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Thermodynamic Analysis of Protein-Ligand Binding Interactions in Complex Biological Mixtures using the Stability of Proteins from Rates of Oxidation (SPROX) Method

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Abstract

The detection and quantitation of protein-ligand binding interactions is critical in a number of different areas of biochemical research from fundamental studies of biological processes to drug discovery efforts. Described here is a protocol that can be used to identify the protein targets of biologically relevant ligands (e.g. drugs like tamoxifen or cyclosporin A) in complex protein mixtures such as cell lysates. The protocol utilizes quantitative, bottom-up, shotgun proteomics technologies (iTRAQ) with a covalent labeling technique, termed Stability of Proteins from Rates of Oxidation (SPROX). In SPROX, the thermodynamic properties of proteins and protein-ligand complexes are assessed using the hydrogen peroxide-mediated oxidation of methionine residues as a function of the chemical denaturant (e.g. guanidine Hydrochloride or urea) concentration. The proteome-wide SPROX experiments described here enable the ligand binding properties of hundreds of proteins to be simultaneously assayed in the context of complex biological samples. The proteomic capabilities of the protocol render it amenable to detection of both the on- and off-target effects of ligand binding.

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AUTHOR CONTRIBUTIONS

G.M.W., P.D.D., Y.X., E.C.S., M.A.G., D.T.T., and J.A. contributed to the development and optimization of the described SPROX protocol. E.C.S. collected and analyzed the SPROX data highlighted in this manuscript. E.C.S., M.A.G., D.T.T., J.A., and M.C.F. drafted the manuscript.

COMPETING FINANCIAL INTEREST STATEMENT

The authors have no competing financial interests.

Supporting information The Supporting Information contains a total of 5 files including: 2 Excel files and 3 tab delimited text files. The "Sample_Data_Set.xlsx" file contains a sample data set on which steps 36–43 were performed. The data set is from the NAD binding study described in reference 5. The three ".txt" files are the two Runcompare input files and one output file generated from the sample data set as described in 44–46. The "Matched_NAD_Binding.xlsx" file contains the sample data set on which steps 47–52 were performed.

Keywords

Protein-ligand; protein-drug; interaction; mass spectrometry; iTRAQ; shotgun proteomics; hydrogen peroxide; H₂O₂; methionine oxidation; denaturant; guanidine; urea; protein folding/unfolding

INTRODUCTION

Stability of Proteins from Rates of Oxidation (SPROX) is a covalent labeling- and mass spectrometry-based method for evaluating the solution phase thermodynamic properties of proteins and protein-ligand complexes. SPROX utilizes the denaturant dependence of the hydrogen peroxide (H₂O₂) mediated oxidation of methionine side chains in proteins (Fig. 1) to measure the folding free energy of proteins and the K_d values of protein-ligand complexes.¹ The SPROX protocol and data analysis methods are similar to those in an amide H/D exchange- and mass spectrometry-based method, termed Stability of Unpurified Proteins from Rates of H/D Exchange (SUPREX), for making protein folding and stability measurements.^{2,3} Both techniques utilize a mass spectrometry readout to generate thermodynamic information about the chemical denaturant-induced equilibrium folding/unfolding properties of proteins in solution. The primary difference between SPROX and SUPREX is that SPROX utilizes the denaturant dependence of the oxidation rates of globally protected methionine residues in a protein (Fig. 1) to generate such thermodynamic information, whereas SUPREX utilizes the denaturant dependence of the H/D exchange rates of globally protected amide groups in proteins to determine the thermodynamic properties of a protein's folding/unfolding reaction.

The SPROX technique was originally developed with an intact protein readout using MALDI and/or ESI mass spectrometry.¹ More recently, the technique has been interfaced with bottom-up LC-MS-based proteomic platforms to facilitate the detection and quantitation of protein-ligand binding interactions on the proteomic scale.^{4,5} The protocol described in this work is focused on such proteomic applications of SPROX. The described protocol has been used in several proof-of-principle experiments in which the proteins in a yeast cell lysate were screened for binding to several small molecule drugs and an enzyme cofactor.^{4,5} These studies demonstrate the utility of the SPROX methodology for detecting both the direct and indirect effects of protein-ligand binding interactions in complex biological mixtures such as cell lysates.

Most large-scale and high-throughput protein-ligand binding assays, such as the yeast two-hybrid assay⁶ and affinity capture techniques,⁷ detect direct binding events. Indirect binding events are more difficult to detect. Such indirect binding events can arise when the direct binding interaction between one protein and a target ligand precludes and/or induces the binding of the first protein to a second protein. Indirect binding interactions go undetected in the yeast two-hybrid assay, and affinity capture techniques are only sensitive to indirect binding interactions in which a direct binding event recruits additional proteins to the protein complex. More recently, a protease protection assay has been developed for the detection of protein-ligand binding interactions on the proteomic scale.^{8,9} While the protease protection assay is amenable to detection of both direct and indirect binding interactions in complex protein mixtures, it does not enable quantitative measurements of protein-ligand binding affinities. The ability to detect and quantify both direct and indirect effects of protein-ligand binding interactions in complex mixtures is a unique capability of the SPROX methodology.

Experimental design

The methodology presented here involves the use of SPROX with a quantitative, bottom-up, shotgun proteomics platform utilizing isobaric mass tags. The described methodology is designed to identify the protein targets of a specified ligand. The methodology has so far been used to identify protein binding interactions of small molecule ligands,^{4,5} and the described protocol is focused on such applications. The protocol could also be used to study the protein binding interactions of other ligand classes (e.g., DNA, RNA, and other proteins). The experimental workflow, which is outlined in Fig. 2, can be divided into four main parts including: I) protein sample preparation, II) SPROX analysis, III) quantitative, bottom-up, shotgun proteomics sample preparation and analysis, and IV) data analysis.

Protein sample preparation—Outlined in this protocol are the steps involved in the SPROX analysis of proteins in a yeast cell lysate. In theory, the same protocol could also be used to analyze proteins in other types of cells and biological tissues. However, an important prerequisite for the method is that the proteins in the biological sample under study be obtained in solution conditions that allow proteins to adopt their native three-dimensional structures. The protocol requires a total of at least 2–3 mg of protein dissolved in ~400 μ L of buffer. Half of the protein sample is used for the (–) ligand sample, and the other half of the protein sample is used to generate the (+) ligand sample, which typically contains micromole quantities of ligand.

The protocol should be amenable to the analysis of wide range of different protein-ligands. However, ligands that are not stable in H_2O_2 are likely to be problematic as are ligands with limited solubility in aqueous buffer systems. The technique requires the preparation of (+) ligand samples in which the test ligand is present at concentrations of at least several hundred micromolar. Even higher ligand concentrations (e.g., >1 mM) are required in the (+) ligand samples to effectively assay the protein binding properties of weak binding ligands (e.g., $K_d > 100 \mu$ M) (see discussion in **Anticipated results**).

SPROX analysis—The second part of the experimental workflow outlined in Fig. 2 is the SPROX analysis, in which H_2O_2 is used to selectively oxidize methionine residues in the protein sample as a function of the chemical denaturant concentration. The reaction time and H_2O_2 concentration are tuned such that the pseudo-first order oxidation reaction of an unprotected methionine residue will proceed for ~3–5 half-lives. The oxidation reaction conditions are relatively mild and generate minimal oxidation at other amino acid residues (e.g., Cys and Trp). After the oxidation reactions in each denaturant-containing buffer proceed for the same specified time, they are quenched with the addition of free methionine.

