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Structural and Functional Characterization of Chlorite Dismutases and Homologous Proteins

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1. Abstract

The heme-enzyme chlorite dismutase (Cld) catalyzes the decomposition of chlorite to chloride and molecular oxygen. In (per-)chlorate respiring bacteria (PCRB), where it was first discovered, Cld degrades the toxic end product of the respiratory chain, thus evading self-toxification. The enzyme and its homologues are found in several distinct bacterial and archaeal phyla with bioinformatics suggesting lateral gene transfer also between these two domains. Nonetheless most strains are not PCRBs, hence the physiological role of many Clds is not as obvious and many do not show chlorite degrading activity (Cld-like proteins). Since the chlorite contamination is a rather recent problem caused by industrial processes the question about the enzyme's original evolutionary purpose arises. Water contamination by chlorite has become a serious issue in the USA and bioremediation by Cld would present an efficient way to detoxify. Some human pathogens contain a cld-gene, including Listeria monocytogenes, whose gene-product appears to be essential, making it a potential drug target.

Attempts have been made to predict Cld-activity based on the sequence and several residues have been suggested to represent signature residues for active Clds. One part of this thesis will deal with mutation studies of these residues on the active Cld from Nitrospira defluvii (a non-PCRB) and the effect on the enzyme's kinetics.

The second part will focus on the search for the function of Cld from Listeria monocytogenes (LmCld). Preliminary work did not show evidence for heme-binding nor Cld-activity so the main focus is on finding another cofactor which might mediate another function.

The third part describes the initial work on Cld from Sulfolobus solfataricus (SsCld), a acido-thermophile archaeon, which lacks the key residue for Cld-activity but binds heme.
2. Zusammenfassung

Das Häm-bindende Enzym Chlorite Dismutase (Cld) katalysiert den Abbau von toxischem Chlorit zu Chlorid und $O_2$. In (Per)Chlorate-respirierenden Bakterien (PCRB), in welchen es entdeckt wurde, baut es das giftige Endprodukt der Atmungskette ab, und entflieht damit der Selbstvergiftung. Das Enzym und seine homologen Verwandten finden sich in verschiedenen bakteriellen und Archeen-Phyla und bioinformatische Untersuchungen suggerieren lateralen Gentransfer auch zwischen diesen Phyla. Trotzdem sind die meisten Stämme keine PCRB, darum ist die physiologische Aufgabe vieler Clds nicht offensichtlich und zeigen keine Chloritabbauaktivität.

Da die Verunreinigung durch Chlorit eher ein seit kurzem bestehendes Problem darstellt, das von industriellen Prozessen verursacht wird, ergibt sich die Frage nach der ursprünglichen evolutionären Funktion. Wasserverunreinigung durch Chlorit wurde zu einem ernsthaften Problem in den USA und Bioremediation durch Cld würde einen effizienten Weg darstellen um zu entgiften. Einige humanpathogene Krankheitserreger besitzen ein cld-Gen, darunter *Listeria monocytogenes*, dessen Cld-Genprodukt essenziell ist, was es zu einem möglichen potenziellen Angriffspunkt für Medikamente macht. Basierend auf der Sequenz wurde versucht, die Cld-Aktivität vorherzusagen und einige Residues wurden vorgeschlagen als Signaturresidues für aktive Clds.


Der zweite Teil befasst sich mit der Suche nach einer Funktion der Cld von *Listeria monocytogenes* (LmCld). Vorausgehende Untersuchungen zeigten keinen Beweis für die Anwesenheit von Häm-Bindung noch für Cld-Aktivität, darum lag der Hauptfokus darauf, einen Kofaktor zu finden, der eventuell eine andere Funktion vermitteln kann.

Im dritten Teil wird die Arbeit an der Cld von *Sulfolobus solfataricus* beschrieben, ein acidothermophiles Archaeon, dem der Schlüsselresidue für Cld-Aktivität zwar fehlt, aber die prosthetische Gruppe Häm bindet.
3. Introduction:

3.1. Oxochlorates

Most perchlorate found on earth is man-made, so the period of time microorganisms were exposed to perchlorate is very short. There is one natural source in Chile with up to 0.3‰ (w/w) perchlorate (Coates, 2004; Kengen and J., 1999). For industrial purposes perchlorate is produced by electrolysis of other oxochlorates and due to its strong oxidative nature is used as a bleaching reagent, anti-microbial agent and as an additive in rocket fuels (Urbansky, 1998; Urbansky, 2002).

The stability chlorine oxoacids in environmental conditions pronounces the need for proper degradation considering their threat to not only human health. Physical and chemical methods to do so proved to be difficult or failed (Coates, 2004) so bioremediation by PCRBS (perchlorate reducing bacteria) is one way to circumvent these obstacles (Wu, 2008).

For complete reduction of perchlorate to chloride microorganisms need two enzymes (see Figure 1). The first two steps of perchlorate reduction are catalyzed by perchlorate reductase (PerR), producing chlorite which is further reduced by the unique enzyme chlorite dismutase (Ueno, 2000). This enzyme is essential for PCRBS to circumvent the toxicity of chlorite. PerRs are homologous to nitrate reductases and Kengen et al. (Kengen and J., 1999) suggest they evolved from them. Other sources speculate they evolved from DMSO (dimethylsulfoxide) reductase by gene duplication (Goblirsch et al., 2011). PerRs are promiscuous enzymes with the ability to reduce nitrate, iodate and bromate besides (per)chlorate. Some PCRBS show ability of nitrate respiration, too. (Coates, 2004).

Figure 1: Perchlorate reduction pathway (with kind permission of Kira Gysel, Diploma Thesis)
3.2. Chlorite Dismutase

The reaction catalyzed by Cld is actually no dismutation but an intramolecular redox reaction. The correct name should be *chloride:oxygen oxidoreductase* or *chlorite O2-lyase* (Hagedoorn, 2002). Still the name chlorite dismutase is mostly used in literature so it will be used throughout this thesis. Chlorite dismutase does not share any sequence similarities or obvious relationships with other well-characterized protein families. Compared to PerR or nitrate reductase substrate specificity Cld appears to be non-promiscuous (Brandon R. Goblirsch, 2010).

First discovered was Cld in PCRBs where the enzyme plays an essential role in the perchlorate reduction pathway (Bender et al., 2002). Yet environmental genomic approaches revealed many homologues in non-PCRBs, found in both bacterial and archaeal phylae (Maixner et al., 2008) and make up their own chlorite dismutase superfamily (Goblirsch et al., 2011).

Cld-activity was confirmed for a small fraction of this protein family, the validated Clds (termed active or canonical Clds in this thesis), but the function of the rest is still unknown (termed Cld-like proteins (Maixner et al., 2008)). Most likely the ability to degrade chlorite was only recently acquired by this protein family, whose members previously had a different purpose. Two factors indicate this: perchlorate is a recent anthropogenic pollutant (only a small number of microorganisms are specialized in perchlorate-respiration and experience selective pressure to decompose chlorite) and secondly in PCRBs the *cld*-gene is localized in the PerR operon, which it entered by horizontal gene transfer (Coates, 2004).

To map the various Clds and Cld-like proteins in a phylogenetic context several signature residues were suggested (Goblirsch et al., 2011; Kostan et al., 2010; Maixner et al., 2008; Mlynek et al., 2011). Structural and functional investigations show the importance of an arginine in the distal pocket of the heme (Arg173 in NdCld) and mutation studies underline its role in catalysis (Kostan et al., 2010). Moreover, they show that due to the conserved fold, the arginine and the corresponding residues are actually found at the same positions in the active site. This arginine and its corresponding residues are used to group Clds in a phylogeny tree (Figure 2, adapted from Kostan et al. (Kostan et al., 2010)). The only validated Clds are found within one branch, i.e. the one whose members all contain an arginine at this site.
Based on these phylogenetic analyses Mlynek et. al. (Mlynek et al., 2011) proposed a classification of validated Clds into two lineages, one is comprised of the canonical Clds from PCRBs – Cld from *Nitrospira defluvii* (NdCld) being the only non-proteobacterial member – and the second lineage containing Cld from *Nitrobacter winogradskyi* (NwCld) and related proteins.

Apart from the validated and proposed active Clds there is a big cluster of related proteins with a glutamine at the position of the arginine. This “glutamine-cluster” contains many diverse bacterial species and also archaea. Also, there are some human pathogens found within, e. g. *Listeria monocytogenes* or *Staphylococcus aureus*.

Knock-out of the *cld*-gene in *Listeria monocytogenes* leaves the organism not viable (Füreder, 2009). Apart from that, neither Cld-activity nor electron density
for heme in X-ray crystallography experiments could be observed (Mlynek, 2010). Since Listeria as a facultative intracellular human pathogen is the cause of Listeriosis, this essential gene-product presents a potential drug target.

Another big cluster with alanine instead of the arginine includes Actinobacteria with e. g. *Mycobacterium leprae* and *Mycobacterium tuberculosis* being the most interesting ones, due to their human pathogenicity. Another branch is made of only archaeal species where the arginine is replaced by a serine.

Clds and Cld-like proteins are distributed over a many bacterial and archaeal phyla and share a common, ancient fold and sequence similarities. Validated Clds and proposed active Clds make up only a very small fraction of this protein family and the function of the rest remains unknown.

### 3.3. Structure of Chlorite Dismutases

Crystal structures show a common and characteristic fold of all Clds and Cld-like proteins. Typically they are arranged as homo-oligomers (mostly homo-pentamers, but also homo-hexamers) where the oligomerisation state appears to have no effect on the catalytic efficiency (Hofbauer et al., 2012; Mlynek et al., 2011). Each subunit is made of two ferredoxin-like domains. The ferredoxin fold contains both α helices and β sheets, with the secondary structure motif β-α-β-α-β-β-α-β. The β strands form an antiparallel β sheet with the α helices packed against one side (Figure 3).

**Figure 3:** Topology diagram of a ferredoxin-like fold (Gysel, 2011)(with kind permission by Kira Gysel)
Clds consist of two structurally similar domains. The C-terminal ferredoxin-like domain coordinates the heme b via a conserved histidine residue on the proximal side of heme. Heme binding and the heme cavity structure is crucial for catalysis. The fold of these subunits is highly conserved, with a root mean square deviation of 1.72 Å between one subunit of an active Cld and a Cld-like protein over 180 Cα atoms (21).

**Figure 4:** Superposition of chain A of NdCld (3NN1, red) with LmCld (Cld-like protein, blue); r.m.s.d. of backbone atoms = 6.977 Å (667 to 667 atoms)
An exception to the structure described here is the already mentioned Cld from *Nitrobacter winogradskyi* (Mlynek et al., 2011). In contrast to the others, this enzyme is one third smaller in the primary sequence and lacks the α-helices of the N-terminal domain. The N-terminal part was probably lost during evolution (Mlynek et al., 2011). The C-terminal ferredoxin-like domain has an active site which is still very similar to other active members of the family. The reduced sequence does not let the subunits form homo-pentamers, NwCld forms homo-dimers with still high Cld-activity.

Cld-like proteins that contain no heme or show no Cld-activity have also been structurally analyzed, mostly of the lineage where glutamine replaces the arginine signature residue. The Cld structure of the thermophile bacterium *Thermus thermophilus* (TtCld) does not show heme, yet *in vitro* reconstitution with heme was partially successful (60% of subunits loaded with heme) which resulted in weak Cld-activity (Ebihara et al., 2005). On the other hand, *in vitro* binding of heme to recombinant LmCld was not observed (Mlynek, 2010). The option that in *Listeria monocytogenes* unidentified factors exist, that could mediate the uptake was tested in this thesis. A representative of the archaeal serine lineage, Cld of *Thermoplasma acidophilum*, was structurally analyzed by a structural genomics consortium and has no heme in the active site. The representative of this branch studied in this thesis from *Sulfolobus solfataricus* (SsCld) was shown to bind heme (see Section 6.9).

