Supporting information

Reaction mechanism for toxin inactivation using sodium hypochlorite.

Mechanisms and kinetic constants for reactions between sodium hypochlorite and proteins \(27,33,34\), amino acids \(26,35,36\), and other small molecules \(37\) have been investigated thoroughly by others. Earlier studies showed that hypochlorous acid \((pK_a 7.59)\) is \(2 - 4\) orders of magnitude more reactive than the hypochlorite anion. Its reactions with proteins and amino acids are consistent with a second order kinetic mechanism, one that is first order with respect to sodium hypochlorite (e.g. hypochlorous acid) and the target molecule \(26,36\).

Reaction rate  =  \(-\frac{d[target]}{dt} = k [target] [HClO]\)

Beginning with this information and considering the molar excesses of sodium hypochlorite over ricin (20- to 200-fold) and abrin (100- to 1000-fold) used in our fluorescence quenching experiments, we predicted that a simple pseudo-first order model would fit our data best. We compared correlations to simple zero-, first-, and second-order kinetic equations using nonlinear regression analysis. Unexpectedly, the highest correlation occurred when modeling reactions that are second order with respect to the toxins (Figure S1A-E).

An elementary reaction mechanism that invokes unreasonably rare ternary collisions between two toxin molecules and excess hypochlorous acid is an impractical rationalization that can be excluded.
Figure S1. Kinetic models for fluorescence quenching of ricin and abrin treated with sodium hypochlorite. Decay in (A) ricin and (B) abrin concentrations detected using fluorescence. First-order models for (C) ricin and (D) ricin. Second-order models for (E) ricin and (F) abrin.
Case I - Alternative pseudo second-order reaction mechanism with excess hypochlorous acid. T, native ricin or abrin AB heterodimeric toxin; Q, toxin with quenched intrinsic fluorescence.

\[
\frac{dT}{dt} = 2 T \frac{k_1}{[\text{HClO}]} \\
\frac{dQ}{dt} = 2 k_1 [T]^2
\]

Another improbable interpretation that is consistent with second-order dependence on the toxin concentration requires that a reversible step involving the toxin and hypochlorous acid precede intermolecular association with a second toxin molecule. The partially oxidized complex reacts with additional hypochlorous acid to yield two oxidized protein products with quenched intrinsic fluorescence, due either to exposure of protein fluorophores to the solvent or to direct reactions of the fluorophores with hypochlorous acid.

Case II - Alternative pseudo second-order reaction mechanism with excess hypochlorous acid. T, native ricin or abrin AB heterodimeric toxin; O, oxidized toxin; D, dimerized toxin with A_2B_2 stoichiometry; Q, toxin with quenched intrinsic fluorescence.

\[
\frac{dT}{dt} = -k_1 [T] + k_{-1} [O] \\
\frac{dO}{dt} = k_1 [T] - k_{-1} [O] - k_3 [T][O] \\
\frac{dD}{dt} = k_3 [T][O] - k_4 [D]
\]
\[
\frac{dQ}{dt} = 2k_4[D]
\]
\[
\frac{dO}{dt} = k_1[T] - (k_{-1} + k_3[T]) [O]
\]
Apply the Bodenstein (steady state) approximation for intermediate O:
\[
\frac{dO}{dt} = 0 = k_1[T] - (k_{-1} + k_3[T]) [O]
\]
\[
[O] = k_1[T] / (k_{-1} + k_3[T])
\]
\[
\frac{dD}{dt} = k_1k_3[T]^2 / (k_{-1} + k_3[T])
\]
If \( k_{-1} \gg k_3[T] \), then
\[
\frac{dD}{dt} \approx (k_1k_3/k_{-1}) [T]^2
\]
If \( k_3 \gg 2k_4 \), then
\[
\frac{dD}{dt} = \frac{dQ}{dt} = (k_1k_3/k_{-1}) [T]^2
\]
There is no evidence for reversible protein oxidation reactions using hypochlorous acid, making this scheme markedly less tenable.

A third alternative involves the temporal and reversible association of toxin heterodimers to form a complex with overall \( A_2B_2 \) subunit stoichiometry. The complex is then oxidized by hypochlorous acid sequentially to release two equivalents of toxin with quenched fluorescence.