Quantitative, bottom-up shotgun proteomics sample preparation and analysis

—The protein material in each denaturant-containing buffer is submitted to a quantitative, bottom-up shotgun proteomics analysis in which the unoxidized and oxidized methionine-containing peptides are quantified as a function of the SPROX buffer denaturant concentration. We have explored the use of several different experimental strategies to quantify the relative abundance of the methionine-containing peptides generated in a proteome-wide SPROX experiment.^{4,5,10,11} The isobaric mass tagging strategy described here, which relies on the attachment of isobaric tags to the N-termini and lysine residues of tryptic peptides, has been the most widely used approach in our work, to date. The described protocol focuses on use of the iTRAQ 8-plex, although the TMT 6-plex can also be used.⁴ The (–) and (+) ligand samples generated in SPROX (see Step III.a in Fig. 2) can be directly analyzed in an LC-MS/MS analysis or subjected to additional fractionation protocols such as a methionine-containing peptide selection strategy prior to LC-MS/MS analysis in order to enhance the peptide and protein coverage.⁵

The LC-MS/MS system described in this protocol is an Agilent Quadrupole-Time of Flight Mass Spectrometer System (Model 6520) equipped with a ChipCube HPLC interface. However, any LC-MS/MS spectrometer system equipped with the requisite data acquisition and analysis capabilities to perform iTRAQ and/or TMT analyses could be utilized. As in any bottom-up, shotgun proteomics analysis, the speed and sensitivity of the mass spectrometry system will determine the peptide and protein coverage that can be attained in the SPROX experiment.

Data analysis—The iTRAQ reporter ion intensities obtained from the methionine-containing peptides identified in the proteomics experiment are used to generate chemical denaturation data for the proteins to which they map. The protein-ligand binding assay described here relies on a differential analysis of the chemical denaturation data obtained for a given methionine-containing peptide when the protein sample is analyzed both in the presence and in the absence of the ligand. Proteins that interact with the ligand either directly or indirectly are those identified with methionine-containing peptides that display transition midpoint (i.e., $C^{1/2}_{\text{SPROX}}$ value) shifts in the presence of ligand (Fig. 2). A sample data set from a typical ligand binding experiment is provided in the supporting information.

MATERIALS

REAGENTS

Part I – Sample Preparation

Yeast ORF Host Strain (Y258) (Open Biosystems)

Peptone No. 4 (VWR cat. no. 89229-568)

Yeast Extract Powder (VWR cat. no. 90000-026)

Dextrose, Anhydrous (VWR cat. no. BDH0230-2.5KG)

Protease inhibitors:

AEBSF (Thermo Scientific Pierce cat. no. 78431)

Bestatin (Thermo Scientific Pierce cat. no. 78433)

E-64 (Thermo Scientific Pierce cat. no. 78434)

Leupeptin (Thermo Scientific Pierce cat. no. 78435)

Pepstatin A (Thermo Scientific Pierce cat. no. 78436)

Bradford Reagent (Thermo Scientific cat. no. 23238)

Part II – SPROX Analysis

Guanidine Hydrochloride (GdmCl) (EMD cat. no. 5010-OP)

Urea (Sigma-Aldrich cat. no. U6504)

Dimethylsulfoxide (DMSO) (Acros cat. no. 41488-5000)

H₂O₂ (30% w/w) (Sigma-Aldrich cat. no. 21,6763)

CRITICAL Store in the dark at 4°C when not in use to minimize decomposition.

Trichloroacetic acid (TCA) (Sigma-Aldrich cat. no. T6399)

Ethanol (VWR cat. no. MK701816)

L-Methionine (Sigma-Aldrich cat. no. M9625)

Part III – Proteomic Sample Preparation and Analysis

Triethylammonium bicarbonate (TEAB) (Sigma-Aldrich cat. no. T7408)

Sodium dodecyl sulfate (SDS) (Sigma-Aldrich cat. no. L4390)

Bradford Reagent (Thermo Scientific cat. no. 23238)

Tris (2-carboxyethyl) phosphine hydrochloride (TCEP HCl) (Thermo Scientific cat. no. 20490)

S-methyl methanethiosulfonate (MTS) (Sigma-Aldrich cat. no. 208795)

Porcine pancreas trypsin, proteomics grade (Sigma-Aldrich cat. no. T6567)

Isopropyl alcohol (VWR cat. no. BDH1133-4LP)

iTRAQ (isobaric mass Tags for Relative and Absolute Quantitation) Reagent-8plex (AB Sciex cat. no. 4390733, at least 1 unit of each reagent is required)

MacroSpin™ Columns (Silica C18, 50–450 µL loading 30–300 µg capacity) (The Nest Group, Inc. cat. no. SMM-SS18V)

*Pf*³™ Methionine Reagent Kit (Part #:X7201, The Nest Group, Inc.)

Acetonitrile (VWR cat. no. BJ015-4)

Trifluoroacetic acid (TFA) (Sigma-Aldrich cat. no. T6508)

Methanol (VWR cat. no. BDH1135-4LG)

Acetic Acid (Mallinckrodt cat. no. V155-06)

β-mercaptoethanol (Sigma-Aldrich cat. no. M6250)

Formic Acid (Thermo Scientific cat. no. 28905)

Water (VWR cat. no. BJ365-4)

EQUIPMENT

Incubator (e.g., Excella E24 Incubator Shaker from New Brunswick Scientific)

Bench top centrifuge with 14,000 × g speed capacity and refrigeration options (e.g., Eppendorf Model 5430R)

Disruptor Genie (Scientific Industries cat. no. SI-D238)

Acid-washed glass beads (425–600 µm in diameter) (Sigma-Aldrich cat. no. G8772)

Ice bucket with ice

UV-vis spectrometer with 595 and 600 nm filter capabilities (e.g., Hewlett-Packard 8452A)

1.7 mL and 2.0 mL microcentrifuge tubes (Genesee scientific cat. no. 22-281 and 22-283)

0.22 µm cellulose acetate 1 L bottle top filter (Corning Inc. cat. no. 430015)

1 L storage bottle (Corning Inc. cat. no. 430518)

0.2 µm filters (Fisher Scientific cat. no. 09-719C)

Refractometer (Bausch & Lomb Optical Co.)

SpeedVac Concentrator (Thermo Scientific)

Vortexer with foam attachment to hold 2 mL microcentrifuge tubes Scientific Industries cat. no. SI-0236)

Centrifuge Sorvall DuPont with GSA rotor capabilities

Agilent Quadrupole - Time of Flight Mass Spectrometer (model # 6520) equipped with a Chip Cube Interface (G4240A), Loading Pump (G1379A), Analytical Pump (G2226A), and Autosampler (G1377A)

Nano HPLC Chip packed with 5 μ m Zorbax 300SB - C18 packing material. Use large capacity chip with a 150 mm $\times$ 75 μ m HPLC column and 160 nL trapping column.

Spectrum Mill Software (Version B.04)

Runcompare Software (Version 1.0) – (Freely available from the corresponding author upon request)

Microsoft Excel Software (Microsoft Corporation)

REAGENT SET UP

YPD media—Combine 10 g of yeast extract, 20 g of peptone and 20 g of dextrose in a 2 L flask and add water to bring the final volume to 1 L. Stir at room temperature until the media is dissolved (approximately 1–2 hr). Filter the media through a 0.22 μ m cellulose acetate 1 L filter into a sterile 1 L container.

