Direct Detection of Nitrotyrosine-Containing Proteins Using an Aniline-Based Oxidative Coupling Strategy

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Supporting Information

1. General Experimental Procedures.

Reagents. Unless otherwise noted, the chemicals and solvents used were of analytical grade and were used as received from commercial sources. Fluorescinamine isomer I, 5(6)-carboxytetramethylrhodamine *N*-succinimidyl ester, tetranitromethane, 2-(4-amino-phenyl)ethylamine and $K_3Fe(CN)_6$ were purchased from Aldrich and used without further purification. Oregon Green 488 carboxylic acid, succinimidyl ester, 5-isomer was purchased from life technologies and used without further purification. Anhydrous sodium dithionite ($Na_2S_2O_4$) was purchased from Aldrich and stored in a desiccator at room temperature until use. Over the years we have noted that the storage of this reagent at reduced temperature leads to its degradation (possibly due to the condensation of water on the cold material when the bottle is opened). If stored improperly, unidentified adducts can form during the reduction step. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F254 plates with visualization by ultraviolet (UV) irradiation at 254 nm and/or potassium permanganate stain. All organic solvents were removed under reduced pressure using a rotary evaporator. Water (dd-H_2O) used as reaction solvent was deionized using a Barnstead NANOpure purification system (ThermoFisher, Waltham, MA). Centrifugations were performed with an Eppendorf Mini Spin Plus (Eppendorf, Hauppauge, NY).

***Mass Spectrometry*.** Protein bioconjugates were analyzed using an Agilent 1200 series liquid chro-matograph (Agilent Technologies, USA) that was connected in-line with an Agilent 6224 Time-of-Flight (TOF) LC/MS system equipped with a Turbospray ion source.

***Gel Analyses*.** For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a mini gel apparatus (Novex), using a 4-12% NuPAGE® 4-12% Bis-Tris gel (Novex). The sample and electrode buffers were prepared according to Laemmli.[1] All protein electrophoresis samples were heated for 5-10 min at 95 °C in the presence of 1,4-dithiothreitol (DTT) to ensure reduction of disulfide bonds. Gels were run for 35 min at 200 V to separate the bands. Commercially available markers (Bio-Rad) were applied to at least one lane of

each gel for assignment of apparent molecular masses. Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue R-250 (Bio-Rad). Gel imaging was performed on a Gel Doc EZ System (Bio-Rad). For fluorescent protein conjugates, visualization was accomplished on Typhoon 9410 variable mode imager (Amersham Biosciences) prior to gel staining. ImageJ was used to determine the level of modification by optical densitometry.

2. Synthetic Procedures.

Synthesis of aniline-biotin substrate (2b). To a solution of EZ-Link NHS-PEG4-Biotin (25 mg, 0.042 mmol, Thermo Scientific) in CH_2Cl_2 was added 2-(4-aminophenyl)ethylamine (6.1 μL, 0.046 mmol) and triethylamine (12 μL, 0.084 mmol). The reaction mixture was stirred for 4 h and then diluted with CH_2Cl_2 and washed with water. The combined organic layers were dried over sodium sulfate and the solvent was removed *in vacuo*. The reaction afforded 25 mg of pale yellow oil (quantitative yield). HRMS (ESI) calculated for $C_{29}H_{48}O_7N_5S$ ($[M+H]^+$) 610.3269, found 610.3259 m/z.

Synthesis of Oregon green 488 and TAMRA aniline. To a solution of the appropriate NHS ester dye (0.0098 mmol) in DMF was added 2-(4-aminophenyl)ethylamine (0.0118 mmol) and triethylamine (0.02 mmol). The reaction mixture was stirred for 18 h, after which it was evaporated to remove DMF. The reaction was purified by reversed phase HPLC. HRMS (ESI) of Oregon Green 488 aniline calculated for $C_{29}H_{19}F_2N_2O_6$ ($[M]^+$) 529.1217, found 529.1209 m/z. HRMS (ESI) of TAMRA aniline calculated for $C_{33}H_{33}N_4O_4^+$ ($[M]^+$) 549.2496 , found 549.2501 m/z.

