Supporting information for:

HaloTag Forms an Intramolecular Disulfide

Kirsten Deprey and Joshua A. Kritzer

Department of Chemistry, Tufts University, Medford, MA 02155, United States

Table of Contents

Materials and Methods

Site-directed mutagenesis

Mutagenesis was performed using the Pfu polymerase turbo system (Agilent). Primer sequences are included in Table S2, and the parent plasmid was the pH6HTN HaloTag expression plasmid (Promega). The PCR product was purified with a PCR purification kit (Qiagen), and then the template strand was digested with DpnI at 37 ºC for 1 hr. PCR amplification was confirmed by agarose gel electrophoresis with a 1% gel in TAE (BioRad), and bands were visualized with EtBr and a UV Illuminator. The HaloTag-coding region was sequenced (Psomagen) to confirm the presence of the correct mutation.

Protein expression and purification

HaloTag expression plasmids were transformed into *Escherichia coli* BL21(DE3) E. coli (NEB) and grown on an LB/agar plate with ampicillin (100 mg/mL) at 37 ºC overnight. Colonies were picked and grown in 10 mL LB broth with ampicillin at 37 ºC overnight. Each sample was transferred to 1 L flask of LB and grown at 37 ºC for 3-6 h, or until the optical density at 600 nm reached 0.6. Cultures were induced with 1mL of IPTG (1 mM) at room temperature overnight, then pelleted at 4,000 rpm for 10 mins. Pellets were lysed using a filtered and degassed buffer containing 50 mM Tris-Cl, 100 mM NaCl, 5 mM imidazole, and 0.1 mM EDTA, with 1 mg/mL lysozyme, 5 mM 2-mercaptoethanol, and protease inhibitor cocktail (Roche). The pellet was sonicated on ice in lysis buffer for 10 min, 30 s on/off. Lysates were clarified at 10,000 rpm for 1 hr at 4 ºC, and the supernatant was purified through batch affinity purification using Ni-NTA resin (Thermo). First, Ni-NTA resin was equilibrated with His-binding buffer (50 mM Tris-Cl, 100 mM NaCl, 5 mM imidazole, and 0.1 mM EDTA). Clarified lysates were incubated with Ni-NTA resin for 1 hr at 4 ºC. Flow through was discarded, and resin was washed with degassed His-wash buffer (50 mM Tris-Cl, 300 mM NaCl, 10 mM imidazole, and 0.1 mM EDTA, with 5 mM 2-mercaptoethanol). 6xHis HaloTag was eluted from resin with His-elution buffer (50 mM Tris-Cl, 50 mM NaCl, 300 mM imidazole, and 0.1 mM EDTA with 5 mM 2-mercaptoethanol). Elution fractions were desalted to remove imidazole and 2-mercaptoethanol into degassed PBS (pH 7.4).

Synthesis of chloroalkane-carboxylic acid

The chloroalkane tag (ct-) used as the HaloTag ligand was prepared according to a previously reported procedure.[1] Briefly, 2-(2-aminoethoxy)ethanol was dissolved in ethanol, cooled to 0 ºC, and ditertbutyl dicarbonate (Boc_2O, 1 eq) was added at 0 ºC to protect the amine. The reaction mixture was stirred for 1.5 hr at room temperature. The ethanol was removed, and the product was extracted with water and dichloromethane (DCM), washed with brine, dried over sodium sulfate, filtered, and concentrated. The Boc-protected product was stirred with sodium hydride (1.1 eq) in N,N-dimethylformamide (DMF) on ice for 20 mins, followed by the slow addition of 1-chloro-6-iodohexane (1.2 eq). The reaction mixture was stirred at 0 ºC for 15 mins and at room temperature for an additional 15 mins. The reaction was quenched with 1 M HCl, extracted with ethyl acetate, washed with brine, dried, filtered, and concentrated. The resulting product was purified by silica column chromatography using a gradient of ethyl acetate and hexanes. The purified product was dissolved in 20% TFA in DCM and stirred at 0 ºC for 2 hr to remove the Boc protecting group. The solvent was removed, and the product was dissolved in MeOH and cooled to 0 ºC. K_2CO_3 was added to the mixture until the pH reached 7-8, then the MeOH was removed. The product was extracted with water and ethyl acetate, washed with brine, dried over sodium sulfate, filtered, and concentrated. For conjugation to N-hydroxysuccinimide (NHS)-labelled molecules, this product, referred to as chloroalkane-amine, was purified by reverse-phase HPLC. For conjugation to amine-labelled molecules, the chloroalkane-amine was dissolved in DCM and placed under nitrogen. Diisopropylethylamine (DIPEA) (1.3 eq) and succinic anhydride (1.8 eq)

were added to the reaction and the reaction was stirred at room temperature for 2 h. The reaction was quenched with 1 M HCl, extracted with DCM, washed with brine, dried over magnesium sulfate, filtered, and concentrated. The final product, the chloroalkane-carboxylic acid, was purified by reverse-phase HPLC and verified by MALDI-TOF MS and ^{1}H NMR as described.[1]

Conjugation of chloroalkane label to molecules

To prepare chloroalkane-labeled fluorescein (ct-fluorescein) and chloroalkane-labeled PEG_4-biotoin (ct-PEG_4-biotin), the chloroalkane-amine (2 eq) was allowed to react with either NHS-fluorescein (1 eq) or NHS-PEG_4-biotin (1 eq), respectively, with diisopropylethylamine (5 eq) in DMF dried over molecular sieves.[2] The reaction mixture was shaken at room temperature overnight. The mixture was purified by preparatory reverse-phase HPLC. Conjugation was verified by MALDI-TOF MS, and purify was confirmed by analytical HPLC. To prepare ct-DD5o and its analogues, the N-termini of the peptides were capped on resin using chloroalkane-carboxylic acid (2.5 eq), benzotriazole-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate (PyBOP) (2.5 eq), and DIPEA (5 eq) in DMF.[2,3] The reaction mixture was shaken at room temperature for 1 hr. The ct-peptide was cleaved from solid support and purified by preparatory reverse-phase HPLC. Conjugation was verified by MALDI-TOF MS and purity was confirmed by analytical HPLC.

Gel shift assays

For the gel shift assays, HaloTag7 (5 μM) was incubated with increasing concentrations of a chloroalkane-tagged biotinylated stapled peptide. For the gel shift assay with a DMSO concentration profile, HaloTag7 (5 μM) was incubated with either PBS or PBS with increasing concentrations of DMSO (1 to 25% DMSO). For the gels corresponding to the FP data, HaloTag7 (5 μM) was incubated with either 20% DMSO in PBS or with 1 or 2 equivalents of 5,5-dithiobis-2-nitrobenzoic acid (DTNB). All samples were incubated at 37 ºC for 3 hr, dialyzed in PBS to remove the DMSO or DTNB using a Slide-A-Lyzer cassette with a 3.5 kDa MWCO. Samples were mixed with Laemmli buffer (Biorad) without the reducing agent additive, heated at 95 ºC for 3-5 min, cooled to room temperature, then resolved on a 10% gel (BioRad). Samples were Gels were stained and de-stained using the Pierce Power Station PowerStain cassette (Thermo).

Western blot

Polyacrylamide gels were prepared as described above. Protein was transferred to a nitrocellulose membrane using Pierce Power Station PowerBlot cassette (Thermo). For the detection of biotin, the membrane was blocked with 2% BSA in 1x TBST at room temperature for 2 hr. Primary anti-biotin, goat (Thermo) and secondary rabbit anti-goat (Thermo) antibody incubation steps were performed with iBind Flex system (Invitrogen) at room temperature for 4 hr at 1:1000 dilutions. Membrane was rinsed with TBST (2 x 1 min), washed with TBS (3 x 5 min), and then incubated with SuperSignal West Pico chemiluminescent substrate (Thermo) for 5 mins. The membrane was developed using a ChemiDoc MP Imaging system (BioRad).

Tryptic digest of HaloTag

HaloTag7 protein (5 μg) was incubated at room temperature for 3 hours in PBS, 20% DMSO or 1 equivalent DTNB, then was dialyzed in PBS to remove the DMSO or DTNB as described above. Samples were heated at 100 ºC for 3 mins, then incubated with 0.5 μg of trypsin gold (Promega) overnight at room temperature. Tryptic peptides were extracted using ZipTips, and tryptic fragments were detected by MALDI-TOF MS and LC-MS.

