The Flavoprotein RCLA is a Hypothiocyanous Acid Reductase

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THE FLAVOPROTEIN RCLA IS A HYPOTHIOCYANOUS ACID REDUCTASE

by

Irina Chapman

A thesis submitted to the Graduate College in partial fulfillment of the requirements for the degree of Master of Science Chemistry Western Michigan University December 2021

Thesis Committee:

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Reactive chlorine species (RCS) - hypochlorous acid (HOCl), hypothiocyanous acid (HOSCN), and chloramines - are powerful antimicrobial oxidants generated by the innate immune system. Bacteria developed defense mechanisms against RCS, which are highly toxic for invading pathogens. One such defense system involves RclA, an enzyme from the flavin disulfide reductase family, which is massively upregulated upon exposure of *Escherichia coli* to RCS. Recent research suggests that RclA functions as a Cu(II) reductase to provide resistance against RCS. However, the exact mechanism used by RclA was subject to debate. In this study, we demonstrate that RclA is unlikely to function as a Cu(II) reductase. Instead, we show that RclA provides resistance against RCS by directly detoxifying HOSCN, reducing it to non-toxic thiocyanate (SCN⁻) using NAD(P)H via a ping-pong kinetic mechanism. RclA catalyzes the reduction of HOSCN with k_{cat} of 182 s⁻¹, and the specificity constant (k_{cat}/K_m) of 9.12 x 10⁷ M⁻¹ s⁻¹, indicating that RclA is a potent and highly specific HOSCN reductase. HOSCN is a primarily sulfhydryl-reactive oxidant, and RclA has two conserved cysteine residues at its active site. Thus, we hypothesize that RclA detoxifies HOSCN before it can target other thiols in the cell, and the enzyme’s active site cysteines are critical for its HOSCN reductase activity. Our study provides a rationale for how bacteria can cope with RCS produced by the immune system. Targeting RclA in bacteria found in oral cavity or lung airway fluids may make pathogenic bacteria more susceptible to the immune response, thus allowing the creation of new strategies for controlling pathogenic infections.
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Irina Chapman
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INTRODUCTION

Flavin-Dependent Enzymes

Flavin-dependent enzymes are a ubiquitous and diverse class of redox enzymes which are involved in many biological and biochemical processes. These enzymes, despite their diversity, share a number of common properties. Flavoenzymes employ flavins – the derivatives of riboflavin (vitamin B2) as redox active prosthetic groups, which in most cases constitute a noncovalent complex where the flavin is permanently bound to the enzyme. The two enzymatically active flavins are flavin mononucleotide (FMN), formed by phosphorylation of the ribityl chain of riboflavin, and flavin adenine dinucleotide (FAD), formed by adenylation of FMN (Figure 1).

FAD is a more common flavin cofactor, used by approximately 75% of flavoenzymes, whereas the remaining 25% use FMN. For both forms of flavin the heterocyclic isoalloxazine ring system serves as the chemically active portion of the cofactor due to its ability to undergo one- or
two-electron transfer reactions (Macheroux et al., 2011). While the side chains of the isoalloxazine ring, such as the adenine moiety of FAD, are not involved in the redox chemistry, they serve as an anchor that links the flavin to the active site (Ghisla & Massey, 1989).

Flavin naturally exists in five different catalytically active redox forms (Figure 2). Catalytically active species of flavin include oxidized flavin (Fl$_{ox}$), flavin semiquinone, reduced flavin, C$_{4a}$-peroxy flavin, and C$_{4a}$-hydroperoxy flavin. Flavin semiquinone is formed by the addition of a single electron to the oxidized form of flavin, whereas a two-electron reduction of oxidized flavin or a single-electron reduction of a semiquinone leads to the formation of a reduced form of flavin. The reduced flavin can engage molecular oxygen, forming peroxy flavin, which upon protonation affords hydroperoxy flavin (Baker Dockrey & Narayan, 2019; Mondal et al., 2020; Romero et al., 2018).

*Figure 2. Catalytically active species of flavin.*
In addition to being a redox agent, flavin’s isoalloxazine moiety is also a chromophore in all flavin derivatives (Mondal et al., 2020). Each redox state of the flavin possesses distinct absorbance spectra. Ligand binding also causes perturbations of the absorbance spectra due to charge transfer interactions that give rise to additional absorbance bands. Due to the tight noncovalent, or, in some cases, covalent binding of the flavin to the active site, the flavin prosthetic group can be considered a part of the enzyme. Therefore, the chemistry of the enzyme can be monitored by observing the absorbance and fluorescence of the flavin using UV-Vis spectrophotometry and stopped-flow techniques (Fagan & Palfey, 2010).

Due to the reactivity and the existence of several oxidation states of the isoalloxazine ring system, flavin-dependent enzymes are capable of catalyzing a diverse range of redox reactions in a wide variety of biological processes. In addition to the form of the cofactor, the mechanism of the enzyme is determined by the protein scaffold, reaction conditions, and the presence of other redox active sites, for example, metallocofactors or a redox-active disulfide (Hammerstad & Hersleth, 2021).

Typically, the catalytic cycle of flavin-dependent enzymes follows the ping-pong kinetic mechanism with distinct oxidative and reductive half-reactions (Fagan & Palfey, 2010) (Figure 3). In the reductive half-reaction, the oxidized flavoenzyme undergoes reduction by the first, electron-rich, substrate. The resulting reduced enzyme is reoxidized by the second, electron-poor, substrate in the oxidative half-reaction. The two half-reactions can be studied independently using the chromophore of the isoalloxazine ring system as an absorbance or fluorescence probe in order to monitor the kinetics and determine the nature of intermediates in both half-reactions (Fagan & Palfey, 2010; Walsh, 1980).
Flavoprotein Disulfide Reductases

Flavoprotein disulfide reductases (FDRs) represent a family of flavin-dependent oxidoreductases that share distinct structural homology and mechanistic features. At the active site of these enzymes, tightly, but noncovalently bound flavin is complemented by a cysteine-based thiol/disulfide redox center. The flavin catalyzes oxidation or reduction of one of the substrates, while the active site cysteines mediate two-electron transfer between the flavin and a second substrate. A reduced cysteine-based center can transfer electrons to oxidized substrates; whilst substrates can drive electrons to an active site cysteine disulfide, with the subsequent transfer of the electrons to the flavin. For most FDRs, the transfer of electrons between flavin and the disulfide occurs via a transient covalent flavin C₄₆-S-Cys adduct (Landry et al., 2021; Miller, 2013).

Figure 3. Typical catalytic cycle of flavin-dependent enzymes.
According to the most recent classification of FDRs, presented by Miller (2013) and Argyrou and Blanchard (2004), flavoprotein disulfide reductases can be subdivided into five distinct groups based on structural, functional, and mechanistic features: Group 1 are classic dithiol/disulfide oxidoreductases with a single CXXXXC disulfide redox center; enzymes in Group 2A exhibit Group 1 structural fold, but possess an additional C-terminal Cys-based redox center; Group 2B FDR enzymes are represented by low Mr thiorexin reductase (TrxR) and structurally related enzymes, which have a CXXC redox center in the pyridine nucleotide-binding domain; Group 3 FDRs feature cysteine sulfenic acid or mixed Cys-S-S-CoA redox center; Group 4 FDRs have Group 1-fold, but catalyze novel reactions; and Group 5 FDR enzymes are sulfide:quinone oxidoreductases which have a si side pair of Cys residues that are widely separated in sequence and located in different domains, but are spatially proximal (Argyrou & Blanchard, 2004; Landry et al., 2021; Miller, 2013).

**Group 1 Structural Fold FDRs**

Group 1 flavoprotein disulfide reductases are represented by glutathione reductase, dihydrolipoamide dehydrogenase, trypanothione reductase, and mycothione reductase. Group 1 FDRs use two redox centers - FAD and a redox-active disulfide - to perform pyridine nucleotide-dependent reduction of their substrates. The mechanism of these enzyme involves electron transfer from NAD(P)H to the second substrate, typically a disulfide substrate, via a tightly, but noncovalently bound FAD. FAD reacts directly only with the pyridine nucleotide substrate, while the cysteine-based disulfide redox center mediates electron transfer between the flavin and the second substrate of the enzyme (Argyrou & Blanchard, 2004; Miller, 2013).

Group 1 FDRs feature four distinct domains: an FAD-binding domain, a pyridine nucleotide-binding domain, a central domain, and an interface domain. The FAD-binding domain
contains the CXXXXC motif of the redox-active disulfide, and the interface domain contains the essential HXXXXE motif which interacts with the CXXXXC motif and promotes the disulfide formation with the histidine residue. All Group 1 FDRs have two identical active sites and are obligate homodimers, as both active sites are composed of the FAD domain, the pyridine nucleotide domain, and the central domain of one monomer and the interface domain of the other monomer. Both the NAD(P)H binding site and the disulfide are found within the FAD-binding domain of the same monomer. Additionally, both monomers contribute to the binding and specificity of the non-pyridine nucleotide substrate via the residues that surround the disulfide redox center. Thus, only a dimer serves as a catalytically active entity (Argyrou & Blanchard, 2004; Baek et al., 2020; Landry et al., 2021; Miller, 2013).

The isoalloxazine ring of the flavin divides the active site of Group 1 FDRs into a reductive half, and an oxidative half. The pyridine nucleotide binding site is located on the re face of the isoalloxazine ring, while the redox-active disulfide is situated on its si face. In the reductive half, the transfer of electrons occurs via the movement of C4-proS hydride from NAD(P)H to N5 of the flavin. In the oxidative half of the active site, the electrons are transferred between the redox-active disulfide and the non-pyridine nucleotide substrate. Thus, the overall movement of electrons occurs from the pyridine nucleotide to FAD, to redox-active disulfide, to the second substrate. Since the substrates for glutathione reductase, dihydrolipoamide dehydrogenase, trypanothione reductase, and mycothione reductase contain disulfides, the reduction of the disulfide substrate in the oxidative half of the active site occurs via dithiol–disulfide interchange acid-catalyzed by the His-Glu pair of the interface domain (Figure 4) (Argyrou & Blanchard, 2004; Miller, 2013).

A distinctive feature of Group 1 FDRs is the existence of a two-electron reduced intermediate - the EH₂ state of the enzyme, in addition to the fully oxidized E₉ox and 4-electron
Figure 4. General reaction mechanism of Group 1 flavoenzyme disulfide reductases.

Reduced EH$_4$ states (Figure 5). All three states, as well as the complexes of E$_{ox}$ and EH$_2$ with bound pyridine nucleotides exhibit distinct spectrophotometric spectra which can be detected using anaerobic spectrophotometric titrations, therefore allowing monitoring of the mechanism of the enzymatic reactions (Argyrou & Blanchard, 2004; Fagan & Palfey, 2010; Miller, 2013).

Due to the presence of two separate binding sites for the oxidative and reductive substrates, Group 1 FDRs are capable of reacting either via a ping-pong mechanism, or via a ternary complex mechanism. In most cases, Group 1 FDRs exhibit preference for a ping-pong mechanism, where the two substrates react separately in the reductive and oxidative half-reactions that involve E$_{ox}$.
and EH₂ forms of the enzyme. However, in some cases the reaction occurs sequentially via a ternary complex mechanism, where both substrates bind and react, followed by the dissociation of the product (Figure 6). Typically, the mechanism switch occurs with the variation of the concentration of substrates and products, or if a mutant form of the enzyme is used. In case of the sequential mechanism, enzymes which physiologically function as reductases oscillate between EH₂ and EH₄ state, whereas physiological dehydrogenases cycle between E⁰ and EH₂ states, becoming inhibited in EH₄ state (Argyrou & Blanchard, 2004; Miller, 2013).