Yeast cell culture and protein pellet preparation—In a test tube, inoculate 10 mL YPD media containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose with a colony of yeast ORF host strain (Y258). Incubate this yeast cell culture overnight at 30°C and 200 rpm. In a 2 L flask combine 2 mL of the overnight culture with the remaining 0.990 L of YPD media. Let the new yeast cell culture grow at 30 °C and 200 rpm until the optical density at 600 nm measures between 0.8 and 1.2 absorbance units (typically after about 6–8 hr). Centrifuge 250 mL portions of the yeast cell culture in 4 centrifuge tubes at 2500 rpm for 10 min at 4 °C. Decant the supernatant in each tube and resuspend each yeast cell pellet in 1 mL of distilled water. Pipette 1 mL aliquots of the cell suspensions into 2 mL plastic microcentrifuge tubes. Centrifuge the cell suspensions in the 2 mL plastic microcentrifuge tubes at 5000 $\times$ g for 10 min at 4 °C and gently remove supernatant. If any of the supernatants are opaque, resuspend the cell pellet in 1 mL of distilled water, centrifuge at 5000 $\times$ g for 10 min at 4 °C, and gently discard the supernatant. Repeat the re-suspension and centrifugation cycle until each supernatant is transparent. Store cell pellets at –20 °C or colder and thaw on ice prior to use. Cell pellets can be stored at –20 °C or colder for several months.

Lysis buffer—Prepare the following stock solutions: 835 mM (200 mg/ml) AEBSF in water, 16 mM (5 mg/ml) Bestatin in methanol, 56 mM (20 mg/ml) E-64 in 50/50 (v/v) ethanol/water, 2.1 mM (1 mg/ml) Leupeptin in water, and 1.5 mM (1 mg/ml) Pepstatin in methanol. Combine 138 μ L, 191 μ L, 5.4 μ L, 62 μ L, and 24 μ L of the Pepstatin A, Leupeptin, E-64, Bestatin, and AEBSF stock solutions (respectively) to make a 20X protease cocktail inhibitor mix in which the Pepstatin A, Leupeptin, E-64, Bestatin, and AEBSF concentrations are 0.2 mM, 0.4 mM, 0.3 mM, 1 mM, and 20 mM (respectively). Combine 25 μ L of the 20X protease cocktail with 500 μ L of 20 mM phosphate buffer, pH 7.4 and mix thoroughly.

Denaturant-containing buffers—Make a 0 M and an 8 M stock solution of GdmCl in 20 mM sodium phosphate buffer, pH 7.4. Filter the solutions through a 0.2 μ m membrane. Combine different ratios of the 0 and 8 M stock solutions to make denaturant-containing

buffers with the following [GdmCl]: 1, 2, 2.6, 3.4, 4.0, 5, 6, and 7 M. Prepare at least 60 μ l of each [GdmCl] containing-buffer. Adjust the pH to 7.4. Measure the refractive index of each GdmCl-containing buffer and determine the exact [GdmCl] of each buffer using equation 1 from reference.¹²

$$[\text{GdmCl}] = 57.147(\text{RI}) + 38.68(\text{RI})^2 - 91.6(\text{RI})^3$$

In equation 1, RI is the refractive index determined by subtracting the refractive index measured for the 0 M buffer from the refractive index measured for the denaturant-containing buffer. Urea-containing buffers can be prepared in a similar manner except stock solutions in the 20 mM sodium phosphate buffer, pH 7.4, can be as concentrated as 9.5 M, and the following series of [urea] should be used: 0, 1.3, 2.7, 3.6, 4.4, 5.3, 6.5, and 8.0 M. The exact [urea] in each buffer can be determined using equation 2 from reference.¹³

$$[\text{Urea}] = 117.66(\text{RI}) + 29.753(\text{RI})^2 + 185.56(\text{RI})^3$$

GdmCl solutions are stable for months at room temperature. Urea solution should be used within 1 week, as urea can decompose to form cyanate and ammonium ions that can chemically modify the amino groups of proteins.¹³

H₂O₂—Remove a 100 μ L aliquot from the 30% (w/w) H₂O₂ solution, and allow it to reach room temperature shortly before use. H₂O₂ solutions can decompose over time and should be stored in the dark at 4 °C when not in use to minimize decomposition. The H₂O₂ solution used in this protocol should be 30% (w/w), and it is best to check that the solution is not decomposed by measuring the exact concentration of the H₂O₂ solution just prior to use. The exact concentration of the H₂O₂ solution can be determined by measuring the absorbance at 240 nm and using a molar extinction coefficient of 43.6 M⁻¹ cm⁻¹ to calculate the H₂O₂ concentration according to Beer's Law.¹⁴ A 30% (w/w) solution of H₂O₂ is 9.8 M and at a 1:1000 dilution should have an absorbance at 240 nm of 0.427 absorbance units using a 1 cm cuvette.

TCA—Prepare 5 mL of a 100% (w/v) aqueous solution of TCA immediately before use in step 16|.

LC-MS/MS mobile phase—Prepare Buffer A (0.1% formic acid in water) and Buffer B (0.1% formic acid in acetonitrile) using solvents that are proteomic grade or better. Never use detergent to clean mobile phase reservoirs.

Ligand stock solution—Prepare a concentrated stock solution of ligand in 20 mM sodium phosphate buffer, pH 7.4. The ligand concentration should be as high as possible in the stock solution, typically between 1 and 10 mM, in order to maximize the transition midpoint shifts of target proteins (see discussion in **Anticipated Results**). The ligand stock solution can also be prepared using organic solvents such as DMSO or ethanol if the ligand has limited solubility in aqueous buffers. If DMSO or other organic solvents are used to prepare the stock solution, the ligand concentration should not be so high that it will not stay solubilized in the (+) ligand samples prepared in steps 9| and 10| below.

EQUIPMENT SET UP

LC-MS/MS mass spectrometer system—The LC-MS/MS mass spectrometer system should be set up with the appropriate data acquisition methods to maximize the peptide and protein coverage in a bottom-up shotgun proteomics experiment and to generate iTRAQ reporter ion intensities that are sufficient for accurate quantitation. The gradient elution profile used on the LC-MS/MS system employed here is: 2% Buffer B over 5 minutes, 2% to 15% Buffer B over 2.5 min, 15% to 45% Buffer B over 78 min, and then 45% to 100% Buffer B over 10 minutes. The LC-MS/MS system is equilibrated with 2% Buffer B for at least 30 min before sample analysis. The HPLC flow rate is 0.4 μ L/min. The capillary voltage is 1850–1900 V. The drying gas is 350 °C at a flow rate of 6 L/min. The skimmer and fragmentor are set to 65 V and 175 V, respectively. The inclusion window width for precursor ions is 4 m/z units. The scan rate is 3 scans per second in the precursor ion mass spectra and 2 scans per second in the product ion mass spectra, and 4 precursor ions are selected for fragmentation per cycle. The collision energy is 3.9 V/100 m/z with a 2.9 V offset.

PROCEDURE

Protein sample preparation – TIMING - 1 hr

- 1| Thaw a yeast cell pellet in the 2 mL microcentrifuge tube on ice and estimate the volume of pellet using the volume marks on the tube. Pipette 500 μ L of pre-chilled lysis buffer into the microcentrifuge tube and mix the contents by pipetting the mixture up and down several times and/or by vortexing.

CRITICAL STEP SPROX requires the protein sample to be in native solution conditions; therefore, cell lysis buffers containing denaturing agents such as detergents should be avoided. Some buffers like HEPES and MOPS also interfere with the oxidation reaction in SPROX and should be avoided. Aprotinin should not be included in the inhibitor mix since it is a protein-based protease inhibitor that can precipitate with the protein sample in step 18| and inhibit the trypsin digestion in step 28|.

- 2| Add glass beads to the resuspended cells in the microcentrifuge tube. The volume of glass beads added should be approximately half the volume of the cell pellet noted in step 1|.
- 3| Agitate the microcentrifuge tube containing the resuspended cells and glass beads using the Disruptor Genie for 20 s and place the microcentrifuge tube on ice for 1 min.
- 4| Repeat step 3| 14 times.
- 5| Centrifuge the microcentrifuge tube containing the lysed cells at 14,000 $\times$ g for 10 min at 4°C.
- 6| Transfer the supernatant into a new chilled 1.7 mL microcentrifuge tube. Be careful not to transfer any of the cellular debris and/or glass beads.