Liquid chromatography and mass spectrometry

LC-MS analysis of the tryptic fragments was performed using an Agilent 1260 Infinity LC system

coupled with Agilent 6530 Accurate Mass Q-TOF. Tryptic fragments were separated on an AdvanceBio Peptide column (2.1 x 150 mm, Agilent), maintained at 55 ºC during separation. The mobile phase consisted of a gradient of water with 0.1% formic acid (solvent A), to acetonitrile with 0.1% formic acid (solvent B), with a flow rate of 0.4 mL min^{-1}. The gradient consisted of isocratic 5% B from 0-1.75 min, then a gradient from 5% B to 45% B between 1.75-16.00 min. Between 16 min and 16.5 min, the gradient increased from 45% B to 95% B, where it was maintained until the end of the sample run. For the mass spectrometry, an electrospray ionization (ESI) source was used in the positive mode within the range of 300-3000 m/z. Spectra were collected at a rate of 5 scans sec^{-1} from 1.75 min to 20 min of the method. Data were analyzed using the BioConfirm Workflow within the Agilent MassHunter Qualitative Analysis software.

Fluorescence polarization assays

For each experiment, a 200 nM solution of HaloTag was prepared in PBS + 0.01% Tween-20. Equal volumes (15 μL each) of protein and 20 nM chloroalkane-fluorescein were added to each well of a black 384-well plate (Corning), and time-dependent FP signal was recorded immediately. The fluorescence polarization was measured over time using a TECAN Spark multimode plate reader with a kinetic loop method prepared to measure FP every 30 s for 30 min, and then every 10 min for an additional 90 min. There was a 1.5-min delay time between the addition of the probe and the first FP measurement, but this delay was accounted for in the analysis.

To oxidize HaloTag7 with DMSO, the protein solution was prepared in buffer with 20% DMSO and incubated at room temperature for 3 hr. To oxidize HaloTag7 without DMSO, O_2 was bubbled through a 5 mL sample PBS + 0.01% Tween-20 for approximately 5 min. Freshly oxygenated buffer was used to prepare the 200 nM protein stock and left to incubate at room temperature for 4 hr. The samples were then exposed to air for an additional 20 hr at room temperature with shaking. To oxidize HaloTag with 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 200 nM of HaloTag in PBS was incubated with 200 nM (1 eq) or 400 nM (2 eq) of DTNB and incubated at room temperature for 3 hr. For the samples that were reduced after oxidation by either method, 1 mM DTT was added and incubated for 5 min before the addition of the probe.

Second-order rate constants were calculated as described.[4–10] Raw values of FP from each sample over time were normalized to the reference sample, with only buffer and probe, and the maximum polarization value for the unoxidized protein sample. To normalize the data across experiments, we subtracted the polarization value of the probe alone from the raw polarization value of each sample measurement. We divided this value by the difference of the maximum of the untreated sample within a given set and the value of the probe alone. This gave us a normalized value for fraction of bound probe (Equation 1).

Equation 1. Normalization of raw FP signal

$$\frac{(\text{sample} - \text{reference})}{(\text{maximum} - \text{reference})}$$

reference: ct-fluorescein, no HaloTag
maximum: ct-fluorescein + HaloTag at t=3 hr

The predicted binding at each individual time point was calculated using Equation 2. The values of 1 and 0.2 for the polarization (max) and the k_{obs}, respectively, were used as variable starting

points during the fit calculation. We then took the squared difference between the observed binding calculated from Equation 1 and the predicted binding calculated from Equation 2.

Equation 2. Calculation of predicted binding with a pseudo first-order rate law[7,8]

$$P(t) = P(max) \times (1 - e^{-k*t})$$

P(t): polarization at t = time
P(max): maximum polarization
k: observed rate constant

Next, we used the Solver Excel plug-in to fit a 1:1 binding curve, minimizing the sum of the squared differences from Equation 3, and subsequently calculating the actual polarization (max) and k_{obs}. This k_{obs} is a first-order rate constant, which can be used in further calculations because the protein was used in a 10-fold excess, essentially making the reaction pseudo-first order.[7,8] To convert the pseudo-first order rate constant to an apparent second-order rate constant, we divided by the concentration of HaloTag, in excess (Equation 3).

Equation 3. Calculation of apparent 2nd order rate constant[9,10]

$$k, \text{apparent } 2^{nd} \text{ order} = \frac{k, \text{pseudo } 1^{st} \text{ order}}{[\text{HaloTag}], \text{ in excess}}$$

The average and standard error of the mean for the rate constant was determined from three independent replicates. Significance was determined using a two-tailed t-test across the three replicates.

Circular dichroism (CD)
A 300 μL sample of protein (20 μM) in PBS was added to a 1 mm quartz cuvette. CD was measured for 3 technical replicates per trial, with three independent trials. Mean residue ellipticity was calculated for each measured wavelength by dividing the millidegree CD signal by the molar concentration and the number of amino acids. Measurements were made with a JASCO J-815 CD Spectrometer.

Table S1. Sequences and molecular weights of ct-peptides.
ct = chloroalkane tag

Molecule	Sequence	Expected MW (g/mol)	Observed MW (g/mol)
ct-DD5o	ct-VcNAcFHIWH *ortho*-xylene staple	1636.4	1637.4
ct-DD5o-biotin	ct-VcNAcFHIWH-K(PEG_4-biotin) *ortho*-xylene staple	2238.2	2239.2
ct-Tat	ct-YGRKKRRQRRR	1879.7	1881.3

Table S2. Primers used for site-directed mutagenesis of the pH6HTN HaloTag7 plasmid (Promega).

Mutation of pH6HTN	For mutagenesis (5 → 3)
Stop codon insertion	FWD: GAT TTC CGG CTA GCC AAC CAC TG REV: TCG AGC GTC GAC AGC CAG
Asp106Asn	FWD: GTC GTC CTG GTC ATT CAC AAC TGG G REV: CAG AGC GGA GCC CCA GTT G
Cys61Ser	FWD: GTT GCA CCG ACC CAT CGC AGC ATT G REV: TAC CGA TCA GGT CTG GAG CAA TGC TGC
Cys262Ser	FWD: GCC AAA AGC CTG CCT AAC AGC AAG G REV: GCC GAT GTC CAC AGC CTT GCT G
Cys[61+262]Ser mutation of Cys61Ser plasmid	FWD: GCC AAA AGC CTG CCT AAC AGC AAG G REV: GCC GAT GTC CAC AGC CTT GCT G

Table S3. Rate constant of HaloTag with chloroalkane-fluorescein, as measured by FP.

HT7 = HaloTag 7, HT8 = HaloTag8

Sample	Apparent 2nd order rate constant ($M^{-1}s^{-1}$)
HT7: Untreated	22000 ± 4700
HT7: DMSO-oxidized	18000 ± 2500
HT7: DMSO-oxidized, then 1 mM DTT	19000 ± 3000
HT7: Untreated	26000 ± 3700
HT7: Air-oxidized	30000 ± 12000
HT7: Air-oxidized, then 1 mM DTT	19000 ± 1900
HT7: Untreated	50000 ± 17000
HT7: DTNB-oxidized, 1 eqv	52000 ± 21000
HT7: DTNB-oxidized, 2 eqv	30000 ± 7000
HT7: DTNB-oxidized, then 1 mM DTT	49000 ± 24000
HT8: Untreated	49000 ± 5500
HT8: DMSO-oxidized	44000 ± 3000
HT8: DMSO-oxidized, then 1 mM DTT	31000 ± 9200
HT8: Untreated	48000 ± 4400
HT8: DTNB-oxidized, 1 eqv	58000 ± 8300
HT8: DTNB-oxidized, 2 eqv	40000 ± 1800
HT8: DTNB-oxidized, then 1 mM DTT	43000 ± 4700