Group 2A flavoprotein disulfide reductases, represented by the high-molecular-weight form of thioredoxin reductase, mercuric reductase, and thioredoxin glutathione reductase, exhibit classical fold characteristic for Group 1 enzymes, and are obligate homodimers, but feature an additional C-terminal cysteine-based redox center which accounts for differences in mechanisms between Group 1 and Group 2A FDRs (Argyrou & Blanchard, 2004; Miller, 2013).

Glutathione Reductase

Glutathione reductase catalyzes NADPH-dependent reduction of glutathione disulfide (GSSG) to its reduced form GSH (Equation 1).

\[
\text{NADPH} + H^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2\text{GSH} \quad \text{Eq.1}
\]
Figure 6. Catalytic cycles for Group 1 flavoprotein disulfide reductases
Reduced glutathione plays a critical role in resisting oxidative stress via nonenzymatic reactions with ROS and maintains the reducing environment of the cell by serving as a reductant of abnormal disulfide bonds between proteins and between small thiol-containing molecules. Glutathione reductase assists with maintaining high levels of GSH relative to GSSG (Argyrou & Blanchard, 2004; Deponte, 2012; Rietveld et al., 1994; Vanoni et al. 1990).

**Structure**

Like other Group 1 flavoenzyme disulfide reductases, GR consists of two nearly symmetrical subunits and is a homodimer, with the residues for each of the two active sites contributed by both monomers, making only the dimer a catalytically active entity. The active site of GR is divided by the isoalloxazine ring of FAD into two distinguished NADPH and GSSG binding sites, which are both located in the FAD-domain of the first monomer. The His-Glu pair that is essential for catalysis of the oxidative half-reaction is contributed by the interface domain of the second monomer. A tyrosine residue at the re side of the FAD shields the flavin in the absence of pyridine nucleotide and assists with the substrate binding (Figure 7) (Argyrou & Blanchard, 2004; Deponte, 2012).

**Mechanism**

Glutathione reductase typically follows the ping-pong kinetic mechanism. The catalytic cycle of GR consists of a reductive half-reaction, where the enzyme oxidizes NADPH to NADP+, getting reduced to the EH₂ state, and an oxidative half-reaction, in which GSSG is reduced to GSH via a thiol/disulfide interchange reaction, while the enzyme is regenerated to its oxidized state. The oxidative half-reaction is acid catalyzed by His-Glu pair contributed by the interface domain of the second monomer (Argyrou & Blanchard, 2004; Deponte, 2012).

In the reductive half-reaction, NADPH binds rapidly to the pyridine nucleotide-binding
Figure 7. Structure of E. coli glutathione reductase. a, Cartoon structure of the GR homodimer with bound FAD and NADP⁺. Monomer A is depicted in green and monomer B is depicted in cyan. FAD is depicted in red, and NADP is depicted in blue. b, Active site structure with bound NADP⁺. The subscripts denote which monomer the residues belong to. (PDB ID: 1GET)

site and reduces FAD to FADH⁺ by a hydride transfer. The electrons from FADH⁺ are moved to the proximal cysteine (charge transfer thiol), forming a C4a-flavin adduct that serves as a charge-transfer complex, while the distal cysteine (interchange thiol) is protonated by the active site histidine. The reductive half-reaction is concluded by the dissociation of NADP⁺ from the two-electron reduced enzyme, and the binding of a second NADPH molecule (Argyrou & Blanchard, 2004; Deponte, 2012).

In the oxidative half-reaction, GSSG binds to the two-electron reduced GRH₂, assisted by conserved active site residues. The interchange thiol of GRH₂, deprotonated by the histidine residue, attacks GS₁ of the substrate, forming an intermolecular mixed-disulfide (MDS) intermediate. The GS₂ is protonated by the histidine, and the resulting GSH molecule leaves the active site. The free thiolate of the charge transfer thiol then attacks the MDS, completing reduction of glutathione, expelling the second GSH molecule, and regenerating GR₀ (Figure 8) (Deponte (Argyrou & Blanchard, 2004; Deponte, 2012; Fagan & Palfey, 2010; Rietveld et al., 1994).
Figure 8. Catalytic cycle of glutathione reductase.

**Dihydrolipoamide Dehydrogenase**

Dihydrolipoamide dehydrogenase (LipDH) functions as the E3 component of the pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched chain 2-oxoacid dehydrogenase, and glycine reductase multienzyme complexes, which are essential for sugar and amino acid metabolism. In vivo, LipDH catalyzes the reoxidation of dihydrolipoamide, which is covalently attached via amide linkage to specific lysine residues in the acyltransferase (E2) components of the multienzyme complexes, or to H-protein in glycine reductase. Physiologically, LipDH functions in the dehydrogenase direction, with its dithiol/disulfide substrate being oxidized, and NAD$^+$ being reduced to NADH. The overall reaction catalyzed by LipDH is shown in Equation 2.
Dihydrolipoamide + NAD\(^+\) \rightarrow \text{Lipoamide} + \text{NADH} + H^+ \quad \text{Eq. 2}

In its isolated form, LipDH functions largely the same as it does within the multienzyme complexes (Argyrou & Blanchard 2001, 2004; Fagan & Palfey, 2010; Patel et al., 2000).

**Structure**

Dihydrolipoamide dehydrogenase is structurally homologous to GR, and is also a native homodimer, which features the motifs characteristic for Group 1 FDRs, such as CXXXXC motif in the FAD-binding domain and the HXXXXE motif in the interface domain. The active site of LipDH is similar to that of glutathione reductase and is divided into NAD\(^+\) and dihydrolipoamide binding sites by the isoalloxazine ring of the flavin. The redox-active disulfide is found on the si face of the FAD along with a His-Glu pair that is involved in acid/base chemistry. Similarly to GR, a tyrosine residue covers the NAD\(^+\)-binding site in the absence of the pyridine cofactor (Figure 9) (Argyrou & Blanchard 2004; Billgren et al., 2010; Fagan & Palfey, 2010).

**Figure 9.** Structure of *B. stearothermophilus* dihydrolipoamide dehydrogenase. a, Cartoon structure of the LipDH homodimer with bound FAD. Monomer A is depicted in green, monomer B is depicted in cyan, and FAD is depicted in red. b, Active site structure. The subscripts denote which monomer the residues belong to. (PDB ID: 1EBD)

**Mechanism**

The chemical mechanism of dihydrolipoamide dehydrogenase in the reductase direction is similar to that of glutathione reductase. However, physiologically LipDH acts as a dehydrogenase,
with the direction of the reaction opposite to that of GR. Therefore, in physiological direction the enzyme is first reduced by dihydrolipoamide, yielding lipoamide, and then reoxidized by NAD$^+$ which is reduced to NADH (Equations 3 and 4).

$$E_{\text{ox}} + \text{Lip (SH)$_2$} \rightleftharpoons \text{EH}_2 + \text{LipS}_2 \quad \text{Eq. 3}$$

$$\text{EH}_2 + \text{NAD}^+ \rightleftharpoons E_{\text{ox}} + \text{NADH} + H^+ \quad \text{Eq. 4}$$

In the reductive half-reaction, dihydrolipoamide binds to the fully oxidized LipDH, and reduces its redox active disulfide bond via formation of a mixed disulfide with the participation of the interchange thiol of the enzyme. The disulfide interchange is followed by charge transfer interaction between the oxidized flavin and a charge transfer thiolate anion, yielding the red-colored two-electron reduced intermediate. The formation of mixed disulfide intermediate and the release of the oxidized substrate are mediated by the general acid/base properties of the histidine residue provided by the interface domain. The reduction of LipDH to its EH$_2$ state is completed upon release of lipoamide and reformation of the redox active disulfide bond.

In the oxidative half-reaction, NAD$^+$ binds to the two-electron reduced form of the enzyme, which promotes an internal charge transfer between the redox active disulfide and the flavin via a transient covalent C4a-flavin adduct. NAD$^+$ oxidizes the flavin by direct hydride transfer, resulting in the reduction of the pyridine nucleotide to NADH and the regeneration of the fully oxidized state of the enzyme (Figure 10) (Billgren et al., 2010; Hopkins & Williams, 1995).

Typically, during catalysis LipDH oscillates between the fully oxidized E$_{\text{ox}}$ state, and the two-electron reduced EH$_2$ state. However, LipDH in some species, specifically E. coli, is susceptible to being reduced by excess NADH or dihydrolipoamide to the fully reduced EH$_4$ state, which is catalytically inactive (Hopkins & Williams, 1995). In this case, NAD$^+$ acts as an activator,
which induces the active form of the enzyme by partially overcoming the inhibition via reversal of this reduction. Additionally, NAD$^+$ prevents accumulation of the inactive four-electron reduced LipDH by reversing the reduction of EH$_2$ form of the enzyme by NADH (Argyrou & Blanchard, 2001; Matthews & Williams, 1976; Sahlman & Williams, 1988; Wilkinson & Williams, 1981).

**Mercuric Reductase**

Mercuric reductase (MerA) is a cytosolic enzyme, which facilitates bacterial resistance to mercury compounds by reducing Hg$^{2+}$ from Hg(SR)$_2$ substrates to the less toxic volatile elemental mercury, Hg$^0$, using NADPH as a source of reducing power. The overall reaction catalyzed by MerA is shown in Equation 5:
RS-Hg-SR + NADPH + H+ → Hg0 + 2RSH + NADP+ \hspace{1cm} \text{Eq. 5}

(Keirsse-Haquin et al., 2017; Fox & Walsh, 1982; Miller, 2013).

Structure

MerA, a Group 2A FDR that possesses the Group 1 structural fold, is strongly homologous to glutathione reductase based on sequence alignment and structural analysis. Like GR, MerA is a native homodimer, although it may also exist as a tetramer (Keirsse-Haquin et al., 2017). In addition to the inner cysteines, however, MerA also possesses C-terminal cysteines, with the two pairs of cysteines being contributed by different monomers. The cysteines are located at the Hg\textsuperscript{2+}-binding site on the \textit{si} face of the FAD, while NADPH binds at the \textit{re} face. Similarly to GR and LipDH, the active site of MerA features a tyrosine residue, which shields the NADPH-binding site in the absence of the substrate. The histidine which mediates acid/base chemistry in GR and LipDH, however, is replaced by another tyrosine in MerA (Fagan & Palfey, 2010). MerA from the \textit{Tn501} operon, as well as all MerA proteins from operons found in \textgamma-proteobacteria, contain an additional N-terminal domain (NmerA) with a GMTCXXC motif characteristic of heavy metal binding domain (Figure 11) (Fagan & Palfey, 2010; Hong et al., 2014; Ledwidge et al., 2005; Miller, 2013).

Mechanism

Since the active site of MerA contains four redox active thiols, the enzyme exhibits four distinct redox states: fully reduced EH\textsubscript{4} state, with the flavin and both disulfides being reduced; EH\textsubscript{2} state, where FAD is oxidized, but the two disulfides are reduced; E\textsubscript{ox} state, where the flavin and the inner disulfide are oxidized, and the C-terminal disulfide is reduced; and a nonactivated E\textsubscript{ox} state, where the flavin and both disulfides are oxidized (Fagan & Palfey, 2010).