### 3.4. Reaction Mechanism

The most remarkable aspect of the reaction catalyzed by active Clds is the de novo formation of molecular oxygen. Only two other enzymes are known to be able to perform this kind of reaction as their primary function, namely water-plastoquinone oxidoreductase of photosystem II and a still uncharacterized enzyme from an anaerobic methane-oxidizer (Ettwig et al., 2010; Renger and Renger, 2008).

The mechanism of chlorite decomposition was proposed to be similar to the mechanism of heme peroxidases and catalases (Lee, 2008) via the intermediate Compound I state (see Figure 5), which was further modified by Kostan et al., Mlynek et al. and Goblirsch et al. (Goblirsch et al., 2011; Kostan et al., 2010; Mlynek et al., 2011).
Figure 5: Reaction mechanism of Cld; starting with ferric pentacoordinated iron; A) reversible collision to form the Michaelis Complex; B) Heterolytic cleavage of the Cl-O bond to form Compound I with Fe(IV); C) Nucleophilic attack of the intermediate hypochlorite and release of the products and recycled Fe(III); D) off-pathway generation of a tryptophan radical and enzyme inactivation; figure adapted from Goblirsch et al. (Goblirsch et al., 2011)

An alternative pathway including a homolytic cleavage of the Cl-O bond and formation of Compound II and radical hypochlorite has been proposed (Coates, 2004). Yet recent findings hint at the mechanism with the intermediate Compound I (Stefan Hofbauer, personal correspondence).

3.5. Catalytically Important Residues

The key residue arginine in active Clds (R173 in NdCld) plays an important role in substrate binding and stabilization of reaction intermediates. Apart from that, its positive charge is thought to position the substrate for proper catalysis (Mlynek et al., 2011). The conserved proximal histidine residue (H160 in NdCld), besides coordinating the prosthetic group, forms a H-bond to a conserved glutamate (E210 in NdCld). This interaction emphasizes the imidazolate character of the histidine, which in turn supports the ferric state of the heme iron. A tryptophan (W146 in NdCld) is thought to act as the electron donor and is also found in all Clds close to the propionate group of heme. Other conserved residues provide a proper environment for heme-binding, and have therefore been suggested to serve as signature residues for active Clds (I137 and L168 in NdCld) (Kostan et al., 2010).
3.6. Sources of Cld for this thesis

The sources of ClDs used here exhibit several interesting aspects. They are all non-PCRBs with *Nitrospira defluvii* being the only organism with an active Cld. Their natural habitats and function within their environments make them interesting targets for intensive studying.

3.6.1. *Nitrospira defluvii*

*Nitrospira defluvii* are nitrite oxidizing bacteria, their genome was deciphered by environmental genomics from an activated sludge enrichment culture (Lucker et al., 2010). They are not close relatives to other nitrifiers and have probably evolved from microaerophilic or anaerobic progenitors. A periplasmic location signal peptide that precedes their *cld*-gene was removed these studies here as in (Kostan et al., 2010).

3.6.2. *Listeria monocytogenes*

*Listeria monocytogenes* is a gram-positive firmicute and the only human pathogen of the genus *Listeria*. *Listeria monocytogenes* as a facultative intracellular bacterium is the cause of listeriosis which still causes death in 20 – 30% of infected humans. The gene-product of their *cld*-gene is essential (Füreder, 2009) and might be targeted for medical treatment of listeriosis.

3.6.3. *Sulfolobus solfataricus*

*Sulfolobus solfataricus* belongs to the phylum of crenarcheota and is a thermoacidophile archaeon. It was first isolated from hot volcanic springs and its optimal growth conditions are at 80°C (60 – 92°C) and pH 2 – 4 (Zaparty et al., 2010). Its Cld is probably cytosolic and represents an interesting target for biochemical characterization since there are genetic tools like RNAi available for in situ functional analyses (Christa Schleper, personal correspondence).
4. Aim of this Thesis

The aim of this thesis was to gain deeper insight into the protein family of chlorite dismutases, its original and today's function. This can be divided into three parts, each dealing with a representative of different phyla.

One part continues the work on an active Cld, comprised of studies on NdCld (Cld from *Nitrospira defluvii*). The main goal was to gain deeper understanding of the Cld-reaction by mutating potentially relevant residues and analyzing their structural and enzymatic properties.

The second part aimed at finding a function for LmCld, which has been shown to be essential for the human pathogen *Listeria monocytogenes*. The starting point was the finding that recombinant LmCld, despite the presence of the heme-coordinating histidine, does not bind heme which is necessary for efficient catalysis of chlorite degradation. We aimed to assess the presence or absence of heme in LmCld from natural source and/or identify another cofactor and subsequently look for the function based on our findings.

The last part is centered on the archaeal lineage of Clds with the representative SsCld (Cld from *Sulfolobus solfataricus*). Proteins of this lineage contain a serine residue at the position of arginine in active Clds and have diverged from bacterial Clds early in evolution, most likely they perform reactions different from chlorite degradation. The goal was to establish recombinant over-expression and purification protocols to further investigate its enzymatic activity and biochemical, structural and biophysical properties.
5. Materials and methods

5.1. Media, Buffers and other Stock Solutions

Table 1: Antibiotics (purchased from Sigma Aldrich) were filtered (0.45 µm) and stored at -20°C

- 1000x Ampicillin/Carbencillin: 100 mg/mL in H₂O
- 2000x Kanamycin: 100 mg/mL in H₂O
- 1000x Chloramphenicol: 34 mg/mL in ethanol

Table 2: Media, buffers and stock solutions

- LB-Medium: 10 g Tryptone, 5 g yeast extract, 10 g NaCl, adjusted to pH 7.0 with NaOH (ready-made, Sigma, 30 g for 1000 mL H₂O); autoclaved
- LB-Agar: 15 g agar in 1 L LB-medium
- TBS: 50 mM Tris (pH adjusted to according value with HCl), 150 mM NaCl
- 0.5M EDTA: EDTA solubilized with solid NaOH to final pH 8
- 50x TAE: 2 M TrisHCl pH 8, 1 M acetic acid, 50 mM EDTA
- TE: 10 mM TrisHCl, 1 mM EDTA
- 10xSDS running buffer: 0.25 M Tris-HCl pH 8.3, 2 M glycine, 1% (w/v) SDS
- 2x SDS loading buffer: 0.125 M TrisHCl pH 6.8, 6 M Urea, 2% SDS, 30% glycerol, 1 M β-mercaptoethanol, 0.1% Bromphenol blue
- TBE: 0.05 M Tris; 0.06 M boric acid; 0.001 M EDTA
- 2x heme-staining loading buffer: 0.125 M TrisHCl pH 6.8, 6 M Urea, 4% SDS, 20% glycerol, 0.1% Bromphenol blue
- Staining Solution: 250 mL ethanol, 80 mL acetic acid, 2.5 g Coomassie brilliant blue, filled to 1000 mL with H₂O
- Heme: 200 mg Hemin (Fluka) dissolved in 1.6 mL 0.5 M NaOH, filled to 40 mL with H₂O
- IPTG: 0.5 M IPTG in H₂O, sterile filtered (0.45 µm), stored at -20°C
5.2. Storage

Proteins were stored at 4°C for short term storage. For long term storage proteins were aliquoted to 100 µL, flash frozen in liquid Nitrogen and stored at -80°C.

DNA was stored in TE at -20°C.

Bacteria from 50 mL O/N culture were dissolved in 10 mL sterile filtered 15% glycerol, flash frozen and stored at -80°C.

Buffers and stock solution were stored at room temperature unless stated otherwise.

5.3. Cloning and Expression

Proteins were expressed in *E. coli* lab strains (Tuner (DE3), BL21 (DE3) or Rosetta2). For plasmid maintenance and mini-preps DH5α was used.

5.4. Expression vectors

The expression vectors all carry a copy of the *lacI* gene for the lac-repressor and thus are all inducible by addition of lactose or the artificial inducer Isopropyl-beta-D-thiogalactopyranoside (IPTG). The vectors include pETM11 with a TEV-cleavable 6x his-tag, pETM30 with TEV-cleavable 6x-his-GST-tag and a modified pET21(+) with a TEV-cleavable strep-tag II.

5.5. Constructs

All the constructs used during the work on this thesis are found in table 8. Most of the NdCld mutants were produced and purified together with Kira Gysel, who presented a more detailed analysis on the effects of mutated signature residues in her diploma thesis (Gysel, 2011).
5.6. Cloning

Site directed mutagenesis was used to create single or double mutants of the NdCld gene, for changing vectors conventional restriction enzymes were used. Restriction enzymes and respective buffers purchased from Fermentas. DNA concentration was determined spectroscopically with NanoDrop 2000c (ThermoFischer Scientific).

All oligonucleotides were ordered at Sigma-Aldrich on a synthesis scale of 0.2 µmol, purified by desalting. The dry oligonucleotides were dissolved in 1x TE in an appropriate volume to reach a concentration of 100 µM.

5.7. Site directed mutagenesis

To introduce point mutations of key residues in NdCld PCR (polymerase chain reaction) was used. For this purpose forward and reverse primers were designed, that carried the changed codon, where the reverse primer being the reverse complement of the forward primer. For double mutants the vector containing the single mutant gene was used as a template whereas for single mutants was the wild type vector.

PCR-protocol includes the 2x PhusionFlash MasterMix (Finnzymes), able to process 4 kb/min. PCR itself was carried out in an Eppendorf Mastercycler gradient PCR machine. To eliminate template DNA the PCR solutions were treated with Dpn1 (Fermentas) at 37°C for 1 h.
**Table 3:** List of primers for the constructs used or created during this thesis together with Kira Gysel (other NdCld mutation primers can be found in Kira Gysel's Diploma Thesis (Gysel, 2011)); changed codons marked red in fwd primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>$T_m$[°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>W145V fwd</td>
<td>5'-GAAGGACGCGGAAGTGTTGGGCACCTGGACCAGG-3'</td>
<td>66</td>
</tr>
<tr>
<td>W145V rev</td>
<td>5'-CCTGGTCCAGTGCCACACTTCGCGGTCTCCC-3'</td>
<td>66</td>
</tr>
<tr>
<td>W145F fwd</td>
<td>5'-CTCCCTATCAAGAAGGACCGGGAAATTTTGGGCACCTG-3'</td>
<td>62</td>
</tr>
<tr>
<td>W145F rev</td>
<td>5'-CAGTGCCAAAAATTCGCCGCTCTTCTCAGTGAGGA-3'</td>
<td>67</td>
</tr>
<tr>
<td>W146Y fwd</td>
<td>5'-GAAGGACGCGGAATGTAAGGCACCTGGACCAGG-3'</td>
<td>64</td>
</tr>
<tr>
<td>W146Y rev</td>
<td>5'-CCTGGTCCAGTGCCACCTGACCTCCGCGGTCTCCC-3'</td>
<td>64</td>
</tr>
<tr>
<td>W145V/W146Y fwd</td>
<td>5'-GAAGGACGCGGAAGTGGCACTGGACCAGG-3'</td>
<td>63</td>
</tr>
<tr>
<td>W145V/W146Y rev</td>
<td>5'-CCTGGTCCAGTGCCACCTGACCTCCGCGGTCTCCC-3'</td>
<td>64</td>
</tr>
<tr>
<td>R173Q fwd</td>
<td>5'-CTGAAGACGGTGAAACAAAAACTGTATCATGGTG-3'</td>
<td>56</td>
</tr>
<tr>
<td>R173Q rev</td>
<td>5'-CGAATGATACAGTTTTTGTTTCACCAGTCCTCAG-3'</td>
<td>56</td>
</tr>
<tr>
<td>NwCld fwd</td>
<td>5'-GCTGAGCCATGGGGACGTTCACAGTCTTCACC-3'</td>
<td>63</td>
</tr>
<tr>
<td>NwCld rev</td>
<td>5'-AGTTTCTCCTCAGTGATACAGTTTTGGTCTCAGTCCTCACC-3'</td>
<td>63</td>
</tr>
<tr>
<td>LmCld fwd</td>
<td>5'-GCTGAGCCATGGGGAGTACGAGTCCTCAGTCCTCACC-3'</td>
<td>64</td>
</tr>
<tr>
<td>LmCld rev</td>
<td>5'-CCCTCCTCCTCAGTGATACAGTTTTGGGATACGAGTCCTCACC-3'</td>
<td>57</td>
</tr>
<tr>
<td>NdCld fwd</td>
<td>5'-GGAGATATACCCCATGGGCGGTACGTCTCAG-3'</td>
<td>55</td>
</tr>
<tr>
<td>NdCld rev</td>
<td>5'-TTCGCGATCATCTACTGTGCAG-3'</td>
<td>55</td>
</tr>
</tbody>
</table>