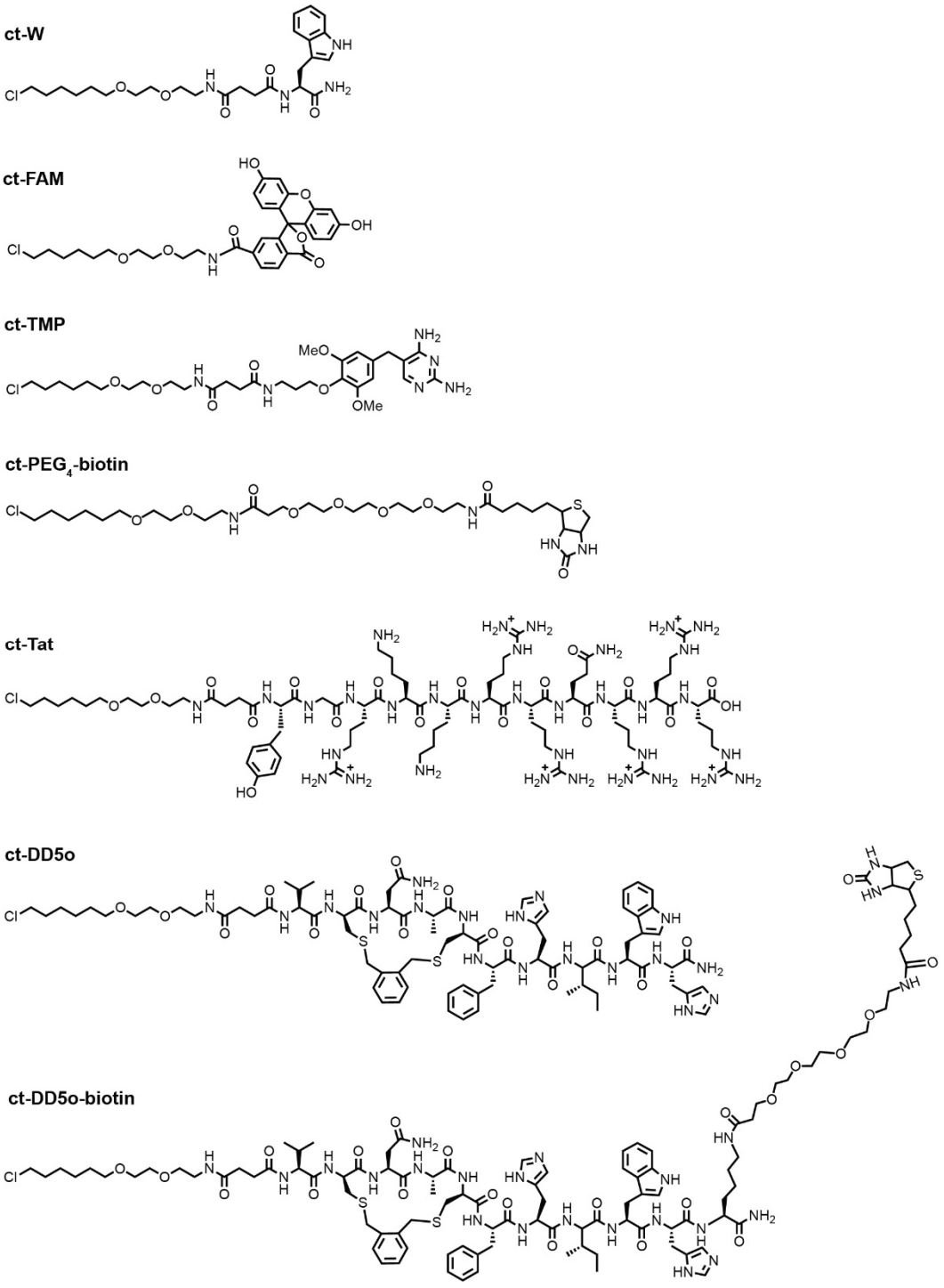

Figure S1. Chemical structures of key compounds.

Key:

Detected tryptic fragments

Undetected tryptic fragments

Fragment detected in HaloTag7 band, but not higher-mobility band

HaloTag band (34 kDa):

MKHHHHHHAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPH
VAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHW
AKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMG
VVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKL
LFWGTPGVLIPPAEAARLAKSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISG

Higher-mobility band (apparent MW roughly 27 kDa):

MKHHHHHHAEIGTGFPFDPHYVEVLGERMHYVDVGPRDGTPVLFLHGNPTSSYVWRNIIPH
VAPTHRCIAPDLIGMGKSDKPDLGYFFDDHVRFMDAFIEALGLEEVVLVIHDWGSALGFHW
AKRNPERVKGIAFMEFIRPIPTWDEWPEFARETFQAFRTTDVGRKLIIDQNVFIEGTLPMG
VVRPLTEVEMDHYREPFLNPVDREPLWRFPNELPIAGEPANIVALVEEYMDWLHQSPVPKL
LFWGTPGVLIPPAEAARLAKSLPNCKAVDIGPGLNLLQEDNPDLIGSEIARWLSTLEISG

Figure S2. Mass spectrometry of the HaloTag7 band and the higher-mobility band. HaloTag7 was treated with DMSO, subjected to SDS-PAGE, and the HaloTag7 band (34 kDa) and the higher-mobility band (apparent molecular weight roughly 27 kDa) were extracted. The protein samples were denatured, reduced and subjected to a tryptic digest and LC-MS/MS analysis. Detected peaks correspond to expected tryptic fragments. Only one sequence was missing from the analysis of the higher-mobility band, and it was centrally located within that protein and could not account for the apparent mass difference.

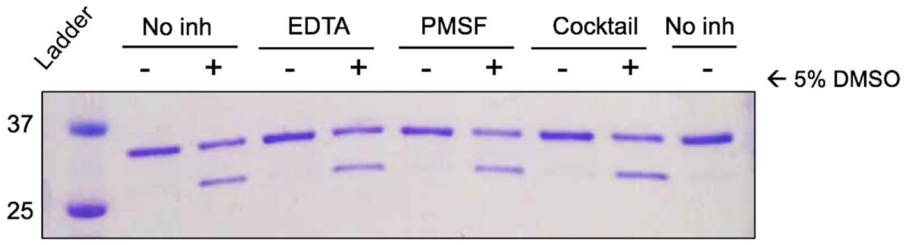

Figure S3. Higher-mobility band is not caused by trace protease activity. Recombinant HaloTag7 (5 μM) was incubated in the presence of protease inhibitors with and without 5% DMSO. Samples were denatured, resolved on a nonreducing SDS-PAGE gel, and stained with Coomassie. The higher-mobility band appears even in the presence of protease inhibitors, suggesting that trace protease activity is not responsible for the higher-mobility band. This gel is representative of two independent replicates.

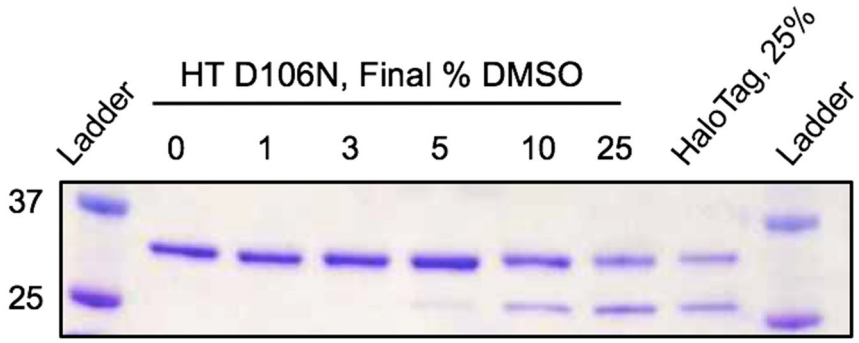

Figure S4. The catalytic aspartate is not required for appearance of the higher-mobility band. A version of HaloTag in which the catalytic aspartate is mutated to an asparagine, HT D106N, was prepared. Recombinant HT D106N HaloTag7 (5 μM) was incubated in the presence of varying concentrations of DMSO. Samples were denatured and resolved on a non-reducing SDS-PAGE gel and stained with Coomassie. The higher-mobility band around 27 kDa is still observed, suggesting that the catalytic aspartate is not involved in formation of the lower band. This gel is representative of three independent replicates.

