SUPPLEMENTAL FIGURES

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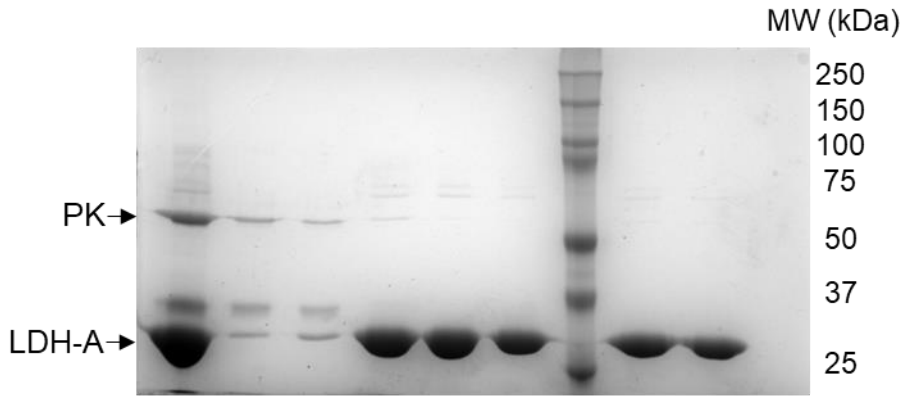
Figure S1: Purification of LDH-A by Cibacron Blue chromatography

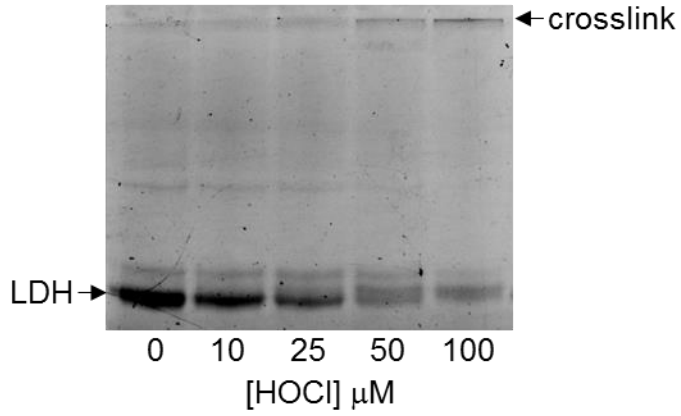


Lane 1: before column; lanes 2-3 0.2 M NaCl washes with PK activity (1 ml fractions); Lanes 4-6, N2-N4 with peak LDH-A activity; lane 7: MW stds; lane 8: combined N2-N5 before desalting; lane 9: desalted pure LDH-A (frozen in aliquots at -80 °C)

Figure S2. IAF labeling of LDH-A oxidized by HOCl

Reaction conditions were identical to Figure 2B except IAF, not M5F, was used to modify cysteines. IAF in DMF was added to achieve a 10-fold excess over LDH-A cysteines (500 μM final). The fluorescence image has been inverted to improve detection of the crosslink.

IAF labeled LDH-A (inverted image)



Coomassie stain of IAF labeled LDH-A

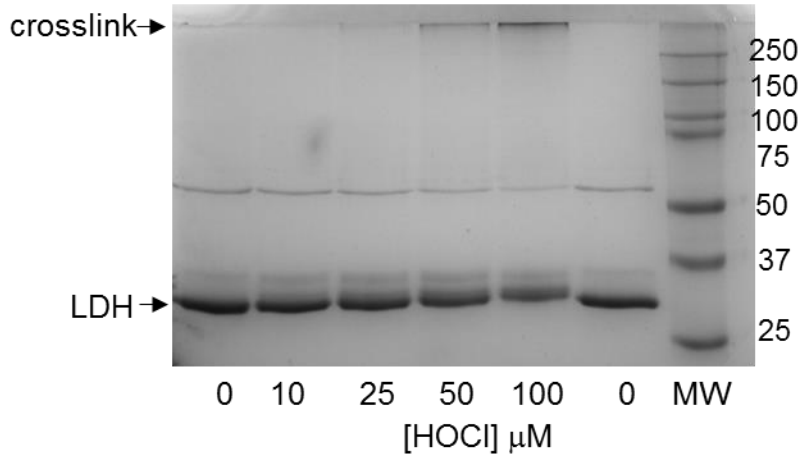
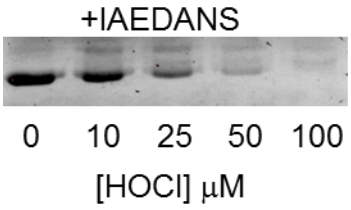


