1 **An increase in surface hydrophobicity mediates chaperone activity in N-chlorinated proteins**

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18 Running title: N-chlorination sites in RidA from *E. coli*

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28 Keywords: N-chlorination, oxidation, oxidative stress, *E. coli*, chaperone

29 ABSTRACT

30 Under physiological conditions, *Escherichia coli* RidA is an enamine/imine deaminase, which promotes
 31 the release of ammonia from reactive enamine/imine intermediates. However, when modified by
 32 hypochlorous acid (HOCl), as produced by the host defense, RidA_{HOCl} turns into a potent chaperone-like
 33 holdase that can effectively protect the proteome of *E. coli* during oxidative stress. We previously
 34 reported that the activation of RidA's chaperone-like function coincides with the addition of at least
 35 seven and up to ten chlorine atoms. These atoms are reversibly added to basic amino acids in RidA_{HOCl}
 36 and removal by reducing agents leads to inactivation. Nevertheless, it remains unclear, which residues in
 37 particular need to be chlorinated for activation. Here, we employ a combination of LC-MS/MS analysis,
 38 a chemo-proteomic approach, and a mutagenesis study to identify residues responsible for RidA's
 39 chaperone-like function. Through LC-MS/MS of digested RidA_{HOCl}, we obtained direct evidence of the
 40 chlorination of one arginine residue (and, coincidentally, two tyrosine residues), while other N-
 41 chlorinated residues could not be detected, presumably due to the instability of the modification and its
 42 potential interference with a proteolytic digest. Therefore, we established a chemoproteomic approach
 43 using 5-(dimethylamino) naphthalene-1-sulfinic acid (DANSO₂H) as a probe to label N-chlorinated
 44 lysines. Using this probe, we were able to detect the N-chlorination of six additional lysine residues.
 45 Moreover, using a mutagenesis study to genetically probe the role of single arginine and lysine residues,
 46 we found that the removal of arginines R105 and R128 leads to a substantial reduction of RidA_{HOCl}'s
 47 chaperone activity. These results, together with structural analysis, confirm that the chaperone activity
 48 of RidA is concomitant with the loss of positive charges on the protein surface, leading to an increased
 49 overall protein hydrophobicity. Molecular modelling of RidA_{HOCl} and the rational design of a RidA
 50 variant that shows chaperone activity even in the absence of HOCl further supports our hypothesis. Our
 51 data provide a molecular mechanism for HOCl-mediated chaperone activity found in RidA and a growing
 52 number of other HOCl-activated chaperones.

53 INTRODUCTION

54 Phagocytosis is a crucial mechanism of our innate immune system used in the defense against and the
55 elimination of pathogens, such as bacteria and fungi. Within the phagosome, a cellular compartment
56 formed from the cytoplasmic membrane during phagocytosis, pathogens are exposed to a complex
57 mixture of different reactive oxygen and nitrogen species, including superoxide radicals, hydrogen
58 peroxide, peroxynitrite, and hypochlorous acid (for comprehensive reviews see Mortaz et al., 2018;
59 Winterbourn et al., 2016).

60 Hypochlorous acid is produced by the heme enzyme myeloperoxidase from H_2O_2 and chlorine. HOCl is
61 a highly reactive oxidizing and chlorinating agent and one of the most potent oxidants that exist in human
62 cells (Albrich et al., 1981). It is known to cause a variety of modifications in virtually all cellular
63 macromolecules including DNA (Prütz, 1996), lipids (Winterbourn et al., 1992; Carr et al., 1996;
64 Deborde and von Gunten, 2008), carbohydrates (McGowan and Thompson, 1989) and proteins. Amino
65 acid modifications caused by HOCl include oxidation of cysteine and methionine residues and
66 chlorination of tyrosine, lysine, arginine, and histidine (Hawkins et al., 2003; Pattison et al., 2012). Often
67 these modifications are irreversible, leading to protein misfolding and aggregation (Hawkins and Davies,
68 1999; Winter et al., 2008; Müller et al., 2014), loss of function, and eventual degradation by proteolytic
69 enzymes (Hawkins and Davies, 1999). Thus, modification of proteins by oxidative stressors and HOCl,
70 in particular, is mostly detrimental to their function. However, in some cases, oxidative modifications
71 can also regulate and activate functions of some proteins to help the cell overcome those oxidative stress
72 conditions. Long-established among the oxidative modifications that can lead to protein activation are
73 reversible oxidations of the side chain of cysteines. Prime examples are transcription factors such as
74 OxyR (Storz et al., 1990; Zheng et al., 1998) or the redox-regulated chaperone Hsp33 (Jakob et al., 1999)
75 in *E. coli*. Both proteins become activated upon the formation of disulfide bonds and, conversely,

reduction of these disulfides leads to their inactivation. More recently we discovered that N-chlorination of basic amino acid side chains, such as lysine and arginine, is another *in vivo*-reversible modification that mediates the activation of a chaperone-like holdase function in the bacterial protein RidA. After treatment with HOCl or monochloramine, we observed an approximately 60 % reduction in the amino group content accompanied by an increase in RidA's hydrophobicity. Consistent with this finding, mass spectrometry (MS) analysis revealed the presence of multiple chlorinated species (Müller et al., 2014). This activation of RidA is independent of the oxidation status of its sole cysteine and is fully reversible by DTT, ascorbic acid, glutathione, and the thioredoxin system.

Other proteins, like the bacterial protein CnoX have been found to use a similar mechanism. After activation with HOCl, CnoX, similar to RidA, turns into an effective chaperone-like holdase that can bind a variety of substrate proteins and prevent their aggregation (Goemans et al., 2018). Moreover, CnoX has a thioredoxin domain and can protect its substrates from irreversible thiol oxidation by forming mixed disulfides with them (Goemans et al., 2018). But not only bacterial proteins are affected by N-chlorination: recently we discovered that human plasma proteins, such as human serum albumin, are activated by N-chlorination and act not only as chaperones to protect damaged host proteins but can also activate immune cells promoting their production of ROS (Ulfig et al., 2019) and mediating their antigen processing (Ulfig et al., 2021).

In this study, we identified the residues facilitating RidA's HOCl-driven activation mechanism. Our data suggest that chlorination of basic amino acids by HOCl is not a process that targets one or two crucial amino acids that act as the "switch" affecting the whole protein. Instead, the N-chlorination of multiple residues changes the electrostatic potential of the molecular surface of RidA allowing it to interact with unfolded substrate proteins.

98 RESULTS

99 *Direct identification of chlorinated sites in RidA after treatment with HOCl revealed chlorination of one*
100 *arginine and two tyrosines*

101 We previously reported that the enamine/imine deaminase RidA from *E. coli* turns into a potent protein
102 holdase upon N-chlorination of its lysine and arginine residues. RidA, modified in this way (RidA_{HOCl}),
103 can protect other cellular proteins from unfolding due to chlorine stress. Previous mass spectrometric
104 analysis of undigested HOCl-treated RidA showed that the reversible addition of at least 7 and up to 10
105 chlorine atoms to amino acid residues is concomitant with chaperone-like holdase activity in this protein
106 (Müller et al., 2014). However, it remains unknown, which residues are modified and which of those are
107 required for activation. If we consider arginine, histidine, and lysine as potential targets for a reversible
108 N-chlorination, RidA has 15 possible N-chlorination sites: 5 arginines, 1 histidine, 8 lysines, and the
109 protein's N-terminus (Figure 1).

1 MSKTIATENAPAAIGPYVQGVDLGNMIITSGQIPVNPKTGEVPADVAAQA
51 RQSLDNVKAIVEAAGLKVGDIVKTTVFVKDLNDFATV NATYE AFFTEHNA
101 TFPARSCVEVARLPKDVKIEIEAIAVRR

Figure 1.

111 The amino acid sequence of the *E. coli* protein RidA (Uniprot entry number: P0AF93). Lysines (K) are
112 highlighted in pink, arginines (R) in purple, and histidine (H) in blue.

113 To elucidate which amino acids are affected by chlorination, we first performed a mass spectrometric
114 analysis of N-chlorinated RidA after proteolytic digest, specifically searching for peptides having an
115 added mass due to chlorination. Among the above-mentioned 15 possible N-chlorination sites, we were
116 only able to identify R51 to be chlorinated in any of the three replicates after digest with trypsin (Table
117 1, Figure 2). The monoisotopic mass of the chlorinated peptide containing R51 was ~ 33.96 Da heavier
118 than the unmodified peptide, corresponding to the monoisotopic mass of a chlorine atom minus the
119 monoisotopic mass of the hydrogen atom it replaced. The same tell-tale mass difference could be
120 observed in 9 fragment y-ions containing R51 (Figure 2C). Additionally, we found two chlorinated

121 peptides containing the two tyrosines present in RidA (Y17, Y91) (Table 1). Tyrosine chlorination by
 122 HOCl is an irreversible modification that occurs with a much slower rate than the chlorination of lysine,
 123 histidine and arginine (Hawkins et al., 2003). But once formed, chlorotyrosine is highly stable
 124 (Winterbourn and Kettle, 2000; Hendrikje Buss et al., 2003). Our previous experiments showed that the
 125 7 to 10 added chlorine atoms observed in full-length MS are virtually all removable by DTT, inconsistent
 126 with chlorinated tyrosine residues. We thus concluded that our finding is, due to the high stability of the
 127 modification in question, probably of auxiliary nature with no functional relevance.

128 We suspected that the low number of identified N-chlorinated residues in our experiment could be caused
 129 by our use of trypsin. Trypsin, the protease most commonly used for the MS analysis of proteins, cleaves
 130 proteins after lysine and arginine, the very residues which we suspected to be modified by HOCl. As
 131 chlorination of these residues might interfere with trypsin's ability to recognize them, we additionally
 132 prepared a digest with chymotrypsin. Nevertheless, we were still not able to detect more N-chlorinated
 133 residues in our chymotryptic digest in any replicate but only re-identified chlorinated tyrosines Y17 and
 134 Y91 (Table 1).

Figure 2. Arginine R51 is chlorinated upon treatment with HOCl. (A, B) Extracted ion chromatograms (XIC) of m/z 659.81, corresponding to the mass of the N-chlorinated peptide TGEVPADVAAQAR₅₁ at a retention time from 25 to 30 minutes. A tryptic digest of untreated RidA produces no ion with the respective m/z (A), while RidA treated with a 10-fold molar excess of HOCl shows a peak at 26.265 min (B). (C) Primary MS spectrum of the N-chlorinated arginine-containing peptide TGEVPADVAAQAR₅₁. (D) Corresponding fragment spectrum of the N-chlorinated peptide contains signals corresponding to 9 y-ions containing the chlorine atom.