3. Protein modification procedures.

General procedure for oxidative coupling in buffered solution. To a solution of nitrophenol modified protein (bovine serum albumin (BSA), chymotrypsinogen, lysozyme, or MS2) in 100 mM phosphate buffer, pH 6.0 was added 2 μL of a freshly prepared 100 mM solution of $Na_2S_2O_4$ in 100 mM phosphate buffer, pH 6.0 (see note about dithionite storage in the General Experimental Section). The solution was briefly vortexed and incubated at room temperature for 20 min. To the solution of reduced protein was added 10 μL of a 2 mM solution of aniline probe and 10 μL of a 800 mM solution of $K_3Fe(CN)_6$, for a final total volume of 100 uL. The solution was briefly vortexed and then incubated at room temperature for 2 h. The reaction mixtures were purified by using a 0.5 mL centrifugal filter with the appropriate molecular weight cut off (MWCO, Millipore). The reaction mixtures were purified by repeated (3-6 times) centrifugation before analysis by SDS-PAGE.

Oxidative coupling in cell lysates. The procedure outlined above for the oxidative coupling in buffered solution was performed in the presence of 72 μg of mammalian cell lysate (untreated, from Jurkat or Ramos cells). The reaction mixtures were purified by repeated (3-6 times) centrifugation before analysis by SDS-PAGE.

4. Additional procedures.

Protein nitration by tetranitromethane. Proteins (200 μM of BSA, chymotrypsinogen, or lysozyme) were dissolved in phosphate buffer saline (PBS, Gibco). Aliquots of the protein solution (0.25 ml) were diluted with an equal volume of PBS. 10 μL of tetranitromethane dissolved in ethanol (4% v/v) was then added to the protein solution. Afterwards, the reaction mixtures were gently shaken at room temperature (~25 ℃) for 1 h. The modified proteins were purified by illustra NAP-5 columns (GE Healthcare).

Cell culture and preparation of cell lysates. Cell lines including Jurkat and Ramos were cultured in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine (Gibco), 100 I.U./ml penicillin, 100 μg/ml streptomycin (Gibco), and 10% (v/v) fetal bovine serum (FBS) (Gibco). All cell lines were maintained at 37 ℃ in a humidified atmosphere containing 95% air and 5% CO_2. Cells were collected and then washed twice in 10 mL of DPBS, with centrifugation at 1300 rpms for 3 min after each wash step to pellet the cells. The cell pellet was resuspended in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris pH 7.4, and protease inhibitors (Calbiochem, set III, diluted 1:100 or EDTA-free complete tablets)) and then disrupted by sonication (5 s on, 5 s off, for 1 min of sonication). The volume of lysis buffer was two times the volume of the cell pellet or 200 μL, whichever was larger. Insoluble material was removed by centrifugation (12000 rpms for 10 min at 4 ℃). Protein concentration was determined using a BCA assay (Pierce).

Precipitation of proteins. To a sample of protein in 100 μL buffer was added 400 μL of cold MeOH, followed by 100 μL of cold $CHCl_3$. The sample was vortexed after addition of each portion of organic solvent. The solution was diluted to 1 mL with 300 μL of cold dd-H_2O and then vortexed. The aqueous and organic layers were separated by centrifugation at 14,000 g for 2 min. The aqueous layer was removed by pipet. To the remaining $CHCl_3$ was added 400 μL of MeOH, and the solution was vortexed. The protein was pelleted by centrifugation at 14,000 g for 3 min. The organic solvent was removed by pipet and the residual solvent was allowed to evaporate at room temperature. The protein was resuspended in an aqueous 1% Triton-X solution before analysis.

Western blot protocol. Protein samples were run on a SDS-PAGE gel and then the contents of the gel were dry-transferred to nitrocellulose membranes using iBlot 2 Dry Blotting System (Novex, P0:7 min, 20-25 V). The blots were blocked overnight at 4 ^{o}C in 10 mL tris-buffered saline with 0.1% Tween-20 (TBST) containing bovine serum albumin (5% w/v). To the blot in blocking solution was added an anti-biotin antibody conjugated to horseradish peroxidase (#7075, cell signaling at 1:5000 dilution). The antibody was incubated with the blot for 30 min at room temperature. The blot was then washed with TBST (3 x 15 min) followed by detection using chemiluminescence using SuperSignal West Pico Chemiluminescent 98 Substrate (Pierce).

For the anti-nitrotyrosine antibody, a similar method was used: to the membrane was added anti-nitrotyrosine antibody (ab110282, abcam at 1:2000 dilution) in blocking solution. The antibody was incubated overnight at 4 ^{o}C. After washing with TBST, the membranes were incubated with a Goat anti-Mouse IgG H&L (ab97040, abcam at 1:50000 dilution) secondary antibody conjugated to horseradish peroxidase in blocking solution.

[1] Laemmli, U. K. *Nature* **1970**, *227*, 680-685.