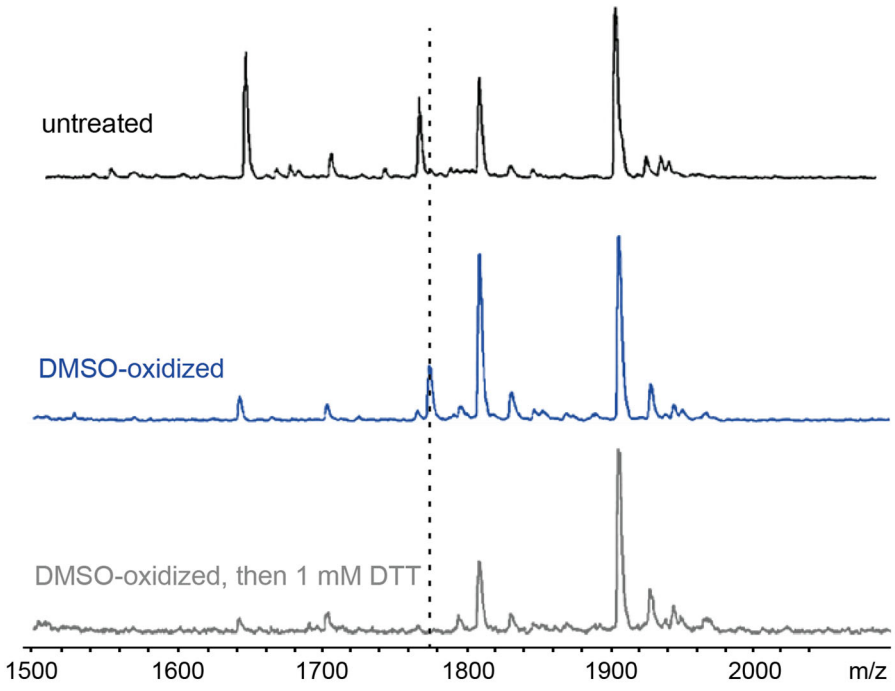

Figure S5. Detection of disulfide-bonded fragment by MALDI-TOF after DMSO oxidation. A tryptic digest was performed on HaloTag7 samples that were untreated, oxidized with 20% DMSO, and oxidized with 20% DMSO followed by reduction with 1 mM DTT. The dotted line corresponds to the molecular weight of the disulfide-containing fragment, which was detected in the DMSO-oxidized sample and disappeared upon addition of DTT. Other peaks correspond to expected tryptic fragments.

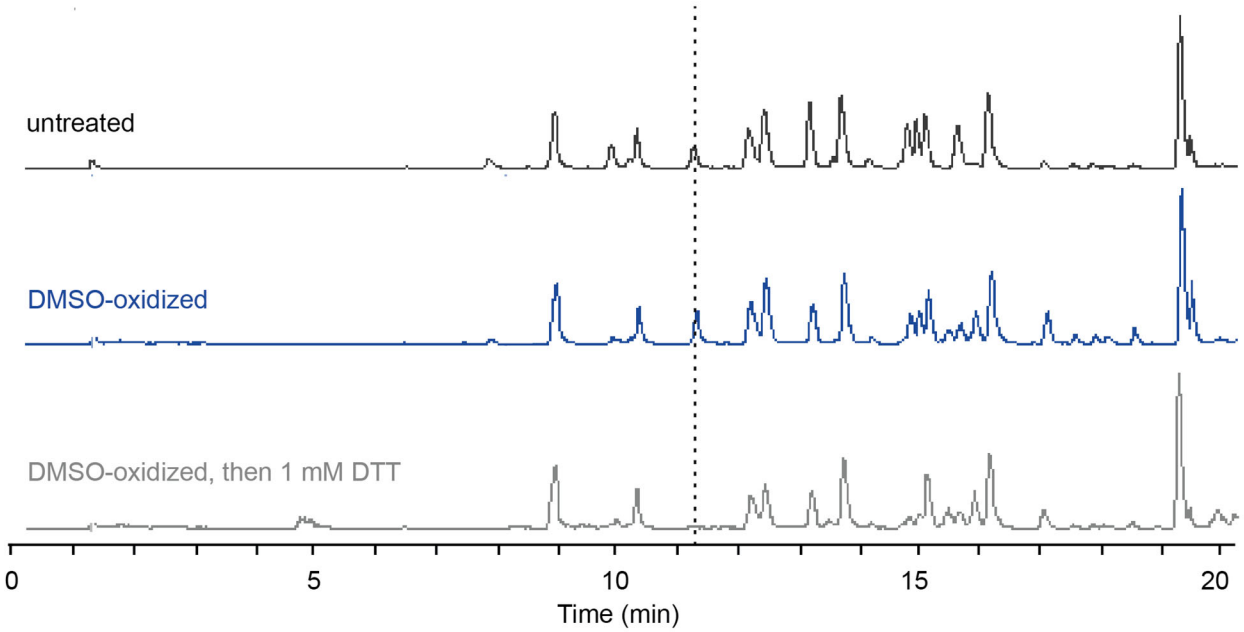

Figure S6. Detection of disulfide-bonded fragment by LC-MS after DMSO oxidation. Base peak chromatograms (BPC) were extracted from the total ion chromatograms detected by LC-MS analysis of a tryptic digest of treated HaloTag7 samples. The dotted line corresponds to a prominent peak that is missing only from the sample treated with DTT at a retention time of 11.2 min. This peak contains the expected *m/z* values of the two cysteine-containing tryptic fragments joined by a disulfide.

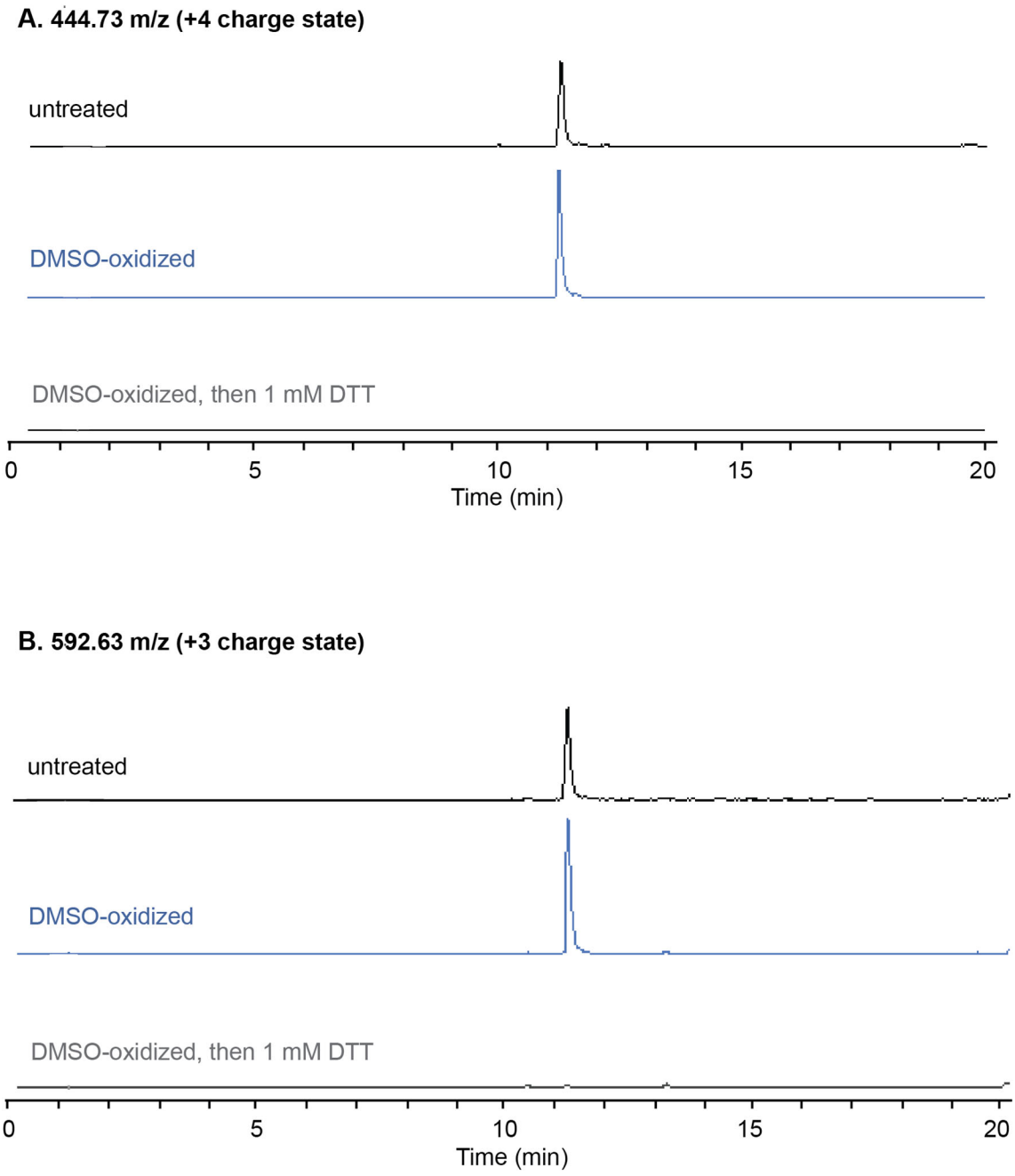

Figure S7. Extracted ion chromatogram of disulfide-bonded fragment after DMSO oxidation. Extracted ion chromatograms (EIC) were extracted from the total ion chromatograms detected by LC-MS of a tryptic digest of DMSO-treated HaloTag7 samples. Samples were scanned for *m/z* values that correspond to the +4 and +3 charge states (**A.** and **B.**, respectively) of the expected fragments joined by a disulfide. These *m/z* values were not detected in the samples that were treated with DTT. The retention time of these extracted ion peaks corresponds to the peak in the BPC that was absent in the sample treated with DTT.