The presence of additional cysteine pairs in MerA accounts for mechanistic differences
between mercuric reductase and Group 1 FDRs. While in Group 1 FDRs the inner cysteines cycle between $E_{\text{ox}}$ and $E_{\text{H2}}$ states, in MerA these cysteines remain reduced throughout the catalytic cycle and participate in $\text{Hg}^{2+}$ binding (Ledwidge et al., 2005; Miller et al., 1989). The catalytic cycle occurs between $E_{\text{H2}}$ state and the states that involve $E_{\text{H2}}$ bound to a reduced or oxidized form of a pyridine nucleotide (Fagan & Palfey, 2010; Miller et al., 1986), although it has been proposed that the catalytic cycle occurs between the $E_{\text{H2}}$ and $E_{\text{H4}}$ states (Argyrou & Blanchard, 2004). N-terminal cysteines scavenge $\text{Hg}^{2+}$ in the cellular milieu and assist with the delivery of mercury substrates to the catalytic core of the protein (Engst & Miller, 1998; Fagan & Palfey, 2010; Hong et al., 2014; Johs et al., 2011; Ledwidge et al., 2005).

For the catalysis to proceed, MerA needs to be primed with NADPH in order to generate the $E_{\text{H2}}$•NADPH intermediate. First, NADP+ from the previous turnover leaves the active site to be replaced with NADPH. NADPH rapidly forms a charge-transfer complex with the oxidized flavin, then a charge-transfer complex is formed between the oxidized flavin and the thiolate...
(Fagan & Palfey, 2010), after which Hg(II) substrate enters the active site (Argyrou & Blanchard, 2004; Keirisse-Haquin et al., 2017).

As suggested by Argyrou and Blanchard (2004), the bound NADPH reduces the FAD to FADH$_2$ to generate the EH$_4$•NADP$^+$•Hg(II) intermediate, which reduces the Hg(II) substrate to Hg$^0$. Johs et al. (2011) propose a mechanism of delivery of the Hg(II) substrate from N-terminal cysteines to C-terminal cysteines via a tricoordinate intermediate, with the subsequent transfer of the substrate from C-terminal cysteines to the inner thiols, where the reduction of Hg$^{2+}$ to Hg$^0$ occurs, followed by the release of the reduced substrate and NADP$^+$ (Johs et al., 2011; Ledwidge et al., 2005). Lian et al. (2014) suggest that the transfer of Hg$^{2+}$ substrate from C-terminal thiols to inner thiols also occurs via a transient tricoordinate intermediate. It is implicated that two tyrosine residues in the active site also play a role in the coordination of the substrate (Figure 12) (Argyrou & Blanchard, 2004).

Due to the necessity of pyridine nucleotide binding for the mercury reduction to occur, the catalytic cycle of MerA occurs via a ternary complex, rather than a ping-pong mechanism (Sahlman et al., 1984), which is another characteristic feature of mercuric reductase that distinguishes it from Group 1 flavoenzyme disulfide reductases.

**RclA - A Reactive Chlorine Species Resistance Enzyme. Overview of Previous Research**

RclA, a protein that is involved in reactive chlorine species resistance in bacteria, has recently been identified as a Group 1-fold flavoenzyme disulfide reductase (Baek et al., 2020; Derke et al., 2020; Parker et al., 2013), due to its structural homology and sequence similarity to other Group 1-fold FDRs. Like typical Group 1-fold FDRs, RclA is a native homodimer, with each monomer consisting of four domains: an FAD domain with a CXXXXC motif for the redox-active disulfide, a pyridine nucleotide domain, a central domain, and an interface domain with the
The FAD domain with redox active Cys-43 and Cys-48, the NADH domain, and the central domain are contributed by one monomer, whilst the interface domain with His-426 and Glu-431 comes from the second monomer. Furthermore, sequence comparison of Salmonella enterica RclA demonstrated the highest homology to mercuric reductase, despite RclA not having an additional disulfide redox center (Baek et al., 2020). Additionally, the active site of RclA contains two lysine residues provided by the first monomer. The lysine residues Lys-13 and Lys-16 are found near Cys-43 and are unique to RclA (Figure 13).

RclA in *E. coli* is encoded by the *rclA* gene, the expression of which, along with the *rclB*
Figure 13. Structure of *E. coli* RclA. a, Cartoon structure of the RclA homodimer with bound FAD. Monomer A is depicted in green; monomer B is depicted in cyan, and FAD is depicted in red. b, Active site structure. The subscripts denote which monomer the residues belong to. (PDB ID: 6KGY)

and *rclC* genes, is controlled by a transcriptional activator RclR of the AraC family. Parker et al. (2013) reported that exposure of *E. coli* to HOCl and N-chlorotaurine, a secondary oxidation product of HOCl in vivo, results in reversible oxidation of RclR’s two key cysteine residues. The oxidation of Cys-21 and the stabilization of the protein due to the formation of a disulfide bond promote the DNA-binding activity of RclR, which, in turn, leads to massive up-regulation of *rclA*, *rclB* and *rclC* genes. While several RCS-sensitive transcription factors have been identified in bacteria, the majority of them are involved in response to multiple stressors, among which are not only RCS, but all reactive oxygen species and electrophiles. RclR, on the other hand, has been identified as a reactive chlorine-specific transcriptional activator. Deletion of any of the three genes controlled by RclR leads to a dramatic increase in RCS sensitivity, suggesting that all three genes - *rclA*, *rclB* and *rclC* are essential for bacterial survival of reactive chlorine stress (Parker et al., 2013).
In the Rel system, RclA is the most phylogenetically conserved protein, which is found almost exclusively in bacteria known to colonize epithelial surfaces (Derke et al., 2020). Derke et al. (2020) hypothesized that RclA plays an important role in host-microbe interactions in many species and investigated the possible function and mechanism of RclA in bacterial response to RCS stress. The experiments with exposure of wild type and ΔrclA mutant E. coli to sublethal HOCl stress, and examination of the ability of E. coli to colonize the intestine of Drosophila melanogaster, confirmed previous results by Parker et al. (2013) that RclA contributes to HOCl resistance and revealed that RclA facilitates early colonization of an animal host and relieves stress caused by host-produced RCS in early stages of host colonization (Derke et al., 2020).

Having observed that E. coli exposure to RCS stress correlates with upregulation not only of rclA, but also of copper resistance genes, and based on homology of RclA to mercuric reductase, Derke et al. (2020) investigated the role of rclA in responding to HOCl stress under growth conditions containing different amounts of copper. The results of these experiments led to a hypothesis that extracellular CuCl2 protects both wild-type and ΔrclA E. coli strains against HOCl, whereas RclA protects E. coli against the combination of HOCl and intracellular copper by reducing copper(II) - the putative substrate of RclA - to copper(I). The hypothesis was formed based on observations that the specific activity of RclA as measured by NADH oxidation increased in the presence of copper, and an accumulation of copper(I) was noted following the reaction between RclA, NADH, and CuCl2. The rate of NADH oxidation by RclA in the presence of copper in vitro was slow, approximately 4.4 min⁻¹, however, the expression of rclA is rapidly induced >100-fold after sublethal doses of HOCl in E. coli. It was therefore hypothesized that the massive upregulation of rclA in vivo could compensate for the low rate of NADH turnover by RclA in the presence of copper observed in vitro. In conclusion, it was suggested that RclA is a
highly stable Cu(II) reductase, the physiological role of which is to resist the stress resulting from the combination of HOCl and copper in the cytoplasm (Derke et al., 2020).

Baek et. al (2020) further probed Derke et al.’s (2020) hypothesis that RclA is a HOCl-induced copper(II) reductase. Having tested a range of putative substrates - GSH (GSSG), lipoic acid, cystine, HOCl, and a variety of metal ions - Baek et al. (2020) observed that only Cu²⁺, Hg²⁺, and Ag⁺ accelerated the oxidation of NADH in RclA. Mercury and silver, however, were deemed physiologically irrelevant, therefore, further experiments focused on Cu(II) as the most feasible substrate out of the ones tested (Baek et al., 2020).

It was observed that NADH oxidation did not occur in anaerobic conditions, therefore, it was suggested that O₂ is required for NADH oxidation in the presence of Cu²⁺. The specific oxygen consumption rate was higher in the presence than in the absence of Cu²⁺, and despite the increased consumption of oxygen, superoxide anion formation was not observed in the presence of copper, while it has been observed in the direct reaction between RclA, oxygen, and NADH. These observations led to a hypothesis that Cu²⁺ catalyzes the reduction of oxygen during the oxidation of NADH, promoting the reaction between oxygen and FADH₂ at the active site of RclA while suppressing the production of superoxide anion. The Kₘ for Cu²⁺ with wild type RclA was found to be 0.54 mM, while Vₘₐₓ was determined to be 1.00 µmole•mg⁻¹•min⁻¹, which corresponds to the k_cat of 0.83 s⁻¹ and the specificity constant (k_cat/K_m) of 1537 M⁻¹ s⁻¹ (Baek et al., 2020).

Based on mutational and structural studies, Baek et al. (2020) suggested that Cys-48 controls Cu²⁺-dependent NADH oxidase activity of RclA, and Cys-43 is involved in the metal specificity and further in the binding of Cu²⁺ at the active site. It was proposed that RclA mediates the reaction shown in Equation 6, and a reaction order of O₂ and Cu²⁺ reduction by RclA was suggested.
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4NADH + 4H⁺ + O₂ + 4Cu²⁺ → 4NAD⁺ + 2H₂O +4Cu⁺  Eq. 6

Overall, Baek et al. (2020) supported Derke et al.’s (2020) hypothesis that RclA is Cu(II) reductase which contributes to the survival of bacteria under HOCl stress in macrophage phagosomes.

**Reactive Chlorine Species**

Reactive chlorine species - powerful antimicrobial agents capable of chlorinating and oxidizing most biological macromolecules - are extensively used for disinfection, primarily in the form of bleach (sodium hypochlorite) and its active ingredient hypochlorous acid. Bacteria encounter RCS not only as disinfectants, but also as a part of innate immune response against invading pathogens (da Cruz Nizer et al., 2020; Gray et al., 2013; Nontaleerak et al., 2020; Parker et al., 2013).

**Formation of Reactive Chlorine Species in Microbial Environments**

As a part of a response to injury or infection, phagocytes, such as neutrophils and macrophages, are recruited to infection sites where they engage in engulfing pathogens into phagosomes. During phagocytosis, these phagocytes increase their oxygen consumption through the activity of an NADPH-oxidase (NOX). NOX generates superoxide anion (O₂⁻), which is dismutated into hydrogen peroxide (H₂O₂) by superoxide dismutases (SOD). Superoxide anion and hydrogen peroxide, in turn, give rise to other reactive oxygen intermediates. Concomitantly with NOX activity, myeloperoxidase (MPO) and other antimicrobial enzymes are delivered into the phagosome, which, along with generation of reactive oxygen/nitrogen species, constitutes the process called respiratory burst (Dahlgren & Karlsson, 1999; Gray et al., 2013, Karupiah et al., 2000; Sultana et al., 2020; Ulfig & Leichert, 2021).

In newly formed neutrophil phagosomes, MPO, which constitutes 5% of the total protein
content of neutrophils, catalyzes the production of the hypohalous acids: hypochlorous acid (HOCl), hypobromous acid (HOBr), and hypothiocyanous acid (HOSCN) from hydrogen peroxide and Cl\(^-\), Br\(^-\), or SCN\(^-\) (Figure 14) (Gray et al., 2013, Ulfig & Leichert, 2021).