**5.8. Polymerase Chain Reaction**

**Table 4:** PCR composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Template</td>
<td>15 ng</td>
</tr>
<tr>
<td>2x PhusionFlash</td>
<td>25 µL</td>
</tr>
<tr>
<td>MasterMix</td>
<td></td>
</tr>
<tr>
<td>add H2O to total of</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

**Table 5:** PCR settings

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature [°C]</th>
<th>Time [sec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>300</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30</td>
</tr>
<tr>
<td>Primer annealing</td>
<td>x°C</td>
<td>45</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>120</td>
</tr>
<tr>
<td>Cycles</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C</td>
<td>300</td>
</tr>
<tr>
<td>Cooling</td>
<td>4°C</td>
<td>hold</td>
</tr>
</tbody>
</table>
5.9. Transformation

100 µL of chemically competent cells (Hanahan, 1983) were put from -80°C to 4°C on ice, transformed with 5-20 ng DNA (or 6-10 µL of DpnI-treated PCR product after site-directed mutagenesis) and left on ice for 20 minutes. After a 45 - 60 sec heatshock (at 42°C), the cells were incubated again for 3 minutes on ice. Subsequently, 900 µL nonselective LB medium were added and the cells could recover at 37°C and 800 rpm for one hour in case of kanamycin and chloramphenicol resistance. Afterwards, the cells were plated onto a selective agar plate and grown in an incubator overnight at 37°C. For ampicillin resistance, cells were directly plated on selective agar.

5.10. Colony PCR

Before sequencing, newly transformed constructs were checked by colony PCR. Therefore single colonies were picked, dissolved in 5 µL sterile ddH₂O, of which 1µL was put on selective agar plates marked with a grid for later identification of the clones. Generic primers for the respective genes were used for amplification. The PCR products were analyzed on an 1 % agarose gel for the presence of a band at the corresponding size. Cells containing the desired insert from the plate were used for inoculation of a small-scale culture for minipreps.

Table 6: Colony-PCR Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell suspension</td>
<td>-</td>
<td>4 µL</td>
<td>-</td>
</tr>
<tr>
<td>2x PhusionFlash</td>
<td>1x</td>
<td>12.5 µL</td>
<td>2x</td>
</tr>
<tr>
<td>MasterMix</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer fwd</td>
<td>0.8 µM</td>
<td>1 µL</td>
<td>10 µM</td>
</tr>
<tr>
<td>Primer rev</td>
<td>0.8 µM</td>
<td>1 µL</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

add H2O sterile to total of 25 µl
Table 7: Colony-PCR settings

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Process</th>
<th>Temperature [°C]</th>
<th>Time [sec]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysis</td>
<td>98°C</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>x°C</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Cycles</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Cooling</td>
<td>4°C</td>
<td>Hold</td>
<td></td>
</tr>
</tbody>
</table>

5.11. Small Scale Plasmid Preparation (Mini-Prep)

For sequencing plasmids were extracted by the Plasmid Mini Kit from Fermentas. 5-10 mL of selective LB were inoculated with a single colony and incubated at 37°C, shaking over night. The dense cell cultures were harvested by centrifugation (15 minutes at 4000 rpm) and the plasmids purified according to the manufacturer's instructions (alkaline lysis method). The DNA was eluted from the spin columns with 30-50 µL TE buffer.

5.12. Subcloning

The expression vector was changed from pETM-11 (for NdCld, provided by Julius Kostan (Kostan et al., 2010)), from pET21b(+) (for LmCld, provided by Stephanie Füreder (Füreder, 2009)) or from pCR8-GW (for SsCld, provided by Andrea Manica) to a modified version of pET21(+) with an N-terminal Strep-Tag II, cleavable by TEV protease. This vector will be referred to as StrepTEVpET21(+) throughout this thesis. The Cld insert was cut out with the restriction endonucleases Ncol (5’CCATGG-3’) and Xhol (5’-CTCGAG-3’). The StrepTEVpET21(+) vector was cut with the same enzymes. The reaction mix consisted of 1 µg vector DNA, 5 µL 10x buffer R (Fermentas), 1 µL Xhol, 1 µL Ncol (both Fermentas, 10 U/µL). Sterile ddH₂O was added to a total volume of 50 µL and the mixture was incubated at 37°C for 1 h.

The digested DNA was purified on an 1 % (w/v) agarose gel containing cSYBR Safe DNA gel stain (Invitrogen, at a ratio of 1:10000) for UV visualization of the DNA. The bands were cut out from the gel, extracted with the GeneJET Gel extraction kit (Fermentas) and subsequently eluted from the spin column in 30 µL 1x TE.
The DNA yield was determined on the Nanodrop. The ligation reaction, which consisted of 1 µL 10x T4 ligation buffer, contained digested vector and insert at a molar ratio of 1:4 with approximately 20 ng of vector, 0.5 µL T4 ligase and sterile ddH₂O was added to a total volume of 10 µL. The ligation reaction was carried out at 16°C over night and the ligation product then transformed into *E. coli* DH5α.

**Table 8:** List of constructs

The following list contains all the constructs used and/or created during the thesis.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Insert</th>
<th>Vector</th>
<th>Tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJK23</td>
<td>NdCld</td>
<td>pETM-11</td>
<td>6x his</td>
</tr>
<tr>
<td>pJK29</td>
<td>LmCl</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>pJK30s</td>
<td>NdCld R173K</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pJK32s</td>
<td>NdCld R173L</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pJK53s</td>
<td>NdCld W146Y</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pJK54s</td>
<td>NdCld W145V</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pJK55s</td>
<td>NdCld W145V W146Y</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pKG1s</td>
<td>NdCld R173Q</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pKG3s</td>
<td>NdCld W146Y R173Q</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pKG4</td>
<td>LmCld</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pKG6</td>
<td>NdCld R173Q E210A</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pKG8</td>
<td>NdCld W145F</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pKG9</td>
<td>NdCld W145V R173E</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pKG10</td>
<td>SsCld</td>
<td>StrepTEVpET21</td>
<td>Strep tag II</td>
</tr>
<tr>
<td>pSF29</td>
<td>NwCld</td>
<td>pET21b(+)</td>
<td>6x his</td>
</tr>
<tr>
<td>pAH1</td>
<td>SsCld</td>
<td>pETM30</td>
<td>6x his-GST</td>
</tr>
</tbody>
</table>

For NdCld constructs (accession no. ACE75544), (Kostan et al., 2010) used a truncated version of the gene, lacking the predicted signal peptide for periplasmatic localisation (i.e. 26 N-terminal amino acids), since the full length construct was not functional.

Cld-like genes from *Listeria monocytogenes* (*lmo2113*, accession no. NC003210, provided by Stephanie Füreder), from *Sulfolobus solfataricus* (accession no. ACX92972.1) and from *Nitrobacter winogradskyi* (accession no. YP_319047) (Mlynek et al., 2011) were used full length. pETM-11 carries the resistance against kanamycin, pET21 the ampicillin resistance.
5.13. Protein Expression

Heterologous protein expression was carried out in *E. coli* lab strains. Precultures were grown from a glycerol stock stored at -80°C by scratching the surface of a frozen stock with a pipette tip and were inoculated in 50 mL selective LB medium.

Protein expression at 18°C and 24°C was carried out in a coolable incubator shaker (Sartorius), at 37°C in a non-coolable incubator shaker (New Brunswick Scientific). The cells were harvested by centrifugation in 1 L centrifuge tubes using an SLC-4000 rotor at 4°C for 15 minutes at 5000 rpm in a coolable centrifuge (Sorvall Evolution RC Superspeed Centrifuge). The cell pellet was resuspended in 10 mL of the supernatant medium, transferred to a 50 mL Falcon tube and centrifuged again at 4000 rpm for 20 minutes at 4°C in an Eppendorf benchtop centrifuge. Pellets were either further processed right away or otherwise flash frozen and stored at -80°C until usage.

5.13.1. NdCld: wild type and mutant expression

*E. coli* BL21 (DE3) Tuner cells carrying an expression vector for a NdCld mutant (see Table 8) were grown in 50 mL selective LB medium (pETM-11: Kanamycin; StrepTEVpET21: Ampicillin) at 37°C, shaking overnight. A 1/100 dilution was used for inoculation of selective LB and the cells were grown at 37°C to an OD₆₀₀ of 0.8. Then the temperature was reduced to 24°C, 50 mg hemin were added (1:1000, see 5.1) and the expression of NdCld induced with 0.5 mM IPTG. Cld was expressed 4 - 8 hours and the cells afterwards harvested by centrifugation, frozen in LN₂ and stored at -80°C.

5.13.2. NdCld: expression without heme ("apoCld")

*E. coli* BL21 (DE3) Tuner cells carrying the pJK23 plasmid (NdCld wild-type) were grown in 50 mL selective LB at 37°C over night. A 1/100 dilution was used for inoculation of selective LB and the cells were grown at 37°C to an OD₆₀₀ of 0.8. The protein expression was then induced with 0.5 mM IPTG and protein was expressed for 4 hours at 37°C. This yields the maximum amount of protein, while keeping the heme content as low as possible. The cells were harvested by centrifugation, the cell pellets flash-frozen in LN₂ and stored at -80°C until further use.
5.13.3. LmClId

E. coli BL21 (DE3) Tuner cells carrying either pJK29h or pKG4s were grown in 50 mL selective LB (kanamycin for pJK29h and ampicillin for pKG4, respectively) at 37°C shaking over night. A 1/100 dilution was used for inoculation of selective LB and the cells were grown at 37°C to an OD$_{600}$ of 0.8. Expression was induced by addition of IPTG to a final concentration of 0.5 mM and the cultures were cooled to 24°C, under agitation (160 rpm) for at least 4 h. Cells were then harvested by centrifugation, the pellets flash frozen and stored at -80°C until further use.

5.13.3. SsClId

E. coli Rosetta 2 pLysS cells transformed with pKG10 were grown in 50 mL LB supplemented with ampicillin and chloramphenicol over night at 37°C and this was used to inoculate selective LB, at 1/100 ratio. Cells grew at 37°C and 180 rpm to an OD$_{600}$ of 0.8 at which point they were induced to express by addition of IPTG to a final concentration of 0.25 mM and 50 mg heme per liter of culture and cooled down to 18°C, under agitation (160 rpm) for at least 8 h. Cells were then harvested by centrifugation, the pellets flash frozen and stored at -80°C until further use.