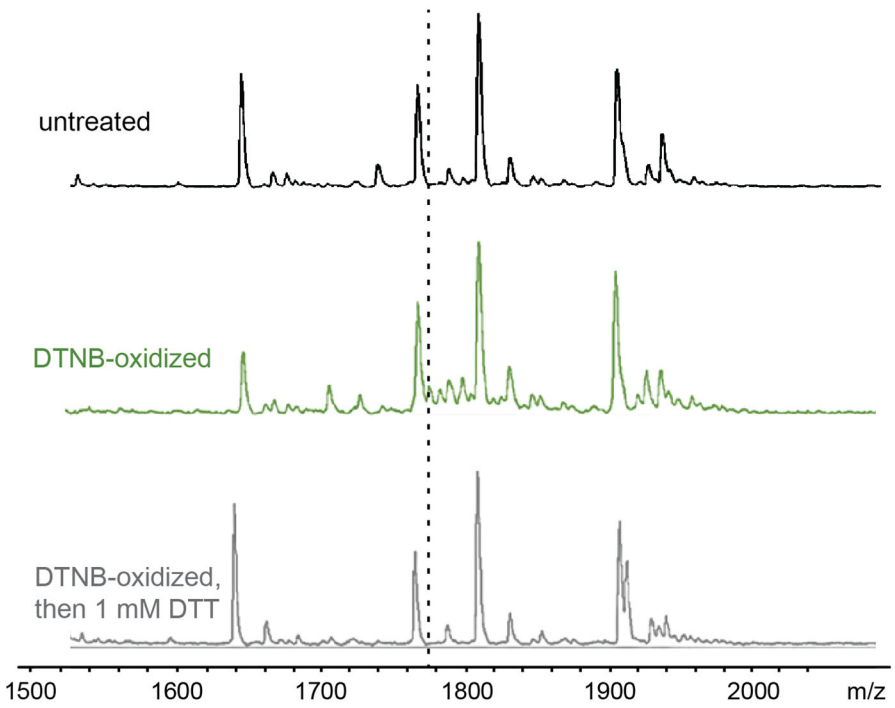

Figure S8. Detection of disulfide-bonded fragment by MALDI-TOF after DTNB oxidation. A tryptic digest was performed on HaloTag7 samples that were untreated, oxidized with 1 equivalent of DTNB, and oxidized with 1 equivalent of DTNB followed by reduction with 1 mM DTT. The dotted line corresponds to the molecular weight of the disulfide-containing fragment, which was detected in the DTNB-oxidized sample and disappeared upon addition of DTT. Other peaks correspond to expected tryptic fragments.

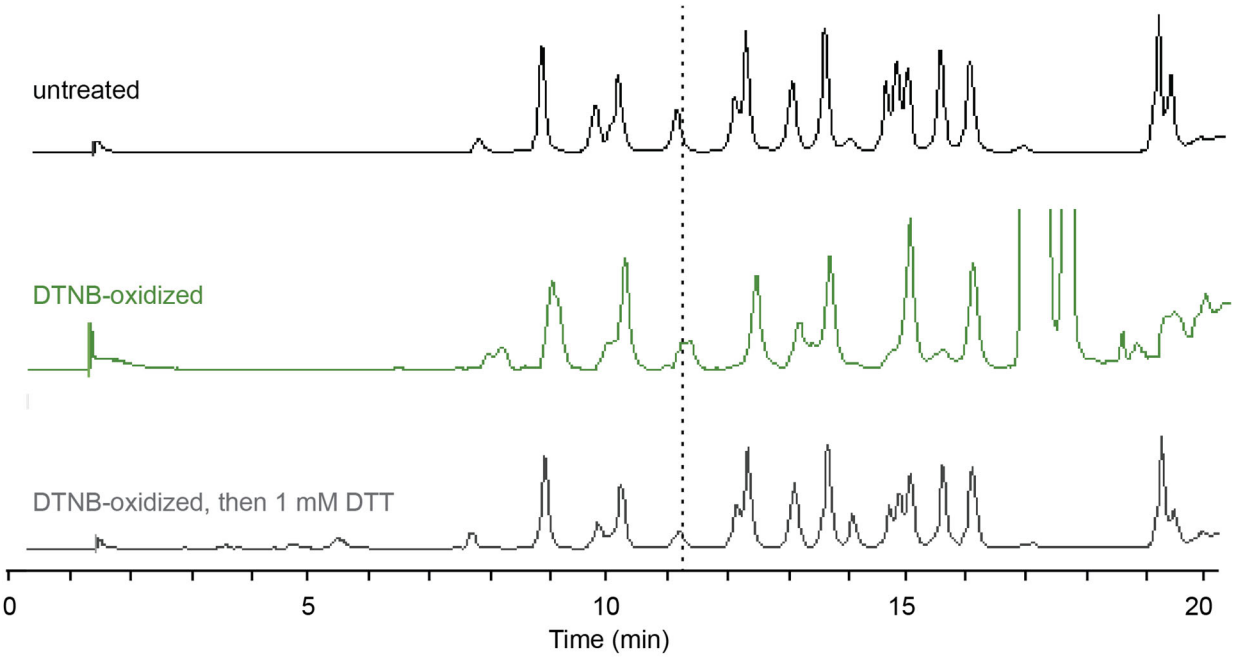

Figure S9. Detection of disulfide-bonded fragment by LC-MS after DTNB oxidation. Base peak chromatograms (BPC) were extracted from the total ion chromatograms detected by LC-MS of a tryptic digest of DTNB-treated HaloTag7 samples. The dotted line corresponds to a prominent peak that is missing only from the sample treated with DTT at a retention time of 11.2 min, that contains the expected m/z values of two tryptic fragments joined by a disulfide.