?TROUBLESHOOTING

- 7| Measure the protein concentration of the lysate using a Bradford Assay to ensure the protein concentration in the lysate is between 6 and 12 mg/mL. Continue immediately to step 8.

SPROX analysis – TIMING - 4 hr

- 8| Divide the lysate into two 180 μ L portions.

- 9| Add 20 μL of the ligand stock solution (prepared in an aqueous buffer or organic solvent such as DMSO depending on the solubility of the ligand) to one portion of the lysate to generate the (+) ligand sample of the lysate, and add 20 μL of the aqueous buffer (or organic solvent) used for the ligand stock solution to the other portion of the lysate to generate the (–) ligand sample of the lysate (see Step I in Fig. 2).
- 10| Equilibrate the (–) and (+) ligand samples of the lysate at room temperature for 30 min.

CRITICAL STEP Ensure that the material in (+) and (–) ligand samples is solubilized.

!TROUBLESHOOTING

- 11| Pipette 25 μL aliquots of each denaturant-containing buffer solution into two series of eight microcentrifuge tubes such that each series of buffer solutions has the following eight concentrations of GdmCl: 1.0, 2.0, 2.6, 3.4, 4.0, 5.0, 6.0, and 7.0 M. If urea is used, pipette 75 μL aliquots of buffer solution with the following eight concentrations: 0, 1.3, 2.7, 3.6, 4.4, 5.3, 6.5, and 8.0 M.

CRITICAL STEP If potential protein-ligand binding interactions are expected to be stabilized by electrostatic interactions (e.g., protein-DNA interactions, protein-RNA interactions, protein-nucleoside interactions, etc.) urea should be used as the chemical denaturant instead of GdmCl.

- 12| Pipette 20 μL aliquots of the (–) ligand sample of the lysate into one series of the eight denaturant-containing buffers prepared in step 11|, and pipette 20 μL aliquots of the (+) ligand sample of the lysate into the other series of eight denaturant-containing buffers prepared in step 11|.
- 13| Equilibrate the 16 protein samples prepared in step 12| for 30 min.
- 14| Add 5 μL of 30% (w/w) H_2O_2 to each of the 16 protein samples, gently drawing the sample up and down the pipette tip several times to ensure that the H_2O_2 is adequately mixed into the solution.
- 15| After 3 min (or 6 min if urea is used as the denaturant), add 1 mL of a 300 mM L-methionine solution to each of the 16 protein samples in order to quench the oxidation reactions.

CRITICAL STEP If other reaction times and H_2O_2 concentrations are used, ensure that the amount of L-methionine used to quench the reaction is in at least a 5-fold molar excess of H_2O_2 .

- 16| Add 200 μL TCA (100%) to each of the 16 protein samples such that the final TCA concentration in each sample is 20% (w/v)

CRITICAL STEP Methionine will precipitate in acetone. DO NOT use acetone for the protein precipitation.

! CAUTION TCA is a highly corrosive acid; handle with appropriate gloves and personal protective equipment.

- 17| Vortex each of the 16 protein samples for 15 s and incubate on ice for at least 2 hr.

PAUSE POINT Samples can be stored overnight at 4°C.

- 18| Centrifuge each of the 16 protein samples at $8,000 \times g$ for 30 min at 4°C to pellet the precipitated proteins. Discard supernatants in each of the 16 protein samples in an appropriate waste container.

?TROUBLESHOOTING

CRITICAL STEP Gently decant the supernatant; do not disturb the pellets.

- 19| Add 300 μL of ice-cold ethanol to the protein pellets in each of the 16 protein samples, gently invert each tube, and remove the supernatant. Be careful not to dislodge the protein pellet.
- 20| Repeat step 19| twice.

PAUSE POINT Washed protein pellets can be stored at -20°C for several weeks before proceeding to the proteomics analysis.

Quantitative, bottom-up proteomics analysis – TIMING - 36–48 hrs

- 21| Allow residual ethanol to evaporate from protein pellets in the 16 microcentrifuge tubes by placing opened tubes in a fume hood for approximately 20 min or using a SpeedVac concentrator for 10 min.
- 22| Add 30 μL of 0.5 M TEAB buffer, pH 8.5, to each of the 16 protein pellets.
- 23| Add 1.5 μL of 2% (w/v) SDS to each protein sample (final concentration of 0.1% (w/v) SDS).
- 24| Vortex, heat at 60°C , and sonicate samples for 10 min each in order to dissolve the protein pellets.
- 25| Remove 5 μL from each sample for use in a Bradford Assay to determine protein concentration in each of the 16 samples. The protein concentration in each sample should be between 1 and 3 mg/mL.

?TROUBLESHOOTING

- 26| Add 2.5 μL of 50 mM TCEP to each sample (final concentration of 5 mM) and incubate at 60°C for 1 hr with shaking to reduce disulfide bonds.

CRITICAL STEP Do not use dithiothreitol (DTT) to reduce protein samples. DTT is not compatible with iTRAQ labeling and methionine enrichment.

- 27| Add 1.5 μL of 200 mM MMTS dissolved in isopropanol to each protein sample (such that the final concentration in each protein sample is 10 mM) and incubate at room temperature for 10 min to alkylate cysteine residues.
- 28| To each protein sample add a volume of 1 mg/mL porcine pancreas trypsin, typically 1.0 – 2.5 μL depending on protein concentration, to achieve a trypsin:protein ratio between 1:20 and 1:100. Incubate samples at 37°C with shaking overnight (12–16 hrs).
- 29| Optional: Check for peptides using a MALDI-TOF MS analysis to verify that the trypsin digestion was successful. Combine 1 μL of digested sample with 9 μL α -cyano-4-hydroxy-cinnamic acid in 50% acetonitrile/0.1% TFA in H_2O (v/v). Spot 2 μL on MALDI sample plate, allow the solvent to evaporate, and collect MALDI mass spectra.

?TROUBLESHOOTING

PAUSE POINT Digested protein samples can be stored at -20°C for a few weeks before proceeding to the iTRAQ labeling.

- 30| Allow iTRAQ reagents to reach room temperature. Vortex and centrifuge at $1000 \times g$ for 30 s.
- 31| Dissolve 1.0 unit of each iTRAQ tag from the iTRAQ 8-plex in 100 μL isopropyl alcohol.
- 32| Label the protein samples in each set of 8 denaturant-containing SPROX buffers with a different iTRAQ tag by combining the digested protein sample with 50 μL (0.5 units) of the corresponding iTRAQ tag (see Fig. 2) prepared in 31|. Let samples react for 2 hrs at room temperature.

CRITICAL STEP Check the pH of each sample using 0.5 μL of sample and pH paper to ensure the pH is 7.5 or greater. A pH of 7.5 or greater is required for optimal iTRAQ labeling efficiency. If the pH is less than 7.5, add up to 5 μL of 0.5 M TEAB buffer until the pH is between 7.5 to 8.5. The concentration of organic solvent in each sample must be at least 60% (v/v).

- 33| Combine the eight different iTRAQ labeled samples from the (–) ligand SPROX analysis into one microcentrifuge tube, and combine the eight different iTRAQ labeled samples from the (+) ligand SPROX analysis into another microcentrifuge tube (see Fig. 2).

PAUSE POINT Samples can be stored at $-20\text{ }^{\circ}\text{C}$ or colder for at least several weeks.

- 34| Prepare the (–) and (+) ligand SPROX samples from step 33| for LC-MS/MS analysis using the C18 tips (**Box 1**) or using the $P^3\text{TM}$ Met resin and silica C18 media (**Box 2**). Use of the $P^3\text{TM}$ Met resin can significantly increase the peptide and protein coverage observed in the experiment (see **Anticipated Results**).