Figure S3: IAEDANS labeling of LDH-A treated with HOCl

The cysteines of control and HOCl-treated LDH-A were also labeled with IAEDANS, which like M5F and IAF, reacts with reduced to cysteines to yield a fluorescently tagged protein. As for M5F and IAF, as the concentration of HOCl increased, the fluorescence of the LDH-A band decreased. In this experiment, HOCl was only present for 20 min, not 30 min.

Because IAEDANS is less fluorescent than IAF, the high molecular weight crosslink was not detected. However, Coomassie staining confirmed the presence of the crosslink for LDH-A samples treated with 50 and 100 μM HOCl.

IAEDANS labeled LDH-A (inverted image)



Coomassie stain of IAEDANS labeled LDH-A

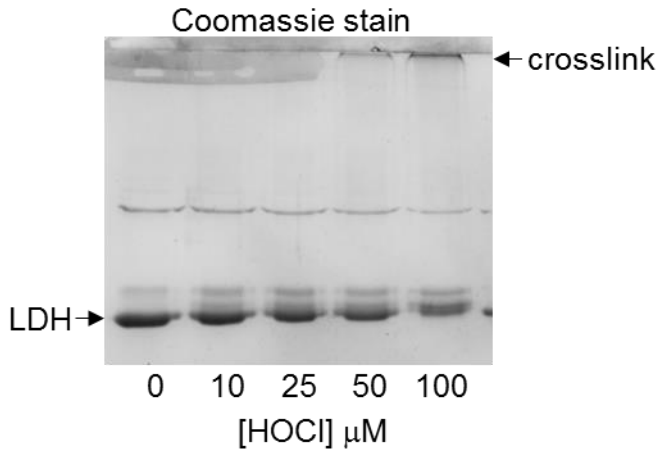
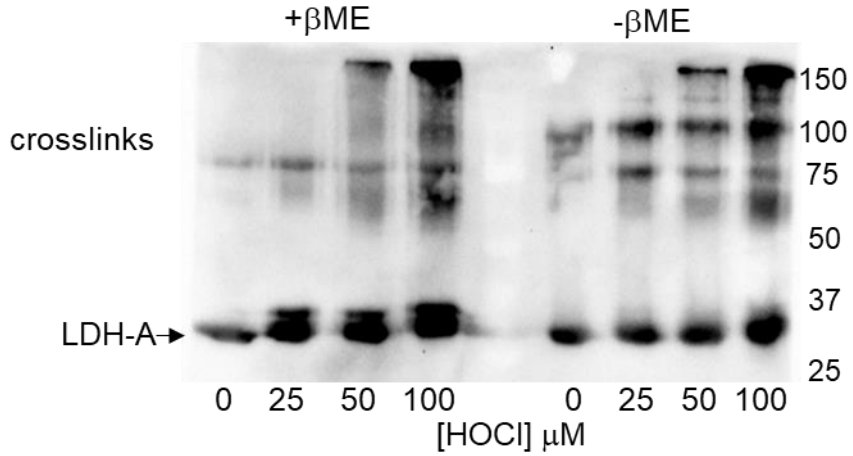
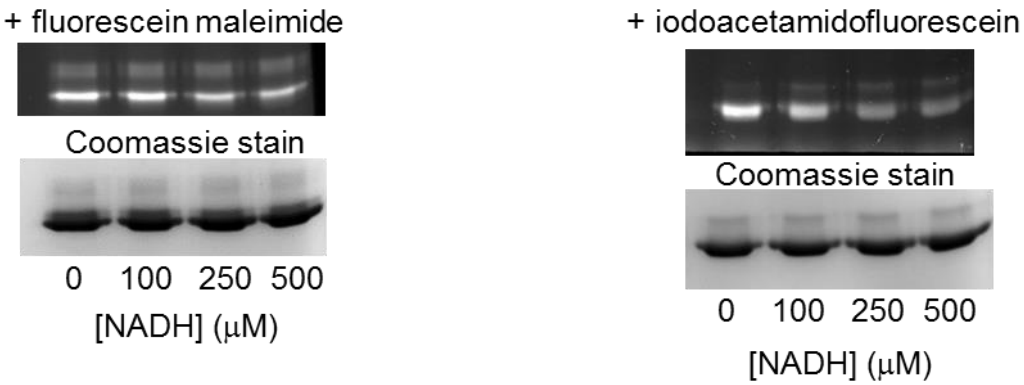