142 **Table 1. Retention times (RT) and properties of unchlorinated and chlorinated peptides identified by LC-MS/MS after tryptic or chymotryptic**
 143 **digest of HOCl-treated RidA.** Methionine sulfoxidation is highlighted in red, and possible sites of chlorination are labeled blue. The PEP-score
 144 (posterior error probability) calculated by MaxQuant indicates the probability that a peptide was falsely identified (Käll et al., 2008; Cox and Mann,
 145 2008)

Protease	Residues	Peptide sequence	Uncolorinated			Chlorinated		
			m/z, charge	RT, min	PEP-score	m/z, charge	RT, min	PEP-score
<i>Trypsin</i>	4-38	TIATENAPAAIGPY _{Cl} VQGVDL- GNM _{ox} IITSGQIPVNK	1189.29, +3	47.17	8.21 x 10 ⁻⁷²	1200.95, +3	49.29	4.28 x 10 ⁻⁴⁷
<i>Trypsin</i>	39-51	TGEVPADVAAQAR _{Cl}	642.83, +2	25.88	4.31 x 10 ⁻²⁵⁷	659.81, +2	26.36	1.81 x 10 ⁻⁵
<i>Trypsin</i>	80-105	TTVFVKDLNDFATVNATY _{Cl} - EAFFTEHNATFPAR	910.12, +4	45.45	5.45 x 10 ⁻²⁰	918.68, +4	48.90	1.62 x 10 ⁻⁶
<i>Chymotrypsin</i>	2-19	SKTIATENAPAAIGPY _{Cl}	802.42, +2	31.27	8.66 x 10 ⁻²⁵⁰	819.40, +2	32.81	3.73 x 10 ⁻²²²
<i>Chymotrypsin</i>	78-91	VKDLNDFATVNATY _{Cl}	785.89, +2	39.82	1.24 x 10 ⁻²⁹⁰	802.87, +2	39.10	2.67 x 10 ⁻¹⁴⁸

147 *DANSO₂H, a novel proteomic probe for N-chlorinated lysine residues in RidA_{HOCI}*

148 Our previous results showed that at least seven and up to ten residues become N-chlorinated in RidA,
149 when it is active as a chaperone. Thus, we were dissatisfied with our limited ability to detect N-
150 chlorinated amino acids using mass spectrometry of proteolytic digests of HOCl-treated RidA. This
151 inability might be due to the inherent instability and high reactivity of N-chloramines. Therefore, we
152 devised a way to stably modify N-chlorinated amino acids.

153 To this end we utilized dansyl sulfinic acid (5-(dimethylamino)naphthalene-1-sulfinic acid, DANSO₂H),
154 a derivative of the well-characterized reagent dansyl chloride (5-dimethylamino)naphthalene-1-sulfonyl
155 chloride, DANSCl), which has been used for a long time to derivatize amines, amino acids, and proteins.
156 It reacts with free amino groups and forms stable, highly fluorescent sulfonamides, that can be separated
157 by HPLC and detected by mass spectrometry. In proteins, DANSCl usually reacts only with the free
158 amino group of lysine and the N-terminus (Hsieh and Matthews, 1985) (Figure 3A). Unlike DANSCl,
159 DANSO₂H is not reactive towards unmodified amino groups. Instead, it reacts with monochloramines,
160 forming the same sulfonamide product as the reaction of DANSCl with corresponding unmodified
161 amines (Figure 3C). This provides us with a method for the selective labeling of N-chlorinated lysine
162 residues. DANSO₂H was synthesized from DANSCl by a reaction with sodium sulfite (Scully et al.,
163 1984). The synthesized molecule had a purity of 95 %. The only contamination present was dansyl
164 sulfonic acid (DANSO₃H) (Figure S2), which is chemically inert and does react neither with amino
165 groups nor N-chlorinated residues (Scully et al., 1984). The synthesized DANSO₂H was then used to
166 modify RidA_{HOCI}. DANSCl was used as a positive control to modify untreated RidA (RidA_{UT}), and, as a
167 negative control, DANSO₂H was incubated with RidA_{UT}. As expected, after treatment with DANSCl,
168 RidA_{UT} showed the typical fluorescence of dansyl-modified proteins (Figure 3B). The same fluorescence
169 albeit to a lesser extent was observed in DANSO₂H-treated RidA_{HOCI} (Figure 3D). The ~30 % lower
170 fluorescence intensity is consistent with our previous finding that only up to 10 out of 15 potential

171 chlorination sites are chlorinated in fully active RidA_{HOCI}. RidA_{UT} treated with DANSO₂H showed
172 virtually no fluorescence, demonstrating the specificity of our probe (Figure 3D).

173
174 **Figure 3. Modification of RidA with dansyl-derived compounds.** (A) Dansyl chloride (DANSCl) reacts with
175 an exemplary amino group in RidA forming a “dansylated” fluorescent sulfonamide. (B) Dansylation of proteins
176 can be detected using fluorescence spectroscopy at Ex/Em: 340/500 nm. The reaction of RidA_{UT} with a 10-fold
177 molar excess of DANSCl yields a fluorescent protein (orange spectrum), while the protein by itself shows no
178 fluorescence (teal spectrum). (C) Dansyl sulfinic acid (DANSO₂H) is a derivative of DANSCl and reacts
179 specifically with N-chlorinated amino groups forming the same “dansylated” fluorescent product found in panel
180 (A). (D) HOCl-treated RidA (RidA_{HOCI}) reacts with a 10-fold molar excess of DANSO₂H producing a fluorescent
181 peak at the expected excitation/emission wavelengths (purple spectrum). Virtually no fluorescent signal was
182 observed with DANSO₂H-treated RidA_{UT} (green spectrum).

183 *N-chlorinated lysines can be identified after labeling of RidA_{HOCI} with DANSO₂H*

184 Since the fluorescence spectroscopic experiments showed promising results, we then digested the
185 DANSO₂H-treated RidA_{HOCI} with chymotrypsin and performed LC-MS/MS analysis. In total, six lysines
186 (K3, K38, K67, K79, K115, K118) were consistently identified as dansylated in all tested samples of
187 DANSO₂H-treated RidA_{HOCI} (Table 2). Masses corresponding to these dansylated peptides were not
188 observed in RidA_{UT}. Unfortunately, arginines seem not to be modified by DANSO₂H, consistent with
189 DANSCl’s preference for free amino groups. Dansylated peptides have a mass difference to the

unmodified peptide of ~ 233.05 Da, which equals the mass of DANSO₂H minus the monoisotopic mass of two hydrogen atoms. The dansyl-modification changes the chemical property of a peptide making it more hydrophobic, causing it to elute at significantly higher acetonitrile (ACN) concentrations in the LC. For instance, unmodified peptide harboring K3 eluted at approx. 18 % ACN, whereas the corresponding dansyl-modified peptide eluted at approx. 27 % ACN (Table 2)

Figure 4. LC-MS/MS analysis of RidA_{HOCl} after treatment with DANSO₂H and subsequent chymotryptic digest. (A, B) Extracted ion chromatogram (XIC) of m/z 918.95, corresponding to the mass of dansylated peptide SK₃TIATENAPAAIGPY at retention times from 40-45 min. **(A)** In the sample derived from DANSO₂H-treated RidA_{UT}, no ion at this m/z was observed. **(B)** A peak at 41.373 min corresponding to the mass of the dansylated peptide could be observed in DANSO₂H-treated RidA_{HOCl}. **(C)** Primary MS spectrum of the DANSO₂H-modified peptide SK₃TIATENAPAAIGPY found in the digest derived from DANSO₂H-treated RidA_{HOCl}, at an m/z of 918.95. **(D)** MS/MS analysis revealed that the modified amino acid is lysine K3 at position 2 of the chymotryptic peptide with one y-ion and thirteen b-ions showing the mass shift associated with dansylation.

204 No dansylated amino acids were found in a RidA_{UT} sample treated with DANSO₂H. With this novel
205 chemoproteomic method we were able to gather direct evidence for the N-chlorination of six lysines in
206 the active RidA_{HOCI} chaperone-like holdase.

207 *Mutagenesis reveals two arginines that play an important role in the activation of RidA's chaperone*
208 *activity*

209 Since DANSO₂H is most likely not suitable for the detection of N-chlorinated arginine residues, we used
210 a mutagenesis approach to test if individual amino acids have a particular impact on the chaperone
211 function of N-chlorinated RidA. We thus engineered a total of 13 RidA variants with every single lysine
212 or arginine residue exchanged to a serine.

213 These 13 RidA variants were expressed and purified from *E. coli* BL21(DE3) and their chaperone activity
214 after treatment with HOCl was then determined. Activity was tested in a 4-fold excess over citrate
215 synthase. The activity of the variants was compared against wild-type RidA_{HOCI} under the same
216 conditions. The activity of most of HOCl-treated mutants was not affected in a significant way by the
217 exchange of a single N-containing amino acid to serine, when compared to HOCl-treated wild-type
218 protein (Figure 5). However, two variants lacking specific arginines (R105S and R128S) showed a
219 significantly reduced chaperone activity when compared to HOCl-treated wild-type RidA (RidA_{WT}).

220 **Table 2. Retention times (RT) and properties of peptides derived from dansyl sulfinic acid (DANSO₂H)-labeling of HOCl-treated RidA after**
 221 **chymotryptic digest and LC-MS/MS.** Methionine sulfoxidation and cysteine sulfonic acid modifications are highlighted in red, and lysines that are
 222 modified with dansyl sulfonic acid are labeled orange. The PEP-score calculated by MaxQuant indicates the probability that a peptide was falsely
 223 identified (Käll et al., 2008; Cox and Mann, 2008)

Protease	Residues	Peptide sequence	Undansylated			Dansylated		
			m/z, charge	RT, min	PEP-score	m/z, charge	RT, min	PEP-score
<i>Chymotrypsin</i>	2-19	SK _{DANS} TIATENAPAAIGPY	802.42, +2	28.57	2.24×10^{-250}	918.95, +2	41.37	1.61×10^{-250}
<i>Chymotrypsin</i>	18-54	VQGVDLGNM _{ox} IITSGQIPVNP- K _{DANS} TGEVPADVAAQARQSL	1263.99, +3	34.61	5.56×10^{-50}	1341.68, +3	41.80	6.63×10^{-38}
<i>Chymotrypsin</i>	67-77	K _{DANS} VGDIVKTTVF	603.86, +2	30.89	5.73×10^{-13}	720.38, +2	41.49	2.26×10^{-8}
<i>Chymotrypsin</i>	78-91	VK _{DANS} DLNDFATVFNATY	785.89, +2	33.37	8.66×10^{-136}	902.42, +2	42.05	6.52×10^{-148}
<i>Chymotrypsin</i>	103-128	PARSC _{so3H} VEVARLPK _{DANS} D- VK _{DANS} EIEAIAVRR	989.56, +3	28.90	1.02×10^{-46}	1144.92, +3	43.91	2.28×10^{-10}

Figure 5. Chaperone activity of RidA variants lacking individual lysine or arginine residues. Chaperone activity was tested in an aggregation assay with citrate synthase. **(A)** HOCl-treated RidA_{WT} (teal) strongly inhibits aggregation of chemically denatured citrate synthase when compared to untreated control (black), as measured by light scattering at 360 nm. All variants, except for R105S (yellow) and R128S (violet), showed activity similar to RidA_{WT}. **(B)** Bar graph data represents means and standard deviations from three independent experiments. Differences in chaperone activity of RidA_{WT} between the variants were analyzed using a one-way ANOVA with Tukey's comparison test (****, $p < 0.0001$). The activity of RidA_{WT} was set to 100 %, and all the data are presented in correlation to this control.