5.13.4. NwClId

E. coli BL21 (DE3) cells transformed with pKG10 were grown in 50 mL LB supplemented with ampicillin over night at 37°C and this was used to inoculate selective LB, at 1/100 ratio. Cells grew at 37°C and 180 rpm to an OD$_{600}$ of 0.8 at which point they were induced to express by addition of IPTG to a final concentration of 0.5 mM and 50 mg heme per liter of culture and cooled down to 18°C, under agitation (160 rpm) over night (ca. 16 h). Cells were then harvested by centrifugation, the pellets flash frozen and stored at -80°C until further use.

5.14. Protein Purification

Purification was done on ÄKTA PURIFIER systems (GE Healthcare) at 4°C. All columns were purchased from GE Healthcare.

Progress of the purification was monitored by measuring absorption at $\lambda_1 = 280$ nm, $\lambda_2$ = between 400 and 410 nm, which corresponds to the soret peak of bound heme and $\lambda_3 = 360$ nm, corresponding to the soret peak of free
heme. Extinction coefficients for $A_{280}$, according to Mlynek et al. (28) and Kostan et al. (21):

NdCld: $\varepsilon = 37930 \text{ M}^{-1} \text{cm}^{-1}$; $E_{0.1\%} (= 1 \text{ g/L}) = 1.382$

NwCld: $\varepsilon = 40450 \text{ M}^{-1} \text{cm}^{-1}$; $E_{0.1\%} (= 1 \text{ g/L}) = 1.984$

mutated NdClds, LmCld, SsCld: extinction coefficient taken from ProtParam (Gasteiger E, 2005)

LmCld: $\varepsilon = 45505 \text{ M}^{-1} \text{cm}^{-1}$; $E_{0.1\%} (= 1 \text{ g/L}) = 1.577$

SsCld: $\varepsilon = 62340 \text{ M}^{-1} \text{cm}^{-1}$; $E_{0.1\%} (= 1 \text{ g/L}) = 2.151$

Protein concentrations were always measured by UV absorption at 280 nm on the Thermo Scientific Nanodrop 2000c Spectrophotometer.

5.15. Purification conditions

5.15.1. Purification of NdCld wild type and mutants

Lysis buffer: 50 mM HEPES-NaOH pH 7.4, 5% (v/v) glycerol, 0.5% (v/v) Triton X-100
StrepTrap elution buffer: 20 mM HEPES-NaOH pH 7.4, 2% (v/v) glycerol, 2.5 mM Desthiobiotin
StrepTrap binding/dialysis/SEC buffer: 20 mM HEPES-NaOH pH 7.4, 2% (v/v) glycerol

5.15.2. Purification of Apo-NdCld

Lysis buffer: 50 mM HEPES-NaOH pH 7.4, 5% (v/v) glycerol, 0.5% (v/v) Triton X-100, 20 mM imidazole
HisTrap binding buffer: 20 mM HEPES-NaOH pH 7.4, 2% (v/v), 20 mM imidazole
HisTrap elution buffer: 20 mM HEPES-NaOH pH 7.4, 2% (v/v) glycerol, 500 mM imidazole
Dialysis/SEC buffer/AEX binding buffer: 20 mM HEPES-NaOH pH 7.4, 2% (v/v) glycerol
AEX elution buffer: 20 mM HEPES-NaOH pH 7.4, 2% (v/v) glycerol, 2 M NaCl
5.15.3. Purification of LmCld

Strep-tagged KG4s:
Lysis buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol
StrepTrap elution buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol, 2.5 mM Desthiobiotin
StrepTrap binding/Dialysis/SEC buffer: 20 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol

6x his-tagged JK29h:
Lysis buffer/HisTrap binding buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol, 20 mM imidazole
HisTrap elution buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol, 500 mM imidazole
Dialysis/SEC buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol

5.15.4. Purification of SsCld

Lysis buffer: 50 mM TrisHCl pH 9, 150mM NaCl, 2% (v/v) glycerol
StrepTrap elution buffer: 50 mM TrisHCl pH 9, 2% (v/v) glycerol, 2.5 mM Desthiobiotin
StrepTrap binding/Dialysis/SEC buffer: 20 mM TrisHCl pH p, 2% (v/v) glycerol

5.15.5. Purification of NwCld

Lysis buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol, 0.5% (v/v) Triton X-100, 20 mM imidazole
HisTrap binding buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol, 20 mM imidazole
HisTrap elution buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol, 500 mM imidazole
Dialysis/SEC buffer: 50 mM TrisHCl pH 8.5, 150mM NaCl, 2% (v/v) glycerol

Cell pellets were thawed and resuspended in 35 mL Lysis buffer per L culture. Cells were broken either by sonication (3 x 5 minutes, 3 cycles, 70% power) or on a french press and the Crude extract subsequently cleared by centrifugation (SS34 rotor) at 4°C, 38,000 g for 15 minutes.
5.16. Strep-Tactin Affinity

For the proteins that were expressed with an N-terminal Strep-Tag II, the clear supernatant was loaded on a clean StrepTrap HP column previously equilibrated with StrepTrap binding buffer, washed with 5 CV binding buffer and subsequently eluted with a step gradient of 2.5 mM Desthiobiotin in the Strep-Trap elution buffer. After analysis by SDS-PAGE, the fractions containing Cld were pooled together.

5.17. Ni-NTA Affinity

For constructs containing a 6x His tag, the clear supernatant was loaded on a clean HisTrap HP column previously equilibrated with HisTrap binding buffer, washed with 5 CV binding buffer and subsequently eluted with a linear gradient of HisTrap elution buffer. After analysis by SDS-PAGE, the fractions containing Cld were pooled together.

5.18. Further purification

After affinity purification the tag was cleaved off with TEV protease. The fractions containing the protein were pooled and transferred to a dialysis bag with a molecular weight cut-off of 12,000 - 14,000 Da, TEV protease added at a 1:30 - 1:50 mass ratio and dialyzed overnight at 4°C against 1 L of dialysis buffer with 1 mL mercaptoethanol. uncleaved protein was then removed by another affinity purification step, where only the flow-through was collected. This step also gets rid of 6x his-tagged TEV in case of Ni-NTA affinity. Cleavage of the tag was monitored by SDS-PAGE. For heme-binding proteins, heme (freshly prepared see section 5.1) was added to the protein solution at a molar ratio of 2:1 heme:protein and left on 4°C for 30 – 60 min. Insoluble heme was then removed by spinning for 30 minutes at 18,000 rpm at 4°C.

The protein solutions were concentrated in an Amicon Ultra centrifugal filter unit (Milipore) with a molecular weight cut-off of 30,000 or 50,000 to a volume of about 5 mL (which also removes most of the remaining TEV protease in case of the Strep-Tactin based purification) and loaded on an equilibrated Superdex 200 16/60 size exclusion column.

Pure protein was further concentrated in an Amicon Ultra centrifugal filter unit to 5-30 mg/mL (depending on solubility), divided to 100 µL aliquots, flash-frozen in liquid nitrogen and stored at -80°C.
For production of apoNdCld an additional anion exchange step before SEC was included to get rid of partially loaded NdCld. Previous observations showed a different behaviour of loaded and unloaded NdCld on an anion exchange column (based on the differences between the ratios of $A_{280}/A_{\text{socret}}$). The sample was loaded on a 6 mL ResourceQ AEX column equilibrated with SEC buffer. A washing step with 5% of AEX elution buffer (corresponds to 100 mM NaCl and approximately 11.5 mS/cm) to remove heme-loaded NdCld. A linear salt gradient over 15 CV to 20% AEX elution buffer (corresponding to 400 mM NaCl) let the desired fractions elute (usually at about 16 mS/cm), confirmed by spectrometrically determining the ratio $A_{280}/A_{\text{socret}}$. NdCld was declared apoCld when $A_{280}/A_{\text{socret}}$ was about 1/10.

5.19. Immuno-precipitation

Binding buffer: TrisHCl pH 7.5, 150mM NaCl
Elution buffer: TrisHCl pH 7.5, 150mM NaCl, 3 M urea, 10 mM DTT

Immuno-precipitation of native LmCld was done by polyclonal rabbit anti-LmCld antibodies (kindly provided by Stephanie Füreder) and magnetic Protein A beads (GE Healthcare).

The *Listeria monocytogenes* lab strain LO28 was grown under anaerobic conditions, since it has been reported that under these conditions the expression of LmCld was slightly upregulated. Cell pellets were frozen and stored at -80°C and kindly provided by Thomas Decker.

Cells were resuspended in binding buffer with appropriate amounts of the protease inhibitor cocktail complete EDTA-free (Roche) (pellet from 1L culture in 20ml) and lysed by sonication (2 x 4 minutes, 3 cycles, 70% power). Cell debris was removed by centrifugation (SS34 rotor) at 4°C, 38,000 g for 15 minutes. This centrifugation step alone led to precipitation in the pull-down step so an additional ultra centrifugation step was applied (100 000 g at 4°C for 1 h).

Magnetic Protein A beads were prepared by equilibration with the binding buffer and subsequent addition of polyclonal anti-serum with the antibodies in excess to prevent binding of other immunoglobulins than immunoglobulin G. Protein A is a membrane bound protein produced by *Staphylococcus aureus* to prevent opsonization by the immune system due to its high affinity for the IgG-Fc domain thereby hindering interaction of the IgG epitopes with their para-
topes and the interaction of their Fc-region with other factors involved in the classical complement pathway.

After binding of IgG to the Protein A beads the beads were washed 3x by adding 500 µL binding buffer and vortexing for 3 – 5 sec and removal of the buffer.

Protein A – IgG beads were then applied to the cleared lysate, to minimize unspecific binding in a ratio of 10µL beads in 8 mL of lysate. The pull-down step was performed on a spinning wheel at 4°C for 4 - 8 h.

The solution was removed and the beads washed 6x with binding buffer, including one wash step with increased NaCl concentration (500 mM instead of 150 mM) to improve purity. Each step consists of addition of 500 µL buffer, gentle vortexing for 3 – 5 sec and removal of the buffer.

Beads with native LmCld bound were either stored in binding buffer at 4°C, cooked in SDS-sample buffer for conventional SDS PAGE, incubated in heme-staining sample buffer for 20 min at room temperature or the protein was eluted by addition of elution buffer. Depending on the next step in line the protein was concentrated when necessary.

The identity of the pulled down protein was verified by mass spectrometry (BSRC Mass Spectrometry and Proteomic Facility St. Andrews).

5.20. Heme staining

2x sample buffer: 0.125 M TrisHCl pH 6.8, 6 M urea, 4% SDS, 20% glycerol, bromphenol blue
Running buffer: 0.05 M Tris, 0.06 M boric acid, 0.001 M EDTA (TBE)
Staining solution: 200 mg o-dianisidine (=DMB, 3',3'-dimethoxybenzidine; Sigma-Aldrich) stirring in 180 mL H₂O; before staining addition of 20 mL of 0.5 M sodium citrate pH 4.4 and 0.4 mL of 30% H₂O₂

The heme staining protocol by Francis and Becker (Francis RT Jr, 1984 ) offers means to stain heme still bound to the electrophoretically separated protein. Here a modified version was used, since the elution from the Protein A beads needed harsher conditions. In these conditions a non-covalently bound heme dissociates and migrates at the dye front. The reported limit of detection of 40 pmol heme was confirmed here.

An equal amount of 2x sample buffer was added to the samples and incubated for 20 min at room temperature. Samples were applied to a normal 15% poly-
acrylamide gel, electrophoresis was performed at 4°C for 1.5 – 2 h with 15mA in the running buffer TBE.