CRITICAL STEP Make sure to remove any salts and detergents from the sample by performing step 34| before LC-MS/MS analysis.

- 35| Perform at least three replicate LC-MS/MS analyses of the (–) and (+) ligand samples prepared in step 34|. Load approximately 1 μg – 10 μg of total peptide on column, based on total protein concentration determined in step 25|. If the methionine enrichment protocol is employed (**Box 2**), it is still necessary to perform at least one LC-MS/MS analysis of the (–) and (+) ligand samples that were prepared using the C18 Clean-Up (**Box 1**) but were not enriched for methionine-containing peptides using the $P^3\text{-Met}$ resin.

?TROUBLESHOOTING

Data analysis – TIMING - 5 hrs—A sample data set which has been analyzed according to steps 36| –52| is included in the Supporting Information.

- 36| Search the LC-MS/MS files generated in step 35| using appropriate software such as Spectrum Mill and export the following search results into Excel: Filename, peptide sequence, identification score, modification sites (e.g., oxidized methionine, iTRAQ label, etc.), retention time, protein accession number and/or name, and the 8 iTRAQ reporter ion intensities (without any applied normalization). The oxidation reaction conditions in SPROX (see steps 14 – 15|) are tuned such that the thioether group in the side chain of methionine residues in proteins is the primary site of oxidation in SPROX. Oxidation at other amino acid side chains that are susceptible to oxidation (e.g., Cys, and Trp) is generally not observed in the SPROX experiment because the oxidation reaction rates associated with these other amino acid side chains is much slower than that of the methionine side chain. In order to ensure that there are no

significant oxidation products involving other amino acid residues, the LC-MS/MS data searches can include potential oxidation sites on various other amino acids (e.g., Cys and Trp).

7TROUBLESHOOTING

CRITICAL STEP The LC-MS/MS data searches for the samples prepared using the Pf^{37M} -Met resin should require that the identified peptides contain a methionine residue. Other important search parameters for all the LC-MS/MS data include fixed modifications of MMTS on cysteine, iTRAQ 8-plex on N-terminus and lysine, and variable modification of oxygen on methionine.

- 37|** Sum and average all the iTRAQ reporter ion intensities for each peptide entry in the Excel file containing the LC-MS/MS search results.
- 38|** For each peptide entry in the Excel file, divide each of the eight reporter ion intensities by the average reporter ion intensity for that peptide to generate a set of so-called N1-normalized iTRAQ reporter ion intensities for each peptide. This N1-normalization procedure accounts for reporter ion intensity differences that are typically observed from peptide to peptide.
- 39|** Filter the peptides in the Excel file to include peptide entries from the (–) ligand samples with high summed iTRAQ intensity values (typically summed values >1000) and sequences identified with high confidence (e.g., FDR <1%) that do not contain a methionine residue.
- 40|** Average all the N1-normalized values obtained for each iTRAQ reporter ion in **39|**. The average N1-normalized values obtained for each iTRAQ reporter ion will be used as the N2-normalization factors for the second normalization in **42|**. It is also important to check the distribution of the N1-normalized values for each iTRAQ reporter ion to ensure that it is centered close to the average and approximates a normal distribution with a coefficient of variation of ~30%. See Fig. 3 for typical result. In theory, the N1-normalized values from all the non-methionine-containing peptides and the resulting N2-normalization factors for each of the iTRAQ reporter ions should be close to 1.0. In practice, the N1-normalized values and resulting N2-normalization factor for a specific iTRAQ reporter ion can be shifted away from 1 due to differential amounts of protein being precipitated and redissolved in steps **16 – 20|** and/or to differential yields of iTRAQ-labeled peptide products in **32|**. N2-normalization factors are typically between 0.8 and 1.2.

7TROUBLESHOOTING

- 41|** Repeat steps **39|** and **40|** for peptides identified in the (+) ligand sample to generate N2-normalization factors for each iTRAQ tag in the (+) ligand sample.
- 42|** Filter the data in the Excel file containing all the data from the LC-MS/MS search results to include peptide entries in the (–) ligand sample with large summed iTRAQ reporter ion intensities (typically sums >1000) that also contain a methionine residue. Divide the N1-normalized values for each of these methionine-containing peptides by the appropriate N2-normalization factor that was determined in step **40|** in order to generate a set of N2-normalized iTRAQ reporter ion intensities for each methionine-containing peptide. This N2-normalization procedure accounts for systematic errors in the experiment that might impact the relative iTRAQ reporter ion intensities observed for a given methionine-containing peptide (e.g., differential amounts of protein being

precipitated and redissolved in steps **16 – 20** and/or to differential yields of iTRAQ-labeled peptide products in **32**.

7TROUBLESHOOTING

- 43** Repeat step **42** using the LC-MS/MS data generated on the (+) ligand sample.
- 44** Make a new Excel spreadsheet containing the following data in consecutive columns: the identified peptide sequences in the (–) ligand sample that contain a methionine residue and have summed iTRAQ reporter ion intensities >1000, the name of the protein to which each peptide maps, the identification score for each peptide identification, the retention time of the peptide, and the eight N2-normalized iTRAQ reporter ion intensities values determined in **42**. Be sure that the oxidized methionine residues in the peptide sequences are clearly distinguished from unoxidized methionine residues (i.e., use m(ox) and M, not m and M), and that all other one letter amino acid abbreviations are capitalized. Some software programs, such as Spectrum Mill, indicate the presence of specific amino acid modification with lower cased letters (e.g., “q” for a deaminated glutamine). All such lower cased letters should be replaced with capital letters. Export the data from this new Excel spreadsheet into a tab delimited text file (i.e., a “*.txt” file).
- 45** Repeat step **44** using the (+) ligand sample data.
- 46** Use Runcompare to average the N2-normalized data generated from replicate product ion mass spectra collected on the same peptide and to determine the methionine-containing peptides that are common to both the (–) and (+) ligand samples. The average retention time and score for each peptide replicate is also included in the Runcompare output files.
- 47** Open the newly created matched file in Excel and examine the distribution of the N2-normalized values for the iTRAQ tags corresponding to the low and high denaturant concentration for the unoxidized methionine-containing peptides that are common to both the (–) and (+) ligand samples. The distribution of N2-normalized values for the tag corresponding to the high denaturant concentration is typically centered around 0.5 and the distribution of N2-normalized iTRAQ values for the tag corresponding to the lowest denaturant concentration is typically centered around 1.5. See Fig. 4a for a typical result. Where the distributions cross (typically ~1) is the N2-normalized value expected at the transition midpoint of a peptide's chemical denaturation data set (Fig. 4b).

7TROUBLESHOOTING

- 48** Repeat step **47** for the oxidized methionine-containing peptides. In this case, the distribution of N2-normalized values for the tag corresponding to the high denaturant concentration should be centered around 1.5 and the distribution of iTRAQ_N2 values for the tag corresponding to the lowest denaturant concentration should be centered around 0.5. Where the distributions cross is the approximate transition mid-point of a theoretical curve, typically ~1.

7TROUBLESHOOTING

- 49** For each of the matched methionine-containing peptides, subtract the N2-normalized values obtained in the (+) sample from the corresponding values obtained in the (–) sample.