The concomitant exchange of R105 and R128 does not further decrease chaperone activity

Since an exchange of arginine 105 or 128 resulted in decreased chaperone activity, a variant harboring both mutations (R105S_R128S) was constructed to investigate if a synergistic effect can be observed. The variants were then also treated with HOCl and used in an 8-fold excess over citrate synthase. However, the chaperone activity of the variant R105S_R128S remained at approximately the same level as the single exchange variants and a further decrease of chaperone activity was not observed (Figure 6).

Figure 6. Chaperone activity of RidA double exchange variant R105S_R128S. Chaperone activity was tested in an aggregation assay with citrate synthase. **(A)** HOCl-treated RidA_{WT} (teal) strongly inhibits aggregation of chemically denatured citrate synthase compared to untreated control (black), as measured by light scattering at 360 nm. The concomitant exchange of R105 and R128 against serine (pink) does not further decrease the chaperone activity of RidA and is comparable to single amino acid exchange variants (R105S (yellow) and R128S (violet)). **(B)** Bar graph data represents means and standard deviations from three independent experiments. Differences in chaperone activity between the variants were analyzed using a one-way ANOVA with Tukey's comparison test (***, $p < 0.001$). The activity of RidA_{WT} was set to 100 %, and all the data are presented in correlation to this control.

The activation of RidA's chaperone-like holdase function depends on an overall change of the molecules electrostatic surface.

Summed up, our proteomic, chemo-proteomic and mutagenesis studies suggest that no single amino acid acts as a discrete “switch”, but rather the modification of multiple N-containing amino acids leads to the activation of RidA's chaperone-like holdase function. Even arginines 105 and 128, whose individual exchanges to the inert amino acid serine had the largest effect on activatability, did not show synergistic effects when both were removed. Chaperones are known for surface patches that enable them to interact with hydrophobic regions of unfolded proteins. Indeed, our previous experiments showed that RidA_{HOCl} did bind a hydrophobic dye much better than RidA_{UT} (Müller et al., 2014). In order to understand how N-chlorination of basic amino acids changes the surface properties of RidA, we predicted the electrostatic surface potential of RidA, using the known X-Ray structure of RidA (Volz, 2008) (Figure 7A). We then computationally substituted lysine and arginine residues with their chlorinated counterparts within the

262 structure data set. Using a customized force field, we were then able to predict the electrostatic surface
263 potential of an N-chlorinated RidA molecule as well (Figure 7B). Strikingly, the electrostatic surface
264 potential shifted towards a more negative potential, more or less over the complete surface of the
265 molecule. We thus concluded that the loss of positive electrostatic surface potential is the underlying
266 molecular reason for RidA_{HOCI}'s chaperone-like properties.

267 *An engineered variant of RidA mimicking RidA_{HOCI}'s surface potential is an active chaperone without*
268 *HOCI treatment*

269 To test our hypothesis, we decided to engineer a variant of RidA that mimics RidA_{HOCI}'s surface
270 potential. To this end, we wanted to mutate all lysine and arginine residues in RidA to more
271 “electroneutral” amino acids. In order to select amino acids that would not lead to a disruption of RidA's
272 structure, we performed a multiple sequence alignment to direct our choice of suitable amino acids. For
273 our mutagenesis, we selected amino acids that occur at the respective position and are mostly uncharged
274 at physiological pH. With the exception of the invariant R105, the arginine in the active deaminase site
275 of RidA, we were able to find a suitable amino acid at all positions. This resulted in a variant, which we
276 termed R0K0_R105 (Figure 8).

277 The predicted electrostatic surface potential of this R0K0_R105 variant showed a high similarity to the
278 predicted surface potential of N-chlorinated RidA_{HOCI} (Figure 7C).

Figure 7. Calculated electrostatic surface potentials of *E. coli* RidA_{UT} (Uniprot entry: P0AF93, PDB entry: 1QU9), RidA_{HOCl}, and the K0R0_R105 variant. The C-terminal R128 was added to the RidA structure, as it was only partially resolved in the crystal structure. **(A)** Electrostatic surface visualization of wild-type RidA. The calculation was performed using the Advanced Poisson-Boltzmann Solver in PyMol using the AMBER force field, as described in the materials and methods section. The coloring scale was chosen from -15 to +5 kT/e according to the legend. Middle panel: the molecule rotated 90 degrees around the x-axis in comparison to the upper panel. Lower panel: the molecule was rotated 90 more degrees around the x-axis. **(B)** Electrostatic surface visualization of RidA_{HOCl} using PyMOL. The charge-bearing proton was removed from all lysine and arginine residues, except for R105, and one of the hydrogens was then replaced by a chlorine atom using bond geometries derived from model compounds. The force field (partial charges of respective N and Cl atoms as well as the van der Waals radius of Cl) was adjusted accordingly. **(C)** Electrostatic surface visualization of K0R0_R105. Amino acid exchanges were introduced in RidA's structure using PyMol's "Mutagenesis" function.

Figure 8. Sequence alignment of wild-type RidA (P0AF93 RidA *E. coli*) with the R0K0_R105 variant and RidA homologs. In the K0R0_R105 variant all lysine and arginine residues were replaced with more electroneutral residues based on amino acids found at those positions in RidA homologs, with the exception of the active-site arginine R105, where no replacement amino acid was found. The order of sequences is based on overall amino acid identity, ranging from 93.75 % (*S. enterica*) to 42.19 % (*A. vinelandii*). Arginine and lysine residues found at the position of the replaced amino acids are highlighted in cyan in the alignment, while amino acids chosen for replacement are highlighted in blue. Other amino acids at those positions are highlighted in grey. The invariant arginine R105 is highlighted in green.

302 The K0R0_R105 variant was then expressed and purified from *E. coli* BL21(DE3) and its chaperone
303 activity in comparison to RidA_{UT} and RidA_{HOCI} was determined. As predicted, the RidA variant
304 K0R0_R105 showed already potent chaperone-like activity without HOCl pre-treatment, and treatment
305 with HOCl did not increase its chaperone activity significantly (Figure 9). Overall, the activity of
306 K0R0_R105 was indeed comparable to HOCl-treated RidA, supporting our hypothesis.

307 **Figure 9. Chaperone activity of the RidA variant K0R0_R105.** Chaperone activity was tested in an aggregation
308 assay with citrate synthase. **(A)** HOCl-treated RidA_{WT} (teal) strongly inhibits aggregation of chemically denatured
309 citrate synthase compared to untreated control (black), as measured by light scattering at 360 nm. The variant
310 K0R0_R105 (purple) is fully active as a chaperone without any HOCl treatment, and N-chlorination of this variant
311 (light blue) does not influence its chaperone activity. **(B)** Bar graph data represents means and standard deviations
312 of three independent experiments. Difference in chaperone activity between the untreated and HOCl-treated
313 K0R0_R105 was analyzed using a one-way ANOVA with Tukey's comparison test. The activity of RidA_{WT} was
314 set to 100 %, and all data are presented in correlation to this control.
315

316 Taken together, our results suggest that chlorination of basic amino acids by HOCl is not a process that
317 targets one or two crucial amino acids that act as a “switch” that affects the whole protein. Instead, the
318 N-chlorination of multiple residues generates a more negatively charged and hydrophobic molecular
319 surface on RidA, allowing for the interaction with unfolded client proteins. This loss of positive surface
320 charge through N-chlorination is a plausible mechanism for the chaperone-like switch that occurs in a
321 growing number of proteins in response to exposure to HOCl and other reactive chlorine species.

322 DISCUSSION

323 After reacting with HOCl or monochloramine, RidA turns into a potent chaperone-like holdase that can
324 bind client proteins. The underlying mechanism was proposed to be the N-chlorination of lysine and
325 arginine residues (Müller et al., 2014). Since then, several other proteins have been discovered that seem
326 to switch to a chaperone-like function upon N-chlorination of basic amino acids. However, it was still
327 unclear if the chlorination of one or several specific residues is decisive for chaperone activity of RidA
328 or rather a general modification of multiple amino acid side chains that affects the overall surface of the
329 protein.

330 Four proteinogenic amino acids are known to be prone to chlorination (Peskin and Winterbourn, 2001).
331 First, lysines have an amino group on their side chain that can react with HOCl, forming
332 monochloramines. The guanidino group of arginine can also be N-chlorinated. These modifications are
333 thought to be reversed by ascorbate. The side chain of histidine can also react with HOCl, forming a
334 short-lived chloramine. Nevertheless, this chloramine has been shown to be more reactive than
335 corresponding chloramines of lysine and arginine. Moreover, it can rapidly transfer chlorine to other
336 amine groups to generate more stable chloramines (Pattison and Davies, 2005). Additionally, histidines
337 are typically less abundant in proteins in comparison to lysines and arginines. For instance, RidA has
338 only one histidine. Lastly, tyrosine is known to become chlorinated. However, the chlorination of
339 tyrosine is an irreversible reaction and leads to the formation of 3-chlorotyrosine (Hawkins et al., 2003).

340 *LC-MS/MS analysis revealed HOCl-modified arginine, along with two modified tyrosines*

341 Previously our laboratory reported that RidA's full chaperone-like activity is coinciding with the addition
342 of up to 10 chlorine atoms. However, the LC-MS/MS analysis of chlorinated RidA after tryptic digest
343 performed in the current study only revealed one N-chlorinated amino acid, arginine R51. There was also
344 mass spectrometric evidence for the chlorination of both tyrosines present in RidA, but, as mentioned

above, this modification is irreversible and thus should not account for the reversible activation of RidA's chaperone function. Chlorination of lysine or arginine could interfere with the tryptic digest, as these are the amino acids recognized by this particular protease. However, digest with an alternative protease, chymotrypsin did not reveal additional chlorination sites. Alternatively, N-chlorination might potentially be lost during the process of sample preparation, since the amino acid-derived monochloramines, even though less reactive, retain some of the oxidizing capacity of HOCl and are highly reactive towards oxidizable components of reaction buffers (Pattison and Davies, 2006; Ashby et al., 2020). Furthermore, N-chlorinated RidA can potentially react with sulfur-containing amino acids found in the proteases used. Therefore, a direct MS-based identification of chlorinated residues is challenging and does not result in the identification of all modified residues.

Goemans et al. conducted a similar experiment where they performed a mass spectrometric analysis of chlorinated CnoX, in which chaperone activity is also activated by HOCl. Although they identified at least 8 chlorines added to the protein mass during mass spectrometry of full-length CnoX, only five chlorinated amino acid residues were identified using LC-MS/MS analysis after protease digest, among them no lysine residue, hinting at the challenges involved in identifying this particular N-chlorinated amino acid by direct MS analysis (Goemans et al., 2018).

