2$  and PAA shed light on both the likely biological roles of Clds and the mechanism by which some Clds catalyze their unusual  $\text{O}_2$  forming reaction. First, sluggish reactivity with  $\text{H}_2\text{O}_2$  in the transient and steady state suggests that this and similar proteins are unlikely to act as functional peroxidases or catalases *in vivo*. This is not due to the inability of the reducing substrate to approach the reactive heme: rapid reaction with ascorbate is observed when peracetic acid is the oxidant, and the expected compound I and II intermediates form. Rather, the lack of a distal base likely limits the ability of the enzyme to form the initial compound 0 ferric-hydroperoxy complex. This structural feature sets DaCld and the whole chlorite dismutase family in strong contrast with their DyP and EfeB structural relatives, which are highly functional peroxidases that react readily with  $\text{H}_2\text{O}_2$ .

The reactions of DaCld with PAA further illustrate how DaCld breaks this O–O bond and suggest how, by analogy, it may be expected to form one. Heterolytic cleavage of the bound peracid O–O bond in DaCld appears to be the preferred reaction over the full range of pHs for which the protein is stable. At weakly acidic pH, compound I is the initially observed intermediate, while at alkaline pHs ( $\geq 8$ ), a mixture of  $[\text{Fe}^{\text{IV}}=\text{O}] + (\text{AA}^\bullet)$  and another ferryl heme species forms an order of magnitude more quickly. These observations give indirect mechanistic support for an  $\text{O}_2$  forming process via a similar microscopic reverse reaction: nucleophilic attack of a hypochlorite leaving group upon a compound I intermediate, ultimately yielding  $\text{O}_2$ .

## ASSOCIATED CONTENT

### Supporting Information

Additional supporting data and a description of the derivation of estimated redox potentials discussed here. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

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## ABBREVIATIONS

compound I,  $[\text{Fe}(\text{IV})=\text{O}(\text{P}^\bullet)]$  or  $[\text{Fe}(\text{IV})=\text{O}(\text{AA}^\bullet)]$ , with the radicals exchange coupled; compound II,  $[\text{Fe}(\text{IV})=\text{O}]$  or  $[\text{Fe}(\text{IV})-\text{OH}]$  (no observable protein radicals);  $[\text{Fe}(\text{IV})=\text{O}] + (\text{AA}^\bullet)$ , ferryl with an uncoupled protein-based radical; Cld, chlorite dismutase; DaCld, *Dechloromonas aromatica* Cld; DyP, dye-decolorizing peroxidase; EfeB, structurally characterized protein from *E. coli* which belongs to a “DyP-type peroxidase family”; RR, resonance Raman; EPR, electron paramagnetic resonance; PAA, peracetic acid; OEC, oxygen evolving complex

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