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Review

Mechanism of chlorite degradation to chloride and dioxygen by the enzyme chlorite dismutase

Irene Schaffner [a], Stefan Hofbauer [a,b], Michael Krutzler [a], Katharina F. Pirker [a], Paul G. Furtmüller [a], Christian Obinger [a,*]

[a] Department of Chemistry, Division of Biochemistry, BOKU – University of Natural Resources and Life Sciences, Muthgasse 18, A-1190 Vienna, Austria
[b] Department for Structural and Computational Biology, Max F. Perutz Laboratories, University of Vienna, A-1030 Vienna, Austria

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ABSTRACT

Heme *b* containing chlorite dismutase (Cld) catalyses the conversion of chlorite to chloride and dioxygen which includes an unusual O—O bond formation. This review summarizes our knowledge about the interaction of chlorite with heme enzymes and introduces the biological role, phylogeny and structure of functional chlorite dismutases with differences in overall structure and subunit architecture. The paper sums up the available experimental and computational studies on chlorite degradation by water soluble porphyrin complexes as well as a model based on the active site of Cld. Finally, it reports the available biochemical and biophysical data of Clds from different organisms which allow the presentation of a general reaction mechanism. It includes binding of chlorite to ferric Cld followed by subsequent heterolytic O—Cl bond cleavage leading to the formation of Compound I and hypochlorite, which finally recombine for production of chloride and O_2. The role of the Cld-typical distal arginine in catalysis is discussed together with the pH dependence of the reaction and the role of transiently produced hypochlorite in irreversible inactivation of the enzyme.

Interaction of chlorite with heme enzymes

Chlorous acid ($HClO_2$) is a weak and unstable acid with a pK_a of 1.96 that easily disproportionates to hypochlorous acid (HClO) and chloric acid ($HClO_3$). By contrast, its conjugate base chlorite (ClO_2^- or $OClO^-$) is stable and is the dominating species at physiological pH. The anion is a strong oxidant [$E^{\circ\prime}$ (ClO_2^-/ClO^-) = 1.175] [1] that interacts with many biomolecules thus exhibiting strong cell damaging effects [2]. Besides unspecific oxidation of cell components, research of the last two decades also showed some specific interactions, especially with heme proteins. It was demonstrated that the anion interacts with heme proteins in their ferrous and ferric states. Chlorite induces the formation of methemoglobin [3] and might act as hydroxylating agent in cytochrome P450 [4]. Among heme peroxidases, chlorite was shown to be utilized by cytochrome *c* peroxidase [5], chloroperoxidase [6] and horseradish peroxidase (HRP) [7] thereby producing chlorinating agents [8–10]. Horseradish peroxidase mixed with chlorite follows the whole peroxidase cycle [11], i.e. $OClO^-$ mediates the two-electron oxidation of ferric HRP [Por...Fe(III)] to Compound I [$Por^{\bullet+}$...Fe(IV)=O] thereby releasing hypochlorous acid which is the chlorinating species (Reaction (1)). Furthermore, chlorite acts as one-electron reductant of both Compound I and Compound II [Por...Fe(IV)=O] forming chlorine dioxide ($^{\bullet}ClO_2$) (Reactions (2) and (3)). In addition, both reaction products also mediate the two-electron oxidation of ferric HRP to Compound I (Reactions (4) and (5)) but cannot serve as electron donors for Compounds I or II.

$$[\mathrm{Por}\ldots\mathrm{Fe(III)}] + {}^{-}\mathrm{OClO} \rightarrow [\mathrm{Por}^{\bullet+}\ldots\mathrm{Fe(IV)}{=}\mathrm{O}] + \mathrm{HOCl}/\ \text{-}\mathit{OCl},\mathrm{H}^{+} \quad \text{(Reaction 1)}$$

$$[\mathrm{Por}^{\bullet+}\ldots\mathrm{Fe(IV)}{=}\mathrm{O}] + {}^{-}\mathrm{OClO} \rightarrow [\mathrm{Por}\ldots\mathrm{Fe(IV)}{=}\mathrm{O}] + {}^{\bullet}\mathrm{ClO_2} \quad \text{(Reaction 2)}$$

$$[\mathrm{Por}\ldots\mathrm{Fe(IV)}{=}\mathrm{O}] + {}^{-}\mathrm{OClO} \rightarrow [\mathrm{Por}\ldots\mathrm{Fe(III)}] + {}^{\bullet}\mathrm{ClO_2} + \mathrm{H_2O} \quad \text{(Reaction 3)}$$