- 50| Plot the distribution of all the differences from all the matched methionine-containing peptides. The plot should resemble a normal distribution centered at or near 0. See Fig. 5 for typical result.
- 51| Determine what range of differences encompass ~70% of the data, and filter the differences to identify peptides that have at least two consecutive differences outside the specified range. Copy these peptides into a new worksheet labeled “Potential Hits”.
- 52| The chemical denaturation data (i.e., the N2-normalized iTRAQ reporter ion intensities) for each peptide in the “Potential Hits” worksheet should be visually inspected to determine which peptides have significant transition midpoint differences (>0.5 M for GdmCl or >1 M for Urea) in the (–) and (+) ligand samples. In the visual inspection of the chemical denaturation data, $C^{1/2}_{\text{SPROX}}$ values are assigned to be the chemical denaturant concentration at which the N2-normalized iTRAQ values transition from the pre- to the post-transition baseline (see Figs. 4b and 5). Normally, the denaturant concentrations corresponding to the N2-normalized iTRAQ values flanking the transition are averaged, and the average value is assigned as the transition midpoint. However, if there is an N2-normalized iTRAQ value of 1.0 ± 0.1 at the transition, then that denaturant concentration is assigned as the transition midpoint. In cases where multiple N2-normalized iTRAQ values of 1.0 ± 0.1 exist at the transition, the denaturant concentrations at all points are averaged, and this average value is assigned as the transition midpoint. Chemical denaturation data sets in which more than one data point (i.e., one N2-normalized iTRAQ value) is not >1.0 or <1.0 in the pre- and post-transition baselines, respectively, are classified as “poor quality” and not assigned a transition midpoint. In cases where only a single normalized reporter ion intensity is inconsistent with the expected pre- and post-transition baseline values, the outlying value is typically removed from the data set and the remaining seven values are used in the visual inspection to assign the transition midpoint. In cases where no transition is observed (i.e., the N2-normalized iTRAQ reporter ion values obtained for a methionine-containing peptide are randomly distributed around 1.0) the peptide may not be derived from a globally protected region of protein structure or the transition midpoint may be greater than the largest denaturant concentration probed.

Anticipated results

The peptide and protein coverage in a proteome-wide SPROX experiment will depend on the protein sample and on the LC-MS/MS instrumentation and data acquisition methods used in the shotgun proteomics analysis. For a specific protein to be assayed for ligand binding, a methionine-containing peptide from the protein must be successfully identified and quantified in both the (–) and (+) ligand samples. Therefore, only a subset of the peptides and proteins identified in the LC-MS/MS analyses are included in the assay. In a typical bottom-up proteomics experiment, approximately 20% of the identified peptides are methionine-containing peptides, and they map to about 30% of the identified proteins.⁴ The methionine-containing peptide enrichment protocol typically increases the fraction of methionine-containing peptides to $>70\%$.^{5,15}

In triplicate LC-MS/MS analyses of SPROX samples both before and after methionine-containing peptide enrichment, typically ~200 methionine-containing peptides from ~100 proteins in a yeast cell lysate can be assayed using the Q-TOF instrument and data-dependent acquisition methods outlined in this protocol. As many as ~400 methionine-containing peptides from ~200 yeast proteins can be assayed by increasing the number of

LC-MS/MS analyses performed on the methionine-containing peptide enriched samples from 3 to 12. The use of faster and more sensitive LC-MS/MS instrumentation is also likely to increase the number of peptides and proteins that can be effectively assayed for ligand binding in the SPROX experiment.

Approximately 30–40% of the methionine-containing peptides assayed in a SPROX experiment yield SPROX data sets with clearly defined pre- and post- transition baselines and easily assigned transition midpoints (Fig. 6a). Another 30–40% of the identified methionine-containing peptides yield SPROX data sets in which one N2-normalized value must be overlooked in order to establish the pre- and post-transition baselines and assign a transition midpoint (Fig. 6). Approximately 30% of the identified methionine-containing peptides yield so-called “poor quality” SPROX data sets that do not have the assumed data set structure and must be eliminated from the analysis.

Hit proteins are identified as those with peptides that have significant ligand-induced $C^{1/2}_{\text{SPROX}}$ value shifts (>0.5 M GdmCl or >1.0 M urea) to either a higher or lower chemical denaturant concentration (Fig. 6b). Such hit proteins are expected to be targets (either direct or indirect) of the ligand under study. The magnitude of a protein's $C^{1/2}_{\text{SPROX}}$ value shift will depend on the binding free energy, which is a function of both the inherent affinity of the protein-ligand binding interaction(s) responsible for the hit and the free ligand concentration used in the SPROX analysis.⁵ In our proof-of-principle studies, a $C^{1/2}_{\text{SPROX}}$ value shift of 1.0 M urea was observed for the binding of NAD⁺ to alcohol dehydrogenase, yielding a K_d value 6 μM ,⁵ and a $C^{1/2}_{\text{SPROX}}$ value shift of 1.5 M GdmCl was observed for the tight binding interaction between cyclophilin and cyclosporin, yielding a K_d value of 26 nM.⁴

The false discovery rate will vary depending on the magnitude of the observed $C^{1/2}_{\text{SPROX}}$ value shift and how many iTRAQ tag differences are used to identify the protein hit in step 52]. Based on a comparison of two (–) ligand sample analyses of the proteins in a yeast cell lysate, we have estimated the false discovery rate to be ~4% for protein hits identified with only two consecutive iTRAQ tag differences and 1% for protein hits identified with 3 or 4 consecutive iTRAQ tag differences.⁵ The accuracy of the iTRAQ quantitation strategy is not sufficient to reliably identify protein hits based on a single iTRAQ tag difference between (–) and (+) ligand samples. The need to select protein hits based on more than a one iTRAQ tag difference and the limited number of data points in chemical denaturation data sets generated by SPROX (i.e., 8 in this work) ultimately limits the range of $C^{1/2}_{\text{SPROX}}$ value shifts that can be reliably detected to those that are >0.5 M GdmCl or >1.0 M urea.

The described protocol is a discovery platform and additional experiments are needed to validate hit proteins and to determine whether or not a hit protein is the result of a direct or indirect binding interaction. The appearance of false positives is largely due to random error associated with the iTRAQ reporter ion intensities measured in the SPROX experiment. Thus, hit proteins can most easily be validated using technical replicates generated by acquiring additional product ion mass spectra on target peptides and/or using biological replicates generated by repeating the experiment. Direct binding interactions can be further validated by performing a protein-ligand binding analysis with the ligand and a purified sample of the hit protein using SUPREX, SPROX, and/or other protein-ligand binding assays. The further validation of indirect binding hits is more challenging, and requires protein specific strategies such as activity-based assays with the unpurified protein and/or pull-down experiments performed on the unpurified protein sample using the protein hit as bait.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1: C18 Clean-Up

The following steps describe the use of commercially available MacroSpin™ columns with silica C18 to concentrate and desalt the iTRAQ-labeled peptide mixtures generated in the SPROX protocol. This concentration and desalting step is critical to the success of the LC-MS/MS analysis. This is a modified version of the manufacturer's protocol.

1. Add 1.4 mL of 0.1% TFA to 40–80 μL (–) and (+) ligand samples from step 33], which should each contain between 30 and 300 μg of total peptide. This should decrease the concentration of the organic solvent in each sample to 5%. Check the pH of the sample using 0.5 μL of sample and pH paper. If the pH is greater than 3, add 1–5 μL of 5% TFA to decrease the pH to 2–3.
2. Obtain two columns of C18 resin one each for the (–) and (+) ligand samples. 3. Add 400 μL of acetonitrile or methanol to each C18 column and vortex to wet column. Place each column in a 2 mL centrifuge collection tube and centrifuge at $2,500 \times g$ for 30 sec. Dispose of column flow through.
3. Add 400 μL of 0.1% TFA to the C18 column and centrifuge at $2,500 \times g$ for 30 s. Dispose of column flow through.
4. Centrifuge sample through the prepared C18 column in three ~500 μL aliquots at $2500 \times g$ for 30 s.
5. Add 400 μL of 0.1% TFA to the C18 column and centrifuge at $2,500 \times g$ for 30 s. Dispose of column flow through.
6. Repeat step 6 twice.
7. Obtain new collection tubes for C18 columns. Add 50 μL of 30% acetonitrile/0.1% TFA and centrifuge at $2,500 \times g$ for 30 s. Column elution contains peptides.
8. Repeat step 8 using 70% acetonitrile/0.1% TFA.
9. Place samples on a SpeedVac Concentrator for approximately 30 min or until sample volume is less than or equal to 50 μL .
10. Add 100–130 μL of 0.1% TFA to each sample to increase total sample volume. **CRITICAL STEP** Make sure the total percentage of organic is less than 10% before LC-MS/MS analysis.
11. **PAUSE POINT** Samples can be stored at 4°C for several days.