Dansyl sulfinic acid can be used to stably modify N-chlorinated lysines

Searching for ways to chemically label N-chlorinated lysine, and thus making it accessible for MS-based analysis, dansyl sulfinic acid (DANSO₂H) caught our attention. It has been previously used to derivatize low molecular weight monochloramines in water and other fluids, allowing for their detection by HPLC (Scully et al., 1984, 1986). We synthesized this probe to label the full-length RidA after its chlorination and found that it exclusively reacts with the HOCl-treated protein. We were able to detect a robust fluorescence signal once N-chlorinated RidA was treated with DANSO₂H, but DANSO₂H did not react

with the untreated protein. Therefore, DANSO₂H allowed for the specific labeling of N-chlorinated proteins. Using LC-MS/MS analysis of DANSO₂H-derivatized RidA_{HOC}, we identified 6 lysine residues that we were not able to detect using a direct MS-based approach. Interestingly, these 6 lysine residues are also the lysine residues most exposed to the solvent (Table 3), suggesting they are particularly accessible to HOCl.

Table 3. Accessible surface area and accessibility of lysines and arginines of RidA. The analysis was performed using Accessible Surface Area and Accessibility Calculation for Protein (ver. 1.2) (<http://cib.cf.ocha.ac.jp/bitool/ASA/>). Relative area is the fraction of the amino acid's total area exposed to the solvent.

Amino acid position	Amino acid	Area (Å ²)	Relative area (0.0 – 1.0)
38	LYS	186.596	0.873
67	LYS	150.440	0.704
115	LYS	146.521	0.686
3	LYS	141.010	0.66
127	ARG	103.542	0.451
128	ARG	91.273	0.326
79	LYS	83.866	0.392
51	ARG	81.596	0.356
118	LYS	75.743	0.354
112	ARG	67.246	0.293
58	LYS	52.885	0.247
105	ARG	14.170	0.062
73	LYS	9.844	0.046

DANSO₂H allows for the irreversible derivatization of monochloramines, increasing their stability and simplifying their detection using LC-MS/MS. DANSO₂H initially reacts with monochloramines to form dansyl chloride, which then, in turn, reacts with the newly freed amino group (Scully et al., 1984; Jersey et al., 1990). The specificity of this probe in complex samples might be limited due to this issue, as there is a chance that the dansyl chloride diffuses from the originally modified amino residue. The second order rate constant of the reaction of dansyl chloride and the ε-aminogroup of lysine is 42 M⁻¹ s⁻¹ (Gray,

1967). Therefore, a two-step procedure with blocking of unmodified amino groups might be considered for complex samples prior to labeling with DANSO₂H.

Two RidA variants lacking either R105 or R128 showed a significantly decreased chaperone activity

To find out, whether specific amino acids play a key role in the activation of RidA's chaperone-like function, all lysines and arginines were individually changed to serine residues through site-directed mutagenesis. The individual variants were then examined regarding their chaperone activity. The exchange of most lysines and arginines did not affect the chaperone activity of RidA. However, the individual exchange of amino acid residues R105 and R128 resulted in diminished chaperone activity after HOCl treatment. This could indicate that these arginines play a prominent role in chaperone activity.

Potentially, these two arginines are particularly accessible to unfolded proteins in N-chlorinated RidA and, therefore, their chlorination might be important for client protein binding. R128 is the C-terminal amino acid of RidA and, based on structural predictions, exposed to the solvent, which makes it especially accessible to unfolded proteins (Table 3). In the bacterial cytoplasm, RidA is present in the form of a trimer (Volz, 2008) and R128, R127, and K3 of every protein subunit form a large positively charged surface patch (visible in the center of the molecule in the upper panel of Fig. 7A), which is predicted to change to a more electroneutral or even negatively-charged patch after N-chlorination (Fig. 7 B, upper panel) and thus might allow for binding of unfolded client proteins.

The other residue, R105, is highly conserved in three of the seven RidA subfamilies that have enamine/imine deaminase activity (Lambrecht et al., 2012; Liu et al., 2016) and was invariable in all RidA homologs that were identified in a blast search in the SwissProt database (Altschul et al., 1990; Bateman et al., 2021) (see also Fig. 8). In *E. coli* and related species, RidA accelerates the release of ammonia from intermediates that result from the dehydration of threonine by IlvA (threonine dehydratase). Arginine 105 plays a particularly important role in the active center of the protein by

407 forming a salt bridge with the carboxylic acid of the substrate (Lambrecht et al., 2012). This amino acid
408 is also located in the immediate vicinity of RidA's only cysteine at position 107. This cysteine is redox-
409 sensitive and was modified during the nitrosative stress response (Lindemann et al., 2013). However, the
410 presence of the cysteine is not important for RidA chaperone activity as the mutant lacking the cysteine
411 was as active as wildtype after chlorination (Müller et al., 2014). R105, through its position in the active
412 site, is probably also accessible to client proteins, when N-chlorinated.

413 We also exchanged both arginines R105 and R128 simultaneously through site-directed mutagenesis. If
414 our argumentation that both arginines are of particular importance for client protein binding were true,
415 we would expect the double mutant to be an even less effective chaperone than the individual mutants.
416 Conversely, the chaperone activity of this variant corresponded approximately to the chaperone activity
417 of the individual exchanges. One explanation would be the distant localization of these two residues,
418 excluding a mutual influence on chaperone-like activity, even if both residues are absent. Alternatively,
419 the lower chaperone-like activity in both single and double mutants might not be due to the lack of an N-
420 chlorination site but the general structural disturbance that an exchange of these residues (especially in
421 the active site of the protein) induces.

422 The latter possibility is further supported by the fact that the second-order rate constant of the reaction
423 of HOCl with arginines is three orders of magnitude lower than the one for the reaction with lysines (k
424 $= 7.9 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ vs. $k = 26 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively) (Pattison and Davies, 2001; Pattison et al., 2012).
425 Hence, lysines are reacting faster with HOCl than arginines. Nevertheless, it is possible that these two
426 residues are particularly reactive with HOCl. Sometimes particular amino acid residues are more reactive
427 than suggested by the reaction rate with model compounds. As such, cysteines are usually oxidized by
428 H_2O_2 with a second-order rate constant of $k = 14.7 \pm 0.35 \text{ M}^{-1} \cdot \text{s}^{-1}$, while certain cysteines in the active site
429 of peroxiredoxins are oxidized at a much higher velocity ($k = 12'000 \text{ M}^{-1} \cdot \text{s}^{-1}$) (Peskin et al., 2013).

Therefore, it might be that R105 and R128 in RidA are particularly susceptible to N-chlorination due to the microenvironment in the protein's structure.

Electrostatic surface modeling of RidA_{HOC} and the K0R0_R105S variant revealed the patterns necessary for RidA's chaperone activity

As shown by our MS-based experiments and our mutagenesis studies, N-chlorination affects multiple basic amino acids (at least 9: lysines K3, K38, K67, K79, K115, K118 and arginines R51, R105, R128). Therefore, positive charges on the surface of a protein molecule are eliminated, causing an increase in hydrophobicity, which was previously determined experimentally using Nile Red (Müller et al., 2014; Ulfig et al., 2019) and is demonstrated by the calculated electrostatic surface potential of an N-chlorinated RidA molecule. Protein hydrophobicity as a driving force for chaperone activity is a known mechanism, and other chaperones are also known to have exposed hydrophobic surfaces that allow for the binding of client proteins (Graf et al., 2004; Mayer and Bukau, 2005; Kumar et al., 2005; Koldewey et al., 2016, 2017). A rationally designed variant, which mimics the electrostatic surface potential of N-chlorinated RidA and is indeed constitutively active as a chaperone-like holdase, substantiates this hypothesis.

CONCLUSION

For the activation of the chaperone function of RidA, a significant increase in the protein surface hydrophobicity must take place. We present here computational and experimental evidence that this is achieved by reversible N-chlorination of nine individual lysine and arginine residues. The N-chlorination of these basic amino acids removes positive charges from the surface of RidA allowing it to bind and protect unfolded client proteins in the presence of high HOCl concentrations as they appear during inflammatory processes in bacteria-host interactions.

451 MATERIALS AND METHODS

452 *Preparation of chlorinating agents*

453 The concentration of NaOCl stock was determined spectrophotometrically using a JASCO V-650
 454 UV/VIS spectrophotometer (JASCO, Tokyo, Japan) at 292 nm using the extinction coefficient $\epsilon_{292} = 350$
 455 $M^{-1}cm^{-1}$. Monochloramine was prepared freshly by dropwise addition of 200 mM NaOCl solution,
 456 dissolved in 0.1 M KOH to 200 mM NH_4Cl solution. After stirring for 5 min, the concentration of the
 457 monochloramine produced was measured spectrophotometrically using the extinction coefficient $\epsilon_{242} =$
 458 $429 M^{-1}cm^{-1}$.

459 *Treatment of RidA with HOCl and monochloramine (MCA)*

460 Chlorination of RidA or RidA variants was achieved by adding 10-fold molar excess of HOCl or MCA
 461 to the protein solution. Samples were incubated for 10 min at 30 °C for HOCl-treatment and 45 min at
 462 37 °C for MCA-treatment. Removal of the residual oxidants was achieved by size-exclusion
 463 chromatography using “Micro Bio-Spin P30 Tris Chromatography Columns” (Bio-Rad, München,
 464 Germany) or Nap-5 columns (GE Healthcare Life Sciences, Amersham, UK) according to the
 465 manufacturer’s instructions. Final protein concentrations were determined by measuring the absorbance
 466 at 280 nm using a JASCO V-650 UV/VIS spectrophotometer with an extinction coefficient of RidA of
 467 $\epsilon_{280} = 2980 M^{-1}cm^{-1}$.

468 *Preparation, tryptic and chymotryptic digest of RidA_{HOCl} for mass spectrometry analysis*

469 RidA was treated with HOCl as described above. 5 μl (approx. 25 μg of protein) of untreated and N-
 470 chlorinated RidA were mixed together with 35 μl ultrapure water and either 10 μl 5x trypsin digestion
 471 buffer (500 mM ammonium bicarbonate, pH 8.0) or 10 μl 5 x chymotrypsin digestion buffer (500 mM
 472 Tris-HCl, 10 mM $CaCl_2$, pH 8.0). Freshly reconstituted trypsin in 50 mM acetic acid (1 mg/ml) or
 473 chymotrypsin in 1 mM HCl (1 mg/ml) was added to the sample for a final 1:20 enzyme-to-protein ratio.

474 The sample was digested at 37 °C for 18 h, and afterward, the peptides were purified using OMIX C18
475 tips. Purified peptides were concentrated to dryness and dissolved in 0.1 % TFA for LC-MS/MS analysis.

476 *Peptide cleanup using OMIX C18 tips*

477 The samples after tryptic or chymotryptic digest were first adjusted to 0.1 % (v/v) TFA using 10 % TFA
478 solution. Then the tips were first equilibrated 2 times with 100 µL 100 % ACN, followed by 2 times
479 washing with 100 µL 50 % ACN, 0.1 % TFA, and lastly 2 times washing with 0.1 % TFA. Afterwards,
480 the sample was loaded onto the tip by pipetting it 8 times slowly up and down without releasing the
481 pipette. The tip was then washed 3 times in 500 µL 0.1 % TFA and the sample was eluted in 40 µL 75
482 % ACN, 0.1 % TFA by pipetting it 8 times.