![Diagram of NOX2 and MPO in a neutrophil phagosome](https://example.com/diagram.jpg)

**Figure 14.** Generation of reactive chlorine species in neutrophil phagosome.

Thomas and Fishman (1986) and van Dalen et al. (1997) reported that under conditions similar to those in plasma, up to half of the H\(_2\)O\(_2\) used by MPO is converted into hypothiocyanite, whereas under conditions similar to those of saliva, where thiocyanate is present at 1–5 mM, hypothiocyanite was the dominant oxidant formed (van Dalen et al., 1997; Thomas & Fishman, 1986). As a substrate for MPO, SCN\(^-\) has a much higher specificity constant, by a factor of 730-fold, and reacts with a faster rate constant than Cl\(^-\) or Br\(^-\) (van Dalen et al., 1997; Pattison et al., 2012). HOSCN can be generated in plasma, saliva, airway epithelial lining fluid (ELF), nasal lining fluid (NLF), milk, tears, and gastric juices via catalysis by such peroxidases as eosinophil peroxidase (EPO), gastric peroxidase (GPO), myeloperoxidase (MPO), salivary peroxidase (SPO),
and thyroid peroxidase (TPO) (Barrett & Hawkins, 2012; Chandler & Day, 2012; Fischer, 2009). Additionally, HOSCN can be generated in the process of oxidant scavenging by SCN⁻, which decreases toxic effects of hypohalous acids and haloamines (Chandler & Day, 2012).

Another group of reactive chlorine species are chloramines, which are formed upon reaction of HOCl with primary and secondary amines (Gray et al., 2013; Pattison et al., 2012). While most chloramines are unstable and rapidly decompose into respective aldehydes, some demonstrate notable stability, such as N-chlorotaurine (NCT) - an abundant chloramine associated with the innate immune system (Gray et al., 2013; Nagl et al., 2000). Because taurine amounts to approximately half of the amino acid pool of neutrophilic granulocytes, where NCT is formed, NCT is the main reaction product in the reaction between HOCl and N-H compounds occurring in the intra- and extracellular environment of leucocytes, as well as a detoxifying agent for HOCl (Gottardi & Nagl, 2010).

**Reactions of RCS with Biological Molecules and the Effect of RCS on Cells**

Overall, reactive chlorine species affect cells by damaging multiple cellular components simultaneously and causing oxidative unfolding and aggregation of essential bacterial proteins, which may lead to cytotoxicity and cell death. However, specific damage varies based on bacterial species, exposure conditions, and the type of RCS involved (Gray et al., 2013).

Among reactive chlorine species, hypochlorous acid is by far the most reactive and the least selective (Gray et al., 2013; Pattison et al, 2012). Due to its neutrality, HOCl can penetrate bacterial cells easily via passive diffusion, and attacks multiple cellular targets. HOCl cleaves peptide bonds of proteins, which results in protein fragmentation, and causes protein unfolding, which, in turn, leads to the irreversible aggregation of essential bacterial proteins (da Cruz Nizer et al., 2020, Winter et al., 2008). The primary targets of HOCl are sulfur-containing compounds
such as cysteine, methionine, or glutathione. The cascade of reactions initiated by HOCl-mediated oxidation of cysteine thiols leads to the formation of oxidized cysteine sulfenic, sulfinic, and sulfonic acids, and formation of disulfide bonds, thiosulfinates and thiosulfonates, which ultimately leads to protein degradation (da Cruz Nizer et al., 2020; Gray et al., 2013).

HOCl affects bacterial growth by inhibiting protein and DNA synthesis, and targets primary and secondary amines of nucleotide bases, which leads to the formation of nitrogen radicals and stable chlorinated nucleotides, consequently causing DNA dissociation (Gray et al., 2013). Reaction of HOCl with lipids forms chlorohydrins, which may disrupt membrane function and structure (da Cruz Nizer et al., 2020; Gray et al., 2013). Additionally, HOCl may intensify the damage caused by other reactive species by affecting bacterial defenses against oxidative stress (da Cruz Nizer et al., 2020).

Reaction of HOCl with primary and secondary amines gives rise to chloramines, which, although much less reactive than HOCl, are nevertheless considered to be reactive chlorine species due to their ability to oxidize and chlorinate biological molecules (Gray et al., 2013; Grisham et al., 1984; Peskin et al., 2005). In addition to lower reactivity compared to HOCl, chloramines exhibit higher selectivity towards thiols with the reactivity depending on thiol pKa, but are involved in methionine oxidation as well, albeit with lower reaction rates. Chloramines form more mixed disulfides than HOCl when added to cells. Chloramines can form nitrogen radicals upon reaction with iron and copper and react with nucleotides and lipids (Gray 2013). The overall effect of chloramines on the cells varies on their stability, reactivity, and cell permeability (Peskin et al., 2005; Winterbourn & Kettle, 2013).

N-chlorotaurine, the most stable and abundant chloramine produced during respiratory burst, has a broad-spectrum microbicidal activity despite its low reactivity (Arnitz et al., 2006;
NCT reacts very fast with thiols, and moderately fast with aromatic compounds, which contributes to its microbicidal activity. At the same time, NCT is a relatively mild oxidant, well-tolerated by host tissue, and protects cells in the inflammatory tissue from cytotoxicity caused by RCS and ROS (Gottardi & Nagl, 2010; Jang et al., 2009; Piao et al., 2011).

Hypothiocyanous acid, although less reactive than HOCl, exhibits high specificity towards thiols, selectively targeting cysteine and selenocysteine residues in both bacterial and host proteins, as well as sulfhydryl groups of thiol-based oxidants. Reaction of HOSCN with sulfhydryl groups results in the formation of sulfenyl thiocyanates, which can hydrolyze to sulfinic acids or form disulfides (Barrett & Hawkins, 2012; Barrett et al., 2012; Chandler & Day, 2012; Pattison et al., 2012; Ulfig & Leichert, 2021). Antibacterial activity of HOSCN is facilitated by its ability to penetrate the bacterial cell wall prior to oxidizing critical metabolic elements and reacting with proteins (Chandler & Day, 2012; Hawkins, 2009). The bacterial targets of HOSCN include glycolytic enzymes that contain essential thiol groups, such as glyceraldehyde-3-phosphate dehydrogenase, hexokinase, glucose-6-phosphate dehydrogenase, and aldolase, which leads to inhibition of glycolysis and, as a result, of bacterial cell growth. Additionally, it has been reported that HOSCN affects glucose transport and respiration, and inhibits the activity of urease, which is essential for the ability of Helicobacter pylori to colonize the stomach (Chandler & Day, 2012; Hawkins, 2009; Pattison et al., 2012).

Antibacterial activity of HOSCN in vivo primarily occurs in the oral cavity, which has a large pool of SCN−. It has also been suggested that HOSCN plays an important role in a number of antimicrobial and antioxidant processes involved in the protection of the airway and lung cells (Barrett & Hawkins, 2012; Chandler and Day, 2012). While HOSCN can be damaging to a variety of mammalian cells, for example, erythrocytes, endothelial cells, and macrophages (Love et al.,
2016), the host cells in the oral cavity and airway are resistant to HOSCN damage, which indicates that conversion of SCN⁻ to HOSCN was developed as an important protective mechanism against pathogen invasion in the mouth and lungs (Barrett & Hawkins, 2021; Pattison et al., 2012).

This thesis focuses on determining the substrate for RclA and the mechanism by which the enzyme participates in bacterial defense against reactive chlorine species.

**MATERIALS AND METHODS**

**RclA Purification and Expression**

The plasmid encoding for RclA WT protein (pRCLA11) and the plasmids encoding for RclA C43A and C48A mutants (pRCLAa and pRCLAb respectively) were a gift from Michael Gray (University of Alabama at Birmingham). The plasmids were purified using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and transformed into *E. coli* BL21 (DE3) Rosetta2 pLysS cells and plated onto LB + chloramphenicol + ampicillin + 0.4% glucose plates. The glucose was necessary to suppress leaky expression of RclA through catabolite repression. Liquid cultures were inoculated from a single colony and grown in LB media containing 0.4% glucose, 6.8 μg ml⁻¹ chloramphenicol, and 100 μg ml⁻¹ ampicillin at 37° C with shaking to an optical density at 600 nm of 0.6. The temperature was then lowered to 20° C and expression induced with 100 μM IPTG. The culture was grown overnight at 20° C. After harvesting by centrifugation, the cells were lysed at 4° C by sonication in 20 mM NaH₂PO₄•H₂O, 280 mM NaCl, 6 mM KCl, pH 7.4 (PBS; RclA lysis buffer) with Benzonase nuclease and cOmplete protease inhibitor cocktail. The lysate was cleared by centrifugation and the supernatant was loaded on a 5-ml StrepTrap HP column pre-equilibrated in RclA lysis buffer. The column was washed with 150 ml of lysis buffer, then RclA was eluted with 50mL RclA lysis buffer + 2.5 mM d-Desthiobiotin. Purified protein was
concentrated, exchanged into 100 mM phosphate, pH 7.4 buffer, or into 20 mM HEPES, 100 mM NaCl, pH 7 buffer and flash-frozen, then stored at −80°C until use.

**Glutathione Reductase Vector Construction, Purification, and Expression**

The gor gene encoding for glutathione reductase was amplified from *E. coli* genomic DNA using primers 5’ AAA AAA GGA TCC ATG ACT AAA CAC TAT GAT TAC ATC G 3' and 5’ AAA AAA CTC GAG TTA ACG CAT TGT CAC GAA CT 3’ and was ligated into the BamHI and XhoI sites of pET28a to generate an N-terminally his-tagged construct. The pET28a-based expression vector for glutathione reductase was transformed into BL21(DE3) *E. coli* and grown in LB media containing 50 μg ml⁻¹ kanamycin at 37°C with shaking to an optical density at 600 nm of 0.6. The temperature was then lowered to 20°C and expression induced with 100 μM IPTG. The culture was grown overnight at 20°C. After collection, the cells were lysed at 4°C by sonication in 50 mM NaH₂PO₄•H₂O, 300 mM NaCl, 20 mM imidazole, pH 8 (GR lysis buffer) with Benzonase nuclease and cOmplete protease inhibitor cocktail. The GR lysate was cleared by centrifugation and the supernatant was loaded onto a Ni-NTA column (G Biosciences) pre-equilibrated in GR lysis buffer. The column was washed with 100 ml of GR lysis buffer, then the protein was eluted with 50mL of 50 mM NaH₂PO₄•H₂O, 300 mM NaCl, 250 mM imidazole, pH 8 (elution buffer). Protein was then exchanged into 25 mM Tris pH 8 buffer and loaded onto a Q-sepharose column equilibrated into the same buffer. The bound protein was eluted by applying a linear gradient of NaCl from 0 to 1.0 M in 25 mM Tris pH 8 buffer. Purified protein was exchanged into 20 mM HEPES, 100 mM NaCl, pH 7 buffer and flash-frozen, then stored at −80°C until use.

**Preparation of Chlorinated Amino Acids Stock Solutions**

N-chlorotaurine (NCT) was obtained from Markus Nagl (Innsbruck Medical University). The concentration of NCT stock solutions was determined using ε(NCT)₂₅₁nm = 397.4 M⁻¹ cm⁻¹
Chlorinated amino acids, with the exception of N-chlorotaurine, were prepared by mixing 20 mM stock of respective amino acid in PBS buffer with 1 mM of HOCl. The concentration of the resulting chlorinated amino acid was tested using a 3,3′,5,5′-Tetramethylbenzidine (TMB) colorimetric assay (Dypbukt et al., 2005).