Proteins and heme were fixed by treatment with 12.5% TCA for 30 min on a benchtop shaker and then washed with ddH2O for 30 min followed by shaking in the staining solution for one hour.

The reaction occurring during staining relies on the intrinsic peroxidase activity of heme, o-dianisidine binds to heme and heme catalyses the transfer of two hydrogen atoms to H2O2, the net reaction being

$$H_2O_2 + 2 DH \rightarrow 2 H_2O + 2 D$$

where DH is the reduced version and D is the oxidized version of the donor.

The donor in this case is o-dianisidine and upon oxidation it changes its color to a brownish red when bound to protein and green when dissociated. This redox reaction is also the reason for the non-reducing conditions in the sample buffer.

This procedure stains both the dissociated heme from proteins to which it was not covalently bound and heme still attached to proteins. Due to the absence of SDS in the running buffer the migration of proteins is slower as compared to the migration in conventional SDS PAGE resulting in impaired separation between proteins present in the respective samples. Therefore a conventional SDS PAGE was always performed in parallel to check for impurities.

After heme staining the gels can optionally be stained by coomassie blue to optically analyze the protein migration. Note that o-dianisidine also has affinity for coomassie blue and will be stained as well.

5.21. Fluorescence based thermal shift assay

The assay relies on measuring fluorescence of the hydrophobic dye SYPRO orange (Invitrogen), which is quenched when surrounded by water molecules (Reinhard L, 2013). Upon heating on the real time PCR thermocycler (BioRad iQ5 Multicolor Real-Time PCR Detection System; 1°C per minute) the protein starts to unfold, thereby exposing hydrophobic patches that are then available for binding of the hydrophobic dye. Interaction between dye and hydrophobic parts of the protein diminishes the quenching effects of water. This way, the more protein is unfolded, the more fluorescent signal is measured and the temperature where half of the protein is unfolded is considered the melting temperature.
Three commercial screens were applied to recombinant LmCld with cleaved Strep-tag II, namely Additive Screen, Silver Bullets and Silver Bullets Bio (all Hampton Research).

4 mg of protein, 2 µL SYPRO orange and SEC buffer to a total of 22.5 µL were dispensed into a RT-PCR plate. 2.5 µL of each condition of the different screens were dispensed by Phoenix liquid handling system (Art Robbins Instruments) and mixed three times. The plates were sealed and centrifuged before measurements.

The melting temperatures were compared and the compounds found to be increasing the $T_M$ of the protein are shown in Figures 6 - 8 and Tables 10 - 12.

5.22. Crystallography

5.22.1. Robotics and Materials

Initial crystallization trials were done by sitting drop vapour diffusion in 96-well plates Phoenix liquid handling system (Art Robbins Instruments).

Optimization of crystallization conditions were done by using an Alchemist II liquid handling robot (Rigaku) for dispensing of non-commercial optimization screens in either 96-well plates and sitting drop vapour diffusion or in 24-well Linbro format hanging drop plates.

96-well sitting drop plates were inspected by a Minstrel DT imaging system (Rigaku) equipped with the Atlantis software for automatic imaging in combination with CrystalTrak software (Rigaku).

Plates used were 96-well IntelliPlates (Art Robbins instruments) with 3 slots for the drops and 24-well pregreased Linbro plates (Crystalgen) which were set up manually.

Crystals were stored in an temperature regulated room at either 22°C or 4°C.

5.22.2. Crystallization of LmCld

Pure recombinant LmCld from strep-affinity purification at 7.33 mg/mL showed positive hits in the SaltRX screen (Hampton) of which two conditions were used for optimization. These were chemically similar, i.e. the pH was slightly alkaline and the precipitating salts were both organic acids (tartrate and citrate,
respectively). According to the diffraction power of the crystals formed, the condition chosen for crystallization was derived from condition 87 of SaltRX (Hampton Research).

In this condition multiple nucleation was observed. To overcome this obstacle glycerol concentration was increased to 8\% (v/v) in order to slow down the vapour diffusion kinetics.

For cryo protection the glycerol concentration was further elevated to 25\% (v/v) by adding mother liquor with glycerol to the drops.

5.22.3. Crystallization of LmCld with additives

The optimized condition was then applied to LmCld together with selected additives (see section 6.1) in the same ratio as in the thermofluor studies. Protein and additives were mixed prior to crystallization and divided in two, one of which was centrifuged at 16,000 g, 4°C and both crystallized in parallel. Crystallization with additives was done in 24-well Linbro plates only. For final crystallization conditions see table of crystallization conditions (Table 9).

For cryo protection the glycerol concentration raised to 25\% (v/v) by adding mother liquid with glycerol to the drops. Transfer to drops with higher concentrations was undesirable, since it might have caused the additives to be washed out.

5.22.4. Crystallization of NdCld W145F

In contrast to other constructs this mutant was crystallized still attached to the strep-tag, hence leaving out the TEV protease in the dialysis step (see section 5.18). The rationale behind this was the observation that degradation took place when treated with TEV whereas without TEV it did not. Initial attempts to crystallize this mutant in conditions similar to wild type and other NdCld mutants (high ammonium sulfate, citrate buffer pH 3.5 – 4.5; derived from condition 13 of JCSG+, Quiagen) gave crystals growing in only two dimensions, i. e. they were plate-shaped.

Screening for better conditions with commercial screens provided a few starting conditions, from those a condition similar to the previously mentioned conditions was selected (condition 15 from SaltRX, Hampton). There, the citric acid was used as precipitant instead of buffering agent together with ammonium, buffered by sodium acetate.
For cryo protection the crystal were soaked in increasing concentrations of ethylen glycol, since the crystals were impaired when using high glycerol concentrations. Stepwise increase of 5% (v/v) of ethylen glycol in mother liquid to a final concentration of 30% (v/v) left the crystals unharmed.

The structure of the W145F mutant was also solved in complex with the inhibitor cyanide, which binds to the heme iron. KCN was dissolved in SEC buffer (see section 5.15.1) and was added in 2-fold excess to the protein solution and incubated for 1 h at room temperature prior to crystallization.
Table 9: Crystallization conditions

<table>
<thead>
<tr>
<th>Construct and additives</th>
<th>Condition</th>
<th>Drop [µL]</th>
<th>Cryo protectant</th>
</tr>
</thead>
<tbody>
<tr>
<td>LmCld</td>
<td>1 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>2:1 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>LmCld BaCl₂ (AS1)</td>
<td>1.15 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>0.5 : 0.5 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>LmCld CdCl₂, CoCl₂, CuCl₂, NiCl₂ (SB42)</td>
<td>1 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>1 : 0.5 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>LmCld YCl₃ (AS9)</td>
<td>1.15 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>1 : 0.5 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>LmCld CdCl₂, CaCl₂, MgCl₂, MnCl₂, ZnCl₂ (SBB46)</td>
<td>1 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>1 : 0.5 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>LmCld GdCl₃, SaCl₃, benzamidineCl, salicin (SB40)</td>
<td>1 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>1 : 0.5 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>LmCld FeCl₃ (AS11)</td>
<td>1.2 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>1 : 0.5 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>LmCld protamine sulfate (SB52)</td>
<td>1.1 M Na K tartrate, 8% glycerol, 0.1 M TrisHCl pH 9</td>
<td>1 : 0.5 hanging</td>
<td>25% (v/v) glycerol</td>
</tr>
<tr>
<td>NdCld W145F</td>
<td>0.7 M Ammonium citrate dibasic, 0.1 M Na acetate pH 4.4</td>
<td>2:1, hanging</td>
<td>30% (v/v) ethylen glycol</td>
</tr>
<tr>
<td>NdCld W145F + cyanide</td>
<td>0.78 M Ammonium citrate dibasic, 0.1 M Na acetate pH 4.53</td>
<td>2:1, hanging</td>
<td>30% (v/v) ethylen glycol</td>
</tr>
</tbody>
</table>

In Brackets you find the screen and number of the respective conditions, with the abbreviations being AS: Additive Screen, Hampton; SB: Silver Bullets, Hampton; SBB: Silver Bullets Bio, Hampton

Additives were used as suggested by the manufacturer. LmCld was used at 7.4 mg/mL, NdCld W145F at 10 mg/mL
5.23. Structure Determination

5.23.1. Data Collection
Diffraction data were collected at synchrotron beamlines (ESRF ID 23-1, BESSY ID14-1). Integration and scaling was done with XDS and XSCALE (Kabsch, 2010).

5.23.2. Molecular Replacement
The phases for all data sets were derived from molecular replacement by the online server software BALBES (Long et al., 2008).

5.23.3. Refinement
Refinement of LmCLd structures was carried out in Phenix Refine (Adams et al., 2010) and Coot (Emsley, 2010). Refinement of NdCLd W145F was refined in Refmac 5 (Collaborative Computational Project, 1994; Murshudov et al., 2011).

5.23.4. Localization of Anomalous Scatterers
Data collection was done at wavelengths close to the absorption edges of the respective metal ions from the additive screens (see section 6.1). mtz files of the solved structure of LmCLd and the ones from data sets taken at appropriate wavelengths were combined in Cad (Collaborative Computational Project, 1994) of the CCP4 software suite. The combined mtz files were used to create an anomalous difference map carried out in FFT (Collaborative Computational Project, 1994) of the CCP4 software suite.

5.24. Steady-state Kinetics
The activity of NdCLd mutants and SsCLd was determined by measuring the oxygen created when the enzyme is processing its substrate, ClO₂ or H₂O₂ respectively. This was measured with a Clark-type electrode (Oxygraph System, Hansatech Instruments, Norfolk, UK) in a stirred water bath at a constant temperature between 30°C and 45°C. Buffers for measurements include 100 mM phosphate buffer, pH 7.0 for NdCLd, 100 mM Sodium Acetate, pH 6.25 and 5.5, an 100 mM TrisHCl, pH 9 for SsCLd. The electrode was equilibrated to 100% O₂ saturation by bubbling O₂ to a blank reaction mixture for 10 minutes
and with 0% O₂ by bubbling with N₂ for another 10 minutes, removing all the oxygen from the solution.

For activity measurements the substrate was injected to the cell in increasing concentrations of 25 - 1000 µM ClO₂ and 5 – 930 mM H₂O₂. The buffer-substrate solution (total volume of 1mL) was purged from all oxygen by bubbling the solution with N₂ until a stable baseline was reached. Subsequently the reaction was initiated by injecting 20 µL of a protein solution into the cell with a hamilton syringe to a final concentration of about 100 nM for NdCld and 500 nM for SsCld.

For calculations only the initial linear slope was used. Production of molecular oxygen (in [M O₂ s⁻¹]) was determined and plotted against chlorite concentrations and H₂O₂ concentration, respectively, in SigmaPlot (Systat Software Inc., v12). For fitting and calculation of the values of the kinetic constants of the enzyme the Michaelis-Menten formula was employed:

\[ K_M = \frac{v_{max}}{2} \text{ [µM]} \]
\[ k_{cat} \text{, the turnover number [s}^{-1}\text{]} \]
\[ \frac{k_{cat}}{K_M} \text{, the catalytic efficiency of the enzyme [M}^{-1}\text{ s}^{-1}\text{]} \]

5.25. Static Light Scattering

SLS was employed to determine the molecular weight and the radius of gyration of purified proteins. In this case the goal was to determine the oligomeric state of the protein (Murphy, 1997).

The system consisted of a miniDawn Treos (Wyatt) light scattering detection system connected to a refractometer (RI-101, MD Scientific) and an HPLC (1260 Infinity HPLC, Agilent).