5. Supporting Figures.

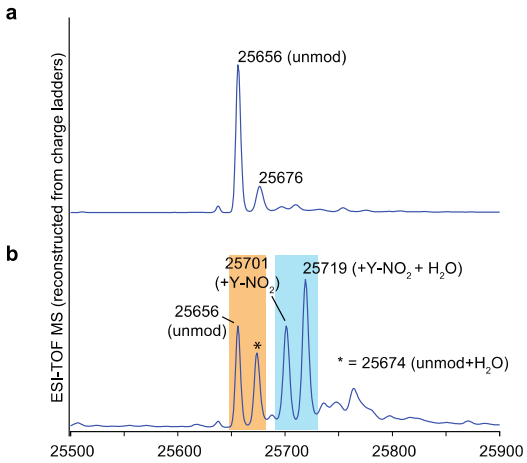

Fig. S1 ESI-TOF MS analysis of (a) unmodified and (b) tetranitromethane modified chymotrypsinogen (Y-NO_2 chymotrypsinogen). Deconvoluted charge ladders are shown. The percent modification was calculated by integration of the shaded regions: 37% unmod (orange), 63% Y-NO_2 chymotrypsinogen (blue).

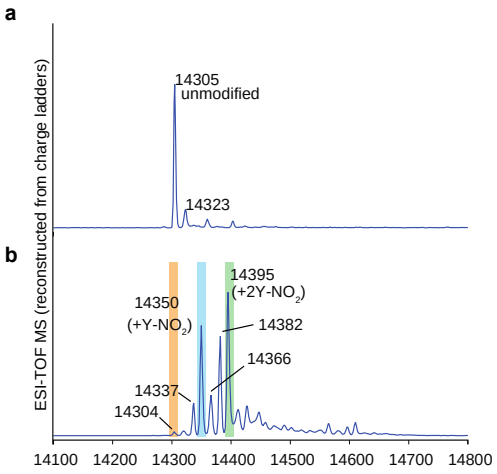

Fig. S2 ESI-TOF MS analysis of (a) unmodified and (b) tetranitromethane modified lysozyme (Y-NO_2 lysozyme). Deconvoluted charge ladders are shown. Both singly- and doubly-modified products were observed. The percent modification was calculated by integration of the shaded regions: 2% unmod (orange), 40% Y-NO_2 lysozyme (blue), and 58% 2(Y-NO_2) lysozyme (green) .

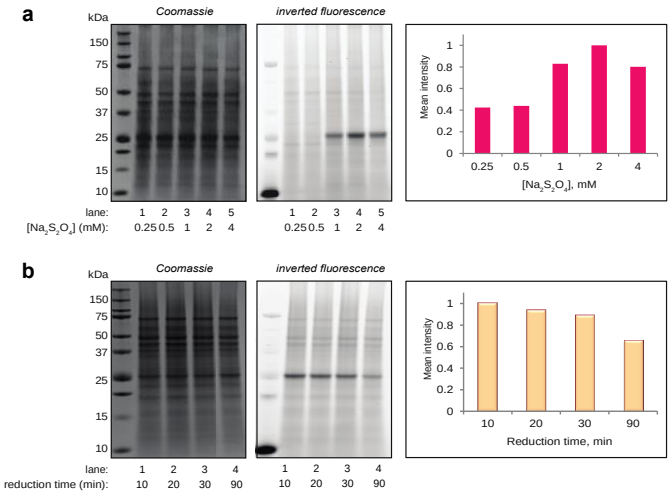

Fig. S3 Optimization of the dithionite-mediated Y-NO_2 reduction step in cell lysates using Y-NO_2 chymotrypsinogen as a model protein. (a) To determine the optimal number of dithionite equivalents, 100 µL reduction reactions were run with 0.8 µM protein and 20 µg of Ramos lysate for 20 min with varying concentrations of sodium dithionite (0.25-4 mM). After reduction, the reaction mixtures were treated with **2a** (200 µM) in the presence of ferricyanide (80 mM) in 100 mM pH 6 phosphate buffer for 2 h. (b) To determine the optimal reaction time, 100 µL reduction reactions were run with 0.4 µM protein in 20 µg of cell lysate with 1 mM sodium dithionite for 10-90 min. After reduction, the reaction mixtures were treated with **2a** (400 µM) in the presence of ferricyanide (80 mM) in 100 mM pH 6 phosphate buffer for 2 h. After purification, the reaction mixtures were analyzed by SDS-PAGE with fluorescence detection. The mean intensities of the modified protein bands were quantified using ImageJ, and these values were used to determine the optimal dithionite concentration.