483 *Liquid chromatography-mass spectrometry (LC-MS/MS)*

484 Dansyl-derivatized or unmodified peptides after tryptic or chymotryptic digest were analyzed via LC-
485 MS/MS with an LTQ Orbitrap Elite (Thermo Fisher Scientific) as follows: 100 ng of the sample were
486 loaded onto a C18 precolumn (100-µm × 2-mm Acclaim PepMap100, 5 µM, Thermo Fisher Scientific)
487 with 2.5 % ACN/0.1 % TFA (v/v) at a flow rate of 30 µl/min for 7 min. The peptides were then loaded
488 onto the main column (75-µm × 50-cm Acclaim PepMap100 C18, 3 µm, 100-Å, Thermo Fisher
489 Scientific) with 95 % solvent A (0.1 % formic acid (v/v)) and 5 % solvent B (0.1 % formic acid, 84 %
490 ACN (v/v)) at a flow rate of 0.4 µl/min. Peptides were eluted with a linear gradient of 5–40 % B (120 min,
491 0.4 µl/min). The 20 most intense peaks were selected for MS/MS fragmentation using collision-induced
492 dissociation in the linear ion trap (charge range +2 to +4, exclusion list size: 500, exclusion duration:
493 35 s, collision energy: 35 eV).

494 The generated raw files after LC-MS/MS were analyzed using Xcalibur Software (Qual Browser 3.1,
495 Thermo Fischer Scientific, Waltham, MA, USA).

496 *Mass spectrometry data analysis using MaxQuant*

497 MaxQuant software (version 1.5.1.0, DE) was used to identify and quantify dansyl- and chlorine-
 498 modified peptides. The following modifications were added to the variable modification list of the search
 499 engine Andromeda: dansylation (+233.05, lysine, arginine, and histidine), chlorination (+33.96, lysine,
 500 arginine, histidine, tyrosine) and trioxidation (+47.98, cysteine). For peptide search using Andromeda,
 501 the *E. coli* K12 proteome database (taxonomy ID83333) obtained from UniProt (4518 proteins, released
 502 September 2019, The UniProt Consortium, 2019) was used. Two miscleavages were allowed, Oxidation
 503 (M), Dansylation (KR), Chlorination (KRYH), Carbamidomethyl (C), Trioxidation (C) were chosen as
 504 variable modification. Identified peptides were assessed using the “peptides.txt” MaxQuant output file.
 505 The availability of modification was monitored using “dansylation-sites.txt”, “chlorination-sites.txt” and
 506 “modificationSpecificPeptides.txt” output files. Data was imported and analyzed using Microsoft Excel
 507 Version 16.20 (Microsoft, Redmond, WA, USA). All peptide modifications were verified by manual
 508 examination of MS/MS spectra using the criteria proposed by Nybo et al., 2018. Described criteria are:
 509 1) identification of unmodified peptide; 2) coverage of modification site by fragment ion series; 3) correct
 510 assignment of peaks in MS/MS spectra (neutral losses: Met + O (−64), Met + 2O (−80), Cys + O (−50),
 511 Cys + 2O (−66), Cys + 3O (−82)); 4) similar fragmentation patterns between modified and unmodified
 512 peptide(s).

513 *Construction of *RidA* variants with single amino acid substitutions*

514 *E. coli* strains, plasmids, and primers used in this study are listed in table 4. Single nucleotides in the *ridA*
 515 gene were exchanged using PCR-based mutagenesis, known as QuickChange PCR. PCR was performed
 516 using 150 ng of pUC19_ *ridA* or pEX_ *ridA*-K0R0 as a template and 125 ng of each specific primer (Table
 517 5) for every single exchange. 20 µL of the PCR product were digested with 20 U of DpnI at 37 °C for
 518 1 h to eliminate the template plasmid. Subsequently, *E. coli* XL-1 blue cells were transformed with the
 519 sample using a standard heat-shock method and plated on LB agar plates supplemented with 100 mg/ml

ampicillin. Plasmid DNA was isolated from single colonies, and successful mutagenesis was verified by sequencing. Afterwards, *ridA* gene variants were subcloned into pET22b(+) expression vector via the restriction sites *NdeI* and *XhoI*. The resulting pET22b(+)-based constructs were transformed into *E. coli* BL21(DE3) cells for the subsequent overexpression of RidA variants.

Table 4: Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or description	Source or Reference
<i>Strains</i>		
<i>E. coli</i> XL1 blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i>	Stratagene
<i>E. coli</i> BL21 (DE3)	<i>fhuA2 [lon] ompT gal (Δ DE3) [dcm] ΔhsdS</i>	
<i>E. coli</i> DH5α	<i>fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	17
<i>Plasmids</i>		
pUC19	2686 bp, Amp ^R	
pUC19_riDA	3083 bp, Amp ^R , <i>ridA</i>	A. Baumann
pUC19_riDA_K3S	3083 bp, Amp ^R , <i>ridA_K3S</i>	This work
pUC19_riDA_K38S	3083 bp, Amp ^R , <i>ridA_K38S</i>	This work
pUC19_riDA_R51S	3083 bp, Amp ^R , <i>ridA_R51S</i>	This work
pUC19_riDA_K58S	3083 bp, Amp ^R , <i>ridA_K58S</i>	This work
pUC19_riDA_K67S	3083 bp, Amp ^R , <i>ridA_K67S</i>	This work
pUC19_riDA_K73S	3083 bp, Amp ^R , <i>ridA_K73S</i>	This work
pUC19_riDA_K79S	3083 bp, Amp ^R , <i>ridA_K79S</i>	This work
pUC19_riDA_R105S	3083 bp, Amp ^R , <i>ridA_R105S</i>	This work
pUC19_riDA_R112S	3083 bp, Amp ^R , <i>ridA_R112S</i>	This work
pUC19_riDA_K115S	3083 bp, Amp ^R , <i>ridA_K115S</i>	This work
pUC19_riDA_K118S	3083 bp, Amp ^R , <i>ridA_K118S</i>	This work
pUC19_riDA_R127S	3083 bp, Amp ^R , <i>ridA_R127S</i>	This work
pUC19_riDA_R128S	3083 bp, Amp ^R , <i>ridA_R128S</i>	This work
pET22b	5493 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag	Novagen
pET22b_riDA_K3S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K3S</i>	This work
pET22b_riDA_K38S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K38S</i>	This work
pET22b_riDA_R51S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_R51S</i>	This work

pET22b_ridA_K58S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K58S</i>	This work
pET22b_ridA_K67S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K67S</i>	This work
pET22b_ridA_K73S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K73S</i>	This work
pET22b_ridA_K79S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K79S</i>	This work
pET22b_ridA_R105S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_R105S</i>	This work
pET22b_ridA_R112S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_R112S</i>	This work
pET22b_ridA_K115S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K115S</i>	This work
pET22b_ridA_K118S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K118S</i>	This work
pET22b_ridA_R127S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_R127S</i>	This work
pET22b_ridA_R128S	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_R128S</i>	This work
pUC19_ridA_R105S_R128S	3083 bp, Amp ^R , <i>ridA_R105S_R128S</i>	This work
pET22b__ridA_R105S_R128S	5751 bp, Amp ^R , <i>ridA_R105S_R128S</i>	This work
pEX_ridA_K0R0	2846 bp, Amp ^R , <i>ridA_K0R0</i>	This work
pEX_ridA_K0R0_R105	2846 bp, Amp ^R , <i>ridA_K0R0_R105</i>	This work
pYP169	2622 bp, Kan ^R , <i>lacZ</i>	Y. Pfänder und B. Masepohl, unpublished
pYP169_ridA_K0R0_R105	3019 bp, Kan ^R , <i>lacZ</i> , <i>ridA_K0R0_R105</i>	This work
pET22b_ridA_K0R0_R105	5751 bp, Amp ^R , T7-Promotor, HIS ₆ -Tag, <i>ridA_K0R0_R105</i>	This work

526 **Table 5: Primers used in this study**

Primer name	Sequence
K3S_fw	CATATGAGCAGCACTATCGCGACGGAAATGC
K3S_rv	CCGTCGCGATAGTGCTCATATGTATATCTCCTTC
K38S_fw	CCCGGTAAATCCGAGCACGGGCGAAGTACC
K38S_rv	GCCCGTGCTCGGATTTACCGGGATCTGAC
R51S_fw	GCACAGGCAAGCCAGTCGCTGGATAACG
R51S_rv	CGACTGGCTTGCTGTGCAGCGACGTC
K58S_fw	GGATAACGTAAGCGCGATCGTCGAAGCCGC
K58S_rv	CGACGATCGCGCTTACGTTATCCAGCGACTG
K67S_fw	GGCCTGAGCGTGGGCGACATCGTTAAACTACCG
K67S_rv	GTCGCCCACGCTCAGGCCAGCGGCTTC
K73S_fw	GGGCGACATCGTTAGCACTACCGTGTTTGTAAAAG
K73S_rv	CGGTAGTGCTAACGATGTCGCCCACTTTCAGG
K79S_fw	CCGTGTTTGTAAAGCGATCTGAACGACTTCGC
K79S_rv	GATCGCTTACAAACACGGTAGTTTTAACGATGTCGCC
R105S_fw	CCCGGCAAGCTCTTGCGTTGAAGTTGCC
R105S_rv	CGCAAGAGCTTGCCGGGAAGGTGGCG
R112S_fw	GTTGCCAGCCTGCCGAAAGACGTGAAG
R112S_rv	CGGCAGGCTGGCAACTTCAACGCAAG
K115S_fw	CGTCTGCCGAGCGACGTGAAGATTGAGATCG
K115S_rv	CTTCACGTCGCTCGGCAGACGGGCAACTTC
K118S_fw	GACGTGAGCATTGAGATCGAAGCGATCGC
K118S_rv	CGATCTCAATGCTCACGTCTTTCGGCAGACG
R127S_fw	CGCTGTTAGCCGCCTCGAGCACCACCAC
R127S_rv	CGAGGCGGCTAACAGCGATCGCTTCGATC
R128S_fw	CGCTGTTTCGTAGCCTCGAGCACCACCACC
R128S_rv	GCTCGAGGCTACGAACAGCGATCGCTTCG
K0R0_NdeI_fw	CCCATATGAGCACCCTATCGCGACGG
K0R0_XhoI_rv	GGCTCGAGGCCCAAGAACAGCGATC
R105_fw	CCTTCCCGGCACGTTCTTGCGTTGAAGTTGCCGC
R105_rv	CAAGAACGTGCCGGGAAGGTGGCGTTGTGTTCGG

527 *Overexpression and purification of RidA variants*

528 For overexpression, a single colony of *E. coli* BL21 (DE3) carrying the respective pET22b(+) plasmid
529 was inoculated in 50 mL LB containing 200 mg/L ampicillin and grown overnight at 37 °C, 120 rpm.
530 The next morning, the overnight culture was used to inoculate 5 L of LB medium, supplemented with
531 ampicillin, and incubated at 37 °C and 120 rpm until the OD₆₀₀ reached 0.5-0.6. At this point, protein

532 expression was induced by the addition of 1 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) to the
533 culture. After 3 h of incubation at 37 °C, 120 rpm, cells were harvested by centrifugation at 7800 x g and
534 4 °C for 45 min and either stored at -80 °C or directly used for the purification procedure.