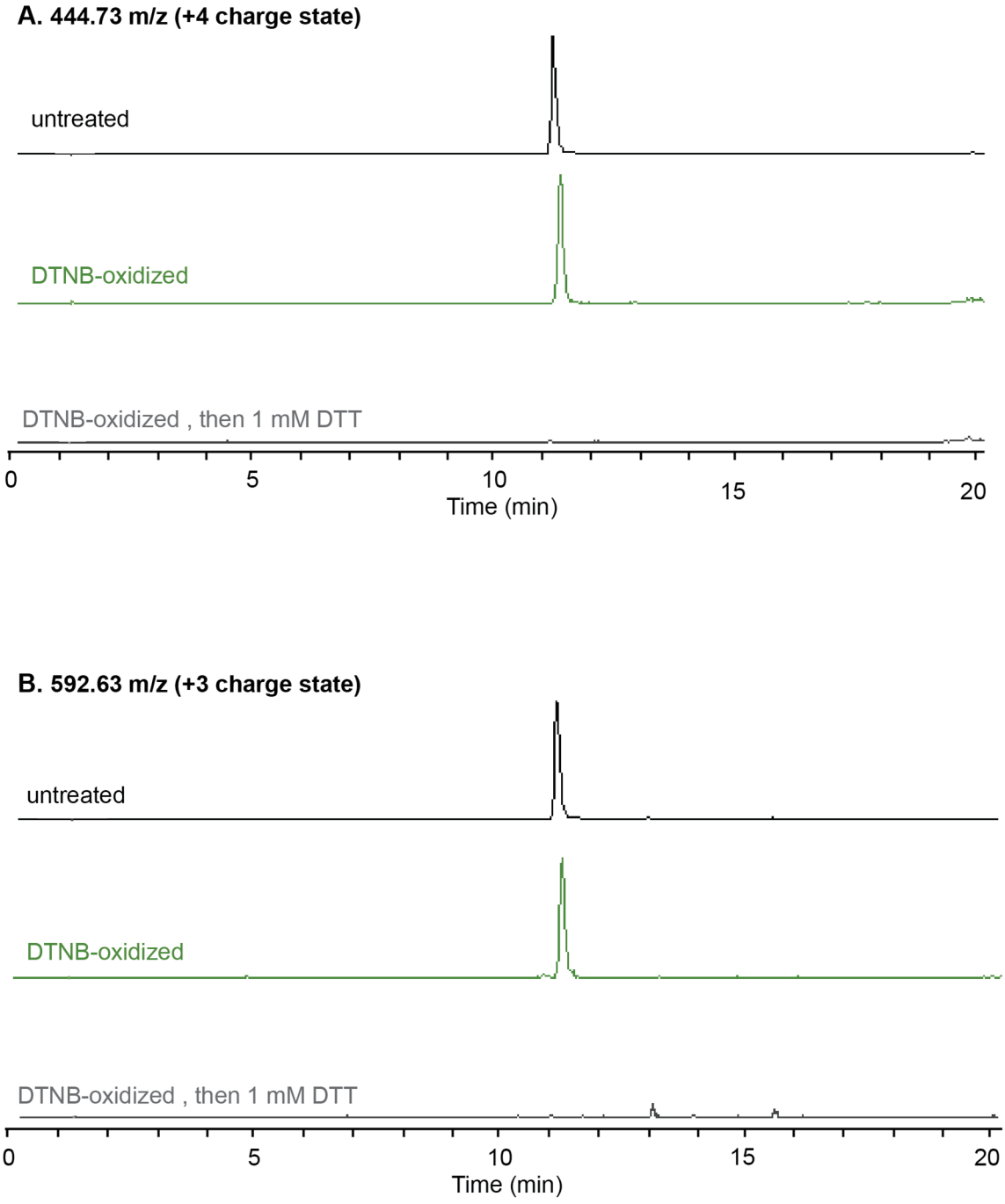

Figure S10. Extracted ion chromatogram of disulfide-bonded fragment after DTNB oxidation. Extracted ion chromatograms (EIC) were extracted from the total ion chromatograms detected by LC-MS of a tryptic digest of DTNB-treated HaloTag7 samples. Samples were scanned for *m/z* values that correspond to the +4 and +3 charge states (**A.** and **B.**, respectively) of the expected fragments joined by a disulfide. These *m/z* values were not detected in the samples that were treated with DTT. The retention time of these extracted ion peaks corresponds to the peak in the BPC that was absent in the DTT-treated sample.

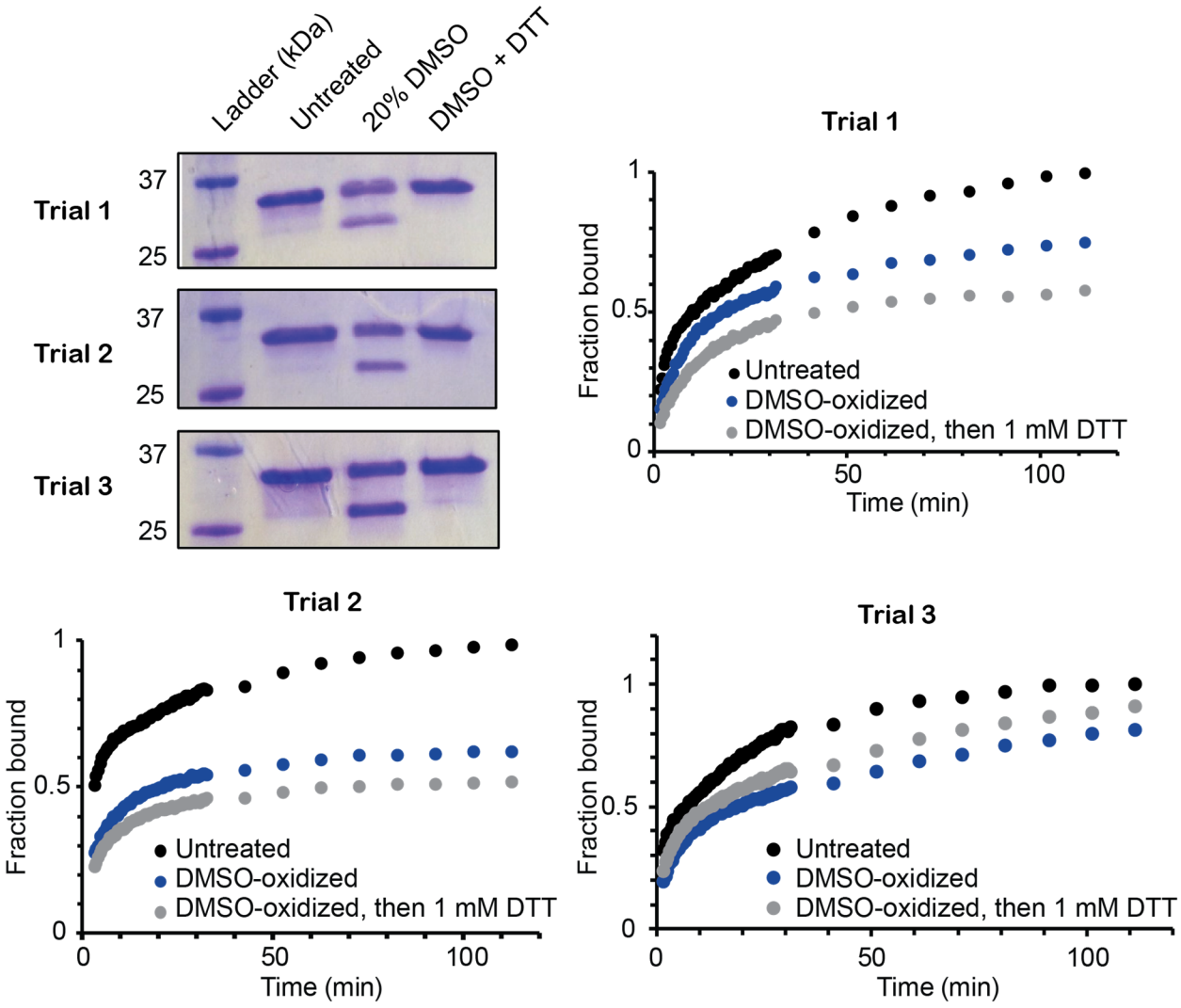

Figure S11. Individual kinetics trials for DMSO-oxidized HaloTag7. Stained gels of HaloTag7 incubated with PBS (untreated), 20% DMSO, or 20% DMSO followed by reduction with 1 mM DTT. Time-dependent FP assays with chloroalkane-fluorescein were performed on the same samples. No significant change in rate was observed among the three conditions. The gels from each trial consistently show a 30-50% oxidation. The compiled data for these three independent replicates is included in Table S3.