Case III - Alternative pseudo second-order reaction mechanism with excess hypochlorous acid. T, native ricin or abrin AB heterodimeric toxin; D, dimerized toxin with \( A_2B_2 \) stoichiometry; \( D^* \), partially oxidized dimeric toxin complex; Q, toxin with quenched intrinsic fluorescence.

\[
\begin{align*}
2 T & \xrightarrow{k_1} D & \xrightarrow{k_2} D^* & \xrightarrow{k_3} 2Q \\
& \quad \text{[HClO]} & \quad \text{[HClO]}
\end{align*}
\]
\[
\frac{dT}{dt} = -k_1[T]^2 + k_{-1}[D]
\]
\[ \frac{dD}{dt} = k_1 [T]^2 - k_1 [D] - k_2 [D] = k_1 [T]^2 - (k_1 + k_2) [D] \]

\[ \frac{dD^*}{dt} = k_2 [D] - k_3 [D^*] \]

\[ \frac{dQ}{dt} = 2k_3 [D^*] \]

Apply the Bodenstein (steady state) approximation for intermediates \( D \) and \( D^* \):

\[ 0 = \frac{dD}{dt} = k_1 [T]^2 - (k_1 + k_2) [D] \]

\[ [D] = \frac{(k_1)(T)}{(k_1 + k_2)} [T]^2 \]

\[ 0 = \frac{dD^*}{dt} = k_2 [D] - k_3 [D^*] \]

\[ [D^*] = \frac{(k_2)(k_3)}{[D]} = \frac{(k_1k_2)}{(k_1k_3 + k_2k_3)} [T]^2 \]

\[ \frac{dQ}{dt} = 2k_3 [D^*] = 2 \frac{(k_1k_2k_3)}{(k_1k_3 + k_2k_3)} [T]^2 \]

This reaction pathway presumes that reactive groups on separate toxin molecules become juxtaposed properly through dimerization to provide critical targets for sequential attacks by hypochlorous acid leading ultimately to yield fully oxidized toxin molecules with quenched fluorescence. The potential for covalent intermolecular protein crosslinks presents itself in this hypothetical model.

The sulfur containing amino acids methionine, cysteine, and cystine are known to be exquisitely sensitive to hypochlorous acid. Pattison and Davies\(^\text{26}\) measured second order rate constants for reactions between hypochlorous acid and amino acids and peptide bonds at pH 7.4 and reported the following series: Met \( (3.8 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) > \) Cys \( (3.0 \times 10^7 \text{ M}^{-1} \text{s}^{-1}) \gg \) cystine \( (1.6 \times 10^6 \text{ M}^{-1} \text{s}^{-1}) \approx \) His \( (1.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}) \approx \) \( \alpha \)-amino group \( (1.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}) > \) Trp \( (1.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}) \approx \) Lys \( (5.0 \times 10^3 \text{ M}^{-1} \text{s}^{-1}) \gg \) Tyr \( (44 \text{ M}^{-1} \text{s}^{-1}) \approx \) peptide backbone amide bonds \( (10 \times 10^{-3} \text{ M}^{-1} \text{s}^{-1}) > \) Gln \( (0.03 \text{ M}^{-1} \text{s}^{-1}) \). Solvent-
accessible reactive groups identifiable in the published tertiary structures for ricin and
abrin provide a means to predict hypochlorite-sensitive amino acids in each polypeptide.

**Figure S2. Chemical reactions and kinetic equations involving excess hypochlorous acid and protein toxins.**

\[
\begin{align*}
K_a & = \frac{[H^+][ClO^-]}{[HClO]} \\
C_t & = [HClO] + [ClO^-] \\
[HClO] & = \frac{C_t [H^+]}{K_a + [H^+]} \\
& - \frac{d}{dt} [\text{toxin}] = k_1 [\text{toxin}] [HClO] \\
& - \frac{d}{dt} [\text{toxin}] = k_2 [\text{toxin}] [HClO] \\
& - \frac{d}{dt} [\text{toxin}] = k_3 [\text{toxin}] [HClO] \\
& - \frac{d}{dt} [\text{toxin}] = k_4 [\text{toxin}] [HClO] \\
& - \frac{d}{dt} [\text{toxin}] = (k_1 + k_2 + \ldots + k_i) [\text{toxin}] [HClO] \\
\end{align*}
\]

if $[HClO] \gg [\text{toxin}]$, then $\frac{d}{dt} [HClO] \approx 0$ and

\[
k'_{\text{obs}} = (k_1 + k_2 + \ldots + k_i) [HClO] \\
- \frac{d}{dt} [\text{toxin}] = k'_{\text{obs}} [\text{toxin}]
Using this theoretical basis, we predicted that the intrinsic fluorescence of ricin and abrin protein toxins would decay in a time-dependent manner consistent with the overall kinetic control of a network of parallel chemical reactions involving hypochlorous acid and various labile groups on the toxins (Figure S2).