**Preparation of HOSCN Stock Solutions**

HOSCN preparation method was adapted from the method employing LPO-catalyzed reaction of SCN− with H₂O₂ described by Nagy et al. (2009). The concentration of LPO stock solutions was determined spectrophotometrically using ε(LPO)₄₁₂nm = 112 mM⁻¹ cm⁻¹. The concentration of H₂O₂ stock solutions was determined spectrophotometrically using ε(H₂O₂)₂₄₀nm = 43.6 M⁻¹ cm⁻¹. The reaction was initiated by adding a small aliquot of H₂O₂ (0.8 mM after mixing) to a mixture of 1.0 μM LPO and 7.5 mM NaSCN in 100 mM pH 7.4 phosphate buffer at 4 °C. Eight additional aliquots of H₂O₂ were added in 1 min intervals (7.2 mM final). After 20 min at 4 °C, 20 μg (300 activity unit)/mL catalase was added to destroy residual H₂O₂. Residual LPO and catalase were removed by centrifugation at 4000 RPM and 4°C using a 10 kDa cutoff Amicon Ultra centrifugal filter device. Concentrations of the stock solutions were determined by measuring the decrease of absorbance at 412 nm after mixing and vortexing the diluted stock a ∼4-5-fold excess of TNB solution. TNB stock solutions were prepared and quantified as previously described by Nagy et al. (2009). This method typically yielded ∼1.6 mM HOSCN/OSCN⁻. To prepare HOSCN stock solutions for ¹³C-NMR experiments, the same method was followed, with ¹³C-labeled KSCN used in place of NaSCN.

**¹³C-NMR Spectroscopy**

All ¹³C-NMR spectra were acquired on a JEOL ECZ-400S, 400 MHz digital FT-NMR spectrometer equipped with a 400MHZ 5MM FG/RO digital auto tune probe: ZNM-03811RO5S-
4S and controlled by Delta 5.3 Software. The samples were prepared in 50 mM phosphate buffer, pH 7, 0.1 % s 1,4-dioxane, 10 % D₂O, at 25 °C using 5 mm NMR tubes. 1,4-dioxane was used as a chemical shift reference (66.6 ppm). Observed $^{13}$C chemical shifts for SCN- and HOSCN were previously measured by Nagy et al. (2006). The concentration of HOSCN in the samples was 1.6 mM, NADH - 5 mM, RclA - 1 µM. Reactions were allowed to proceed for 15 minutes before adding to the samples NaOH. Due to instability of hypothyocyanous acid, 100 mM NaOH was added to diminish spontaneous decomposition of HOSCN during the ~1 hr time required for data collection. 1,012 scans were taken for each sample over the course of ~1 hr at 20 °C using 5 mm NMR tubes.

**Stopped-Flow Experiments**

For all anaerobic experiments, the solutions were made anaerobic in glass tonometers by repeated cycles with vacuum and anaerobic argon (Moran, 2019). Stopped-flow experiments were carried out on a TgK Scientific SF-61DX2 KinetAsyst stopped-flow instrument (with Kinetic Studio software) that had been equilibrated previously with a glucose/glucose oxidase solution to make the internal components of the system anaerobic. When needed, RclA was reduced in the anaerobic tonometer by adding NAD(P)H from the tonometer’s side arm after making the solution anaerobic. Following the reduction, the redox status of the flavin was spectrophotometrically monitored using a Shimadzu UV-1900 UV–vis spectrophotometer (UV Probe software). The stopped-flow experiments were monitored either using the instrument’s multi-wavelength charge-coupled device detector (1.6-ms data interval time) or a single-wavelength detector with photomultiplier tube.

**Buffers**

All experiments with Cu(II) were done in 20 mM HEPES, 100 mM NaCl, pH 7 buffer. All
experiments with NCT were done in 20 mM HEPES, 100 mM NaCl, pH 7 buffer. All experiments with HOSCN were done in 100 mM phosphate, pH 7.4 buffer.

**Enzyme Assays**

Relative NAD(P)H oxidation rates by various substrates without and with wild-type or mutant RclA, or wild-type glutathione reductase were evaluated by steady state kinetic assays aerobically or anaerobically using the change in absorbance of NADH at 340 nm as a readout. A Shimadzu UV-1900 UV–vis spectrophotometer (UV Probe software) was used to monitor the absorbance change. For anaerobic assays, NAD(P)H solution was made anaerobic in a glass tonometer, with the enzyme and the substrate in two side arms separated from the pyridine nucleotide solution. After the solution was made anaerobic, the enzyme and the substrate were added in one step. For aerobic assays, the reactions were initiated by first injecting 200 μM of the substrate into the solution containing ~200 μM NAD(P)H. For the reaction with an enzyme, the enzyme was added following the addition of the substrate. The concentration of the enzyme for the experiments with NCT and other amino acids was 1 μM. For the experiments with HOSCN, the concentration of the enzyme was 10 nM. The experiments were repeated in triplicate. The initial rates of the reactions were fitted to a line in KaleidaGraph (Synergy Software).

**Oxidative Half-Reaction Kinetics**

Oxidative half-reaction studies were carried out anaerobically in a stopped-flow spectrophotometer. RclA solution was made anaerobic in a glass tonometer, with 0.9 eq. of NADH in a side arm separated from the enzyme solution. After the solution was made anaerobic, NADH from the side arm was added to the enzyme solution to pre-reduce the active site cysteines. For the experiment with NCT, ~20 μM RclA was loaded onto the instrument and mixed with 1.6 mM NCT (all concentrations after mixing). The reaction was monitored for the flavin absorbance change.
between 250 and 700 nm over the course of 0.8-80 seconds. Kinetic traces were fit to sums of exponentials using KaleidaGraph to determine the observed rate constants. The bimolecular rate constants were determined graphically from the slope of the \( k_{\text{obs}} \) vs NCT concentration. For the experiment with HOSCN, \(~18\) \( \mu \)M RclA was loaded onto the instrument and mixed with \( 18\) \( \mu \)M or \( 50\) \( \mu \)M of HOSCN (all concentrations after mixing). The signals at 0 time for zero substrate and \( 18\) \( \mu \)M HOSCN at 1.6 ms after initiating the reaction were compared to reveal that the reaction between RclA and HOSCN was over during the dead time of the instrument.

**Stopped-Flow Steady State Kinetic Assays**

Steady state kinetic assays used to determine \( V_{\text{max}} \) and \( K_M \) for the reactions of RclA or glutathione reductase with NCT or HOSCN were carried out in a stopped-flow spectrophotometer. For anaerobic experiments, the enzyme solution was made anaerobic in a glass tonometer, with NAD(P)H in a side arm separated from the enzyme solution. After the solution was made anaerobic, NAD(P)H from the side arm was added to the enzyme solution. The enzyme+NAD(P)H solution was then loaded onto the instrument and mixed with varying concentrations of the substrate. For aerobic experiments, the enzyme solution and the pyridine nucleotide solution were loaded onto the instrument in separate syringes, then mixed with varying concentrations of the substrate. The change in absorbance of NADH at 340 nm was used as a readout. The initial velocities for four traces per each concentration of the substrate were plotted against the substrate concentration to obtain a Michaelis-Menten plot using KaleidaGraph, from which the \( V_{\text{max}} \) and \( K_M \) were determined.

For NCT, the experiment was carried out anaerobically, with \(~10\) \( \mu \)M RclA, \(~100\) \( \mu \)M NADH and 0.1-49.8 mM of NCT (all concentrations after mixing). For HOSCN, the experiment
was carried out aerobically, with ~10 nM RclA, ~170 µM NADH, and 1-100 µM HOSCN for RclA or 50-250 µM HOSCN for GR (all concentrations after mixing).

**Spectrophotometric Titrations**

Spectrophotometric titrations were carried out on a Shimadzu UV-1900 UV–vis spectrophotometer (UV Probe software). Absorbance spectra were recorded after each addition of the ligand. For NAD⁺ titration, ~20 µM RclA in 20 mM HEPES, 100 mM NaCl, pH 7 buffer was treated with dithiothreitol (DTT) in order to pre-reduce the active site disulfide to thiols. Then the enzyme solution was titrated with NAD⁺ aerobically until the concentration of NAD⁺ reached 2.25 mM, after which the enzyme started aggregating. For NADH titration, the enzyme was pre-reduced with DTT anaerobically in a glass tonometer using a gas-tight Hamilton syringe. Then the enzyme solution was titrated with NADH anaerobically until the concentration of NADH reached 52.21 µM, after which the signal stopped changing significantly.

To determine the $K_d$, the change in absorbance at 600 nm for NAD⁺ was plotted against [NAD⁺], and the plot was fit to a hyperbola in KaleidaGraph using Equation 7.

$$A = \frac{\Delta_A \times [S]}{K_d + [S]} + A_0 \quad \text{Eq. 7}$$

The change in absorbance at 420 nm for NADH was plotted against [NADH], and the plot was fit a square hyperbola to Equation 8, where $\Delta \varepsilon_{\text{max}}$ is the maximum change in extinction coefficient, $E_0$ is the initial enzyme concentration, and $L_0$ is the ligand concentration.

$$\Delta A = \Delta \varepsilon_{\text{max}} \left[ \frac{E_0 + L_0 + K_d - \sqrt{(E_0 + L_0 + K_d)^2 - 4E_0L_0}}{2} \right] \quad \text{Eq. 8}$$

**Binding Kinetics**

The kinetics of NAD⁺ and NADH binding were monitored anaerobically on a stopped-flow spectrophotometer. Experiments were performed by mixing 10 nM RclA with 50-400 µM NAD⁺, or 400 µM NADH (all concentrations are after mixing) in 100 mM phosphate buffer, pH 7.4, at
25°C. For NADH binding occurred too fast, with the reaction being over within the dead time of the stopped flow instrument (1.6 ms). For NAD+, the change in flavin absorbance was monitored at 420 nm. Traces were fit to single exponential to obtain the observed rate constant. Association and dissociation rate constants were determined graphically from the slope of the \( k_{\text{obs}} \) vs. [NAD+] and the y-intercept respectively.

**Mechanism Studies**

To determine the kinetic mechanism of RclA, the enzyme was characterized under steady state conditions. Initial velocity experiments were carried out aerobically in a stopped-flow spectrophotometer by measuring NADH oxidation rate via monitoring the change in absorbance at a single wavelength of 340 nm. 10 nm RclA was loaded onto the instrument and mixed with a fixed concentration of NADH (25, 50, 90, and 177 μM) and varying concentrations of HOSCN (1-100 μM, all concentrations after mixing). The initial velocities for four traces per each HOSCN concentration were plotted against the substrate concentration to obtain Michaelis-Menten plots and double reciprocal (Lineweaver-Burk) plots using KaleidaGraph (Synergy Software).

**RESULTS**

**Copper Experiments**

**RclA Aggregates upon Addition of Copper**

During our initial attempts to characterize the reaction between RclA and CuCl₂, we discovered that the addition of 0.2 mM CuCl₂ to 20 μM solution of RclA caused the enzyme to precipitate extensively. Adding EDTA caused the precipitated protein to go back into the solution, indicating that this precipitation is mediated by an interaction between copper(II) and RclA.
Histidine is known to coordinate copper(II) (Strange et al., 1987), and using a buffer containing 20 mM histidine prevented the copper-mediated precipitation of RclA altogether.