Separation of different oligomeric species was done on a Superdex 200 10/300 analytical SEC column which was equilibrated with SEC buffer until the baseline stabilized. NwCld was also dissolved in NdCld SEC buffer (buffer exchange with BioRad MicroSpin Column). Protein solutions were diluted to 2 mg/mL (with the exception NWcld was used as at 1 mg/mL) and 70 µL were used for each run. Protein elution was monitored by absorption at 280 nm and the correlating molecular mass determined by static light scattering.
5.26. UV-vis spectroscopy

Heme binding and content were monitored on a UV-vis spectrophotometer Hitachi U-3900 with quartz cuvettes of 10 mm pathlength.
6. Results

6.1. Thermal Shift Assay

Purified recombinant LmCld was used for thermal shift measurements in which the thermal stability of the protein was determined. Comparison of the melting temperature $T_M$ of the protein in different solutions was conducted to identify potential specifically binding additives.

Since there is no known function for any Cld-like protein and LmCld appears to be essential for *Listeria monocytogenes* (Füreder, 2009), the question arises if there is another cofactor that aids in the protein's enzymatic activity. If a cofactor exists that specifically binds to LmCld, the assumption that specific binding would lead to stabilization was the basis for this shot-in-the-dark experiment.

**Figure 6**: Thermofluor of LmCld with Additive Screen
Table 10: Stabilizing conditions of Additive Screen

<table>
<thead>
<tr>
<th>Reagent Nr.</th>
<th>Formula</th>
<th>$T_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BaCl$_2$</td>
<td>68°C</td>
</tr>
<tr>
<td>2</td>
<td>CdCl$_2$</td>
<td>62.5°C</td>
</tr>
<tr>
<td>9</td>
<td>YCl$_3$</td>
<td>62.5°C</td>
</tr>
<tr>
<td>10</td>
<td>ZnCl$_2$</td>
<td>62.5°C</td>
</tr>
<tr>
<td>12</td>
<td>NiCl$_2$</td>
<td>61°C</td>
</tr>
<tr>
<td>14</td>
<td>Pr(III) acetate</td>
<td>62.5°C</td>
</tr>
<tr>
<td>Control</td>
<td>NaCl</td>
<td>53°C</td>
</tr>
</tbody>
</table>

Figure 7: Thermofluor of LmCld with Silver Bullets

Table 11: Stabilizing conditions of Silver Bullets

<table>
<thead>
<tr>
<th>Reagent Nr.</th>
<th>Formula</th>
<th>$T_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>GdCl$_3$, SaCl$_3$, benzamidineCl, salicin</td>
<td>55°C</td>
</tr>
<tr>
<td>41</td>
<td>CaCl$_2$, MgCl$_2$, MnCl$_2$, ZnCl$_2$</td>
<td>65°C</td>
</tr>
<tr>
<td>42</td>
<td>CdCl$_2$, CoCl$_2$, CuCl$_2$, NiCl$_2$</td>
<td>65.5°C</td>
</tr>
<tr>
<td>52</td>
<td>Protamine sulfate</td>
<td>70.5°C</td>
</tr>
</tbody>
</table>
**Figure 8:** Thermofluor of LmCld with Silver Bullets Bio

![Melt Curve](image)

**Table 12:** Stabilizing conditions of Silver Bullets Bio

<table>
<thead>
<tr>
<th>Reagent Nr.</th>
<th>Formula</th>
<th>$T_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Protamine sulfate, g-Strophanthin, Benzamidine hydrochloride, D-Fructose 1,6-diphosphate trisodium salt octahydrate, Oxamic acid</td>
<td>55°C</td>
</tr>
<tr>
<td>20</td>
<td>Protamine sulfate, Ellipticine, D-(+)-Trehalose dihydrate, 6-Phosphogluconic acid trisodium salt, D-(+)-Glucose</td>
<td>65°C</td>
</tr>
<tr>
<td>46</td>
<td>CaCl$_2$, CdCl$_2$, MgCl$_2$, MnCl$_2$, ZnCl$_2$</td>
<td>65.5°C</td>
</tr>
<tr>
<td>76</td>
<td>Pyridoxal hydrochloride, Nicotinamide, Batyl Alcohol, Glutaric acid, N-Acetyl-L-glutamic acid</td>
<td>70.5°C</td>
</tr>
</tbody>
</table>

Both the Silver Bullets and the Silver Bullets Bio are designed in a redundant way, meaning that a specific compound is found in more than one condition. This makes it possible to identify the stabilizing compound in the mixture. For example, mixtures containing the arginine-rich polypeptide protamine showed stabilizing effects to different degrees. The highest $T_M$ was measured in the Silver Bullets condition 52, where protamine sulfate is the only compound.
Condition 76 of Silver Bullets Bio gave a high T<sub>M</sub> too, but none of the other conditions containing one of those compounds showed improvement in the protein’s stability. This mixture was therefore not selected for crystallization.

Several conditions containing one kind of salt from Additive Screen could be found in mixtures in the Silver Bullets screens. Due to low availability of protein the mixtures were selected for co-crystallization.

The main question arising from this experiment was, if the stabilizing compounds bind specifically or have unspecific stabilizing effects. The attempt to answer this was done by co-crystallizing selected conditions with LmCld (see section 6.1).

FeCl₃ of condition 11 from Additive Screen did not give an interpretable curve in the thermofluor measurements. Nevertheless it was used for co-crystallization because of the results from ICP-MS (see Section 6.5.3).

6.2. Structure of recombinant LmCld

Recombinant LmCld was crystallized before by Georg Mlynek (Mlynek, 2010), showing high similarity to the active Cld from *Nitrospira defluvii*. Both are pentameric proteins with the same fold, i.e. two ferredoxin-like domains arranged in the same way (see Figure 12). In contrast to NdCld, LmCld does not show any electron density in the active site cavity of the C-terminal ferredoxin domain. Although the heme-coordinating proximal histidine is present, some other residues appear in positions that might clash with heme when bound (see figure 9), assuming no structural rearrangements. Moreover, the α-helix containing the conserved histidine is slightly more tilted towards the cavity, leaving less room for potential heme-binding.

By steady-state kinetic measurements of mutants, the key residue for active Clds was shown to be Arg173 in NdCld (Kostan et al., 2010). All validated Clds contain arginine at the corresponding position. The arginine alone is not enough for Cld-activity, the presence of heme is critical for the protein to exert catalysis. In the case of LmCld the residues comprising the potential heme environment do not favour its binding (figure 9 and 10) and therefore it cannot be catalytically active.
Figure 9: Residues of LmClld clashing with heme. A superposition of LmClld (green) and NdClld (magenta); Coordinating histidine, key residue arginine and heme of NdClld and the clashing residues Y147, G178, I215 and M219 of LmClld are shown.
Moreover, LmCld contains a flexible stretch of aminoacids (residues 111 – 122) that in these data show electron density in 3 out of 5 subunits at low sigma levels. The loop is located at the entrance site of the cavity. Its density cannot be used to build the backbone in compliance with Ramachandran restraints. Probably the loop becomes slightly more ordered at crystallographic interfaces which is the case for these three subunits.

When NdCld was purified via Ni-NTA affinity the elution conditions include imidazole which binds to the heme iron and also acts as a weak inhibitor and can be observed in the crystal structure (PDB: 3NN1; (Kostan et al., 2010)). Georg Mlynek (Mlynek, 2010) crystallized the 6x his-tagged construct of LmCld which led to the assumption that these conditions might impair the protein’s ability to keep heme bound.

Here the strep-tagged LmCld was crystallized to minimize potential errors caused by chemicals binding to heme.

The protein was expressed, purified and crystallized as described in materials and methods. Diffraction data was collected at BESSY ID 14-1 and the structure was solved to a resolution of 2.21 Å and an $R_{free} = 0.264$ (see Tables 13 and 14).
Nonetheless, also in this structure no electron density was found at positions where heme is located in homologous structures. There might still be the possibility that in *Listeria monocytogenes* additional factors help incorporating heme that are absent in *E. coli*.

To test whether or not the absence of heme is an artefact of recombinant expression, several mass spectrometry methods and X ray fluorescence were applied to the native LmCld (see section 6.5).

**Figure 11:** LmCld crystal

![LmCld crystal](image)

**Table 13:** Data Collection of LmCld

<table>
<thead>
<tr>
<th>Source</th>
<th>BESSY ID 14-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>0.9184Å</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
</tr>
<tr>
<td>Unit cell</td>
<td>a = 240.35 Å, b = 108.68 Å, c = 78.17 Å; β = 98.9°</td>
</tr>
<tr>
<td>Molecules/a.u.</td>
<td>5</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>99212 (16031)</td>
</tr>
<tr>
<td>Resolution</td>
<td>46.48 – 2.21 Å (2.34 - 2.21)</td>
</tr>
<tr>
<td>Completeness</td>
<td>98.9% (96.0%)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>2.6 (2.8)</td>
</tr>
<tr>
<td>I/s(I)</td>
<td>7.3 (3.1)</td>
</tr>
<tr>
<td>R&lt;sub&gt;meas&lt;/sub&gt;</td>
<td>14.8% (53.1%)</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt;</td>
<td>18.7% (94.4%)</td>
</tr>
</tbody>
</table>
**Table 14:** Refinement Statistics LmCld

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{work}}$</td>
<td>0.240</td>
</tr>
<tr>
<td>$R_{\text{free}}$</td>
<td>0.264</td>
</tr>
<tr>
<td>RMSD bonds</td>
<td>0.014 Å</td>
</tr>
<tr>
<td>RMSD angles</td>
<td>1.172°</td>
</tr>
<tr>
<td>Avg. B-factor</td>
<td>40.8 Å²</td>
</tr>
</tbody>
</table>

**Figure 12:** Superposition of chain A of LmCld (red) and NdCld (3NN1, blue)
6.3. Structure of LmCld with protamine
The thermal shift assay showed a stabilizing effect of a polypeptide called protamine, a small arginine-rich peptide. When crystallized in the same conditions, the addition of protamine leads to a different crystal packing, i.e., P2₁ as compared to C2 without the peptide. The peptide itself appears not to be ordered in the crystal, thereby not showing any electron density.

The structure was solved to a resolution of 2.1 Å and superposition with the crystal structure without protamine shows no big difference with an r.m.s.d. of 0.223 Å over the backbone atoms of one chain. In contrast to the structure without protamine, where the flexible loop between residues 110 and 123 could never be modeled, one additional residue (111) in chains B, C and D (residues 111 and 112) and two in chain E could be modeled.

**Figure 13:** Superposition of LmCld (red) and LmCld-protamine (cyan); r.m.s.d. of backbone atoms = 0.223 Å (834 to 834 atoms)
Table 15: Data collection statistics LmCld with protamine

<table>
<thead>
<tr>
<th>Source</th>
<th>BESSY ID 14-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>0.918410 Å</td>
</tr>
<tr>
<td>Space group</td>
<td>P2_1</td>
</tr>
<tr>
<td>Unit cell</td>
<td>a = 78.06 Å; b = 128.40 Å; c = 78.22 Å; β = 105.9°</td>
</tr>
<tr>
<td>Molecules/a.u.</td>
<td>5</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>161585 (17049)</td>
</tr>
<tr>
<td>Resolution</td>
<td>37.18 – 2.09 Å (2.15 – 2.09)</td>
</tr>
<tr>
<td>Completeness</td>
<td>99.7% (99.9%)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.19 (4.29)</td>
</tr>
<tr>
<td>I/s(I)</td>
<td>6.5 (2.0)</td>
</tr>
<tr>
<td>CC1/2</td>
<td>98.2* (70.1*)</td>
</tr>
<tr>
<td>R meas</td>
<td>20.3% (79.0%)</td>
</tr>
<tr>
<td>Temperature</td>
<td>100 K</td>
</tr>
</tbody>
</table>

Table 16: Refinement statistics LmCld with Protamine

| R work | 0.217 |
| R free | 0.261 |
| RMSD bonds | 0.014 Å |
| RMSD angles | 1.452° |
| Avg B-factor | 22.9 Å² |

6.4. LmCld with anomalous scatterers

Most stabilizing compounds identified in the thermal shift assay were divalent cations (see section 6.1). Almost all of them exhibited X-ray absorption at wavelengths close to their absorption edge. By appropriate choice of wavelength their positions in the crystal can be determined by exploiting their anomalous signal.