$$[\mathrm{Por}\ldots\mathrm{Fe(III)}] + \mathrm{HOCl} \rightarrow [\mathrm{Por}\ldots\mathrm{Fe(IV)}{=}\mathrm{O}] + \mathrm{Cl}^{-} \quad \text{(Reaction 4)}$$

$$[\mathrm{Por}\ldots\mathrm{Fe(III)}] + {}^{\bullet}\mathrm{ClO_2} \rightarrow [\mathrm{Por}^{\bullet+}\ldots\mathrm{Fe(IV)}{=}\mathrm{O}] + {}^{\bullet}\mathrm{ClO} \quad \text{(Reaction 5)}$$

* Corresponding author. Fax: +43 1 47654 6050.
E-mail address: christian.obinger@boku.ac.at (C. Obinger).

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Interestingly, heme peroxidases from another superfamily (i.e. the peroxidase-cyclooxygenase superfamily) [12] react differently with chlorite. Both myeloperoxidase and lactoperoxidase are rapidly and irreversibly inactivated by chlorite in the micromolar concentration range [13]. Those enzymes cannot heterolytically cleave chlorite in a controlled manner (as reflected by Reaction (1)) and heme bleaching as well as iron release are observed. As a consequence, no chlorinating species are released. Thus these peroxidases cannot cycle despite the fact that Reactions (2) and (3) are still functional [13].

Finally, in 1996 van Ginkel and co-workers [14] discovered a new heme *b* containing enzyme which they called chlorite dismutase (Cld)[1]. Based on sequence analysis as well as first structural and functional characterizations, it became clear very soon that it belongs to a new and until then unknown heme protein family that interacts with chlorite in a different and specific way. It was shown to efficiently decompose chlorite into chloride (Cl^-) and dioxygen (O_2). Thereby, a covalent oxygen–oxygen bond is formed, a unique biochemical reaction that in addition is only catalyzed by the water-splitting manganese complex of photosystem II of oxygenic organisms (cyanobacteria and plants) and an enzyme of an anaerobic methane-oxidizing bacterium [15].

Biological role, phylogeny and structure of chlorite dismutases

The first chlorite dismutases were found in facultative anaerobic perchlorate-reducing bacteria (PCRB) that can utilize perchlorate (ClO_4^-) and chlorate (ClO_3^-) as terminal electron acceptors in the absence of oxygen. Upon chlorate reduction, chlorite is formed intracellularly which in these organisms is degraded by Cld to harmless chloride and O_2 [16–18]. Thus, the first Clds that were studied originated from the perchlorate-reducing bacteria *Azospira oryzae* (GR-1) [14,17], *Ideonella dechloratans* [19], *Dechloromonas aromatica* [20] and *Pseudomonas chloritidismutans* [21]. But later on – most interestingly – homologous enzymes with chlorite dismutase activity have also been found and characterized in non-PCRB like the nitrite-oxidizing bacteria "*Candidatus* Nitrospira defluvii" [22] and *Nitrobacter winogradskyi* [23]. Further phylogenetic analysis showed that *cld* genes are present in numerous bacterial and archaeal phyla, indicating they represent rather ancient sequences [24,25]. Nevertheless, these Clds from non-PCRB degrade chlorite with catalytic efficiencies comparable to those of PCRB (see below) despite the fact that ClO_2^- is not produced during their metabolism. This raises the question about the natural substrate for Clds since the reservoirs of chlorite on Earth are very rare [26] and most chlorite present in our environment is of anthropogenic origin [18,27]. This discussion is still ongoing.

As outlined in detail by Zamocky et al. [28] in this issue, functional chlorite dismutases comprise one family within the peroxidase-chlorite dismutase superfamily together with Cld-like proteins [29,30] and dye-decolorizing peroxidases (DyPs) [31,32]. This was first described by Goblirsch et al. in 2011 [33]. Phylogenetic as well as structural analyses showed the occurrence of two lineages of functional Clds [25,28,33] differing in overall sequence length and subunit topology (Fig. 1a–c). Table 1 summarizes the available crystal structures of Clds and Cld-like proteins (December 2014). The first topology ("long Clds"), i.e. Clds from Lineage I, includes Clds from *A. oryzae* strain GR-1(AoCld) [34], *D. aromatica* (DaCld) [35] and "*Candidatus* Nitrospira defluvii" (NdCld) [24]. The subunit of these proteins consists of a heme free N-terminal and a heme *b* containing C-terminal ferredoxin-like fold (Fig. 1c). Representatives of Lineage I show hexameric [34] or pentameric [24,35,36] crystal structures stabilized by hydrogen bonds and salt bridges. In solution, homopentameric [24,35,36], homotetrameric [21,34] or even dimeric structures [37] were described depending on buffer conditions (pH, ionic strength) and protein concentration.