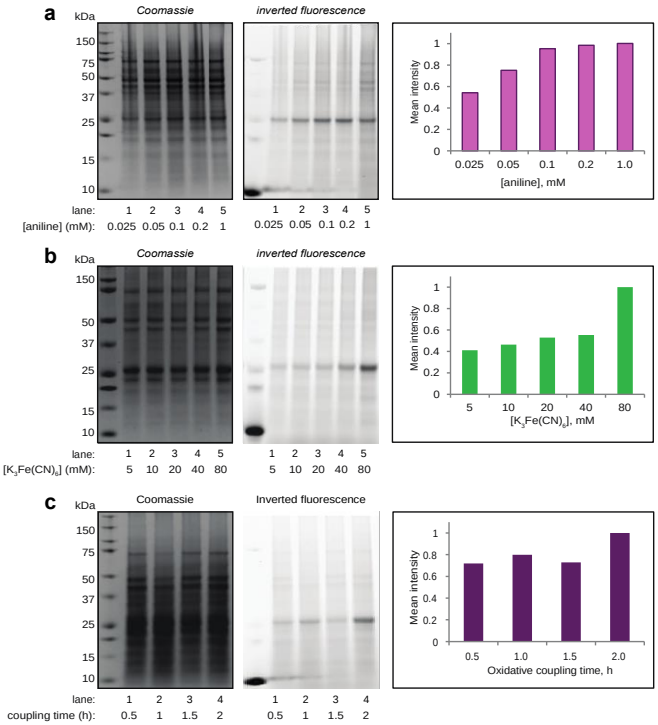

Fig. S4 Optimization of the oxidative coupling in cell lysates with Y-NO_2 chymotrypsinogen as a model protein. The equivalents of the aniline coupling partner (a), the concentration of oxidant (b), and the duration of oxidative coupling (c) were optimized separately. (a) To determine the optimal equivalents of aniline coupling partner, 100 µL reactions were run with 0.8 µM protein and 20 µg of Ramos cell lysate with 1 mM sodium dithionite for 20 min. After reduction, the reaction mixtures were treated with varying concentrations of **2a** (25-1000 µM) in the presence of ferricyanide (80 mM) in 100 mM pH 6 phosphate buffer for 2 h. (b) To optimize the oxidant concentration, 100 µL reactions were run with 0.8 µM protein and 20 µg of cell lysate with 2 mM sodium dithionite for 20 min. After reduction, the reaction mixtures were treated with varying concentrations of **2a** (200 µM) in the presence of ferricyanide (5-80 mM) in 100 mM pH 6 phosphate buffer for 2 h. (c) To optimize the oxidative coupling time, 100 µL reactions were run with 0.8 µM protein and 20 µg of cell lysate with 1 mM sodium dithionite for 20 min. After reduction, the reaction mixtures were treated with varying concentrations of **2a** (400 µM) in the presence of ferricyanide (80 mM) in 100 mM pH 6 phosphate buffer for 0.5-2 h. After purification, the reaction mixtures were analyzed by SDS-PAGE with fluorescence detection. The mean intensities of the modified protein bands were quantified using ImageJ, as shown in the graphs.