**RclA Does Not React Rapidly with CuCl₂**

To prevent aggregation of the enzyme, a histidine-containing buffer was used during an experiment in which we evaluated the reaction rate of 200 µM CuCl₂ with 20 µM RclA containing pre-reduced FAD. The changes in the visible flavin absorbance spectrum were monitored anaerobically using stopped-flow spectrophotometry, however, no obvious change in flavin absorbance was observed over the course of the 80 second experiment. When the stopped-flow experiment was repeated without histidine using the same concentrations of RclA and CuCl₂, the results of the experiment were challenging to interpret due to enzyme aggregation, which caused massive increase in absorbance at all wavelengths over time along with the decrease in flavin signal.

To rule out the possibility that histidine may have interfered with the reaction between CuCl₂ and RclA, we ran kinetic assays of the reaction between Cu(II), RclA and NADH without histidine, while also reducing the concentration of RclA to 2 µM to reduce precipitation of the enzyme. The change in absorbance at 340 nm associated with NADH oxidation was used as a readout. Without histidine, copper indeed enhanced NADH consumption, as previously reported (Figure 15) (Baek et al., 2020; Derke et al., 2020). However, precipitation of the enzyme was still observed, and the enhanced reaction rate was slow compared with other flavin disulfide oxidoreductases (Fagan & Palfey, 2010; Fox & Walsh, 1982; Gleason et al., 1990; Pillay et al., 2010; Rietveld et al., 1994; Sahlman & Williams, 1989). Most notably, NADH oxidation by 200 µM copper occurred faster in the absence of RclA than in its presence, which was not observed in a prior study on RclA (Derke et al., 2020). It is currently unclear why our results differ from that
of the prior study. Our new results, in addition to the information described earlier from the literature led us to question the notion that Cu(II) is the physiological substrate for RclA. We therefore evaluated other candidates as the physiological substrate for RclA. RclA provides resistance to *E. coli* against reactive chlorine species produced by the host’s immune system, which are most potent at oxidizing cellular thiols to a disulfide or sulfenic/sulfonic acid. RclA is a group I flavin disulfide reductase and these enzymes (including RclA) have two cysteine residues in their active site that are used to reduce their substrates using NAD(P)H as the reductant. We therefore hypothesized that the physiological substrate for RclA may be a reactive chlorine species, allowing the enzyme to detoxify the reactive chorine species by coupling its reduction to NAD(P)H oxidation using the enzyme’s FAD and active site cysteines.

Reactive chlorine species produced by the mammalian immune system include HOCl, chloramines, and HOSCN, and we set out to evaluate these as potential substrates for RclA. HOCl was too reactive to evaluate, though, as it spontaneously reacts with NADH too quickly to
determine if RclA accelerated the reaction between the two compounds. Chloramines and HOSCN react slowly with NADH in the absence of a catalyst, and we were therefore able to evaluate them as potential substrates for RclA.

**N-chlorotaurine Experiments**

**N-Chlorotaurine Acts as a Possible Substrate for RclA**

N-chlorotaurine (NCT) is by far the most abundant chloramine produced by the mammalian immune system, reaching concentrations of 10-50 μM in neutrophils (Nagl et al., 2000) to help destroy invading pathogens and to protect macrophages from apoptosis (Piao et al., 2011). In order to investigate the behavior of N-chlorotaurine as a possible substrate, we first ran a series of simple steady state assays using the 340 nm absorbance change associated with NADH oxidation as a readout. The experiments revealed that NCT slowly oxidized NADH spontaneously, but upon addition of 1 μM RclA to the mixture of 200 μM NADH and 200 μM NCT, the rate of NADH consumption dramatically increased (Figure 16). Similar results were observed for NADPH consumption rate. The assays were conducted both aerobically and anaerobically, and the results revealed that the NADH oxidation rate by NCT and RclA under anaerobic conditions was similar to the aerobic experiment, indicating that O₂ was not involved in the enhanced NADH oxidation rate by NCT and RclA.

In an attempt to characterize the oxidation of RclA by NCT directly, a further experiment was conducted using stopped-flow spectrophotometry. Since NADH reduces the active site disulfide to thiols in the reductive half-reaction, and the second substrate is expected to re-oxidize the thiols to disulfide, 0.9 equivalents of NADH were used to pre-reduce the active site disulfide to achieve the EH₂ state (Figure 17a). Following the reduction, the reaction of RclA with NCT was carried out in a stopped-flow spectrophotometer. The experiment was conducted anaerobically to
Figure 16. Comparison of NADH oxidation rates by NCT without and with RclA. Both reactions were evaluated by steady state kinetic assays using the change absorbance of NADH at 340 nm as a readout, and both were initiated by first injecting 200 µM of NCT into the solution containing 200 µM NAD(P)H. For the reaction with RclA 1 µM of enzyme was added following the addition of NCT.

prevent re-oxidation of the enzyme by oxygen. Since no excess NADH was present, it was possible to assess the reaction of NCT with the cysteines of RclA without the interference of turnover. Monitoring the enzyme’s absorbance spectrum over time in the instrument revealed that NCT indeed re-oxidized the thiols back to disulfide (from the EH₂ state to Eox), which was consistent with the expectation for the oxidative half reaction (Figure 17b). It was observed that NCT oxidized the cysteines in two kinetically resolvable events, the first of which was responsible for 90% of total signal change. For both events, the observed rate constant increased linearly with NCT concentration, indicating that they each correspond to a bimolecular reaction.

For both events, the bimolecular rate constants were determined graphically from the slope of the kobs vs [NCT]. For the minor event, the bimolecular rate constant was found to be 115 M⁻¹ s⁻¹ (Figure 17d). For the major kinetic event the bimolecular rate constant was found to be 4165 M⁻¹ s⁻¹ (Figure 17c), which was 20 times higher than the published second-order rate constant of
205 M\(^{-1}\) s\(^{-1}\) for the reaction of NCT with cysteine (Peskin & Winterbourn, 2001), indicating that
NCT reacts more quickly with RclA’s active site cysteines than it does with free cysteine.

Figure 17. Oxidative half-reaction studies, RclA, NADH, and NCT. a, RclA was reduced by 0.9 eq. of NADH under anaerobic conditions, then mixed with 1.6 mM NCT. b, The reaction was monitored for the absorbance change by stopped-flow spectrophotometry, revealing that NCT reoxidized the active site thiols back to disulfide (from the EH\(_2\) state to E\(_{ox}\)). c, \(k_{\text{obs}}\) for the major kinetic event was plotted vs. [NCT] to obtain the second-order rate constant of 4165 M\(^{-1}\) s\(^{-1}\). d, \(k_{\text{obs}}\) for the minor kinetic event was plotted vs. [NCT] to obtain the second-order rate constant of 115 M\(^{-1}\) s\(^{-1}\).
To further elucidate the kinetic parameters of the reaction between NCT, RclA, and NADH, steady state kinetic assays were performed anaerobically in a stopped-flow spectrophotometer using the absorbance at 340 nm for NADH oxidation as a readout. The experiment was performed using a fixed NADH concentration (200 µM) and various concentrations of NCT (Figure 18a). The initial velocities for each concentration of NCT were plotted against the substrate concentration to obtain a Michaelis-Menten plot, which allowed us to determine the following kinetic parameters: $K_m$ of 12.7 mM, $k_{cat}$ of 33 s$^{-1}$, and the specificity constant of $2.60 \times 10^3$ M$^{-1}$ s$^{-1}$ (Figure 18b). While the $k_{cat}$ is relatively high, the specificity constant is quite low due to the large $K_m$. Furthermore, the $K_m$ of NCT for RclA vastly exceeds NCT concentrations of 10–50 µM found in vivo (Nagl et al., 2000).

Figure 18. Biochemical analysis of the reaction between RclA, NADH, and N-chlorotaurine using stopped-flow spectrophotometry. a, RclA was mixed with NADH under anaerobic conditions, then mixed with varying concentrations of NCT. b, Biochemical analysis of the reaction between RclA, NADH, and N-chlorotaurine using stopped-flow spectrophotometry. The $V_{max}$ and $K_m$ values were calculated by fitting the data with a Michaelis-Menten equation.
Among Other Biologically Relevant Chloramines, Only Glycine Chloramine Exhibits Reaction Rates Comparable to Those of NCT with RclA

A range of other chlorinated amino acids, such as glycine, glutamate, arginine, alanine, phenylalanine, aspartate, glutamine, lysine, and histidine were also tested as possible substrates because HOCl reacts with free amines to form the respective chloramines. The chlorinated amino acids were prepared by mixing the respective amino acid with 1 mM HOCl, with a presumption that the concentration of the resulting chlorinated amino acid would be also close 1 mM. The presumption was tested for glutamate chloramine using a TMB colorimetric assay (Dypbukt et al., 2005), revealing that the concentration of the chlorinated amino acid prepared using the method described above indeed yielded the concentration close to the expected value.

To test the reaction rates of the chlorinated amino acids, we ran simple steady state assays using the 340 nm absorbance change for NADH oxidation as a readout, adding 200 µM of the respective amino acid first to the chlorinated amino acid, and then 1 µM RclA. Among the tested chlorinated amino acids, only glycine chloramine accelerated NADH oxidation significantly, albeit not as dramatically as NCT (Figure 19). Considering both NCT and chloroglycine molecules are significantly smaller in size than the other tested chlorinated amino acids, the results suggest at only NCT and chloroglycine are small enough to fit into the active site of RclA.

Glutathione Reductase Reacts with NADPH and N-chlorotaurine with the Rate Comparable to that of RclA

To test whether NCT reduction is a function attributed specifically to RclA, we performed steady state assays with another Group 1 flavin disulfide reductase - glutathione reductase. Since glutathione reductase exhibits preference for NADPH, while RclA did not demonstrate a strong preference towards either of the pyridine nucleotides, the experiments were conducted with both
Figure 19. NADH, chlorinated amino acids, and RclA reaction rates. NADH oxidation rates with various chlorinated amino acids evaluated by steady state kinetic assays using the change absorbance of NADH at 340 nm as a readout. The reaction was initiated by first injecting 200 µM of the respective chlorinated amino acid into the solution containing 200 µM NADH, then adding 1 µM RclA.

NADH and NADPH for enhanced comparison.

The results of the kinetic assays revealed that while the reaction rate with NADH and NCT was five times lower for glutathione reductase than for RclA (Figure 20a), the rates were comparable when NADPH was used as a reductant (Figure 20b). This observation suggests that NCT is not a specific substrate for RclA but will generically react with flavin disulfide reductase enzymes.

**Hypothiocyanous Acid Experiments**

**RclA Reacts Rapidly with HOSCN, with a Rate Considerably Higher than Previously Considered Substrates**

To probe the hypothesis that HOSCN could act as a substrate for RclA, we first ran a simple steady state assay using the 340 nm absorbance change for NADH oxidation as a readout. HOSCN was added to the NADH solution first, followed by the addition of RclA. The experiment revealed
Figure 20. Reaction rate comparison, N-chlorotaurine with RclA and glutathione reductase. \(\text{a, Comparison of NADH oxidation rates by NCT and RclA or glutathione reductase. b, Comparison of NADPH oxidation rates by NCT and RclA or glutathione reductase. Both reactions were evaluated by steady state kinetic assays using the change absorbance of NADH at 340 nm as a readout, and both were initiated by first injecting 200 \(\mu\)M of NCT into the solution containing 200 \(\mu\)M NAD(P)H, then adding 1 \(\mu\)M RclA.}

that HOSCN reacted with RclA dramatically faster than NCT. The reaction rate with 10 nM enzyme determined from the kinetic trace was \(2.64 \times 10^{-6}\) M/s. Dividing the initial velocity by the concentration of the enzyme gave an apparent turnover number of 264.38 s\(^{-1}\).