The second subunit topology ("short Clds") is only found in functional Clds of Lineage II (Fig. 1b and c). The only representative with known X-ray structure is the Cld from *N. winogradskyi* (NwCld) [23]. It shows a smaller subunit size that lacks almost the entire N-terminal domain of the first group described above. However, the heme-binding ferredoxin-like fold including the catalytic amino acids is highly similar to the C-terminal domain of "long Clds". Chlorite dismutases from *Cyanothece* sp. PCCC7425 [38] and *Klebsiella pneumoniae* MGH 78578 [39] belong to Lineage II and are homodimeric proteins.

The C-terminal parts of Lineage I and II representatives, which include the heme cavity and substrate access channel, are almost superimposable (Fig. 1c). On both heme sides the same amino acids are found at almost identical positions (Fig. 1d). On the distal side, the only conserved charged residue in close distance to the heme is an arginine, which has been shown to be catalytically important. On the proximal side, a conserved hydrogen bonding network is formed by a histidine, a glutamate, a lysine and two tryptophans. As a consequence, the reduction potential of the Fe(III)/Fe(II) couple at pH 7.0 is almost identical for long and short chlorite dismutases. The respective $E^{\circ\prime}$ values of pentameric NdCld (Lineage I) and dimeric NwCld (Lineage II) were determined spectroelectrochemically to be at −120 mV for the ferric high-spin enzymes and about −400 mV for the respective cyanide complexes [40]. The ferric forms of the high-spin enzymes are enthalpically favored, while the entropic contribution partly compensates for enthalpic stabilization. It has to be mentioned that variations of the $E^{\circ\prime}$ values described above are found in the literature but this might derive from different applied electrochemical methods [1,19,49].

The distal ligand of ferric Clds is typically a water molecule. In the crystal structures it can be exchanged by molecules of the crystallization buffer. In some structures thiocyanate, nitrite and cyanide act as distal ligands (Table 1 and Fig. 2). Despite the fact of almost identical heme cavity architecture (see above), severe differences in spectral signatures of Clds have been reported at comparable pH values. This could be related to differences in conformational stability at the heme cavity (see below). Closely related AoCld, IdCld and DaCld exhibit relatively broad Soret maxima at around 393 nm, Q-bands at around 510 and 535 nm as well as charge transfer bands at 645 nm at neutral pH. These bands indicate the presence of 5-coordinated high-spin heme iron. All three proteins show a clear pH dependence of the UV–visible spectral signatures with a hydroxyl-coordinated low-spin complex (sharpened red-shifted Soret band) at alkaline pH [17,19,20]. These findings are also reflected by RR data on DaCld [20]. In the acidic pH region DaCld loses its heme at around pH 4, exhibiting a spectrum of free heme with a Soret maximum at 375 nm [20]. Resonance Raman spectroscopy of wild-type DaCld also demonstrated that the hydrogen bond between the proximal histidine and the conserved glutamate (Fig. 1) is rather weak compared to other His-coordinated heme proteins. By contrast, PcCld, NdCld, NwCld and CCld show Soret maxima between 403 and 408 nm and CT1 bands between 630 and 640 nm, indicating dominating

[1] *Abbreviations used:* Cld, chlorite dismutase; AoCld, chlorite dismutase from *Azospira oryzae* GR-1; CCld, chlorite dismutase from *Cyanothece* sp. PCC7425; DaCld, Cld from *Dechloromonas aromatica*; GasCld; putative Cld from *Geobacillus stearothermophilus*; IdCld, Cld from *Ideonella dechloratans*; KpCld, Cld from *Klebsiella pneumonia*; NdCld, Cld from "*Candidatus* Nitrospira defluvii"; NwCld, Cld from *Nitrobacter winogradskyi*; PcCld, Cld from *Pseudomonas chloritidismutans*; TaCld, putative Cld from *Thermoplasma acidophilum*; TtCld, putative Cld from *Thermus thermophilus*; LmCld, Cld-like protein from *Listeria monocytogenes*; DyP, dye-decolorizing peroxidase; PCRB, perchlorate reducing bacteria; $E^{\circ\prime}$, standard reduction potential; MD, molecular dynamics; PDB, protein data bank.