Of the 14 conditions that increased the thermal stability of LmCld six were chosen for crystallization that covered all the stabilizing components. Additionally, LmCLd was co-crystallized with Fe(III) although it did not show stabilizing
effects. In the very sensitive ICP-MS approach traces of iron were detected (see section 6.5.3), that is why it was also selected.

For crystallization conditions see table 9.

The search for a putative cofactor for LmCld by screening additives was based on the assumption that a specific cofactor would bind at specific sites and also stabilize the structure.

Only one additive was found to bind at specific sites, which was Ba$^{2+}$. For all the other anomalous scatterers the stabilizing effects seems to be unspecific and those could not be located.

Addition of BaCl$_2$ led to an increase in thermal stability from 53°C to 68°C. Ba$^{2+}$ was found to bind once to each subunit, always in the same position. It replaces a water molecule in the tight turn between β-strands 2 and 3, coordinated by backbone carbonyl-oxygens and by Asp69 (see Figure 14). Since the aspartic acid in this turn is conserved also in other ClDs (both validated and non-validated), it can be speculated that those might also be stabilized by Ba$^{2+}$.

Nonetheless the biological relevance of this binding is probably negligible. The tight turn between beta-strands 2 and 3 is approximately 28 Å away from the conserved histidine, which marks the reaction site for canonical ClDs.

At the energy used for these data (6,200 keV, see Table 17) the sulfur atoms are also visible at a sigma level of 4, whereas Ba$^{2+}$ still gives signal above sigma = 8. The anomalous signal for sulfur atoms provides an intrinsic proof of the power of this method.
Figure 14: Location of \( \text{Ba}^{2+} \) co-crystallized with LmCld contoured at 4 sigma

Table 17: Contributions to anomalous scattering of barium and sulfur at 6200 keV

<table>
<thead>
<tr>
<th></th>
<th>( f' )</th>
<th>( f'' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barium</td>
<td>-5.75</td>
<td>12.53</td>
</tr>
<tr>
<td>Sulfur</td>
<td>0.38</td>
<td>0.89</td>
</tr>
</tbody>
</table>

6.5. Native LmCld

LmCld expressed in its natural environment, i.e. in the *Listeria monocytogenes* lab strain LO28, was purified by immuno-precipitation as described in section 5.19. The presence of heme was tested by several means. In order to check for factors that might not be available during recombinant expression in *E. coli* several mass spectrometry approaches and X-ray fluorescence measurement were employed.
6.5.1. Heme-staining of native LmCld

Native LmCld was eluted from the beads in heme-staining sample buffer and run on a polyacrylamide gel with the buffer TBE with subsequent heme staining as described in section 5.20. In parallel the same samples were applied to conventional SDS-PAGE. The absence of SDS in the running buffer TBE results in weak penetration of the separation gel and therefore weak separation. Nonetheless the method is very sensitive with a limit of detection of 40 pmol heme. This was confirmed by a dilution series of NdCld (on the right part of the gels). Heme migrates at the dye front and appears green after the staining procedure. Comparing the protein amounts needed for sufficient signal, the immuno-precipitated LmCld in lane annotated “Native LmCld” would give a high signal if heme was actually bound, even at a low ratio. For comparison also myoglobin with non-covalently bound heme and cytochrome c covalently bound to heme were applied.
Figure 15: Heme-staining of native LmCld; top left: Coomassie blue staining after heme-staining (bottom, same gel); top right: same samples in conventional SDS-PAGE

6.5.2. X Ray Fluorescence

The spectra of eluted native LmCld, recombinant LmCld and NdCld were collected at ESRF on ID 14-4 by Sandor Brockhauser. NdCld shows peaks at Fe-edges (at 6.4 and 7.1 keV, see Figure 16) whereas neither native nor recombinant LmCld does. LmCld samples show peaks at 2.62 keV which indicates the not surprising presence of chlorine (LmCld in TBS and NdCld in HEPES buffer). All samples show peaks for sulfur at 2.3 keV, possibly calcium at 3.69
keV, as well as an indication to the presence of possibly titanium, iodine or tellurium at 4.51 keV.

**Figure 16**: X-ray fluorescence spectra of NdCl\(_d\) (top left), recombinant LmCl\(_d\) (top right) and native LmCl\(_d\) (bottom)

6.5.3. Inductively Coupled Plasma Mass spectrometry

A more sensitive method to detect trace elements is ICP-MS (Kretschy et al., 2012). The ionization process atomizes the sample and can in principle detect any element heavier than nitrogen. Measurements were done at BOKU Vienna, Department for Analytical Chemistry (special thanks to Stephan Hann and group for advice in sample preparation and measurements).

Native LmCl\(_d\), due to the immuno-precipitation together with IgG, was measured after a SEC column which removed trace elements only present in the buffer and not binding to the protein. Intriguingly, co-eluting with the peak of sulfur (measured as its oxide \(^{48}\)SO; which indicates presence of protein) iron was detected (see Figure 18). This was not the case when only IgG was measured as a negative control (see Figure 17).
Figure 17: $^{48}$SO IgG negative control SEC-ICP-MS

Figure 18: $^{56}$Fe trace of native LmCld SEC-ICP-MS

At a retention time of 13.1 min a high signal of iron was detected. Considering the retention time of IgG of 13.4 min this indicates to be the target LmCld together with IgG. Low signals of cobalt and aluminum were also detected (not shown).

6.5.4. Hydrophobic cofactor mass spectrometry

An approach to identify a cofactor that fits the hydrophobic active site cavity and confirm the absence of heme was to scan all components present in the sample by nanoESI-MSMS (BSRC Mass Spectrometry and Proteomic Facility St. Andrews). Native LmCld was digested off the beads with trypsin and the sample applied to a reverse phase column, on-line with nESI. The expected monoisotopic mass of heme is 616 m/z which gave no significant signal (Figure 19). Figure 20 shows the total ion count of the whole run. The extracted masses could not be assigned to a known cofactor.
6.6. Structure of NdCld W145F

Continuing the work of Kira Gysel (Gysel, 2011), another interesting NdCld mutant was crystallized and its structure determined by X-ray diffraction. The putative electron donor Trp145 was mutated to phenylalanine and its steady-state kinetics indicate that substrate binding is not hindered yet the catalytic efficiency suffers substantially from this mutation (Hofbauer, unpublished data). This decrease in activity is not caused by alteration of the structure, the phenylalanine is in the same plane as the tryptophan in the wildtype structure (see Figure 21).

Mutations of Arg173 of NdCld alters crystal packing from the primitive hexagonal space group P3$_2$1 of wildtype and mutants in which other residues were mutated to the base centered monoclinic space group C2 (Gysel, 2011; Kostan et al., 2010). Interestingly, the mutation of Trp145 to phenylalanine also resulted in C2 crystals.
Again the observation that despite being structurally highly similar (r.m.s.d. of backbone atoms = 0.265 Å (867 to 867 atoms)) to the wildtype structure the resolution does not exceed 2.8 Å (compared to 1.8 Å of the wildtype structure) when I/sigma (I) is used as the cut-off parameter.

Here the XDS parameter C1/2 (Karplus and Diederichs, 2012) was used and made it possible to reach a slightly higher resolution of 2.58 Å at R_free = 0.27.

**Figure 21:** NdCl_d W145F (3NN1, blue) superposed with wildtype NdCl_d (green)
Table 18: Data collection statistics of NdCld W145F

<table>
<thead>
<tr>
<th>Source</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>0.8726 Å</td>
</tr>
<tr>
<td>Space group</td>
<td>C2</td>
</tr>
<tr>
<td>Unit cell</td>
<td>a = 138.30 Å, b = 112.62 Å, c = 120.32 Å, β = 118.48°</td>
</tr>
<tr>
<td>Molecules/a.u.</td>
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</tr>
<tr>
<td>Unique reflections</td>
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<tr>
<td>Resolution</td>
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<tr>
<td>Completeness</td>
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<td>Redundancy</td>
<td>3.24 (2.19)</td>
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<tr>
<td>I/s(I)</td>
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<tr>
<td>CC1/2</td>
<td>99.9* (44.0*)</td>
</tr>
<tr>
<td>R \text{meas}</td>
<td>6.8% (134.8%)</td>
</tr>
<tr>
<td>Temperature</td>
<td>100 K</td>
</tr>
</tbody>
</table>

Table 19: Refinement statistics of NdCld W145F

| R \text{work}       | 0.197                      |
| R \text{free}       | 0.229                      |
| RMSD bonds          | 0.013 Å                    |
| RMSD angles         | 1.489°                     |
| Avg B-factor        | 79.7 Å²                    |

6.7. Structure of NdCld W145F in complex with cyanide

The structure of NdCld W145F was also solved in complex with its inhibitor cyanide. Surprisingly the addition of inhibitor did not improve the statistics, still the two structures are highly similar with an r.m.s.d. = 0.230 Å (881 to 881 atoms).
Table 20: Data collection statistics of NdCld W145F with cyanide

<table>
<thead>
<tr>
<th>Source</th>
<th>ESRF ID 23-1</th>
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<tbody>
<tr>
<td>Wavelength</td>
<td>0.8726 Å</td>
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<tr>
<td>Space group</td>
<td>C2</td>
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<tr>
<td>Unit cell</td>
<td>a = 136.16 Å b = 113.40 Å c = 118.80 Å; β = 117.9°</td>
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<tr>
<td>Unique reflections</td>
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<tr>
<td>Resolution</td>
<td>43.45 – 2.58 Å (2.64 - 2.58)</td>
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<tr>
<td>Completeness</td>
<td>89.9% (44.8%)</td>
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<tr>
<td>Redundancy</td>
<td>7.19 (3.63)</td>
</tr>
<tr>
<td>I/s(I)</td>
<td>10.2 (0.4)</td>
</tr>
<tr>
<td>CC1/2</td>
<td>99.8* (13.7*)</td>
</tr>
<tr>
<td>R meas</td>
<td>14.9% (376.7%)</td>
</tr>
<tr>
<td>Temperature</td>
<td>100 K</td>
</tr>
</tbody>
</table>

Table 21: Refinement statistics of NdCld W145F with cyanide

| R_work                  | 0.214                               |
| R_free                  | 0.269                               |
| RMSD bonds              | 0.015 Å                             |
| RMSD angles             | 1.644°                              |
| Avg B-factor            | 85.1 Å²                             |

6.8. Oligomeric state of different Clds

The $M_W$ and therefore the oligomeric state of various Clds was measured as described in section 5.25. Mlynek et al. (Mlynek et al., 2011) solved the structure of NwCld which is a truncated version of active Cld by X ray diffraction and found it to be dimeric. Kira Gysel (Gysel, 2011) reported the hexameric structure of apoNdCld whereas the holoform wildtype and mutants are arranged as pentamers in the crystal. To test whether these findings were crystallographic artefacts the $M_W$ in solution was monitored by SLS. This was done
in collaboration with Kira Gysel and also reported in her Diploma Thesis (Gysel, 2011).