With these encouraging results, we carried out stopped-flow spectrophotometry experiments to further establish kinetic parameters of the reaction between HOSCN, RclA, and NADH. The experiment was conducted aerobically, monitoring the change in NADH absorbance at a single wavelength of 340 nm. Performing the experiment at a single wavelength allowed detection of signal change for lower concentrations of the substrate, thus achieving higher accuracy in determining the kinetic parameters. The initial velocities for four traces per each concentration of HOSCN were plotted against the substrate concentration to obtain a Michaelis-Menten plot, which allowed us to determine the following steady state kinetic parameters: \(K_m\) of 2 \(\mu\)M, \(k_{cat}\) of...
182 s\(^{-1}\), and the specificity constant (\(k_{\text{cat}}/K_m\)) of \(9.12 \times 10^7\) M\(^{-1}\) s\(^{-1}\), approaching substrate diffusion, which is between \(10^8\) and \(10^9\) M\(^{-1}\) s\(^{-1}\) (Figure 21). The reaction rate between HOSCN and RclA, measured using steady state assay, far exceeded the rates with previously considered substrates: CuCl\(_2\), NCT, and glycine chloramine (Figure 22).

**Figure 21.** Biochemical analysis of the reaction between RclA, NADH, and HOSCN using stopped-flow spectrophotometry. The \(V_{\text{max}}\) and \(K_m\) values were calculated by fitting the data with a Michaelis-Menten equation.

**Figure 22.** Comparison of NADH oxidation rates by RclA and HOSCN, NCT, N-chloroglycine, or Cu (II). All four reactions were evaluated by steady state kinetic assays using the change absorbance of NADH at 340 nm as a readout, and all four were initiated by first injecting 200 µM of the respective substrate into the solution containing 200 µM NAD(P)H, then adding 10 nM RclA.
RclA Reacts with HOSCN Significantly Faster than Glutathione Reductase

Having obtained convincing kinetic data for HOSCN as a substrate for RclA, we tested glutathione reductase as a possible HOSCN reductase as well, as we previously did with NCT. Since glutathione reductase exhibits preference for NADPH, while RclA did not demonstrate a strong preference towards either of the pyridine nucleotides, the experiments were conducted only with NADPH. First, we ran a simple steady state assay for both enzymes, which showed that NADPH oxidation rate was significantly higher for HOSCN with RclA than with glutathione reductase (Figure 23a). Next, we conducted an aerobic stopped-flow experiment to obtain the kinetic parameters for the reactions of glutathione reductase with HOSCN and NADPH. NADPH absorbance traces were monitored at 340 nm, and the initial velocities for four traces per each HOSCN concentration were plotted against the substrate concentration to obtain a Michaelis-Menten plot. The following kinetic parameters were determined from the resulting plot: \( K_m \) of 81.2 \( \mu \)M, \( k_{cat} \) of 6.07 s\(^{-1}\), and the specificity constant of 7.48 \( \times \) 10\(^4\) M\(^{-1}\) s\(^{-1}\) (Figure 23b). Compared to RclA, the \( K_m \) for glutathione reductase is 40 times higher, the \( k_{cat} \) is 30 times lower, and the specificity constant is over a thousand times lower. These results strongly suggest that HOSCN reduction is a function attributed specifically to RclA and is not a general property of flavin disulfide reductase enzymes.

Active Site Cysteines are Essential for the Reaction between RclA, HOSCN, and NADH

To elucidate the importance of the active site cysteines, C43 and C48, in the reaction of HOSCN with RclA, we conducted kinetic experiments with C43A and C48A mutants of RclA using absorbance change at 340 nm associated with NADH oxidation as a readout. The observed reaction rates for both mutants were close to zero (Figure 24), indicating that both C43 and C48 are essential for the reaction between RclA, NADH, and HOSCN to occur.
**Figure 23.** Reaction rate comparison, HOSCN with RclA and glutathione reductase a, Comparison of NADPH oxidation rates by HOSCN and RclA or glutathione reductase. Both reactions were evaluated by steady state kinetic assays using the change absorbance of NAD(P)H at 340 nm as a readout, and both were initiated by first injecting 200 µM of NCT into the solution containing 200 µM NAD(P)H, then adding 10 nM RclA. b, Biochemical analysis of the reaction between glutathione reductase, NADH, and HOSCN using stopped-flow spectrophotometry. The $V_{\text{max}}$ and $K_m$ values were calculated by fitting the data with a Michaelis-Menten equation.

**Figure 24.** Comparison of NADH oxidation rates by HOSCN and wild type RclA, C43A mutant RclA, or C48 mutant RclA. All three reactions were evaluated by steady state kinetic assays using the change absorbance of NADH at 340 nm as a readout, and all three were initiated by first injecting 200 µM of HOSCN into the solution containing 180 µM NAD(P)H, then adding 10 nm RclA.
**SCN⁻ is the Product of the Reaction between RclA, HOSCN, and NADH**

We expect that thiocyanate (SCN⁻) would be the product formed upon reduction of HOSCN by RclA. To test this hypothesis, we compared the \(^{13}\)C-NMR spectra of HOSCN prior to and following the reaction with RclA and NADH. \(^{13}\)C-labeled KSCN was used in the preparation of HOSCN for the experiment in order to increase signal. Due to instability of hypothiocyanous acid, 100 mM NaOH was added to diminish spontaneous decomposition of HOSCN during the \(~1\) hr time required for data collection. In figure 25, before the reaction, the peak at 127.69 ppm corresponds to HOSCN, and at 133.55 to SCN⁻ (Nagy at al., 2006); SCN⁻ was always present in the sample because we were unable to completely convert all the SCN⁻ to HOSCN using the lactoperoxidase-based preparation method. After the reaction, the peak at 127.69 disappears, and the peak at 133.54 increases in intensity, which indicates that the product of the reaction is indeed SCN⁻. Addition of NADH alone did not convert HOSCN to SCN⁻.

![Figure 25](image.png)

*Figure 25.* SCN⁻ is the product of the reaction between RclA, HOSCN, and NADH. \(^{13}\)C NMR spectra of SCN⁻ and HOSCN. Upon addition of RclA and NADH, the signal for HOSCN decreased and the signal for SCN⁻ increased, indicating that HOSCN was converted to SCN⁻. Addition of NADH alone did not convert HOSCN to SCN⁻.
**Reaction between Reduced RclA and HOSCN Occurs Rapidly**

In an attempt to determine the contribution of the oxidative half-reaction to the overall reaction rate, we carried out an anaerobic stopped-flow experiment for the reaction of HOSCN with RclA pre-reduced to the EH\textsubscript{2} state using 0.9 equivalents of NADH, similarly to the experiment we conducted with NCT. Monitoring the traces of enzyme absorbance revealed that both at 18 µM (equivalent to that of the enzyme) and 50 µM HOSCN, re-oxidation of the thiols back to disulfide occurred within the dead time of the stopped-flow instrument, which is 1 millisecond (Figure 26). Presuming that the reaction between RclA and HOSCN is bimolecular, $k_{\text{obs}}$ should be at least 1000 s\(^{-1}\) with an instrument dead time of 1 ms, which means that $k_{\text{on}}$≥5.50x10\(^7\) M\(^{-1}\) s\(^{-1}\). This value is 700 times higher than the published value of 7.8×10\(^4\) M\(^{-1}\) s\(^{-1}\) for the second-order rate constant for the reaction between HOSCN and L-cysteine (Skaff et al., 2009), indicating that RclA’s active site cysteines react with HOSCN dramatically faster than the reaction of free cysteines with HOSCN.

![Figure 26](image.png)

**Figure 26.** Reaction between reduced RclA and HOSCN occurs rapidly. Signal comparison at 0 time for zero substrate and 18.18 µM HOSCN at 1.6 ms after initiating the reaction demonstrates that the reaction is complete during the dead time of the stopped flow instrument, as the very first trace that can be obtained in the presence of the substrate corresponds to the oxidized form of the enzyme.
Release of NAD$^+$ is Likely the Rate-Determining Step in the Reaction between RclA, HOSCN, and NADH

The direct reaction between reduced RclA and HOSCN occurs with an apparent rate constant that is much larger than $k_{cat}$ for the reaction between RclA, NADH, and HOSCN. This difference in reaction rates led us to hypothesize that the release of NAD$^+$, the product of the reductive half-reaction, could serve as the rate-determining step. To determine the binding affinity of RclA for NAD$^+$ and NADH, we performed spectrophotometric titrations, taking advantage of the change in flavin absorbance that occurs when NADH or NAD$^+$ binds to the enzyme. Prior to the experiment, RclA was treated with 1 mM dithiothreitol (DTT) in order to pre-reduce the active site disulfide to thiols, thus preventing electron transfer from NADH to the disulfide. For the first experiment, DTT-reduced RclA was titrated with NAD$^+$ aerobically until the concentration of NAD$^+$ reached 2.25 mM, at which point the enzyme started aggregating (Figure 27a). For the second experiment, the pre-reduced enzyme was titrated with NADH anaerobically until the concentration of NADH reached 52.21 µM, after which the signal stopped changing significantly (Figure 27b).

The change in absorbance at 600 nm for NAD$^+$ was plotted against [NAD$^+$] to determine the binding affinity, and the plot was fit to a hyperbola in KaleidaGraph using Equation 7.

$$A = \frac{\Delta A \times [S]}{K_d + [S]} + A_0$$  \hspace{1cm} \text{Eq. 7}

The change in absorbance at 420 nm for NADH was plotted against [NADH], and the plot was fit a square hyperbola to Equation 2, where $\Delta \varepsilon_{max}$ is the maximum change in extinction coefficient, $E_0$ is the initial enzyme concentration, and $L_0$ is the ligand concentration.

$$\Delta A = \Delta \varepsilon_{max} \left[ E_0 + L_0 + K_d - \sqrt{(E_0 + L_0 + K_d)^2 - 4E_0L_0} \right]$$  \hspace{1cm} \text{Eq. 8}
The $K_d$ for NAD$^+$ was determined to be 102.17 µM (Figure 27c), and the $K_d$ for NADH was found to be 223.82 nM (Figure 27d). These experimentally determined values indicated tight binding of the substrate - NADH - and much weaker binding of the product - NAD$^+$. 

To investigate the kinetics of NAD$^+$ and NADH binding, we performed a series of stopped-flow experiments, monitoring the change in flavin absorbance at 420 nm. While for NAD$^+$ binding was slow enough to track the reaction rate (Figure 28a, b), for NADH binding occurred too fast,
with the reaction being over within the dead time of the stopped flow instrument (Figure 28c).

Traces were fit to single exponential to obtain the observed rate constant, which was found to be increasing linearly with NAD$^+$ concentration (Figure 28d), consistent with a bimolecular reaction. Association and dissociation rate constants could be determined graphically from the slope of the $k_{\text{obs}}$ vs. [NAD$^+$] and the y-intercept respectively. These values were found to be 8.3 x $10^5$ M$^{-1}$ s$^{-1}$ for $k_{\text{on}}$, and 115.8 s$^{-1}$ for $k_{\text{off}}$, giving the K$_d$ of 139.5 µM (Figure 28e), which was close to the K$_d$ determined through titration. Notably, the rate constant for NAD$^+$ dissociation of 115.8 s$^{-1}$ is similar to the $k_{\text{cat}}$ determined in steady state assays, suggesting that NAD$^+$ dissociation may be rate limiting during turnover by RclA.