Box 2: Enrichment of methionine-containing peptides with Pr^3 -Met resin

The following steps describe the use of a commercially available Pr^3 -Methionine Reagent Kit to increase the number of methionine containing peptides identified and quantified in the LC-MS/MS analysis of the iTRAQ-labeled peptide mixtures generated in the SPROX experiment. The final steps in this protocol include a C18-sample cleanup procedure that is similar to that in **Box 1** but employs the C18 media provided with the Pr^3 -Methionine Reagent Kit. This is a modified version of the manufacturer's protocol.

Sample Preparation

- 1 Take between 160 –240 μ L of the (–) and (+) ligand samples (each containing up to a maximum of 100 μ g total peptide) from step 33], and reduce the sample volumes to less than 50 μ L using a SpeedVac Concentrator.
- 2 Add H₂O to achieve a final volume of 75 μ L in each sample.
- 3 Add 25–30 μ L 100% (glacial) acetic acid to each sample to adjust the pH to 2–3. Mix each sample thoroughly.

!CAUTION Glacial acetic acid is volatile/flammable and should be handled under a fume hood. Glacial acetic acid is also a severe health hazard and proper personal protective equipment should be worn.

Pr^3 -Met Pretreatment

- 4 Centrifuge the tube containing the methionine resin at $2,000 \times g$ for 1 min to settle all material at the bottom of the tube.
- 5 Punch a hole in the red cap using the top cap puncher provided in the kit.
- 6 Place a blue cap on the end of the spin tube and then add 200 μ L methanol to the spin tube. Place the red cap on top.
- 7 Vortex the spin tube for 15 min at a speed such that the liquid level in the spin tube is within 2 cm from the bottom of the tube.

CRITICAL STEP Always place the red cap on the spin tube before vortexing.

- 8 Remove the spin tube from the vortex and punch a hole in the blue cap using the provided bottom cap puncher provided in the kit. Remove the blue cap and place the spin tube in the collection tube. Remove the red cap and then centrifuge at $2,000 \times g$ for 1 min. Discard the liquid in the collection tube into an appropriate waste container.
- 9 Place the spin tube back into the collection tube. Pipette 400 μ L H₂O into the spin tube and replace the red cap. Holding the red cap in place, vortex the spin tube for 15 s at a speed in which the liquid level barely reaches the red cap. Remove the red cap and centrifuge at $2,000 g$ for 1 min.
- 10 Repeat step 9 twice.

Methionine Peptide Capture

- 11 Place an unused blue cap on the end of the spin tube before adding the sample to the spin tube. Firmly replace the red cap. Vortex the spin tube with the sample for 1.5 hr.

Wash

- 12** Add 100 μL 0.2 M β -mercaptoethanol in 25% acetic acid to the spin tube. After replacing the red cap, vortex for 30 min.

CRITICAL STEP 0.2 M β -mercaptoethanol in 25% acetic acid should be made fresh and discarded after use.

!CAUTION β -mercaptoethanol is volatile/flammable and should be handled under a fume hood. β -mercaptoethanol is also a severe health hazard and proper personal protective equipment should be worn.

- 13** Punch a hole in the blue cap and remove the blue cap from the spin tube. Place the spin tube into the collection tube, remove the red cap, and centrifuge at $2,000 \times g$ for 1 min. Discard or save sample flow through at 4°C .
- 14** Vortex the spin tube with the following solutions for 15 s so that the volume is within 2 cm from the bottom of the spin tube. Centrifuge at $2,000 \times g$ for 1 min after each addition and discard the flow through in an appropriate waste container.

400 μL 0.2M β -mercaptoethanol in 25% Acetic Acid	3 times
400 μL 70% ACN/0.1% TFA	3 times
400 μL H_2O	3 times

Release of Methionine Peptides

- 15** Place a new blue cap on the end of the spin tube. Pipette 86 μL 1.0 M ammonium bicarbonate, pH 9.0–9.1, and 14 μL of β -mercaptoethanol to each spin tube. Firmly replace the red cap.

CRITICAL STEP 1.0 M ammonium bicarbonate should be made immediately prior to use and the pH should be adjusted to between 9.0 and 9.1 using a pH meter. The pH of the ammonium bicarbonate solution is critical for elution of the methionine-containing peptides from the resin.

- 16** Vortex the spin tube for 2 hrs.

Sample Collection

- 17** Lightly tap the spin tube on the bench top three times. Punch a hole in the blue cap and gently remove the blue cap. Place the spin tube into a new collection tube. Remove the red cap and centrifuge the spin tube at $2,000 \times g$ for 1 min.
- 18** Place a new blue cap on the spin tube and add 50 μL 20% acetonitrile/0.1% TFA to the spin tube. Replace the red cap and vortex the spin tube for 15 min.
- 19** Tap the spin tube on the bench top three times and then punch a hole in the blue cap. Remove the blue cap and place the spin tube into the collection tube. Centrifuge at 2000 g for 1 min.
- 20** Pipette 400 μL of 2% TFA into the spin tube and replace the red cap. Hold the red cap in place and vortex for 15 s until the liquid barely reaches the level of the cap. Tap the spin tube on the bench top three times, then remove the red cap and place the spin tube in the collection tube. Centrifuge at 2,000 g for 1 min.

- 21** Repeat step 20.

Sample Clean-Up

- 22** Discard the glass beads in the spin tube and thoroughly rinse the spin tube with H₂O until all the glass beads have been removed.
- 23** Add 400 µL H₂O to the spin tube and replace the red cap. Hold the cap in place and vortex for 15 s. Centrifuge at 2,000 × g for 1 min and discard the flow through.
- 24** Repeat step 23.
- 25** Add 400 µL of methanol to the spin tube and replace the red cap. Holding the red cap in place, vortex for 15 s. Centrifuge at 2,000 × g for 1 min and discard the flow through.
- 26** Repeat step 23.
- 27** Add 400 µL methanol to the C18 resin provided with the methionine enrichment kit. Transfer the resin in methanol to the spin tube and vortex for 15 s with the red cap in place. Centrifuge at 2,000 × g for 1 min and discard the flow through.
- 28** Add 400 µL 2% TFA to the spin tube and centrifuge at 2,000 × g for 1 min. Discard the flow through.

CRITICAL STEP Do not vortex the spin tubes after the resin has been compacted.

- 29** Add half of the collected sample solution (475 µL) to the spin tube and centrifuge at 2,000 × g for 1 min. Discard the flow through.
- 30** Repeat step 29 with the remaining sample volume.
- 31** Add 400 µL of 2% TFA to the spin tube and centrifuge at 2,000 × g for 1 min. Discard the flow through.
- 32** Obtain a clean collection tube. Add 50 µL of 70% acetonitrile/0.1% TFA to the spin tube and centrifuge at 2,000 × g for 1 min. The elution contains the methionine-containing peptides. Do not discard the elution.
- 33** Repeat step 32 twice.

PAUSE POINT Sample can be stored at 4°C for several days.

- 34** Place sample on a SpeedVac concentrator for 20–30 min to reduce the sample volume to less than 50 µL.