Figure S4: Western blot of LDH-A treated with HOCl +/- βME



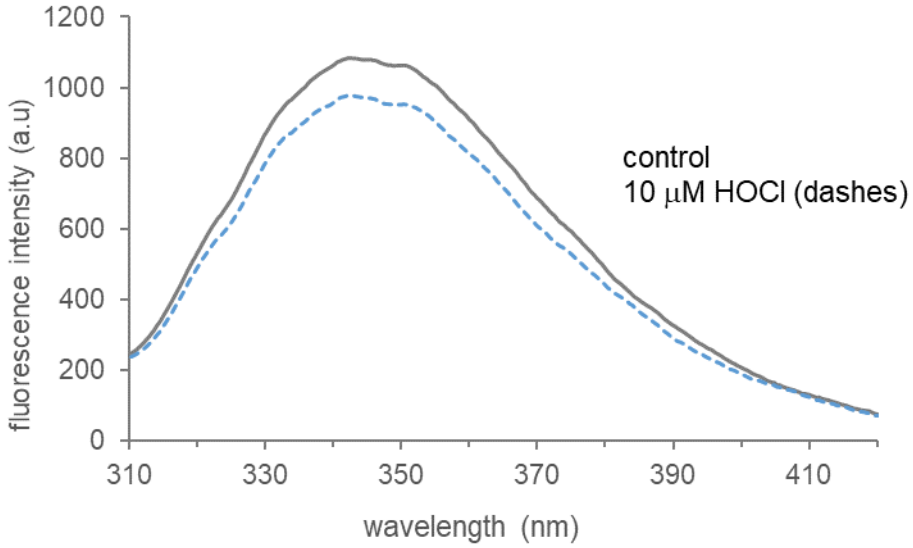
LDH-A samples were prepared as described in Figure 2B. Following separation by SDS-PAGE on 8% polyacrylamide gels, proteins were transferred to PVDF membranes, blocked with 5% milk in PBS-T for 30 min and probed with a rabbit monoclonal anti-LDH-A antibody (1:2000). The LDH-A/antibody complex was detected using a goat anti-rabbit HRP conjugate (1 hr, 1:10,000) and Pierce West Pico chemiluminescent substrate. Chemiluminescence was captured using the Bio-rad Chemi-doc XRS imaging system.

Figure S5: Preincubation of LDH-A with NADH decreases cysteine labeling



LDH-A (10 μM) was incubated with 0.5 mM NADH in 10 mM PB pH 7.4 for 15 min at 20 °C prior to addition of the fluorescent tag. M5F or IAF (500 μM) in DMF was added to achieve a 10-fold molar excess relative to protein cys and samples were incubated at 37 °C for an additional 30 min. Proteins were resolved by SDS-PAGE on 10% gels under reducing conditions and gel images were captured using a Bio-Rad Chemi-doc XRS imaging system. The intensity of the fluorescein-labeled protein bands was measured using Bio-Rad Image Lab software.

Figure S6. LDH-A tryptophan emission at 10 μM HOCl vs control



LDH-A (10 μM) was incubated with 10 μM HOCl in 10 mM PB pH 7.4 for 30 min at 20 °C and tryptophan fluorescence was detected as described in Figure 5. This representative figure shows that 1 equivalent of HOCl resulted in a 10-15% decrease in emission.

Figure S7: Fluorescence emission of HOCl treated LDH-A

LDH-A (10 μM) was treated with 150 μM HOCl in a total volume of 600 μl for 30 min at 20 °C. S-methyl-cysteine (0.5 mM) was added to scavenge unreacted HOCl. NaOH (30 μl, 1 M) was added to deprotonate tyrosines. These conditions (concentrations of ratio of oxidant to LDH-A) were employed because they are the same those used for SDS-PAGE (Figures 2 & 3) and kinetics (Figure 2D & 4C).