**RclA Follows a Ping Pong Kinetic Mechanism with NADH and HOSCN as Substrates**

To elucidate the kinetic mechanism of RclA, a characterization of the enzyme activity was performed under steady state conditions. HOSCN concentrations were varied at fixed concentrations of NADH, with the enzyme concentration of 10 nM. Four fixed concentrations of NADH - 25, 50, 90, and 177 µM - were used, and the concentrations of HOSCN varied between 1 and 100 µM. Initial velocity experiments were carried out by measuring NADH oxidation rate via monitoring the change in absorbance at a single wavelength of 340 nm using stopped-flow spectrophotometry. Double reciprocal plots of the initial rates of RclA reaction with NADH and HOSCN were linear and yielded a parallel line pattern. Figure 15e demonstrates the parallel line behavior of the double reciprocal plots of the experimental data set, which is consistent with a ping-pong kinetic mechanism. Michaelis-Menten plots of the reactions between RclA and varying concentrations of HOSCN at fixed concentrations of NADH show a nearly unchanged K$_m$ along with $V_{\text{max}}$ that increases with NADH concentrations, which also suggests a ping pong mechanism.
(Figure 29 a-d). Plotting $V_{\text{max}}$ against the NADH concentration used allowed us to determine a $K_m$ for NADH of 35.1 µM (Figure 29f).

![Figure 28. NAD$^+$ and NADH binding kinetics.](image)

- a, b, Signal comparison at 0 time for zero substrate and 400 µM NAD$^+$, as well as the signal comparison at three time points for 50 µM NAD$^+$, demonstrate that the binding is slow enough to track the reaction rate.
- c, Signal comparison at 0 time for zero substrate and 400 µM NADH demonstrates that the reaction is complete during the dead time of the stopped flow instrument. The small change in absorbance that is observed is due to the reduction of the flavin by NADH.
- d, Traces at 420 nm for different concentrations of NAD$^+$ were fit to single exponential to obtain $k_{\text{obs}}$. e, Association and dissociation rate constants were determined graphically from the slope of the $k_{\text{obs}}$ vs. [NAD$^+$] and the y-intercept respectively. These values were found to be $8.3 \times 10^5$ M$^{-1}$ s$^{-1}$ for $k_{\text{on}}$ and 115.8 s$^{-1}$ for $k_{\text{off}}$, giving the $K_d$ of 139.5 µM.
Figure 29. RclA follows a ping pong kinetic mechanism with NADH and HOSCN as substrates. **a-d**, Michaelis-Menten plots of the reaction between 10 nm RclA and four fixed concentrations of NADH (25, 50, 90, and 177 μM) with varying concentrations of HOSCN (1-100 μM) demonstrate nearly unchanged $K_m$ along with $V_{max}$ increasing with NADH concentrations, which suggests a ping pong mechanism. **e**, Double reciprocal plots of the experimental data set demonstrate the parallel line behavior, which is consistent with the ping-pong mechanism. **f**, Plotting $V_{max}$ against the NADH concentration used gives a $K_m$ for NADH of 35.1 μM.
DISCUSSION, FUTURE DIRECTIONS, AND CONCLUSIONS

Reactive chlorine species, which are naturally generated during the mammalian innate immune response, are highly toxic for invading pathogens. Therefore, bacteria developed defense mechanisms against these RCS. While it has been known that RclA is involved in bacterial defense against RCS (Baek et al., 2020, Derke et al., 2020; Parker et al., 2013) the kinetic data obtained in previous research and by us for the previously proposed substrate – Cu(II) – led us to question the hypothesis that copper is a physiological substrate for RclA.

Since reactive chlorines species mostly target cellular thiols, and RclA, as a Group 1 FDR, has two cysteine residues in its active site which may be used to detoxify cysteine-targeting substrates, we hypothesized that a reactive chlorine species may be a physiological substrate for RclA. Therefore, in this study, we set out to test several RCS as potential substrates for RclA. We omitted HOCI due to its high reactivity and evaluated less reactive physiologically relevant RCS – N-chlorotaurine and hypoiodocyanous acid.

The kinetic parameters of the reaction between RclA, NADH, and NCT indicate that NCT is unlikely a physiological substrate for RclA. While the $k_{cat}$ of 33 s$^{-1}$ is relatively high, the $K_m$ of 12.7 mM significantly exceeds the physiological concentrations of NCT. Furthermore, the comparatively low specificity constant of $2.60 \times 10^3$ M$^{-1}$ s$^{-1}$, coupled with the observation that the reaction rates of glutathione reductase, NADPH, and NCT, were comparable to those of the reaction between RclA, NADPH, and NCT, suggested that NCT is not a specific substrate for RclA.

The kinetic parameters obtained for the reaction of RclA with HOSCN – $K_m$ of 2 μM, $k_{cat}$ of 182 s$^{-1}$, and the specificity constant ($k_{cat}/K_m$) of $9.12 \times 10^7$ M$^{-1}$ s$^{-1}$, which approaches the rate of diffusion – strongly suggest that hypoiodocyanous acid is a physiological substrate for RclA. While
currently known Group 1 flavoprotein disulfide reductases typically catalyze the reduction of disulfide substrates using the reducing power of NAD(P)H, RclA utilizes the same enzymatic machinery to detoxify HOSCN by reducing it to SCN⁻, functioning as a HOSCN reductase via a ping-pong kinetic mechanism. Thus, it is suggested that FAD catalyzes oxidation of NADH in the reductive half of the active site, whereas the active site cysteines in the oxidative half transfers two electrons to HOSCN, with the overall movement of electrons occurring from NADH to FAD, to redox-active cysteines, to HOSCN. The reaction makes sense physiologically, as HOSCN is a primarily sulfhydryl-reactive oxidant. Thus, using the reductive power of NADH, RclA detoxifies HOSCN before it can target other thiols in the cell.

Our hypothesis that RclA participates in cellular defense against HOSCN stress is supported by in vivo data by Meredith et al. (2021). In their experiments, they exposed wild-type E. coli and an isogenic rclA knockout strain to varying sublethal concentrations of HOSCN over 24 hours. Figure 30 demonstrates that in the absence of HOSCN in the media, the growth of the two bacterial strains was identical, however, at higher HOSCN concentrations, wild-type E. coli recovered from the stress and entered log-phase growth more quickly than the rclA knockout mutant. In another experiment, rclA was expressed from a plasmid, complemented back into the knockout strain, and then exposed to high concentrations of HOSCN. The complemented knockout strain exhibited high resistance to HOSCN stress and was able to recover from up to 1 mM HOSCN, which is significantly higher than the physiological concentration reported in human body fluids (Meredith et al., 2021).

In vivo experimental results by Meredith et al. (2021) also support our hypothesis that active site cysteines C43 and C48 are essential for the reaction between RclA, NADH, and HOSCN to occur. The data collected in in vivo experiments demonstrated that while
Figure 30. *rclA* responds to HOSCN exposure *in vivo*. Wild-type *E. coli* and *rclA* knockout strains were exposed to varying concentrations of HOSCN. The absorbance was measured at 600 nm periodically for 24 hours. Experiments were performed in biological triplicate with the averages shown and error bars representing standard deviation (Meredith et al., 2021).

*rclA* knockouts complemented with wild-type RclA were resistant to HOSCN, the *rclA* knockouts complemented with either RclA\textsuperscript{C43A} or RclA\textsuperscript{C48A} were not able to survive exposure to higher concentrations of HOSCN (Figure 31).

Our results suggest that the reaction between RclA, NADH and HOSCN follows the ping-pong kinetic mechanism, with RclA being reduced by NADH in the reductive half-reaction and then reoxidized by HOSCN in the oxidative half-reaction, while reducing HOSCN to SCN\textsuperscript{−} (Fig. 32). However, the reaction intermediates and the mechanism of action of the active site cysteines C43 and C48, as well as the role of other active site residues, such as H426, E431, K13 and K16, remain to be elucidated. Mutagenesis studies could be used as a method for both determining the
Figure 31. *In vivo* results suggest that active-site cysteines are required for HOSCN reduction by RclA. Knockout strains of *E. coli* *rclA* complemented with pBAD30 plasmids expressing wild-type or mutated forms of *rclA* were exposed to HOSCN with absorbance measured at 600nm for 24 hours. Experiments were performed in biological triplicate with error bars representing standard deviation (Meredith et al., 2021).

Figure 32. Proposed catalytic cycle of RclA
reaction intermediates and the roles of the active site residues. In the past research, a “mixed disulfide fishing” method has been used to visualize covalent disulfide-bonded reaction intermediates for thioredoxin reductase and thioredoxin superfamily enzymes, followed by the identification of the reaction intermediates by mass spectrometry and X-ray crystallography (Kouwen et al., 2008, Sturm et al., 2009). The method involves mutagenesis of one of the active site cysteines and the characterization of the trapped intermediate. A similar method involving mutagenesis of C43 or C48 can be employed for trapping the intermediate of the reaction between RclA and HOSCN, which may be a sulphenic acid. The resulting intermediate can be identified and characterized using mass spectrometry and X-ray crystallography. This method can also provide clarification for the specific role of each of the active site cysteines.

Mutagenesis of other active site residues, such as H426, E431, K13 and K16, can shed light on the significance of these residues in the reaction mechanism. We hypothesize that H426 assists with protonation of OSCN\(^-\) in order to increase its reactivity, as at a physiological pH OSCN\(^-\) predominates over HOSCN (Davies, 2011), which makes it significantly less reactive than HOCl. K13 and K16 may be involved with facilitating the orientation of the substrate. In order to test this hypothesis, \(K_m\) and \(k_{cat}\) of the reaction of K13 and K16 mutants with HOSCN would be compared to the respective kinetic parameters of the reaction of HOSCN and the wild-type enzyme. It is expected that \(K_m\) would be much higher than in the reaction with wild-type enzyme, but \(k_{cat}\) would not change significantly if the lysines are indeed involved in the orientation of the substrate.

Our results suggest that the release of NAD\(^+\) is the rate-determining step in the reaction between NADH, RclA, and HOSCN, as the rate constant for NAD\(^+\) dissociation of 115.8 s\(^{-1}\) is similar to the \(k_{cat}\) of the overall reaction between RclA, NADH, and HOSCN, determined in steady state assays, suggesting that NAD\(^+\) dissociation may be rate limiting during turnover by RclA.
However, since a discrepancy exists between the $k_{off}$ for NAD$^+$ and the $k_{cat}$ of the overall reaction, further experiments are required to clarify the kinetic data for NADH and NAD$^+$ binding. Performing the experiment at a lower temperature, such as 4°C, would slow down the reaction rate, allowing to monitor the reaction progress with higher accuracy. Additionally, it is suggested to determine the binding kinetics for NADP$^+$ and NADPH, to compare the kinetic data for both pyridine nucleotide substrates.

In conclusion, we demonstrated that RclA is a HO SCN reductase, which detoxifies HO SCN by coupling its reduction to SCN$^-$ with NAD(P)H oxidation through the use of the enzyme’s FAD and active site cysteines and follows a ping-pong kinetic mechanism. While further studies are required to elucidate the reaction intermediates and specific roles of active-site residue, our study provides a rationale for how bacteria can cope with reactive chlorine species produced by mammalian immune system, especially in the lungs and oral cavity. Targeting $rclA$ or RclR in bacteria found in oral cavity or lung airway fluids may make pathogenic bacteria more susceptible to the immune response, thus allowing to create new strategies for controlling pathogenic infections.
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