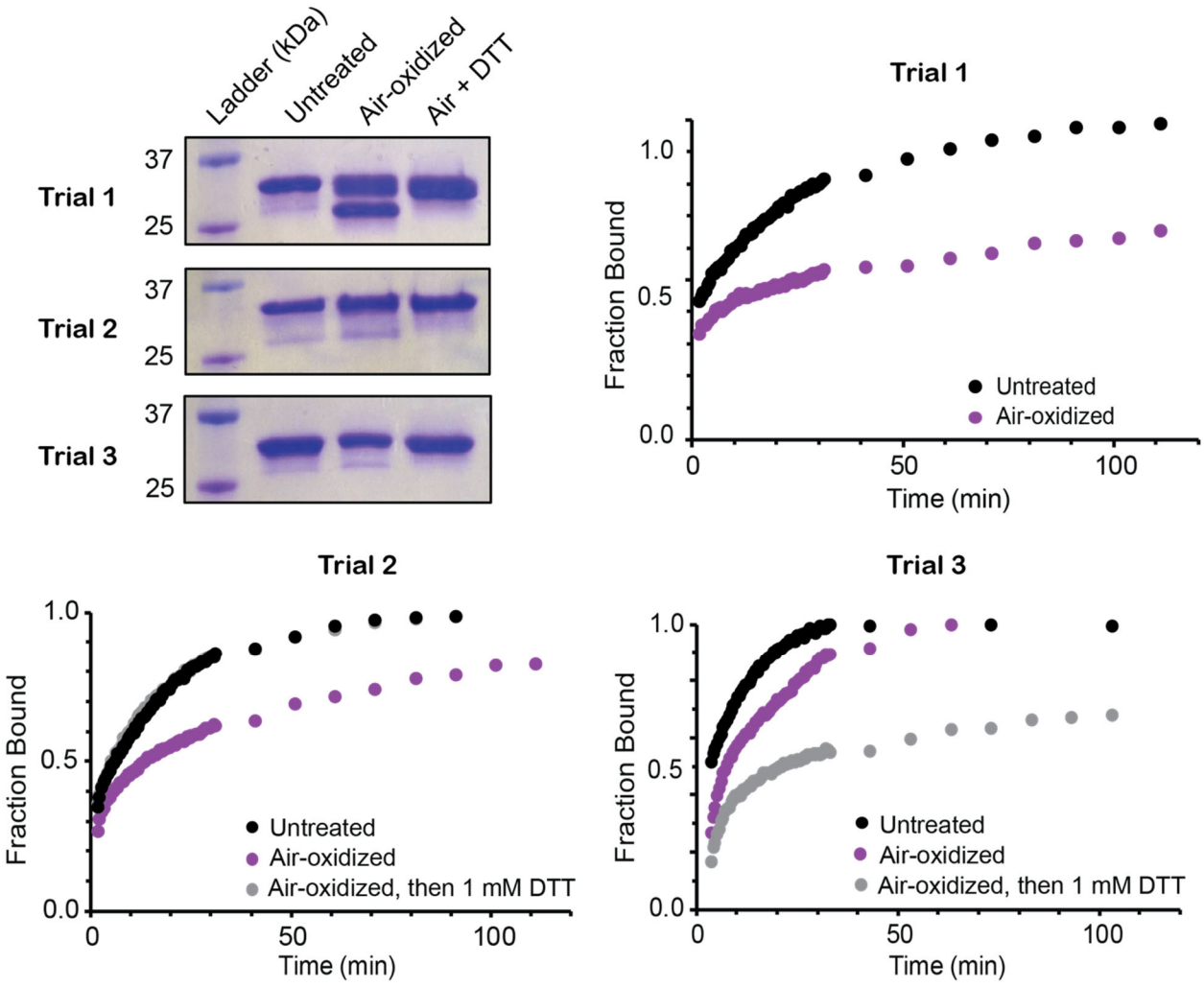

Figure S12. Individual kinetics trials for air-oxidized HaloTag7. Stained gels of HaloTag7 incubated with PBS (untreated), freshly oxygenated buffer with air exposure, or oxygenated buffer and air exposure followed by reduction with 1 mM DTT. Time-dependent FP assays with chloroalkane-fluorescein were performed on the same samples. The degree of oxidation was less reproducible using air oxidation compared to DMSO oxidation. The compiled data for these three independent replicates is included in Table S3.

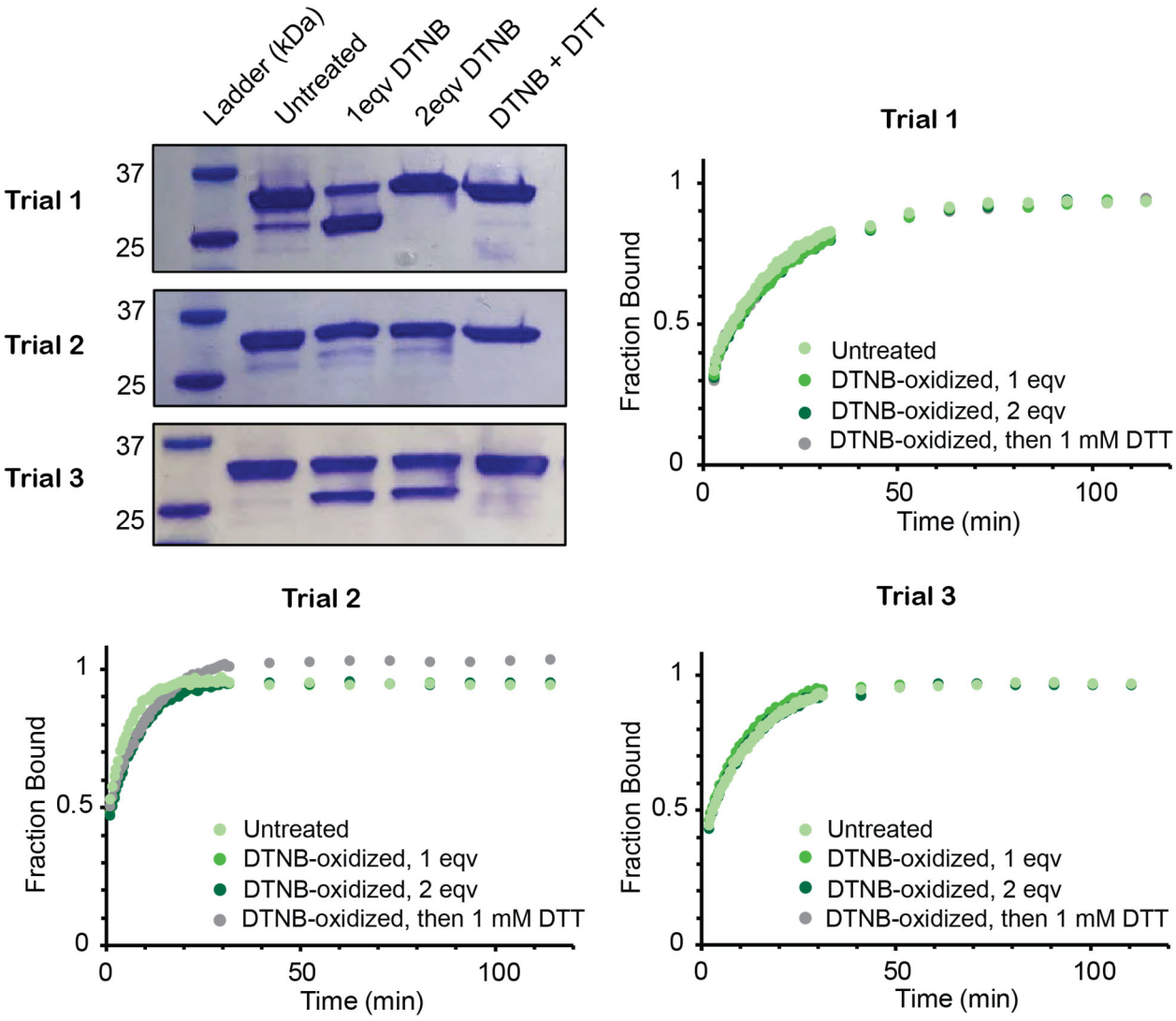

Figure S13. Individual kinetics trials for DTNB-oxidized HaloTag7. Stained gels of HaloTag7 incubated with PBS (untreated), 1 equivalent or 2 equivalents of DTNB, or 1 equivalent of DTNB followed by reduction with 1 mM DTT. Time-dependent FP assays with chloroalkane-fluorescein were performed on the same samples. The gels from each trial show a 10-70% oxidation, as estimated by the intensity of the lower band. The compiled data for these three independent replicates is included in Table S3.