535 The resulting cell pellet was washed once with lysis buffer (50 mM sodium phosphate, 300 mM NaCl,
536 10 mM imidazole, pH 8.0) containing 1 ml of EDTA-free protease inhibitor mixture (Roche Applied
537 Science). Cells were disrupted by passing the cell suspension three times through a Constant systems cell
538 disruption system TS benchtop device (Score Group plc, Aberdeenshire, UK) at 1.9 kbar and 4 °C,
539 followed by the addition of PMSF to a final concentration of 1 mM.

540 The cell lysate was centrifuged at 6700 x g, 4 °C for 1 h, and the supernatant was vacuum filtered through
541 a 0.45 μ m filter. The filtrate was loaded onto a Ni-NTA affinity column. The column was washed with
542 10 mL washing buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0). For
543 purification of the R0K0_R105 variant, washing buffer was additionally supplemented with 1 M NaCl
544 and 0.1 % SDS. Purified proteins were stored at -80 °C and K0R0_R105 was stored at room temperature.
545 Protein concentrations were determined using a JASCO V-650 spectrophotometer using the extinction
546 coefficient $\epsilon_{280}=2,980 \text{ M}^{-1}\text{cm}^{-1}$.

547 *Protein aggregation assay with citrate synthase*

548 Citrate synthase was chemically denatured in 4.5 M GdnHCl, 40 mM HEPES, pH 7.5 at room
549 temperature overnight. The final concentration of denatured citrate synthase was 12 mM.

550 To monitor initial aggregation of citrate synthase, 20 μ l of denatured protein were added to 1580 μ l of
551 40 mM HEPES, pH 7.5 to a final concentration of 0.15 μ M after 20 s of measurement. To test the
552 chaperone activity, RidA or RidA variants were added prior to the addition of citrate synthase to the
553 buffer at different molar excess (0.5-16-fold) over dimeric citrate synthase. The increase in light

554 scattering was monitored for 240 s using a JASCO FP-8500 fluorescence spectrometer equipped with an
555 EHC-813 temperature-controlled sample holder (JASCO, Tokyo, Japan). Measurement parameters were
556 set to 360 nm (Em/Ex), 30 °C, medium sensitivity, slit width 2.5 nm (Em/Ex). Relative chaperone
557 activity of different RidA variants was calculated based on the difference between initial and final light
558 scattering. The chaperone activity of wild-type chlorinated RidA was set to 100 %.

559 *Synthesis of N,N-Dimethyl-1-amino-5-naphthalenesulfonic acid (Dansyl sulfonic acid, DANSO₂H)*

560 Dansyl sulfonic acid (DANSO₂H) was synthesized from dansyl chloride, as described in Scully et al.,
561 1984 with minor modifications. Dansyl chloride (5 g) was added to a continuously stirred aqueous
562 solution of sodium sulfite (10.7 g in 50 mL) warmed to 70 °C. The reaction temperature was kept at
563 80 °C for 5 h. After the solution was cooled, DANSO₂H was precipitated from the product mixture by
564 acidifying the solution to pH 4 with concentrated sulfuric acid. The precipitate was then filtered. The
565 precipitate was then dried in a vacuum desiccator over silica gel. The powder was re-dissolved in a cold,
566 aqueous solution of NaOH (2.8 M). The resulting solution was filtered and titrated to pH 4.0 using
567 sulfuric acid. The resulting DANSO₂H was dried again in a vacuum desiccator and stored in the light-
568 protected vial at 4 °C. The purity of the resulting dansyl sulfonic acid (retention time 6.48 min) was
569 confirmed by HPLC (Fig. S1) and was 95 %. The 5 % contamination by a corresponding sulfonic acid
570 (retention time 6.55 min) does not affect the derivatization of chloramines (Scully et al., 1984; Alpmann
571 and Morlock, 2008).

572 *Derivatization of RidA with dansyl sulfonic acid and dansyl chloride*

573 200 mM DANSO₂H solution was prepared freshly by dissolving the powder in 200 mM NaHCO₃ buffer,
574 pH 9.0, and 10 % (w/w) DANSCl stock solution (370 mM) (abcr, Karlsruhe, Germany) in acetone was
575 directly used.

576 RidA was chlorinated with HOCl as described above. Using an NAP-5 gel filtration column, residual
 577 HOCl was removed, and the buffer was exchanged to 200 mM NaHCO₃ buffer, pH 9.0. 250 μM
 578 RidA_{HOCl} or RidA_{UT} were incubated with a 50-fold molar excess of DANSCl or DANSO₂H for 1h, 37
 579 °C, 1300 rpm. The derivatizing agent was removed using an NAP-5 gel filtration column. Successful
 580 derivatization of RidA by monitoring the fluorescence of the resulting sulfonamide was determined by a
 581 fluorescence emission scan from 360–600 nm in a JASCO FP-8500 fluorescence spectrometer with the
 582 following parameters: 340 nm excitation, 2.5 nm slit width (Ex/Em) and medium sensitivity.

583 *Preparation and chymotryptic digest of dansylated proteins for mass spectrometry analysis*

584 5 μL (approx. 8 μg of protein) of each dansylated sample prepared above were mixed together with 25 μL
 585 ultrapure water and 10 μL 5 x chymotrypsin digestion buffer (500 mM Tris-HCl, 10 mM CaCl₂, pH 8.0).
 586 pH of the resulting solution was monitored to be around 8.0. Then, DTT was added to the solution to a
 587 final concentration of 10 mM and incubated at 60 °C for 45 min. After the sample was cooled to room
 588 temperature, 3.5 μL of freshly prepared 500 mM iodoacetamide solution in pure water were added to a
 589 final concentration of 20 mM. The sample was incubated at room temperature for 30 min, protected from
 590 light. To quench the alkylation reaction, 1 μL of 500 mM DTT was added, and the volume of the solution
 591 was adjusted to 50 μL using pure water. Chymotrypsin, reconstituted to a concentration of 1 mg/mL in 1
 592 mM HCl, was then added to the sample for a final 1:20 enzyme-to-protein ratio. The reaction mixture
 593 was incubated at 37 °C for 18 h. The sample was then desalted using OMIX C18 tips (Agilent
 594 Technologies, USA) according to the manufacturer's instructions. Purified peptides were concentrated
 595 to dryness and dissolved in 0.1 % TFA for LC-MS/MS analysis.

596 *Protein structure modification and electrostatic surface modelling of RidA, RidA_{HOCl}, and K0R0_R105*

597 *E. coli* RidA crystal structure was accessed using PDB entry number 1QU9 and visualized using PyMOL
 598 2.3.4 (Schrödinger, New York, NY, USA). This structure was converted to a PQR file using the

599 PDB2PQR webserver (Jurrus et al., 2018) under an assumed pH of 7.0, using PROPKA to assign
600 protonation and the AMBER forcefield to assign partial charges and atomic volumes to the structure's
601 atoms. Using the resulting PQR file as input, PyMol's APBS plugin was used to calculate the electrostatic
602 surface potential.

603 To model the N-chlorinated RidA_{H₂OCl}, the PQR file of RidA was manipulated using a custom python
604 script (Supplemental Material S2). This script removes charge bearing protons from specified lysine and
605 arginine residues, rectifies binding angles (in case of deprotonated arginine) and replaces one nitrogen-
606 attached hydrogen atom in these residues with a chlorine atom using the following bond geometries and
607 partial charges: Charge of the chlorine atom: 0.07, charge of the nitrogen atom: -0.61 (based on values
608 for CH₃NHCl (Heeb et al., 2017)), radius of the chlorine atom 1.75 Å (Bondi, 1964), length of the N-Cl
609 bond: 1.784 Å (based on values for NH₂Cl (Harmony et al., 1979)). The resulting manipulated PQR file
610 was then used to calculate the electrostatic surface potential of RidA_{H₂OCl} using the APBS plugin of
611 PyMol.

612 The structure of RidA variant R0K0_R105 was modeled using the mutagenesis function of PyMOL. The
613 specified amino acids in wild-type RidA were exchanged as follows:

614 K3 → T3
615 K38 → A38
616 R51 → H51
617 K58 → G58
618 K67 → S67
619 K73 → S73
620 K79 → T79

621 R112 → A112

622 K115 → L115

623 K118 → L118

624 R127 → L127

625 R128 → G128

626 The electrostatic surface potential for this variant was then calculated as outlined for the wildtype

627 above (conversion of the pdb-file to a PQR file using the PDB2PQR webserver under an assumed pH

628 of 7.0, using PROPKA to assign protonation and the AMBER forcefield and subsequent use of the

629 ABPS plugin in PyMol).

630 CONFLICT OF INTEREST

631 The authors declare no conflict of interest.

632 ACKNOWLEDGMENTS

633 LIL acknowledges funding from the DFG Priority Program 1710 “Dynamics of Thiol-based Redox
634 Switches in Cellular Physiology” through grant LE2905/1-2. LIL, EH, and JEB would like to thank
635 the DFG Research Training Grant 2341 “Microbial Substrate Conversion” for supporting this
636 work, and JEB further acknowledges funding from the DFG CRC1316-1 and BA 4193/7-1.

637 AUTHOR CONTRIBUTIONS

638 MV, JF, AM, LIL conceived and designed the study. NL assisted in and performed protein
639 expression and purification experiments. MV performed mass spectrometry and fluorescence
640 spectroscopy experiments and evaluated MS data. MV and LIL synthesized and characterized the
641 DANSO₂H probe. YS and KSC designed and assisted in the synthesis of the DANSO₂H probe. JF
642 designed and performed site directed mutagenesis experiments, mass spectrometry experiments,
643 and citrate synthase aggregation assays. AM designed site directed mutagenesis experiments and
644 the K0R0_R105 variant and performed citrate synthase aggregation assays. KR and BS performed
645 mass spectrometry measurements and assisted in MS data handling. MK, CJ, TJ, EH, JEB consulted
646 on the computational structure prediction and calculation of the electrostatic surface potential of
647 RidA, RidA_{HOC1} and K0R0_R105. LIL and MV calculated the electrostatic surface potentials. MV,
648 AM, JF, LIL wrote the manuscript, all other authors consulted on the manuscript.