The newly purified SsCld was found to elute close to the void volume on an Superdex200 16/60 SEC column. Compared to pentameric NdCld this indicates a higher oligomeric state. To measure the exact $M_W$ SsCld was also applied to SLS.

The $M_W$ of NwCld in solution corresponds to the expected dimeric $M_W$ (Figure 22) of approximately 45 kDa. ApoNdCld appears in solution as a pentamer with $M_W = 135$ kDa (Figure 23), meaning that the hexameric crystal structure was caused by the crystallization conditions. SsCld on the other hand was found to have a $M_W$ between 210 and 220 kDa corresponding to at least a heptameric or even octameric organization (would be ca 230 kDa).

Although no heptameric or octameric structure has been reported by now, it can be speculated that higher oligomerization is important for stability. Hofbauer et al. (Hofbauer S, 2012) reported a large difference in thermal stability between the dimeric NwCld ($T_M = 53^\circ$C) and the pentameric NdCld ($T_M = 92^\circ$C). The thermophile archaeon *Sulfolobus solfataricus* grows optimally at 80°C (Zaparty et al., 2010), at this temperature requiring high thermostability for all its components.
Figure 22: SLS profile of dimeric NwClD (Gysel, 2011)

Figure 23: SLS profile of pentameric apoNdClD (Gysel, 2011)
6.9. Heme content of SsCld

The Reinheitszahl RZ of heme-binding proteins can be determined by UV-vis spectroscopy. It is defined as $A_{\text{soret}}/A_{280}$ and reflects the ratio in which heme binds to the protein. The theoretical Reinheitszahl (100% heme occupation) of SsCld is 1.6, the Soret peak is at 401 nm and the RZ was determined to be 1.3 (see Figure 25) meaning that 82% of the proteins subunits are occupied with heme. The Soret peak is red-shifted compared to NdCld with a Soret maximum at 408 nm, indicating a different heme environment.

Reduction of SsCld by addition of dithionite results in a shift of the Soret maximum from 401 nm to 417 nm. Similar soret maxima of reduced and oxidized heme proteins have been reported (Maixner et al., 2008; Stenklo et al., 2001; van Ginkel, 1996).
Figure 25: UV-vis spectrum of SsCld

For comparison, the UV-vis spectrum of NdCld was taken.

Figure 26: UV-vis spectrum of NdCld
6.10. Steady-state kinetics

The function of Cld-like proteins is still not known. A representative of the archaeal phylum containing a serine residue instead of arginine in active Clds is SsCld. The protein was purified as described in section 5.15, its heme content determined (see section 6.9) and its Cld- and catalase-activity was measured (see Section 6.10).

SsCld, lacking the key residue arginine of active Clds, showed no production of molecular oxygen when the substrate chlorite was added. Since the measurements were taken at 35°C, residual Cld-activity might still play a small role at the optimal temperature of *Sulfolobus solfataricus* growth (80°C). Due to the experimental requirements of the Clark-type electrode the highest temperature available (45°C) was used from this point on.

When adding H₂O₂ as a substrate at 45°C, O₂ production can be observed in a concentration- and pH-dependent manner. This means that the reaction is being catalyzed by SsCld, although the production rate is still weak compared to canonical catalases. The highest activity was measured at pH 9 (see figure 27). Again, this weak activity might get stronger when approaching the natural habitat's temperature of *Sulfolobus solfataricus*. K_{cat} and K_{M} were not calculated because of too few data points.
**Figure 27:** Temperature- and pH-dependent catalase activity of SsCld; Oxygen release plotted against H$_2$O$_2$ concentration Top: pH 5.5; middle pH 6.25; bottom: pH 9
6.10. Steady-state kinetics of the NdCld mutant W145F

The enzymatic parameters of NdCld W145F were measured by Stefan Hofbauer as described in section 5.24.

Table 22: Comparison of steady-state kinetics of active Clds and mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>KM [µM]</th>
<th>Kcat [s⁻¹]</th>
<th>Kcat/KM [M⁻¹ s⁻¹]</th>
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<tbody>
<tr>
<td>NdCld (wt) + imidazole</td>
<td>58 +/- 37</td>
<td>35</td>
<td>6.0 x 10⁵</td>
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<tr>
<td>NdCld (wt)</td>
<td>52 +/- 7</td>
<td>42</td>
<td>8.1 x 10⁵</td>
</tr>
<tr>
<td>NdCld W145F</td>
<td>172 +/- 10</td>
<td>20</td>
<td>1.2 x 10⁵</td>
</tr>
<tr>
<td>Azospira oryzae GR1 Cld</td>
<td>170</td>
<td>1200</td>
<td>7.1 x 10⁵</td>
</tr>
</tbody>
</table>

The mutation of the putative electron donor Trp145 to phenylalanine does not hinder substrate binding. The turnover rate however suffers from this exchange and Kcat/KM drops five fold. This could be the result of decreased availability of electrons necessary for the reaction.
7. Discussion

The function of Cld-like proteins is still an open question. With the representative of the lineage including human pathogens, Cld-like protein from *Listeria monocytogenes*, it was first shown that heme is not a cofactor even in *Listeria monocytogenes* itself and that the absence of this prosthetic group in crystal structures is no artefact of recombinant production. Mimicking the active site of LmCld (W146Y and R173Q) in the active Cld from *Nitrospira defluvii* drops the enzymatic activity but this double mutant is still able to decompose chlorite probably due to the ability to bind heme (Gysel, 2011). The absence of heme in LmCld is most likely caused by the tilting of alpha-helix 3' and other residues that sterically hinder heme binding (Y147, G178, I215, M219; see section 6.2). Although the results presented here may seem contradictory, as XRF did not show presence of iron but the more sensitive method ICP-MS did, the mass over charge ration (m/z) of heme was never observed in any mass spectrometry measurement of native LmCld. Apart from that, no heme could be detected by heme-staining of native LmCld. In addition, neither a mass shift for covalently bound heme nor other post translational modifications of native LmCld was found. The origin of the signal for elemental iron in ICP-MS remains unclear and will need complementary methods to be proved since it apparently does not stem from heme. The related Cld from *Thermus thermophilus*, which also contains a glutamine key residue, could be reconstituted with heme (60% of subunits loaded when reconstituted at high temperatures) and shows then a weak Cld- and catalase-activity (Ebihara et al., 2005). This suggests that probably the whole “glutamine-cluster” of Clds might not bind heme and have a different function.

The screening for potential cofactors with recombinant LmCld was not successful, though general aspects of the correlation between crystallizability and thermal stability can be considered. Dupuex et al. (Dupeux F, 2011 ) reported increased likelihood for crystallization of proteins when crystallized in buffer conditions that elevated the protein's melting temperature. The statistics of LmCld and LmCld co-crystallized with the poly-cationic peptide protamine reveal that if better diffraction data is required, screening additives and different buffer conditions to increase thermal stability might be worth a try. For example, the overall atomic displacement parameters (or average B factors) of the LmCld-protamine structure are only half the value of LmCld crystallized alone. Yet the flexible loop from residues 111 – 123 was still not ordered in the protamine structure although in 3 of chains one residue more and in one chain two residues more could be modeled in agreement with the observed electron density in compliance with Ramachandran restraints.
The coordination of the barium ion in the tight turn between β strand 2 and 3 seems to be the only specific interaction mediated by divalent cations that are able to stabilize LmCld. The turn is made up by 5 residues and the ion is probably coordinated by backbone carbonyl-oxygens and the side chain oxygen of aspartic acid. If iron binds to the protein not as part of heme it would probably also be coordinated by “hard chelators” like the backbone carbonyl-oxygens. Still the biological function of such an interaction will be hard to interpret and the conservation of the cavity and the coordinating histidine have to be taken into consideration. The aspartic acid involved in Ba\(^{2+}\) coordination is conserved over many Clds and Cld-like proteins, providing means for potential stabilization and increased crystallizability also of other Clds.

LmCld was found to exhibit weak catalase-activity, too (Füreder, 2009). Due to the presence of canonical catalase in *Listeria monocytogenes* this function is probably negligible. Here we present weak catalase-activity of SsCld which in contrast to LmCld is able to bind heme. Intriguingly, all archaea containing a cld-gene are aerobic and do not have a gene for canonical catalase (Christa Schleper, personal correspondence). To test the activity at the temperature of optimal growth for *Sulfolobus solfataricus* a different protocol for measuring catalase-activity at high temperatures will have to be employed. The higher order in oligomerization of SsCld will have to be confirmed by complementary methods like X-ray diffraction structural analysis and the properties of the subunit-interfaces revealed might answer how higher oligomerization can influence the protein's stability. This would expand the results of (Hofbauer S, 2012).

The structure of mutated NdCld reported in this thesis is more of a confirmation that the decrease in enzymatic activity is not caused by structural deviations but by the decreased availability of electrons needed for the Cld-reaction. With this mutant it was possible to monitor the reaction since the turnover rate is much lower but binding of substrate is not impaired. It was possible to spectroscopically observe the intermediate state and also to induce the reaction when starting with the intermediate hypochlorite (Stefan Hofbauer, personal correspondence). The goal for crystallographers to trap the intermediate in a crystal will still be difficult due to remaining activity and resulting bleaching also with this mutant.
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### 11. Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AEX</td>
<td>Anion exchange chromatography</td>
</tr>
<tr>
<td>AoCld</td>
<td>Chlorite dismutase from <em>Azospira oryzae</em></td>
</tr>
<tr>
<td>AS</td>
<td>Additive Screen</td>
</tr>
<tr>
<td>Cl</td>
<td>Chlorite dismutase</td>
</tr>
<tr>
<td>DaCld</td>
<td>Chlorite dismutase from <em>Dechloromonas aromatica</em></td>
</tr>
<tr>
<td>DMB</td>
<td>3’,3’-dimethoxybenzidine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable region of IgG</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
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<td>Turnover rate</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
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<td>LB</td>
<td>Lysogeny broth</td>
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<td>LmCld</td>
<td>Chlorite dismutase from <em>Listeria monocytogenes</em></td>
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<tr>
<td>LN2</td>
<td>Liquid nitrogen</td>
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<td>m/z</td>
<td>Mass over charge ratio</td>
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<td>NdCld</td>
<td>Chlorite dismutase from <em>Nitrospira defluvi</em></td>
</tr>
<tr>
<td>nESI</td>
<td>nano Electrospray ionization</td>
</tr>
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<td>Ni-NTA</td>
<td>Nickel-ntriloacetic acid</td>
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<td>(Per)chlorate reductase</td>
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<td>Reverse phase</td>
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<td>Size exclusion chromatography</td>
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<td>TE</td>
<td>Tris, EDTA buffer</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
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<td>Melting temperature</td>
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<td>X-ray fluorescence</td>
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12. Acknowledgements

I want to thank everybody involved in my project, Kristina for providing a well functioning lab and the entire chlorite dismutase group: Kira, Julius, Georg, Christian, Paul, Stefan, Holger, Andrea and Christa. I would like to emphasize the importance of my tutors Georg, with whom it was always a pleasure talking about new developments in crystallography, Julius, who always had an open mind for my scientific problems and Kira, who introduced me to many methods used for this thesis. It was a pleasure working with you and letting your fascination infect me. Anita was always helpful with advice and action. Thanks to Claudia and every other member of my lab who supported me in data acquisition and processing. I would not have come this far without your help.

Thanks also to the NMR group for great coffee break conversations and barbecue, Stephanie Füreder for the marvelous antibodies, the groups of Christa Schleper, Thomas Decker and Stephan Hann.

Last but not least I want to thank my family and my girlfriend for supporting me throughout my studies.
Curriculum Vitae

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Publications


Presentations at Conferences

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