The structure of ricin refined to 2.5 Å was evaluated using Jmol software to identify functional groups that are exposed to solvent molecules (Figure S3). Ricin possesses three methionine thioether functional groups that are partially accessible to solvent but abrin contains four. These thioethers react very rapidly with hypochlorous acid to form stable methionine sulfoxide derivatives. Although more polar than methionine, methionine sulfoxides located on the surfaces of proteins do not normally induce major changes in tertiary structure. Oxidation of buried methionine residues, however, is more likely to lead to significant structural reorganization. Ricin and abrin each contain one exposed cystine disulfide but no cysteine free thiol groups that are accessible to hypochlorite. Cysteine thiol groups can be oxidized by hypochlorite to yield cystine disulfides or cysteic acid. Further hypochlorite-mediated oxidation of cystine cleaves the disulfide bond to form two cysteic acid residues, a modification that often causes major disruptions in protein structure and function. Imidazole nitrogens of histidine react with hypochlorous acid to form reactive and short-lived chloramines that are particularly efficient in secondary intramolecular chlorine transfer reactions with methionines, cysteines, cystines, tryptophans, lysines, and tyrosines. Intramolecular chlorine transfer reactions involving histidine chloramines occur at rates that are only 5- to 25-fold slower than corresponding primary reactions with hypochlorous acid.
The primary amines found at the amino termini of peptides and the ζ-nitrogens of lysines also form unstable chloramine derivatives that may decompose generate a variety of oxidation products. Ricin and abrin each contain three exposed ε-nitrogens on histidine residues, two exposed amino termini, and eight exposed ζ-nitrogens on lysines. The indole rings of tryptophan residues can be attacked by hypochlorous acid at the β-carbon to yield various products, including 2-hydroxyindole and 2-oxoindole derivatives. All β-carbon atoms of tryptophans are buried within the structures of ricin and abrin.

Therefore, initial attacks of excess hypochlorous acid would be expected to occur at 3-4 exposed methione thioether groups to yield more polar methionine sulfoxides. Next, oxidation to cysteic acid of the 1-3 sulfur atoms present in exposed cystine disulfide bonds would introduce electrostatically charged groups that should destabilize tertiary structure further. Hypochlorite should then attack three exposed imidazole nitrogens and the amino termini of the toxin A and B subunits to yield reactive chloramine derivatives that could participate in further secondary reactions. Because all tryptophan β-carbons are protected from initial attack by the tertiary structure of these toxins, potential reactions involving tryptophan would require additional time for protein refolding to occur. Comparing the range of second-order rate constants measured in our study for reactions between sodium hypochlorite and ricin or abrin \((2,000 – 5,700 \text{ M}^{-1} \text{s}^{-1})\) and the published rate constant for reactions with lysine \((5000 \text{ M}^{-1} \text{s}^{-1})\), it appears that only minor participation from lysine would be predicted.
Figure S3. Solvent-accessible labile groups for ricin. Structures expected to be reactive with hypochlorous acid (Met, Cys, His, Lys, and Trp residues plus polypeptide amino termini) within the structure of ricin refined to 2.5 Å (PDB ID: 2AAI; ricin A subunit is pictured in blue, ricin B subunit is pictured in violet) were selected using Jmol software (marked in red). Solvent-accessible (1.4 Å radius) portions of labile groups are depicted as orange surfaces.
Hypochlorous acid is typically regarded as a nonselective protein oxidant. Thus, the presumptions included in Cases II and III that intermolecular collisions between toxin molecules are part of the overall reaction pathway seem awkward. To test this assumption, numerous attempts were made to trap and detect higher molecular weight protein toxin complexes. Ricin was incubated with 10- to 100-fold molar excesses of sodium hypochlorite, quenched using excess L-methionine following 0-5 min incubation, and analyzed by gel electrophoresis. We failed to detect higher molecular weight covalent or noncovalent toxin complexes in any of our repeated attempts (data not shown).

The absence of additional evidence corroborating a reaction mechanism that is second-order with respect to toxins, and coupled with results of published studies that showed first-order dependence on the concentrations of N-blocked amino acids targets for hypochlorous acid, led us to compare two step first-order kinetic equations to simple first- and second-order models (Figure S4). Two step first-order kinetic equations fit the data much better than simple first-order models and significantly better than simple second-order equations (P < 0.001). We feel that this model is justified considering the variety of exposed labile functional groups on ricin and abrin that are available for attack by hypochlorous acid.
Figure S4. Two-step first-order kinetic model for toxin fluorescence quenching.

Reactions with ricin at 5°C (A) and 25°C (B) or abrin at 25°C (C) were modeled using:

\[
Y = (f)(Y_{top} - Y_{bottom})e^{-k_1t} + (1-f)(Y_{top} - Y_{bottom})e^{-k_2t} + Y_{bottom}
\]

where \( f \) = fraction of the fluorescence due to the faster phase of the reaction.