REFERENCES

- Albrich, J. M., McCarthy, C. A. and Hurst, J. K.** (1981). Biological reactivity of hypochlorous acid: Implications for microbicidal mechanisms of leukocyte myeloperoxidase. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 210–214. doi: 10.1073/pnas.78.1.210.
- Alpmann, A. and Morlock, G.** (2008). Rapid and sensitive determination of acrylamide in drinking water by planar chromatography and fluorescence detection after derivatization with dansulfinic acid. *Journal of Separation Science* **31**, 71–77. doi: 10.1002/jssc.200700391.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J.** (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410. doi: 10.1016/S0022-2836(05)80360-2.
- Ashby, L. V., Springer, R., Hampton, M. B., Kettle, A. J. and Winterbourn, C. C.** (2020). Evaluating the bactericidal action of hypochlorous acid in culture media. *Free Radical Biology and Medicine* **159**, 119–124. doi: 10.1016/j.freeradbiomed.2020.07.033.
- Bateman, A., Martin, M. J., Orchard, S., Magrane, M., Agivetova, R., Ahmad, S., Alpi, E., Bowler-Barnett, E. H., Britto, R., Bursteinas, B., Bye-A-Jee, H., Coetzee, R., Cukura, A., Silva, A. Da, Denny, P., Dogan, T., Ebenezer, T. G., Fan, J., Castro, L. G., Garmiri, P., Georghiou, G., Gonzales, L., Hatton-Ellis, E., Hussein, A., Ignatchenko, A., Insana, G., Ishtiaq, R., Jokinen, P., Joshi, V., Jyothi, D., Lock, A., Lopez, R., Luciani, A., Luo, J., Lussi, Y., MacDougall, A., Madeira, F., Mahmoudy, M., Menchi, M., Mishra, A., Moulang, K., Nightingale, A., Oliveira, C. S., Pundir, S., Qi, G., Raj, S., Rice, D., Lopez, M. R., Saidi, R., Sampson, J., Sawford, T., Speretta, E., Turner, E., Tyagi, N., Vasudev, P., Volynkin, V., Warner, K., Watkins, X., Zaru, R., Zellner, H., Bridge, A., Poux, S., Redaschi, N., Aimo, L., Argoud-Puy, G., Auchincloss, A., Axelsen, K., Bansal, P., Baratin, D., Blatter, M. C., Bolleman, J., Boutet, E., Breuza, L., Casals-Casas, C., de Castro, E., Echioukh, K. C.,**

673 **Coudert, E., Cuhe, B., Doche, M., Dornevil, D., Estreicher, A., Famiglietti, M. L.,**
674 **Feuermann, M., Gasteiger, E., Gehant, S., Gerritsen, V., Gos, A., Gruaz-Gumowski, N.,**
675 **Hinz, U., Hulo, C., Hyka-Nouspikel, N., Jungo, F., Keller, G., Kerhornou, A., Lara, V., Le**
676 **Mercier, P., Lieberherr, D., Lombardot, T., et al. (2021).** UniProt: The universal protein
677 knowledgebase in 2021. *Nucleic Acids Research* **49**, D480–D489. doi: 10.1093/nar/gkaa1100.

678 **Bondi, A. (1964).** Van der waals volumes and radii. *Journal of Physical Chemistry* **68**, 441–451. doi:
679 10.1021/j100785a001.

680 **Carr, A. C., Van Den Berg, J. J. M. and Winterbourn, C. C. (1996).** Chlorination of cholesterol in
681 cell membranes by hypochlorous acid. *Archives of Biochemistry and Biophysics* **332**, 63–69. doi:
682 10.1006/abbi.1996.0317.

683 **Cox, J. and Mann, M. (2008).** MaxQuant enables high peptide identification rates, individualized
684 p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature Biotechnology* **26**,
685 1367–1372. doi: 10.1038/nbt.1511.

686 **Deborde, M. and von Gunten, U. (2008).** Reactions of chlorine with inorganic and organic
687 compounds during water treatment-Kinetics and mechanisms: A critical review. *Water Research*
688 **42**, 13–51. doi: 10.1016/j.watres.2007.07.025.

689 **Goemans, C. V., Vertommen, D., Agrebi, R. and Collet, J. F. (2018).** CnoX Is a Chaperedoxin: A
690 Holdase that Protects Its Substrates from Irreversible Oxidation. *Molecular Cell* **70**, 614–627. doi:
691 10.1016/j.molcel.2018.04.002.

692 **Graf, P. C. F., Martinez-Yamout, M., VanHaerents, S., Lilie, H., Dyson, H. J. and Jakob, U.**
693 (2004). Activation of the Redox-regulated Chaperone Hsp33 by Domain Unfolding. *Journal of*
694 *Biological Chemistry* **279**, 20529–20538. doi: 10.1074/jbc.M401764200.

695 **Gray, W. R. (1967).** [12] Dansyl chloride procedure. *Methods in Enzymology* **11**, 139–151. doi:
696 10.1016/S0076-6879(67)11014-8.

697 **Harmony, M. D., Laurie, V. W., Kuczkowski, R. L., Schwendeman, R. H., Ramsay, D. A., Lovas,**
698 **F. J., Lafferty, W. J. and Maki, A. G. (1979).** Molecular structures of gas phase polyatomic
699 molecules determined by spectroscopic methods. *Journal of Physical and Chemical Reference*
700 *Data* **8**, 619–722. doi: 10.1063/1.555605.

701 **Hawkins, C. L. and Davies, M. J. (1999).** Hygochlorite-induced oxidation of proteins in plasma:
702 Formation of chloramines and nitrogen-centred radicals and their role in protein fragmentation.
703 *Biochemical Journal* **340**, 539–548. doi: 10.1042/0264-6021:3400539.

704 **Hawkins, C. L., Pattison, D. I. and Davies, M. J. (2003).** Hypochlorite-induced oxidation of amino
705 acids, peptides and proteins. *Amino Acids* **25**, 259–274. doi: 10.1007/s00726-003-0016-x.

706 **Heeb, M. B., Kristiana, I., Trogolo, D., Arey, J. S. and von Gunten, U. (2017).** Formation and
707 reactivity of inorganic and organic chloramines and bromamines during oxidative water treatment.
708 *Water Research* **110**, 91–101. doi: 10.1016/j.watres.2016.11.065.

709 **Hendrikje Buss, I., Senthilmohan, R., Darlow, B. A., Mogridge, N., Kettle, A. J. and**
710 **Winterbourn, C. C. (2003).** 3-Chlorotyrosine as a marker of protein damage by myeloperoxidase
711 in tracheal aspirates from preterm infants: Association with adverse respiratory outcome. *Pediatric*
712 *Research* **53**, 455–462. doi: 10.1203/01.PDR.0000050655.25689.CE.

713 **Hsieh, W. T. and Matthews, K. S. (1985).** Lactose Repressor Protein Modified with Dansyl Chloride:
714 Activity Effects and Fluorescence Properties. *Biochemistry* **24**, 3043–3049. doi:
715 10.1021/bi00333a036.

716 **Jakob, U., Muse, W., Eser, M. and Bardwell, J. C. A. (1999).** Chaperone activity with a redox
717 switch. *Cell* **96**, 341–352. doi: 10.1016/S0092-8674(00)80547-4.

718 **Jersey, J. A., Choshen, E., Jensen, J. N., Johnson, J. D. and Scully, F. E. (1990).** N-Chloramine
719 Derivatization Mechanism with Dansylsulfonic Acid: Yields and Routes of Reaction.
720 *Environmental Science and Technology* **24**, 1536–1541. doi: 10.1021/es00080a013.

721 **Jurrus, E., Engel, D., Star, K., Monson, K., Brandi, J., Felberg, L. E., Brookes, D. H., Wilson, L.,**
722 **Chen, J., Liles, K., Chun, M., Li, P., Gohara, D. W., Dolinsky, T., Konecny, R., Koes, D. R.,**
723 **Nielsen, J. E., Head-Gordon, T., Geng, W., Krasny, R., Wei, G. W., Holst, M. J.,**
724 **McCammon, J. A. and Baker, N. A. (2018).** Improvements to the APBS biomolecular solvation
725 software suite. *Protein Science* **27**, 112–128. doi: 10.1002/pro.3280.

726 **Käll, L., Storey, J. D., MacCoss, M. J. and Noble, W. S. (2008).** Posterior error probabilities and
727 false discovery rates: Two sides of the same coin. *Journal of Proteome Research* **7**, 40–44. doi:
728 10.1021/pr700739d.

729 **Koldewey, P., Stull, F., Horowitz, S., Martin, R. and Correspondence, J. C. A. B. (2016).** Forces
730 Driving Chaperone Action In Brief. *Cell* **166**, 369–379. doi: 10.1016/j.cell.2016.05.054.

731 **Koldewey, P., Horowitz, S. and Bardwell, J. C. A. (2017).** Chaperone-client interactions: Non-
732 specificity engenders multifunctionality. *Journal of Biological Chemistry* **292**, 12010–12017. doi:
733 10.1074/jbc.R117.796862.

734 **Kumar, M. S., Kapoor, M., Sinha, S. and Reddy, G. B. (2005).** Insights into hydrophobicity and the
735 chaperone-like function of α A- and α B-crystallins: An isothermal titration calorimetric study.
736 *Journal of Biological Chemistry* **280**, 21726–21730. doi: 10.1074/jbc.M500405200.

737 **Lambrecht, J. A., Flynn, J. M. and Downs, D. M. (2012).** Conserved Yjgf protein family deaminates
738 reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme
739 reactions. *Journal of Biological Chemistry* **287**, 3454–3461. doi: 10.1074/jbc.M111.304477.

740 **Liu, X., Zeng, J., Chen, X. and Xie, W. (2016).** Crystal structures of RidA, an important enzyme for
741 the prevention of toxic side products. *Scientific Reports* **6**,. doi: 10.1038/srep30494.

742 **Mayer, M. P. and Bukau, B. (2005).** Hsp70 chaperones: Cellular functions and molecular mechanism.
743 *Cellular and Molecular Life Sciences* **62**, 670–684. doi: 10.1007/s00018-004-4464-6.

744 **McGowan, S. E. and Thompson, R. J. (1989).** Extracellular matrix proteoglycan degradation by

745 human alveolar macrophages and neutrophils. *Journal of Applied Physiology* **66**, 400–409. doi:
746 10.1152/jappl.1989.66.1.400.

747 **Mortaz, E., Alipoor, S. D., Adcock, I. M., Mumby, S. and Koenderman, L.** (2018). Update on
748 neutrophil function in severe inflammation. *Frontiers in Immunology* **9**, 2171. doi:
749 10.3389/fimmu.2018.02171.

750 **Müller, A., Langklotz, S., Lupilova, N., Kuhlmann, K., Bandow, J. E. and Leichert, L. I. O.**
751 (2014). Activation of RidA chaperone function by N-chlorination. *Nature Communications* **5**,
752 5804. doi: 10.1038/ncomms6804.

753 **Nybo, T., Cai, H., Chuang, C. Y., Gamon, L. F., Rogowska-Wrzesinska, A. and Davies, M. J.**
754 (2018). Chlorination and oxidation of human plasma fibronectin by myeloperoxidase-derived
755 oxidants, and its consequences for smooth muscle cell function. *Redox Biology* **19**, 388–400. doi:
756 10.1016/j.redox.2018.09.005.

757 **Pattison, D. I. and Davies, M. J.** (2001). Absolute rate constants for the reaction of hypochlorous acid
758 with protein side chains and peptide bonds. *Chemical Research in Toxicology* **14**, 1453–1464. doi:
759 10.1021/tx0155451.