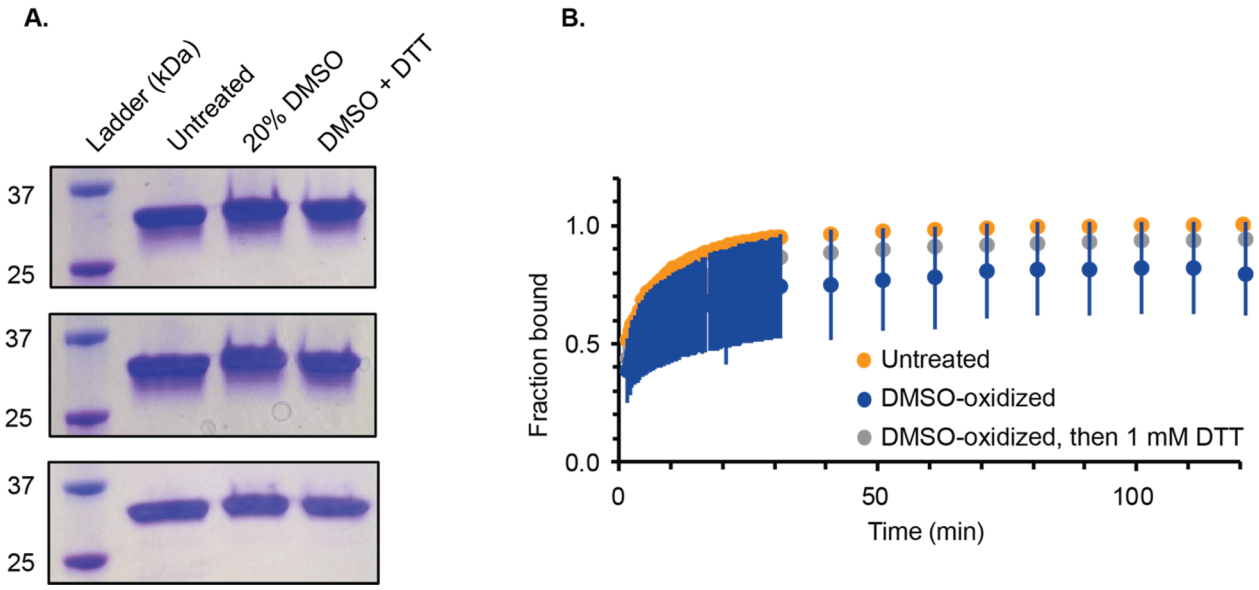

Figure S14. Individual kinetics trials for DMSO-oxidized HaloTag8, which is resistant to oxidation. **A.** Stained gels of HaloTag8 (5 μM) incubated with PBS (untreated), 20% DMSO, or 20% DMSO followed by the addition of 1 mM DTT. No higher-mobility band is observed, indicating that no oxidation has occurred. **B.** Time-dependent FP assays with chloroalkane-fluorescein were performed on the same samples. FP data are averages and standard error of the mean from three independent replicates.

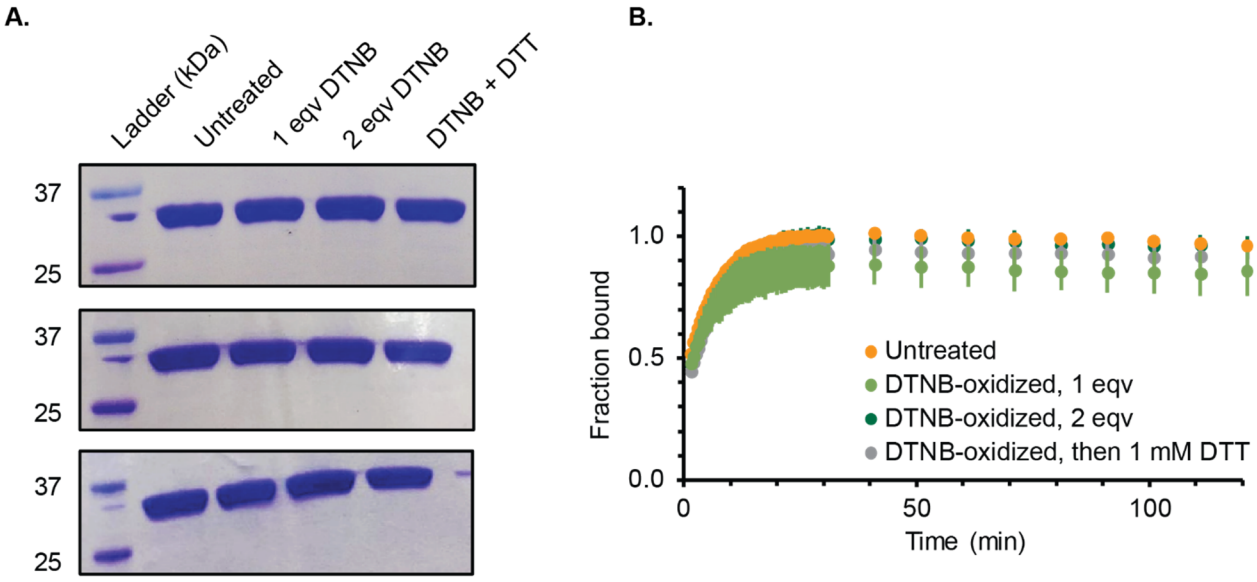

Figure S15. Individual kinetics trials for DTNB-oxidized HaloTag8, which is resistant to oxidation. A. Stained gels of HaloTag8 (5 μM) incubated with PBS (untreated), 1 equivalent or 2 equivalents of DTNB, or 1 equivalent of DTNB followed by reduction with 1 mM DTT. No higher-mobility band is observed, indicating that no oxidation has occurred. **B.** Time-dependent FP assays with chloroalkane-fluorescein were performed on the same samples. FP data are averages and standard error of the mean from three independent replicates.

References

(1) Peraro, L., Deprey, K. L., Moser, M. K., Zou, Z., Ball, H. L., Levine, B., and Kritzer, J. A. (2018) Cell Penetration Profiling Using the Chloroalkane Penetration Assay. *J. Am. Chem. Soc. 140*, 11360–11369.
(2) Deprey, K., and Kritzer, J. A. (2020) Quantitative measurement of cytosolic penetration using the Chloroalkane Penetration Assay. *Methods Enzymol.*
(3) Peraro, L., Zou, Z., Makwana, K. M., Cummings, A. E., Ball, H. L., Yu, H., Lin, Y. S., Levine, B., and Kritzer, J. A. (2017) Diversity-Oriented Stapling Yields Intrinsically Cell-Penetrant Inducers of Autophagy. *J. Am. Chem. Soc. 139*, 7792–7802.
(4) Los, G. V, Encell, L. P., Mcdougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., Wood, M. G., Learish, R., Ohana, R. F., Urh, M., Simpson, D., Mendez, J., Zimmerman, K., Otto, P., Vidugiris, G., Zhu, J., Darzins, A., Klaubert, D. H., Bulleit, R. F., and Wood, K. V. (2008) HaloTag: A Novel Protein Labeling Technology for Cell Imaging and Protein Analysis. *ACS Chem. Biol. 3*, 373–382.
(5) Kaushik, S., Prokop, Z., Damborsky, J., and Chaloupkova, R. (2017) Kinetics of binding of fluorescent ligands to enzymes with engineered access tunnels. *FEBS J. 284*, 134–148.
(6) Encell, L. P., Friedman Ohana, R., Zimmerman, K., Otto, P., Vidugiris, G., Wood, M. G., Los, G. V, McDougall, M. G., Zimprich, C., Karassina, N., Learish, R. D., Hurst, R., Hartnett, J., Wheeler, S., Stecha, P., English, J., Zhao, K., Mendez, J., Benink, H. A., Murphy, N., Daniels, D. L., Slater, M. R., Urh, M., Darzins, A., Klaubert, D. H., Bulleit, R. F., and Wood, K. V. (2012) Development of a Dehalogenase-Based Protein Fusion Tag Capable of Rapid, Selective and Covalent Attachment to Customizable Ligands. *Curr. Chem. Genomics 6*, 55–71.
(7) Simonin, J. P. (2016) On the comparison of pseudo-first order and pseudo-second order rate laws in the modeling of adsorption kinetics. *Chem. Eng. J. 300*, 254–263.
(8) Lin, J., and Wang, L. (2009) Comparison between linear and non-linear forms of pseudo-first-order and pseudo-second-order adsorption kinetic models for the removal of methylene blue by activated carbon. *Front. Environ. Sci. Eng. China 3*, 320–324.
(9) Corbett, J. F. (1972) Pseudo first-order kinetics. *J. Chem. Educ. 49*, 663.
(10) Schnell, S., and Mendoza, C. (2004) The condition for pseudo-first-order kinetics in enzymatic reactions is independent of the initial enzyme concentration. *Biophys. Chem. 107*, 165–174.