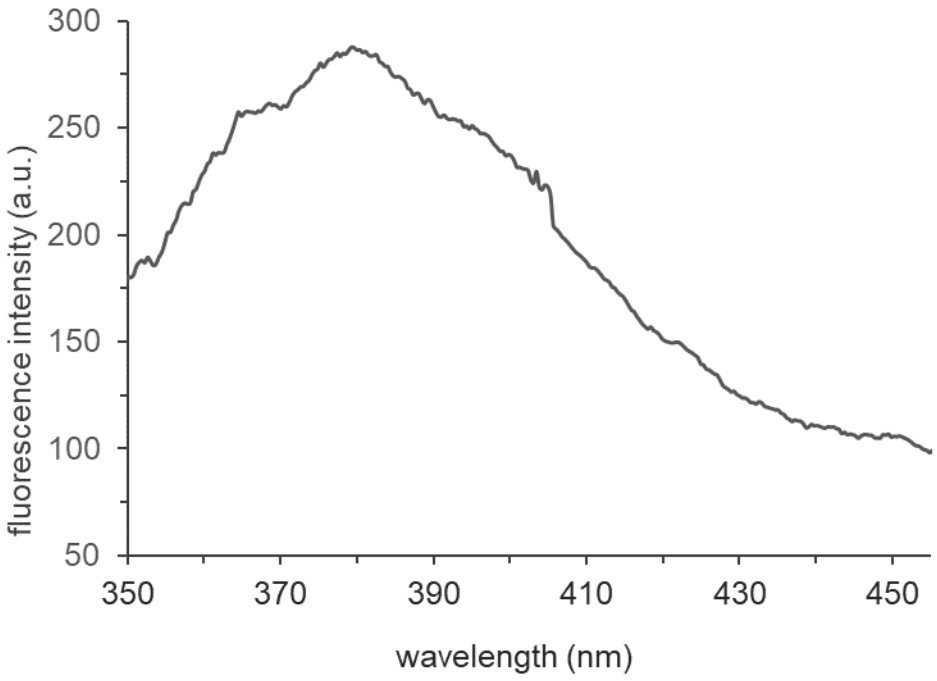


Figure S8: Effect of nitrite on HOCl oxidation of LDH-A

LDH-A (10 μM), in 10 mM PB pH 7.4 was treated with 100 μM HOCl in the presence of $NaNO_2$ for 30 min at 20 °C. For the nitrite/HOCl samples, nitrite was premixed with LDH-A before HOCl addition. Nitrite5x/HOCl and nitrite10x/HOCl contained 500 μM and 1 mM nitrite, respectively. For the “mixed” sample, drops of nitrite and HOCl were combined on the tube wall immediately before mixing with LDH-A.

LDH-A was treated with HOCl, or the mixtures of HOCl and nitrite described above. Following addition of HOCl scavenger, LDH-A cysteines were labeled with M5F as described in Figure 3A. After fluorescence imaging (top), gels were stained with Coomassie blue (bottom).