- 35** Add 100–150 µL 0.1% TFA to the sample and mix well.

PAUSE POINT Sample can be stored at 4°C for several days.

- 36** Heat samples at 60°C for 10 min. Centrifuge at 14,000 × g for 10 min and transfer supernatant to a glass vial with an insert for mass spectrometry analysis.

Fig. 1.

a) Schematic representation of the oxidation primary reactant (i.e., the methionine amino acid side chains in proteins) and the primary product (i.e., the oxidized methionine amino acid side chains) in the H_2O_2 -mediated oxidation reaction in SPROX. **b)** The mechanism by which the methionine amino acid side chains in proteins are oxidized in the SPROX protocol. In the folded protein (represented by the closed circle), methionine residues that are buried in solvent inaccessible regions of a protein's three-dimensional structure are protected from oxidation. In the unfolded protein (represented by the wavy line) these once protected methionine residues become exposed to solvent and are free to react with the H_2O_2 . The k_{open} and k_{closed} values are the rate constants associated with the protein (or protein domain's) unfolding and refolding reactions; and k_{ox} is the intrinsic exchange oxidation rate of an unprotected (i.e., solvent exposed) methionine residue. Provided that $k_{\text{closed}} \gg k_{\text{ox}}$, the overall rate at which the globally (or sub-globally) protected methionine residues in proteins are oxidized can be related to the equilibrium constant for the unfolding and refolding reaction of the protein (or protein domain) and ultimately used to evaluate the protein folding free energy as described in reference 1.

Fig. 2.

Schematic representation of experimental workflow. Adapted with permission (<http://www.pnas.org/site/misc/rightperm.shtml>) from reference 4. The numbers adjacent to the tubes in Step II are the m/z values of the reporter ions for the different iTRAQ reagents that are used to label the samples.

Fig. 3.

Distributions of the N1-normalized reporter ion values for two different iTRAQ tags obtained using the non-methionine containing peptides from a (+) ligand SPROX sample. The calculation of N1-normalized reporter ion values is described in **38**. The data shown are for the 114 (broken line) and 121 (solid line) tags that correspond to the 0 and 2.4 M GdmCl concentrations, respectively, in a SPROX experiment. The average and standard deviation of the values for the 114 and 121 tags are 1.12 ± 0.36 and 0.95 ± 0.39 , respectively.

Fig. 4.

a) Distributions of the N2-normalized iTRAQ reporter ion values obtained for the unoxidized methionine-containing peptides in the (–) and (+) ligand samples at the 0 M (solid line), and 3.0 M (broken line) GdmCl concentrations used in a SPROX experiment. The calculation of N2-normalized reporter ion values is described in steps 42 – 43]. The vertical dotted line represents the N2-normalized value that best separates the two distributions. **b)** Theoretical SPROX data expected for an unoxidized methionine-containing peptide based on the distributions in **a)** The horizontal dotted line represents the N2-normalized value that best separates the two distributions in **a)** Actual SPROX data sets for methionine-containing peptides are generally represented as bar graphs (see Fig. 6), and have transition midpoints that are shifted to higher or lower denaturant concentrations depending on the stability of the protein to which the peptide maps.

Fig. 5. Distribution of the N2-normalized iTRAQ reporter ion value differences observed between the (+) and (−) ligand samples in a proteome-wide SPROX experiment in which the proteins in a yeast cell lysate were analyzed for binding to tamoxifen. Included in the distribution are all the iTRAQ reporter ion value differences for all the tags on all the methionine-containing peptides. Approximately 70% of the differences are greater than −0.5 and less than 0.5.

Fig. 6.

Chemical denaturation data sets from a proteome-wide SPROX experiment in which the proteins in a yeast cell lysate were analyzed for binding to tamoxifen. **a)** Data obtained on a methionine-containing peptide (TQDLLLDVAPLSLGIETAGGVMTK) from SSA1p, which was not identified as a hit because the difference in $C^{1/2}_{\text{SPROX}}$ values between the (–) and (+) ligand samples was < 0.5 M. **b)** Data obtained on a methionine-containing peptide (VETGVIKPGMVVTFAPAGVTTEVK) from translation elongation factor EF-1 α , which was identified as a hit because the difference in $C^{1/2}_{\text{SPROX}}$ values between (–) and (+) ligand data was > 0.5 M. In **a)** and **b)** the light and dark shaded bars represent data from the (–) and (+) ligand samples, respectively. Data points indicated with an * were omitted for $C^{1/2}_{\text{SPROX}}$ value assignments. The averaged data from at least two product ion mass spectra are shown, and the error bars represent one standard deviation. The light and dark arrows point to the denaturant concentrations at the $C^{1/2}_{\text{SPROX}}$ values of the (–) and (+) ligand data sets, respectively.

Table 1

| Troubleshooting table

Step	Problem	Possible reason	Solution
6	Solution is opaque	Cellular debris still in solution	Repeat step 5
10	Precipitate observed in (+) ligand sample	Ligand is not soluble at the working concentration	Reduce the ligand concentration in the ligand stock solution and go back to step 1
18	Pellet not visible	No protein precipitated	Vortex for 15 sec and spin down at 8–10,000 × g for 30 min
		Nature of the sample	Continue with protocol
25	Protein concentration < 1 mg/mL	Sample lost in precipitation and/or redissolution in steps 16–20	Go back to step 1
29	No peptide ion signals detected in MALDI mass spectrum	Problem with trypsin digestion	Repeat step 28
			Check that protein sample does not contain trypsin inhibitor
35	Total ion chromatogram dominated by selected singly charged ions such as m/z 379.2, 391.2, 469.4, 491.2, 683.1, 453.3, and 343.2	Sample not adequately cleaned-up prior to LC-MS/MS analysis	Repeat step 34
36	Some peptides identified in LC-MS/MS searches with oxidized Cys, Trp, and/or other amino acids	Problem with H ₂ O ₂ oxidation reaction	Continue with data analysis, but remove any such oxidized peptides from the data
	All Cys and/or Trp-containing peptides identified are oxidized	Cell lysate may contain a component (e.g., iron) that catalyzes H ₂ O ₂ oxidation reaction	Cell lysate should be prepared without problem component
40	The distribution for a given iTRAQ tag is not centered at 1, but distribution is still narrow (i.e., relative standard deviation = ~30%)	The iTRAQ labeling reaction in step 32 did not go to completion	Be sure to perform N2-normalization procedure in step 42
		Differential amounts of protein precipitated and/or redissolved in steps 16–20	Be sure to apply N2-normalization procedure in step 42
	The distribution for a given iTRAQ tag is not centered at 1 and distribution is wide (i.e., relative standard deviation = >40%)	Problem with iTRAQ labeling reaction	Perform data analysis without using data from problem iTRAQ tag
		Differential amounts of protein precipitated and redissolved in steps 16–20	Perform data analysis without using data from problem iTRAQ tag
42	Very few unoxidized methionine-containing peptides are identified even using Pi ³ Met resin	Problem with H ₂ O ₂ oxidation reaction	Go back to step 1
		Air oxidation of sample post SPROX analysis	Go back to step 1

Step	Problem	Possible reason	Solution
47	The distributions for the tags corresponding to the high and low denaturant concentrations are not well separated	Problem with H ₂ O ₂ oxidation reaction	Go back to step 1 and check that HEPES or MOPS, were not used to prepare the protein samples and that the H ₂ O ₂ solution is 30% (w/w) (see REAGENT SETUP)
48	The distributions for the tags corresponding to the high and low denaturant concentrations are not well separated	Problem with H ₂ O ₂ oxidation reaction	Go back to step 1 and check that the H ₂ O ₂ solution is 30% (w/w) see REAGENT SETUP
		Air oxidation of sample post SPROX analysis	Go back to step 1