760 **Pattison, D. I. and Davies, M. J.** (2005). Kinetic analysis of the role of histidine chloramines in
761 hypochlorous acid mediated protein oxidation. *Biochemistry* **44**, 7378–7387. doi:
762 10.1021/bi0474665.

763 **Pattison, D. and Davies, M.** (2006). Reactions of Myeloperoxidase-Derived Oxidants with Biological
764 Substrates: Gaining Chemical Insight into Human Inflammatory Diseases. *Current Medicinal*
765 *Chemistry* **13**, 3271–3290. doi: 10.2174/092986706778773095.

766 **Pattison, D. I., Davies, M. J. and Hawkins, C. L.** (2012). Reactions and reactivity of
767 myeloperoxidase-derived oxidants: Differential biological effects of hypochlorous and
768 hypothiocyanous acids. *Free Radical Research* **46**, 975–995. doi:

769 10.3109/10715762.2012.667566.

770 **Peskin, A. V and Winterbourn, C. C.** (2001). Kinetics of the reactions of hypochlorous acid and
771 amino acid chloramines with thiols, methionine, and ascorbate. *Free Radical Biology and*
772 *Medicine* **30**, 572–579. doi: 10.1016/S0891-5849(00)00506-2.

773 **Peskin, A. V., Dickerhof, N., Poynton, R. A., Paton, L. N., Pace, P. E., Hampton, M. B. and**
774 **Winterbourn, C. C.** (2013). Hyperoxidation of peroxiredoxins 2 and 3: Rate constants for the
775 reactions of the sulfenic acid of the peroxidatic cysteine. *Journal of Biological Chemistry* **288**,
776 14170–14177. doi: 10.1074/jbc.M113.460881.

777 **Prütz, W. A.** (1996). Hypochlorous acid interactions with thiols, nucleotides, DNA, and other
778 biological substrates. *Archives of Biochemistry and Biophysics* **332**, 110–120. doi:
779 10.1006/abbi.1996.0322.

780 **Scully, F. E., Yang, J. P., Mazlna, K. and Bernard Daniel, F.** (1984). Derivatization of Organic and
781 Inorganic N-Chloramines for High-Performance Liquid Chromatographic Analysis of Chlorinated
782 Water. *Environmental Science and Technology* **18**, 787–792. doi: 10.1021/es00128a012.

783 **Scully, F. E., Mazina, K., Sonenshine, D. and Kopfler, F.** (1986). Quantitation and identification of
784 organic N-chloramines formed in stomach fluid on ingestion of aqueous hypochlorite.
785 *Environmental Health Perspectives* **Vol. 69**, 259–265. doi: 10.2307/3430395.

786 **Storz, G., Tartaglia, L. A. and Ames, B. N.** (1990). Transcriptional regulator of oxidative stress-
787 inducible genes: Direct activation by oxidation. *Science* **248**, 189–194. doi:
788 10.1126/science.2183352.

789 **Ulfig, A., Schulz, A. V., Müller, A., Lupilov, N. and Leichert, L. I.** (2019). N-chlorination mediates
790 protective and immunomodulatory effects of oxidized human plasma proteins. *eLife* **8**, doi:
791 10.7554/eLife.47395.

792 **Ulfig, A., Bader, V., Varatnitskaya, M., Lupilov, N., Winklhofer, K. F. and Leichert, L. I.** (2021).

793 Hypochlorous acid-modified human serum albumin suppresses MHC class II - dependent antigen
794 presentation in pro-inflammatory macrophages. *Redox Biol* **43**:101981. doi:
795 10.1016/j.redox.2021.101981.

796 **Volz, K.** (2008). A test case for structure-based functional assignment: The 1.2 Å crystal structure of
797 the yjgF gene product from Escherichia coli. *Protein Science* **8**, 2428–2437. doi:
798 10.1110/ps.8.11.2428.

799 **Winter, J., Ilbert, M., Graf, P. C. F., Özcelik, D. and Jakob, U.** (2008). Bleach Activates a Redox-
800 Regulated Chaperone by Oxidative Protein Unfolding. *Cell* **135**, 691–701. doi:
801 10.1016/j.cell.2008.09.024.

802 **Winterbourn, C. C. and Kettle, A. J.** (2000). Biomarkers of myeloperoxidase-derived hypochlorous
803 acid. *Free Radical Biology and Medicine* **29**, 403–409. doi: 10.1016/S0891-5849(00)00204-5.

804 **Winterbourn, C. C., van den Berg, J. J. M., Roitman, E. and Kuypers, F. A.** (1992). Chlorohydrin
805 formation from unsaturated fatty acids reacted with hypochlorous acid. *Archives of Biochemistry*
806 *and Biophysics* **296**, 547–555. doi: 10.1016/0003-9861(92)90609-Z.

807 **Winterbourn, C. C., Kettle, A. J. and Hampton, M. B.** (2016). Reactive Oxygen Species and
808 Neutrophil Function. *Annual Review of Biochemistry* **85**, 765–792. doi: 10.1146/annurev-
809 biochem-060815-014442.

810 **Zheng, M., Åslund, F. and Storz, G.** (1998). Activation of the OxyR transcription factor by reversible
811 disulfide bond formation. *Science* **279**, 1718–1721. doi: 10.1126/science.279.5357.1718.

812


```
#####
####  #####  #####  #####  #####  #####  #####  #####
#  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #
#  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #  #
#####  #####  #####  #####  #####  #####  #####
#####

for line in pgrfile:
    mystring = line
    row = mystring.split()
    # print (row)
    # print ('my array has ', len(row), 'elements')
    if row[0] == "ATOM":

#####

#  #  #  #####  #####  #####
#  #  #  #  #  #  #  #  #  #  #
#  #  #  #####  #####  #####
#  #  #  #  #  #  #  #  #  #
#####  #  #####  #####  #####

#####

if row[3] == "LYS":
    if row[5] in residue_to_exclude:
        print (mystring, end = "")
    elif row[2] == "NZ":
        # print ("!NZ", end = " ")
        xNZ = float(row[6]) #remember those X, Y, Z coordinates to calculate the correct bond length later
        yNZ = float(row[7])
        zNZ = float(row[8])
        print (row[0], " ", row[1], " ", row[2], " ", row[3], " ", row[4], " ", row[5], " ", row[6], " ", row[7], " ", row[8], " ", NZLys_charge, " ",
row[10])
    elif row[2] == "HZ1":
        # print ("!HZ1", end = " ")
        print (row[0], " ", row[1], " ", "HZ", " ", row[3], " ", row[4], " ", row[5], " ", row[6], " ", row[7], " ", row[8], " ", row[9], " ", row[10])
    elif row[2] == "HZ2":
        # print ("!HZ2", end = " ")
        xHZ2 = float(row[6]) #I also need those X, Y, Z coordinates to calculate the correct bond length
        yHZ2 = float(row[7])
        zHZ2 = float(row[8])
        # Calculate bond length (or rather the correct XYZ coordinates for CLZ)
        # This is a complicated one. Here I first need to define the line equation in the form
        #
        # x(CLZ) = x(NZ) + (x(NZ)-x(HZ2))*t
        # y(CLZ) = x(NZ) + (x(NZ)-x(HZ2))*t
        # z(CLZ) = x(NZ) + (x(NZ)-x(HZ2))*t
        #
        # Now I have to find t. This I do with an equation that I painstakingly calculated on a sheet of (word-)paper.
        t = -((NCLys_bondlength**2)/((xNZ - xHZ2)**2 + (yNZ - yHZ2)**2 + (zNZ - zHZ2)**2))*0.5
        # print ("t is ", t)
        #now for the calculation of the new X,Y,Z values
        xCLZ = round((xNZ + ((xNZ-xHZ2)*t)), 3)
        yCLZ = round((yNZ + ((yNZ-yHZ2)*t)), 3)
        zCLZ = round((zNZ + ((zNZ-zHZ2)*t)), 3)
        # print ("xNZ: ", xNZ, "xHZ2: ", xHZ2, "xCLZ: ", xCLZ)
        # print ("yNZ: ", yNZ, "yHZ2: ", yHZ2, "yCLZ: ", yCLZ)
        # print ("zNZ: ", zNZ, "zHZ2: ", zHZ2, "zCLZ: ", zCLZ)
        # And now a calculation of the distance
        distance = ((xNZ - xCLZ)**2 + (yNZ - yCLZ)**2 + (zNZ - zCLZ)**2)**0.5
        # print ("And the distance is : ", distance)
        print (row[0], " ", row[1], " ", "CLZ", " ", row[3], " ", row[4], " ", row[5], " ", xCLZ, " ", yCLZ, " ", zCLZ, " ", CLZLys_charge, " ",
CLZLys_radius)
    elif row[2] == "HZ3":
        # print ("!HZ3", end = " ")
        print ("X ", mystring, end = "")
    else:
        print (mystring, end = "")

#####

#  #####  #####  #####  #####  #####
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#  #  #  #  #####  #####  #####

#####

elif row[3] == "ARG":
    if row[5] in residue_to_exclude:
        print (mystring, end = "")
    elif row[2] == "CZ":
        xCZ = float(row[6]) #remember those X, Y, Z coordinates to straighten out the bond from NH1 to only one hydrogen
        yCZ = float(row[7])
        zCZ = float(row[8])
        print (mystring, end = "")
    elif row[2] == "NH1":
        xNH1 = float(row[6]) #remember those X, Y, Z coordinates, too, to straighten out the bond from NH1 to only one hydrogen
        yNH1 = float(row[7])
        zNH1 = float(row[8])
        print (mystring, end = "")
    elif row[2] == "NH2":
        xNH2 = float(row[6]) #remember those X, Y, Z coordinates to calculate the correct NCL bondlength
        yNH2 = float(row[7])
        zNH2 = float(row[8])
        print (row[0], " ", row[1], " ", row[2], " ", row[3], " ", row[4], " ", row[5], " ", row[6], " ", row[7], " ", row[8], " ", NH2Arg_charge, " ",
row[10])
    elif row[2] == "HH11":
        # print ("X ", mystring, end = "") #remove this H
    elif row[2] == "HH12":
        #straighten this guy out. Do it by putting the now single H on the line made by NH1 and CZ
        #similar to above. First calculate the t (this time with a different sign, the H atom is in the other direction and not replacing one of those atoms)
        t = ((NHArg_bondlength**2)/((xNH1 - xCZ)**2 + (yNH1 - yCZ)**2 + (zNH1 - zCZ)**2))*0.5
        #then use the t to calculate the new coordinates for the single H
        xHH1 = round((xNH1 + ((xNH1-xCZ)*t)), 3)
        yHH1 = round((yNH1 + ((yNH1-yCZ)*t)), 3)
        zHH1 = round((zNH1 + ((zNH1-zCZ)*t)), 3)
        # And now a calculation of the distance
        distance = ((xHH1 - xNH1)**2 + (yHH1 - yNH1)**2 + (zHH1 - zNH1)**2)**0.5
        # print ("And the distance is : ", distance)
        #
```

[illegible]