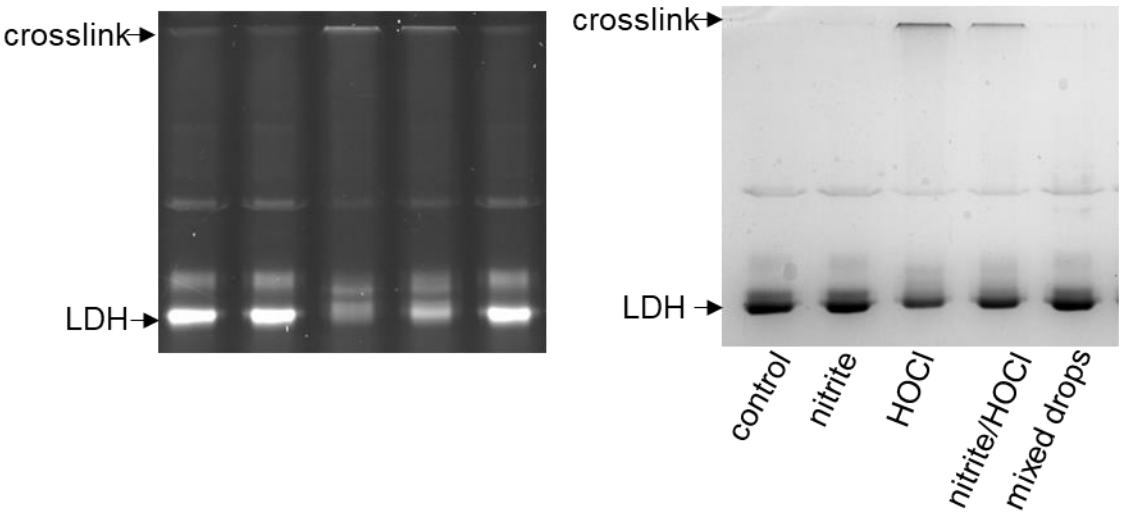
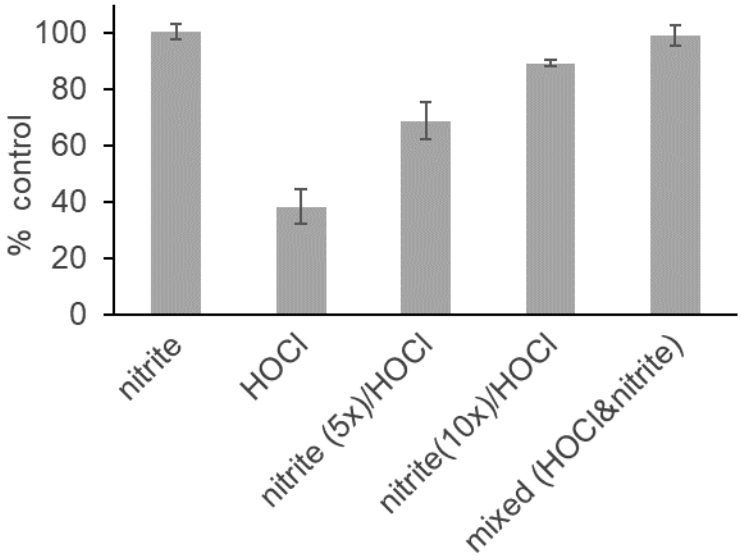


Figure S9: Effect of nitrite on LDH-A inhibition by HOCl
Samples were prepared as in Figure S8. S-methyl-cysteine (0.2 mM) was added to scavenge excess HOCl. LDH-A samples were diluted 1:10 in 20 mM Tris pH 8.6 and assayed for LDH-A activity as described in Figure 2D. Data represent the average of three independent experiments +/- standard error.



In the presence of nitrite ion, HOCl oxidation of tyrosine analogs and protein-bound tyrosines yields different product profiles. Using N-acetyl-tyrosine as their target, Eiserich et al reported that nitrite ion mixed with HOCl increased dityrosine and nitrotyrosine while decreasing chlorotyrosine relative to HOCl alone. Further, the addition of HOCl to a mixture of nitrite and target yielded a different product profile relative to when the nitrite and HOCl were premixed immediately prior to reaction with target. Nitrotyrosine was not detected when the same HOCl, nitrite and LDH-A ratios were used.

Figure S10: LDH-A protection from $^1O_2$ by ascorbate, NADH and GSH

Oxidant & $^1O_2$ scavenger	LDH-A activity (% of control)
none	100
+ $^1O_2$	22 ± 4
+ $^1O_2$ + 50 μM ascorbate	79 ± 3
+ $^1O_2$ + 100 μM ascorbate	91 ± 4
+ $^1O_2$ + 25 μM NADH	93 ± 2
+ $^1O_2$ + 50 μM NADH	100 ± 3
+ $^1O_2$ + 500 μM GSH	37 ± 3
+ $^1O_2$ + 1 mM GSH	48 ± 4

LDH-A (10 μM) in 10 mM PB pH 7.4 was treated with 1.5 μM MB and 60 sec of red light at 20 °C. All scavengers were premixed with LDH-A prior to light exposure. HOCl-treated LDH-A samples (15 μl) were diluted 1:10 in 20 mM Tris pH 8.6 immediately after light exposure. Assays (200 μl) contained 20 mM Tris pH 8.6, 20 nM LDH-A (5 μl of 1:10 diluted LDH-A), 0.5 mM NADH and 0.50 sodium pyruvate. The oxidation of NADH was monitored at 340 nm in a 96-well plate for 4 min at 30 °C. Rates were determined from the initial linear portion of the reaction.