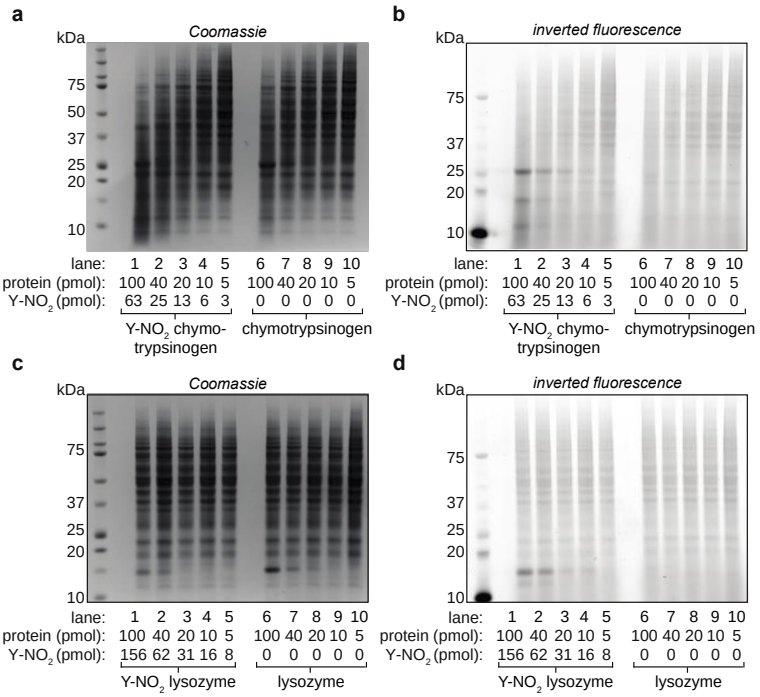

Fig. S5 Detection of Y-NO_2 containing proteins (Y-NO_2 chymotrypsinogen and Y-NO_2 lysozyme) in cell lysates (24 µg Ramos lysate loaded per lane). Portions of each protein were added to 72 µg of cell lysate in 100 µL of phosphate buffer, pH 6. The samples were reduced with 2 mM sodium dithionite for 20 min, and then treated with 200 µM **2a** and 80 mM ferricyanide for 2 h. After ultrafiltration, the reactions were analzed by SDS-PAGE with (a,c) Coomassie staining and (b,d) fluorescence detection (inverted image shown).

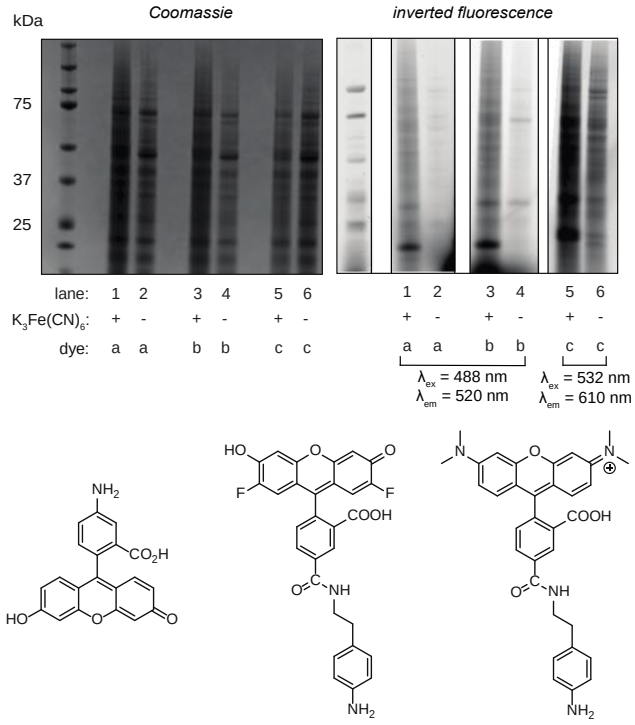

Fig. S6 Screen of alternative fluorescent aniline dyes. The efficiencies of three aniline dyes were compared by reacting a 100 µL portion of 1 µM MS2-T19(Y-NO_2) and 72 µg cell lysate with 2 mM sodium dithionite for 20 min, followed by the addition of 200 µM aniline dye either with (+) or without (–) 80 mM ferricyanide in 100 mM pH 6 phosphate buffer. The reaction mixtures were purified by ultrafiltration and analyzed by SDS-PAGE with fluorescence detection.

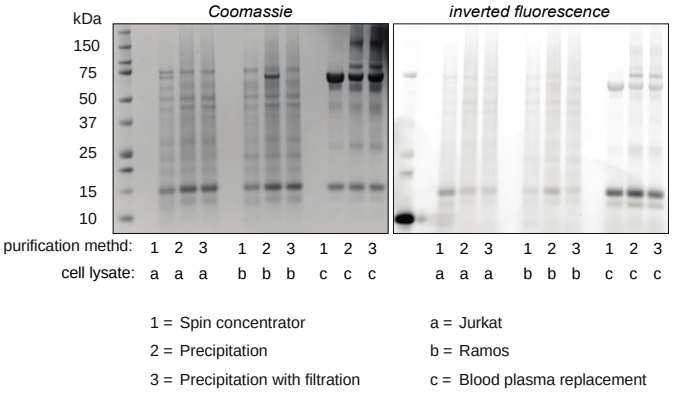

Fig. S7 Several methods for the purification of reactions performed in complex biological samples were tested. To compare the different purification methods the oxidative coupling based detection of Y-NO_2 lysozyme was performed in various biological mixtures including (a) Jurkat cell lysates, (b) Ramos cell lysates, and (c) a commercial blood plasma replacement. The detection reactions were performed with 10 µM Y-NO_2 lysozyme in 72 µg biological mixture with 2 mM sodium dithionite for 20 min and then 200 µM **2a** with 80 mM ferricyanide in 100 mM pH 6 phosphate buffer for 2 h. The reaction mixtures were purified by ultrafiltration (with a 3 kDa molecular weight cutoff spin concentrator), by two rounds of precipitation with MeOH/$CHCl_3$, or by two rounds of precipitation followed by ultrafiltration. The resulting samples were analyzed by SDS-PAGE with fluorescence detection.